

# Molecular insights into the control of transcription initiation at the *Staphylococcus aureus agr* operon

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## ABSTRACT

Central to the regulation of the diverse array of virulence factors encoded by the opportunistic human pathogen *Staphylococcus aureus* is the accessory gene regulator (*agr*) operon. The *agr* operon contains two transcription units; RNAII, encoding a quorum-sensing system including the master transcriptional activator AgrA, and RNAPIII, the effector RNA molecule that regulates virulence gene expression. RNAII and RNAPIII are transcribed from the divergent promoters P2 and P3, respectively. Due to the sub-optimal spacer length of the P3 promoter, it is widely believed that transcription from P3 is dependent on AgrA. A fully native *S. aureus in vitro* transcription system was setup to provide the first insight into the molecular and mechanistic characterisation of the regulation of transcription initiation at the *agr* operon. Surprisingly, results revealed that, in the absence of AgrA, RNA polymerase (RNAP) can interact with P2 and P3 equally well, however the transcription-competent open promoter complex (RPO) forms more readily at P2 than P3. AgrA was demonstrated to increase the occupancy of P2 and P3 by RNAP, to increase transcription initiation at both promoters, with a more pronounced effect at P3. The P3 promoter is unusual as it has a genuine extended -10 motif and a near to consensus -35 promoter element. "Scrambling" of the -35 element or shortening of the sub-optimal spacer to the optimal 17 nucleotides both significantly increased transcription initiation at P3, by facilitating the rate of isomerisation of the initial RNAP-P3 complexes to the RPO, in a strictly extended -10 promoter element dependent manner. Therefore, two possible mutually inclusive mechanisms by which AgrA activates transcription at the *agr* operon P3 promoter are proposed. In addition, the involvement of the transcription factor SarA on transcription from P2 and P3 was investigated. Overall, this study provides the first molecular insights into how transcription is regulated at the *agr* operon in *S. aureus*.

## **DECLARATION OF AUTHORSHIP**

I confirm that this thesis entitled “Molecular insights into the control of transcription initiation at the *Staphylococcus aureus agr* operon” is written entirely by myself and that the research to which it refers to is my own. I confirm that any ideas or quotations from the work of others has always been clearly referenced and that all main sources of help have been acknowledged.

Jonathan Mark Reynolds

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## ABBREVIATIONS

|                 |                                                   |
|-----------------|---------------------------------------------------|
| $^{32}\text{P}$ | Radio-labelled Phosphate                          |
| Å               | Angstrom                                          |
| A               | Adenine                                           |
| a.a.            | Amino acid                                        |
| <i>agr</i>      | Accessory gene regulator operon                   |
| <i>agr</i> IR   | Accessory gene regulator operon intergenic region |
| AHL             | N-acylhomoserine lactone                          |
| AI              | Autoinducer                                       |
| AIP             | Auto-inducing peptide                             |
| AP              | Acetyl phosphate                                  |
| bp              | Base pairs                                        |
| C               | Cysteine                                          |
| Ci              | Curie                                             |
| dNTP            | Deoxy nucleotide triphosphate                     |
| DTT             | Dithiothreitol                                    |
| ECF             | Extracytoplasmic function                         |
| <i>E. coli</i>  | <i>Escherichia coli</i>                           |
| EDTA            | Ethylenediaminetetraacetic acid                   |
| EMSA            | Electrophoretic mobility shift assay              |
| E               | RNAp catalytic core                               |
| E $\sigma$      | RNAp holoenzyme                                   |
| g               | Relative centrifugal force                        |
| G               | Guanine                                           |
| HK              | Histidine protein kinase                          |

|                  |                                        |
|------------------|----------------------------------------|
| HTH              | Helix-turn-helix                       |
| kb               | Kilobase                               |
| min              | Minute(s)                              |
| nm               | Nanometre                              |
| OD               | Optical density                        |
| p                | Promoter                               |
| RNA              | Ribonucleic acid                       |
| tRNA             | transfer RNA                           |
| RNAp             | DNA-dependent RNA-polymerase(s)        |
| aRNAp            | Archeal RNAp                           |
| bRNAp            | Bacterial RNAP                         |
| eRNAp            | Eukaryotic RNAp                        |
| rpm              | Revolutions per minute                 |
| RPc              | Closed promoter complex(es)            |
| RPo              | Open promoter complex(es)              |
| RR               | Response regulator                     |
| <i>S. aureus</i> | <i>Staphylococcus aureus</i>           |
| sec              | Second(s)                              |
| SDS              | Sodium dodecyl sulphate                |
| T                | Thymine                                |
| TBS              | Tris-buffered saline                   |
| TCS              | Two-component system                   |
| TEMED            | N',N',N',N'-Tetramethylethylenediamine |
| Tris             | Tris(hydroxymethyl)aminomethane        |
| U                | Uridine                                |

|              |                                 |
|--------------|---------------------------------|
| v/v          | Volume:Volume                   |
| w/v          | Weight:Volume                   |
| $\alpha$     | RNAp $\alpha$ subunit           |
| $\alpha$ CTD | RNAp $\alpha$ C-terminal domain |
| $\alpha$ NTD | RNAp $\alpha$ N-terminal domain |
| $\beta$      | RNAp $\beta$ subunit            |
| $\beta'$     | RNAp $\beta$ Prime Subunit      |
| $\mu$ Ci     | Microcurie                      |
| $\sigma$     | Sigma subunit                   |
| $\omega$     | RNAp omega Subunit              |

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## **CHAPTER ONE**

### **1. Introduction**

#### **1.1. Foreword**

The regulation of virulence in bacterial pathogens is complex, allowing the bacterium to adapt to changing conditions at the site of infection. One such bacterium, and the organism of focus in this thesis, is *Staphylococcus aureus*. The regulation of the wide array of virulence factors encoded by *S. aureus* has contributed to its rise as a leading source of hospital acquired and community acquired infections. The accessory gene regulator operon (*agr*) is widely regarded as the master regulatory operon of the diverse array of *S. aureus* virulence factors. The *agr* operon orchestrates the growth phase and cell density dependent expression of virulence factors through a quorum sensing system. The *agr* operon is strongly regulated at the transcriptional level by a wide range of transcriptional regulators. Advances in our understanding of the *agr* operon could lead to a greater understanding of *S. aureus* infection and contribute towards uncovering potential targets for its control. This thesis provides some insights into the molecular mechanisms that govern the expression and regulation of the *agr* operon in *S. aureus* at the gene transcriptional level.

#### **1.2. *Staphylococcus aureus***

The bacterium *S. aureus* is a gram-positive coccus that permanently inhabits approximately 30% of the healthy population as a human commensal. *S. aureus* lives on the skin but primarily in the squamous epithelium of the anterior nares in the nasal cavity (VandenBergh, Yzerman et al. 1999). It is recognised as a major human pathogen that causes conditions from minor skin infections to more life-threatening conditions such as acute endocarditis; as shown by the 1,253 deaths involving *S. aureus* in the United Kingdom in 2009 (Office for National Statistics 2010). The success of *S. aureus* as a pathogen is facilitated by a vast array of surface-associated and secreted virulence factors.

##### **1.2.1. *S. aureus* pathogenesis**

The first stage in the pathogenesis of *S. aureus* infections is colonisation. This step involves the adherence of the bacteria to the host tissue, targeting the plasma and matrix proteins such as fibrinogen, fibronectin and collagen (Kochwa, Litwak et al. 1977). In *S. aureus* this is

mediated by a family of surface associated adhesins called MSCRAMMs (microbial surface components recognising adhesive matrix molecules), which are present on the staphylococcal cell surface. More specifically the MSCRAMMs fibronectin binding proteins A and B (FnbpA and FnbpB) play an important role as virulence factors via adhesion to the host fibronectin, elastin and fibrinogen, with both being essential for invasion (Greene, McDevitt et al. 1995; Fowler, Wann et al. 2000). FnbpA is structurally related and shares a similar domain organisation to other staphylococcal MSCRAMMs including clumping factor A (ClfA) and serine-aspartate repeat protein G (Sdr-G), which all specifically interact with host fibrinogen (Rivera, Vannakambadi et al. 2007). Additionally, variation in the fibrinogen and elastin A domains of FnbpA and more recently in FnbpB alters drug targets and highlights the challenges in targeting the MSCRAMMs for new antibiotics (Loughman, Sweeney et al. 2008; Burke, McCormack et al. 2010).

Colonisation can be followed by either local infection or systemic dissemination/sepsis through access to the blood (Lowy 1998). Here *S. aureus* evades the host immune response with an array of secreted and surface exposed virulence factors. One such surface exposed virulence factor is the well-studied Protein A, which disrupts the classical complement pathway through interaction with IgG (Uhlen, Guss et al. 1984; Cedergren, Andersson et al. 1993). Other major surface virulence factors include Clumping factor A (ClfA), which uses two mechanisms to help resist phagocytic uptake. Firstly ClfA binds, and is coated by, fibrinogen via its N-Terminal A domain as a shield from the deposition of complement and opsonising antibodies to the bacterial cell surface (Higgins, Loughman et al. 2006). Secondly ClfA binds to the complement regulator factor I to increase the cleavage of the activated central complement component C3b into inactive C3b (iC3b) (Hair, Ward et al. 2008).

The secreted virulence factors produced by *S. aureus* are primarily expressed during the post-exponential and stationary phases of growth post infection (Tseng, Zhang et al. 2004). These consist of a number of exoenzymes including nucleases, proteases and glycerol ester hydrolipase (lipase) involved in nutrient availability (Gotz, Verheij et al. 1998; Dinges, Orwin et al. 2000). Secreted virulence factors also include an array of exotoxins to target neutrophils and resist phagocytosis and to promote sepsis syndrome (Marrack and Kappler 1990; Bhakdi and Tranum-Jensen 1991). One such exotoxin and superantigen is the well-

studied toxic shock syndrome toxin 1 (TSST-1) (Schlievert, Shands et al. 1981). TSST-1 acts through the binding of its N-terminus to the outside of the peptide-binding groove of the major histocompatibility complex class II (MHC II) binding site, on a cell presenting an antigen, and crosslinks it to a T-cell receptor (Hurley, Shimonkevitz et al. 1995; Kum, Wood et al. 1996). This induces over-reactivity of the host immune response including T-cell proliferation, enhanced expression of cell-adhesion molecules and abnormally high levels of cytokines and chemokines. This results in a condition called toxic shock syndrome.

### **1.2.2. The emergence of drug resistance in *S. aureus***

The emergence of multi drug resistance strains of *S. aureus* such as methicillin resistant *S. aureus* (MRSA) and vancomycin resistant MRSA (VMRSA) have highlighted the adaptivity of *S. aureus* to its surroundings. After the introduction of penicillin for treatment of *S. aureus* infections in 1940, it only took two years until the discovery of the first penicillin resistant isolate (Kirby 1944). Similarly MRSA was reported two years after the introduction of methicillin for treatment of *S. aureus* in 1959 (Barber 1961; Eriksen 1961). Therefore highlighting the need for continued discovery of new antibiotic agents and/or antibacterial targets for the management of infections caused by *S. aureus*.

The resistance of *S. aureus* to methicillin and all other  $\beta$ -lactam antibiotics is due to the acquisition of the *mecA* gene. The *mecA* gene is found on the staphylococcal cassette chromosome named the SSCmec (Sjostrom, Lofdahl et al. 1975). The SSCmec is a mobile genetic element and is inserted on the *S. aureus* chromosome near the origin of replication. *S. aureus* strains encoding the *mecA* gene produce an additional penicillin binding protein (PBP) called PBP2a. Native PBPs are enzymes found in the cytoplasmic membrane that are involved in bacterial cell wall assembly (Sauvage, Kerff et al. 2008). Native PBPs bind to  $\beta$ -lactam antibiotics, which cause disruption in the synthesis of the bacterial peptidoglycan layer. Unlike native PBPs, PBP2a has a lower affinity for the  $\beta$ -lactam antibiotics and so takes over peptidoglycan layer synthesis in the presence of the  $\beta$ -lactams, therefore promoting *S. aureus* survival (Fuda, Hesek et al. 2005).

### **1.2.3. The formation of biofilms by *S. aureus***

It has been estimated that biofilms are involved in 60% of all microbial infections (Lewis 2001). There are a number of advantages to bacteria for life in biofilms including defence against host defence mechanisms and antibiotics. This occurs via a number of mechanisms including; the creation of barrier slime matrices (Dibdin, Assinder et al. 1996), pockets of stationary phase dormant cells that can still uptake antibiotics (Spoering and Lewis 2001) and subpopulations of resistant phenotypes (Suci and Tyler 2003). Another advantage for bacteria in biofilms is increased colonisation in niche locations to increase nutrient availability. One mechanism involved is the ability of biofilms to make structural changes to move towards nutrient sources (Stoodley, Dodds et al. 1998). There are also further advantages of community life such as gene transfer and the sharing of the metabolic burden.

*S. aureus* is prevalent on implanted medical devices (e.g. catheters and orthopaedic implants) and host tissue as biofilms, which can lead to chronic infection (Tsukayama, Estrada et al. 1996; Zimmerli and Ochsner 2003). Biofilm production in *S. aureus* requires the regulation and expression of a number of genes. Essential genes involved in biofilm formation include the *icaADBC* operon involved in intercellular adhesion synthesis through the synthesis of the polysaccharide intercellular adhesin (PIA), clumping factor A (*clfA*), the haemolytic toxin (*hla*), *dltA* involved in D-alanine esterification of teichoic acids and the alternate sigma factor-stress response  $\sigma^B$  (Jefferson 2004). In order for bacteria to form and maintain biofilms they are required to act in unison through the regulation of the genes involved in biofilm formation. This is controlled through the secretion and recognition of signalling molecules, named 'quorum sensing'.

### **1.3. Quorum sensing in bacteria**

In order for bacteria to adapt and survive in changing conditions individual bacterial cells need to co-ordinate their gene expression in local populations (Gray 1997). Many bacteria co-ordinate their gene expression through the secretion and recognition of low-molecular weight signalling molecules. These self-coded molecular signalling molecules are termed "autoinducers". Autoinducers are produced at basal levels and increase in concentration with an increase in growth of the bacterial cell population. As the autoinducer concentration

increases to a critical level they activate receptors in the bacterial cell, leading to altered gene expression allowing the bacteria to respond its changing growth conditions. This bacterial population density dependent response is termed “quorum sensing”(QS) (Fuqua, Winans et al. 1994).

Autoinducers that induce their own synthesis are termed “autoactivators” and those that do not induce their own synthesis are termed “autocrine regulators”. Both autoactivators and autocrine regulators are used as signalling molecules in the bacterial response to increased population size, however only autoactivators that induce their own synthesis lead to an exaggerated population density dependent quorum response (Fuqua, Winans et al. 1994). Autocrine regulators also respond to population density, however the lack of self-induction does not lead to a sharp activation threshold as seen with autoactivators (Korem, Sheoran et al. 2003).

There are three main classes of bacterial autoinducers (AI) that have been identified. The first are N-acylhomoserine lactones (AHLs), which are produced in over 70 species of Gram-negative bacteria (Williams, Winzer et al. 2007). AHLs are able to diffuse freely into the cell and are detected by specific cytoplasmic receptor proteins. Upon activation by the AHL they undergo dimerisation, which allows promoter binding at target genes for transcriptional regulation through activation and repression (Fuqua, Winans et al. 1994). The second are peptides found in Gram-positive bacteria named autoinducing peptides (AIPs) (Sturme, Kleerebezem et al. 2002). AIPs do not usually enter the cell, therefore most bind to transmembrane receptors at the cell surface. This leads to activation of signal transduction pathways resulting in activation or repression of gene targets mostly at the level of transcription. AIPs are autoactivators as they activate their own synthesis. One well studied example of this is the AgrD AIP of the *agr* operon in *S. aureus* (Novick, Projan et al. 1995). The third class of AIs are S-4,5-dihydroxy-2,3-pentanedione (DPD)/autoinducers termed AI-2, which are regarded as an interspecies QS signalling molecule as they are utilised by both Gram negative and Gram positive bacteria (Xavier and Bassler 2005).

#### 1.4. Accessory gene regulator (*agr*) operon

*S. aureus* controls gene expression as a quorum through the expression and secretion of small peptides. In *S. aureus* the major quorum sensing system encoding an AIP is the accessory gene regulator (*agr*). The *agr* operon is widely regarded as the major regulatory switch for the cell density dependent regulation of virulence factors in *S. aureus* (Novick, Ross et al. 1993; Morfeldt, Taylor et al. 1995). The *agr* operon is ~3 kb in length, encodes two RNA transcripts RNAII and RNAIII transcribed from the divergent promoters P2 and P3 and is subject strict to transcriptional regulation (Arvidson and Tegmark 2001; Bronner, Monteil et al. 2004). RNAII encodes a quorum sensing system, incorporating four genes *agrB*, *agrD*, *agrC* and *agrA* (Figure 1). *agrD* encodes the quorum signalling molecule of the operon, whereas *agrB* encodes a transmembrane protein involved in the processing and secretion of AgrD to the AIP. *agrC* and *agrA* encode a two-component system (TCS), where AgrC is a the sensor histidine kinase that detects the quorum dependent increase of the signalling molecule AIP and is autophosphorylated. *agrA* encodes the response regulator of the TCS, which is phosphorylated by the activated AgrC-P, allowing it to bind to a pair of direct repeats in the *agr* intergenic region (*agr* IR) and upregulate transcription from the P2 and P3 promoters (Koenig, Ray et al. 2004).

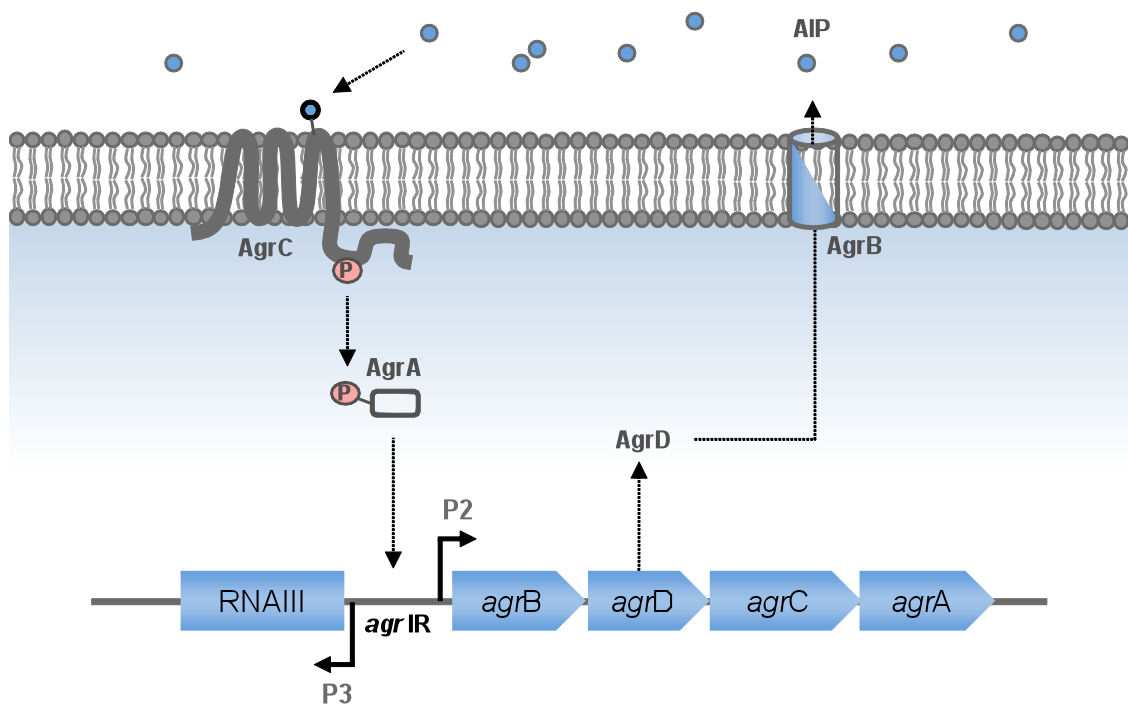


Figure 1: The *agr* operon of *S. aureus*.

A schematic showing the auto-activation of the *agr* operon. The *agrD* gene product (AgrD) is C-terminally processed by the transmembrane protein AgrB and N-terminally processed by SpsB for secretion as the autoinducing peptide (AIP). The AIP is sensed by the transmembrane domain of the receptor AgrC, conferring phosphorylation of the AgrC cytoplasmic HK domain. AgrC then activates AgrA by phospho-transfer, allowing phosphorylated AgrA to activate transcription from both the *agr* P2 and P3 promoters. Transcription from P3 encodes the effector molecule of the *agr* operon, the regulatory RNA, RNAIII. Enhanced transcription from P2 facilitates the autoactivation circuit.

Transcription from P3 encodes the regulatory RNA molecule RNAIII, which is the effector molecule of the *agr* operon and also encodes the peptide  $\delta$ -haemolysin. RNAIII regulates virulence genes mainly at the translational level to upregulate extracellular virulence factors and downregulate cell-surface associated virulence factors, see section 1.4.5. Therefore it provides a quorum dependent regulatory switch for virulence factor expression. The importance of the *agr* operon in the regulation of virulence factors is highlighted by the global transcription profile comparisons between wild-type and *agr* deletion strains of *S. aureus*, which determined that 104 genes are up-regulated and 34 genes are down-regulated in an *agr* dependent manner (Dunman, Murphy et al. 2001).

The *agr* operon was first identified by Peng *et al* in 1988, after a number of studies identifying the growth phase dependent expression of virulence factors such as  $\alpha$ ,  $\beta$  and  $\delta$  haemolysin (Abbas-ali and Coleman 1977; Recsei, Kreiswirth et al. 1986). The conservation

of the *agr* operon in the genus *Staphylococcus* highlights its importance as a global regulator of virulence factors (Morfeldt, Tegmark et al. 1996). The *agr* operon also has functional homologs in several other pathogenic bacteria including *Listeria monocytogenes*, where a mouse infection model strain with an inactivation mutation in *agrA* had 10 times reduced virulence (Autret, Raynaud et al. 2003); and *Enterococcus faecalis*, where inactivation mutations of components of the *fsr* locus, like its *agr* homolog in *S. aureus*, reduces biofilm formation (Qin, Singh et al. 2000).

*In vivo* studies using *agr* deletion strains have highlighted the critical role of the *agr* operon in staphylococcal pathogenesis. One study carried out using a staphylococcal osteomyelitis model found that the disruption of the *agr* operon resulted in a significant reduction in the severity and incidence of osteomyelitis (Gillaspy, Hickmon et al. 1995). In a murine arthritis model *agrA* deletion strains displayed a 6 fold drop in arthritis when compared to wild-type (Abdelnour, Arvidson et al. 1993). Due to evolutionary divergence within the staphylococci, variations have been found in the *agr* B-D-C region, named the hyper-variable region, which has led to division into at least four specificity groups in the *S. aureus* *agr* operon (Ji, Beavis et al. 1997; Jarraud, Lyon et al. 2000). The *agr* specificity groups are divided based upon their ability to inhibit or to cross-activate, by their peptides, *agr* expression. The four specificity groups have been named *agr* I-IV.

#### **1.4.1. AgrB**

AgrB is an integral transmembrane endopeptidase, consisting of four transmembrane  $\alpha$ -helices and two transmembrane hydrophilic regions (Zhang, Gray et al. 2002), whose major role in the *agr* operon is in AIP biosynthesis. AgrD, the peptide precursor of AIP, first associates with the cytoplasmic membrane through its N-terminal amphipathic  $\alpha$ -helix (Zhang, Lin et al. 2004). AgrB then interacts with, and C-terminally processes, AgrD through cleavage involving conserved histidine and cysteine residues of AgrB (Zhang and Ji 2004; Qiu, Pei et al. 2005). Following C-terminal cleavage, the remaining AgrD peptide fragment then binds as a thioester to the cysteine at position 84 of AgrB. The thiolactone ring of the AIP is then formed through the cysteine residue of AgrD at position 28 catalysing thioester exchange. The peptide precursor is then released from AgrB and transported to the outer membrane by an unknown mechanism, before N-terminal processing by SpsB to remove the

N-terminal amphipathic helix (Kavanaugh, Thoendel et al. 2007). The AIP is then free to be detected by AgrC.

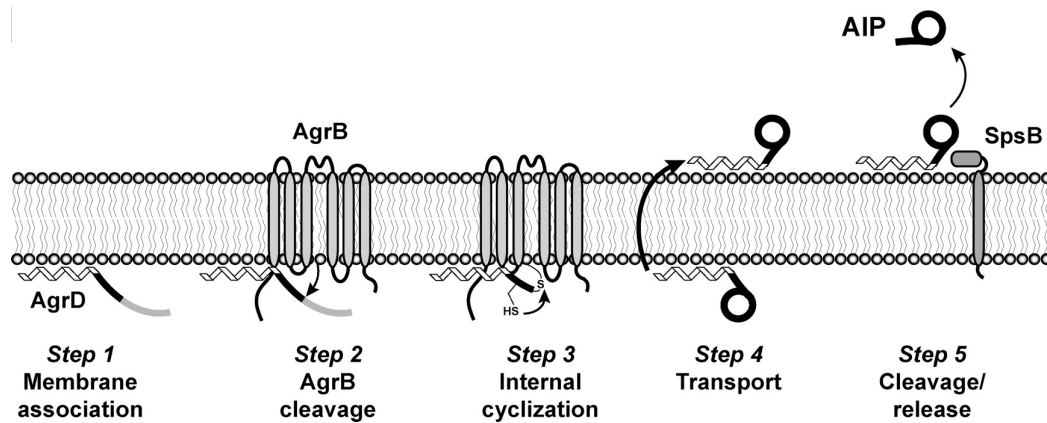


Figure 2: AIP biosynthesis model.

A schematic of the AIP biosynthetic pathway model. In step 1 the AgrD N-terminal associates with the cytoplasmic membrane. In step 2 AgrB cleaves the C-terminal tail of AgrD. In step 3 the AgrD fragment is bound to AgrB as a thioester, before the AgrD cysteine residue attacks the thioester bond to form the thiolactone ring and displace the peptide. In step 4 the AIP precursor is transported to the outer surface of the membrane by a yet to be revealed mechanism. In step 5 the AIP precursor is cleaved by SpsB to remove the amphipathic helix and the AIP is released. Figure from (Thoendel and Horswill 2009).

#### 1.4.2. AgrD

The gene product of *agrD*, once processed by AgrB and SpsB, encodes the AIP of the *agr* operon.

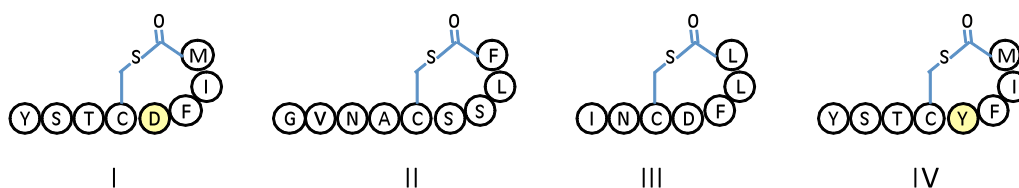


Figure 3: AIP I-IV amino acid sequence and structure.

Diagrammatic representation of the four *S. aureus* AIPs, with the amino acid sequence displayed. Amino acids are presented as spheres with the single amino acid aspartic acid to tyrosine residue change between AIP-I/IV highlighted in yellow.

The AIPs of the *agr* operon contain a thiolactone ring and are split into the four specificity groups, in which the AIP is able to activate its cognate receptor. The AIPs of the four specificity groups vary by amino acid sequence (AIP I-IV) and also structure, as shown in

Figure 3 (Ji, Beavis et al. 1997; Kalkum, Lyon et al. 2003). Only one amino acid sequence change, aspartic acid (D) to tyrosine (Y), is required to change AIP I to AIP IV group specificity (P, Affas et al. 2001). The build-up of the AIP is detected by the extracellular signal receptor AgrC, leading to the activation of AgrC.

#### **1.4.3. AgrC**

AgrC is the histidine protein kinase (HK) of the two component partners AgrA and AgrC (Peng, Novick et al. 1988; Novick, Projan et al. 1995). AgrC is a transmembrane signal receptor HK that senses the AIP and is activated through a highly sequence specific interaction (Balaban and Novick 1995). AgrC contains two domains, an N-terminal extracellular sensing domain and a C-terminal cytoplasmic signalling domain (Lina, Jarraud et al. 1998). The extracellular sensing domain contains the AIP binding site and is highly variable (Wright, Lyon et al. 2004). The extracellular sensing domain consists of six transmembrane regions, including three extracellular loops, and contains the determinants of specificity for AIP activation and binding (Wright, Lyon et al. 2004). A number of studies have determined key residues in this specificity. In one study, the construction of AgrC chimeras (AgrC-I-IV) with the sensor domains exchanged and modified to determine specificity, indicated that *agr* group specificity is determined by just five amino acid positions on the second extracellular loop (Wright, Lyon et al. 2004; Chen, Tsou et al. 2009). A further study of the AgrC sensor domain chimeras including amino acids substitutions at five divergent residues indicated that just one single amino acid substitution can alter group specificity (Geisinger, George et al. 2008). Interestingly, one residue (I171) identified on the third extracellular loop has been shown to play an important role in ligand mediated inhibition (Geisinger, Muir et al. 2009). The mutation of I171 was shown to eliminate the sensitivity of the AgrC receptor to inhibition by noncognate ligands, leading to broadened activation and reduced sensitivity to inhibition.

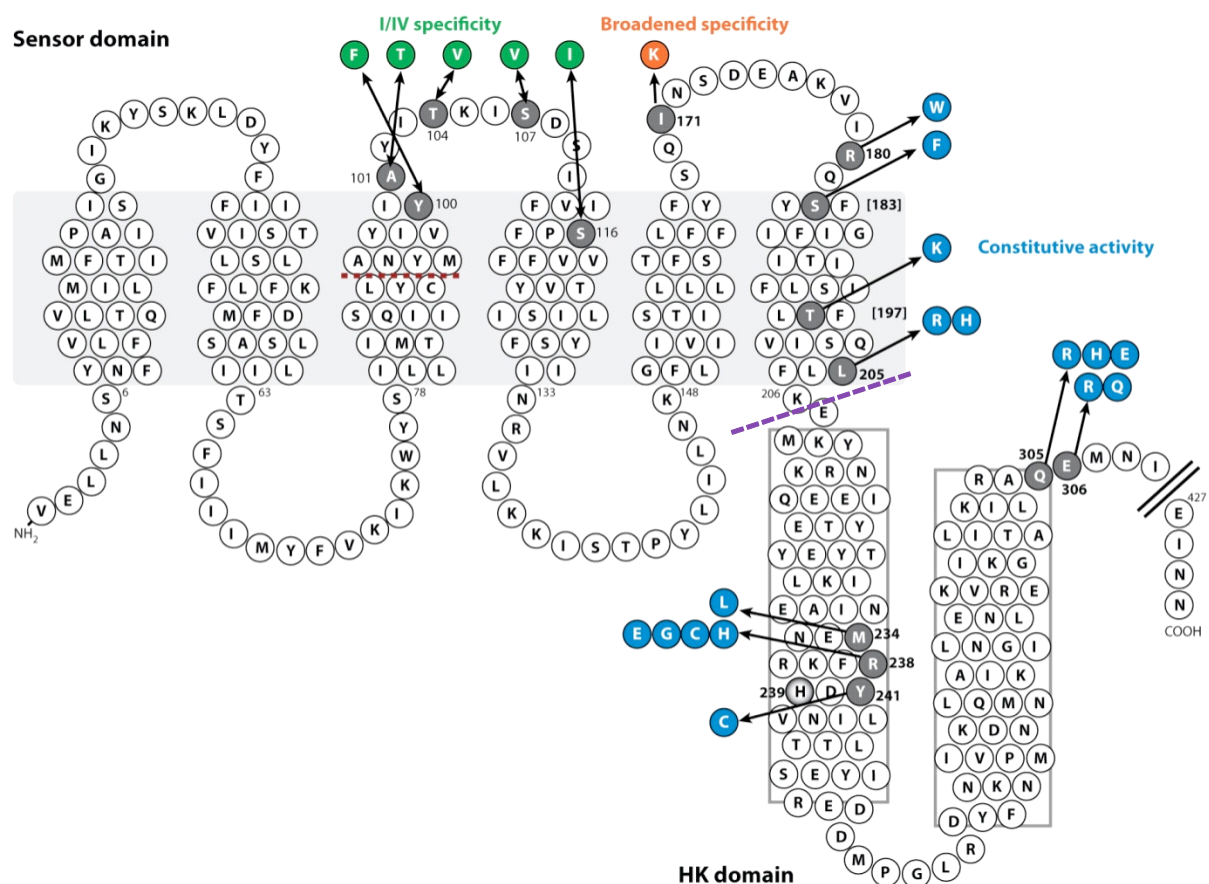


Figure 4: AgrC amino acid sequence and structure.

Diagrammatic representation of the AgrC signal receptor amino acid sequence and structure. Amino acid residues are shown in spheres and the red dotted line indicates the site of intergroup sensor domain chimera construction. AgrC-IV residues replaced to determine group specificity are highlighted in green. The replacement of the I171 residue to K171 to broaden activity is shown in red and amino acid residues resulting in constitutive activity are highlighted in blue. The purple dashed line indicates the start of the HK domain, potentially used for *in vitro* phosphorylation of AgrA. Figure from (Novick and Geisinger 2008).

The sensor domain transmits the ligand-induced activating signal to the C-terminal cytoplasmic signalling domain. This mechanism of signal transmission has barely been revealed. The C-terminal cytoplasmic signalling domain is a highly conserved HK domain. The co-immunoprecipitation and intermolecular complementation of co-expressed mutant receptors with inactivated HK motifs demonstrated that AgrC activation, like many other HKs occurs through dimerisation and trans-autophosphorylation. (George Cisar, Geisinger et al. 2009). This allows the forward flow of the phosphate from a conserved histidine residue to activate the response regulator of the *agr* operon, AgrA. In one study random mutagenesis was used to isolate AgrC mutants that have constitutive activity (Geisinger,

Muir et al. 2009). Four residues were identified in the cytoplasmic domain, M234, R238, Y241 and Q305, with the first three clustered around the dimerisation histidine phosphotransfer (DHp) subdomain.

#### 1.4.4. AgrA

AgrA is the response regulator (RR) of the *agr* TCS (AgrC/AgrA). TCSs are abundant multistep signalling pathways that convey input stimuli into an output response via a phosphotransfer reaction. In a prototypical TCS pathway the phosphoryl group is transferred from the conserved histidine residue in the dimerisation and histidine transfer domain (DHp) of the HK, to a conserved aspartate residue in the RR receiver domain (REC), see Figure 5. This is aided by the catalytic and ATP binding domain (CA) of the HK, which binds ATP and catalyses autophosphorylation of the histidine residue (Laub and Goulian 2007).

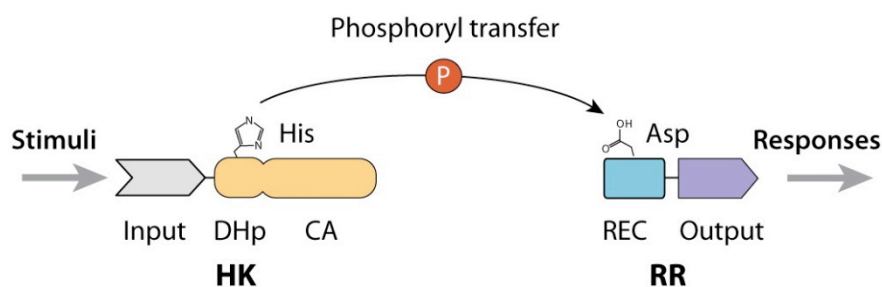


Figure 5: Two-component system (TCS) pathway.

Schematic diagram of a prototypical TCS pathway. The TCS pathway couples the input stimuli and output responses via the conserved phosphoryl transfer from the highly conserved kinase core (DHp and CA) to the receiver domain (REC) of the RR. Adapted from (Gao and Stock 2009).

The RR AgrA consists of an N-terminal CheY-like receiver domain and a C-terminal DNA binding domain that belongs to the LytR-family. The CheY receiver domain consists of a compact globular five stranded parallel  $\beta$ -sheet core, which is flanked by five  $\alpha$  helices (Volz and Matsumura 1991). Within the CheY domain are five conserved active site residues important for phosphorylation and conformational changes. The AgrA receiver domain receives the phosphoryl group from the AgrC sensor kinase at the site of phosphorylation. The site of phosphorylation has been suggested to be a conserved aspartate residue based on its homology with the phosphorylation site of CheY (Lee, Cho et al. 2001).

Phosphorylation of the CheY receiver domain has been shown to cause long-range structural perturbations in NMR analysis (Lowry, Roth et al. 1994; Halkides, Zhu et al. 1998).

The changes induced by phosphorylation alter the molecular surface of the regulatory domain through altering topological and electrostatic features, rather than through large changes in the secondary structure. For example, the NMR structure of the CheY homolog NtrC receiver domain demonstrated that phosphorylation leads to the exposure of a hydrophobic surface involved in signal transmission, due to the slight repositioning of secondary structure elements (Kern, Volkman et al. 1999). However, structural studies of activated response regulators are challenging due to the short lifetime of the phosphorylated state (Nohaile, Kern et al. 1997). This had led to problems in capturing the crystal structure of activated CheY, with the use of structural mutants aiming to capture CheY in a constitutively active state (Zhu, Rebello et al. 1997; Dyer, Quillin et al. 2004). The challenges in capturing the phosphorylated state of a RR have led to the concept that the response regulator exists in a dynamic equilibrium between the (inactive) unphosphorylated and (active) phosphorylated like conformations. This was demonstrated by a study of unphosphorylated CheY in complex with a FlIM peptide, which showed that CheY was present not just in an inactive conformation but also in an intermediate conformation (Dyer and Dahlquist 2006). Consistent with this hypothesis, a study of the crystal structures of OmpR/PhoB subfamily of RRs demonstrated that a subpopulation of the unphosphorylated RR receiver domain exist in an active confirmation (Barbieri, Mack et al. 2010).

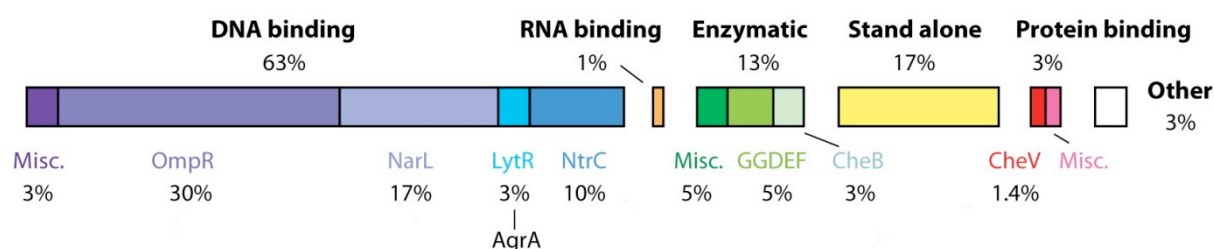


Figure 6: The distribution of RR effector domains.

A horizontal bar representing the distribution of diverse effector domains of prokaryotic RRs. Each RR effector domain is grouped into a RR family according to its functions. Each domain is expressed as % of all prokaryotic RRs. The AgrA RR is presented as an example of the LytR family of RRs. Stand alone effector domains do not contain a receiver domain. Adapted from (Gao and Stock 2009).

The LytTR DNA binding domain, of the LytR family, is relatively uncharacterised and rare, being present in only 3% of response regulators analysed (Galperin 2006), as displayed in Figure 6. The LytTR domain shows no similarity to the winged-helix or helix-turn-helix (HTH) domains of other previously characterised RRs and is therefore a novel type of DNA-binding domain (Nikolskaya and Galperin 2002), see Figure 8. Other LytTR containing response regulators that regulate virulence factor and toxin production include AlgR from *Pseudomonas aeruginosa*, which modulates exopolysaccharide alginate production in chronic pneumonia (Lizewski, Lundberg et al. 2002) and CdtR from *Clostridium difficile*, which regulates binary toxin production (Carter, Lyras et al. 2007). The LytTR DNA binding domain is the effector domain of AgrA that binds to target sites to regulate transcription initiation once in an active conformation.

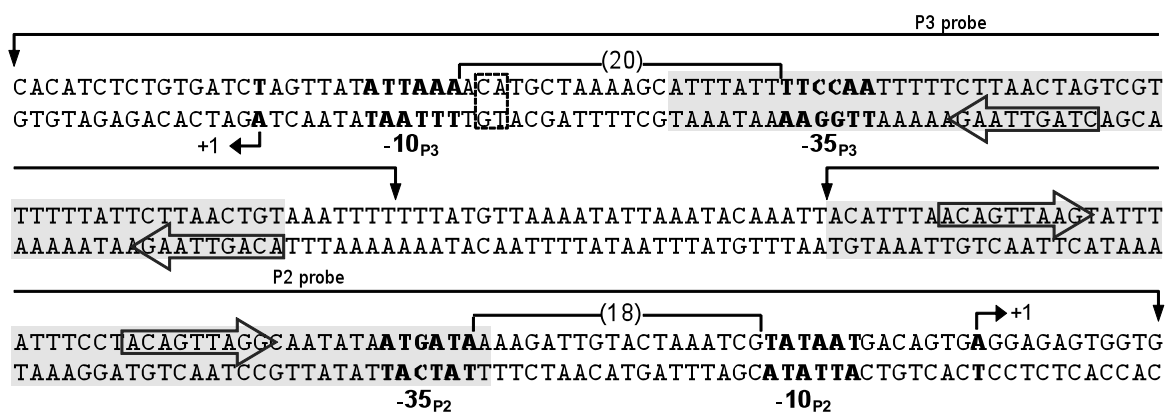


Figure 7: AgrA binding sites at the *agr* intergenic region.

The sequence of the *agr* P2 and P3 promoters from *S. aureus* NCTC 8325 are shown with the consensus -10 and -35 hexamers highlighted in bold. The region protected by AgrA in DNase I protection assays are shaded in grey and the direct repeats bound by AgrA are highlighted by arrows. The promoter consensus spacer length and the transcription start site (+1) are labelled. The extended -10 motif at the P3 promoter is boxed with a dashed line. The sequences for the *agr* P2 and P3 probes are indicated (used in chapters 3-7).

The AgrA response regulator recognises a pair of direct repeats separated by 12-13 bp at both the *agr* P2 and P3 promoters, see Figure 7 (Nikolskaya and Galperin 2002). AgrA binds to the novel elements, with the consensus sequence [TA][AC][CA]GTTN[AG][TG], at the P2 promoter with high affinity, however with a lower affinity to the P3 promoter (Koenig, Ray et al. 2004). The region protected by bound AgrA, determined in DNase I protection assays, overlaps the -35 elements of both the P2 and P3 promoters (Koenig, Ray et al. 2004), indicating a possible mode of transcriptional regulation where AgrA interacts with the RNA

polymerase (RNAP) enzyme to help form the closed promoter complex (RPc) and/or the open promoter complex (RPo) (see section 1.5.5 and 1.5.6.4). Electrophoretic mobility shift assays (EMSA) determined that phosphorylation by the small phosphodonor, acetyl phosphate, increased the affinity of AgrA to both the P2 and P3 promoters, however 10-fold more at P2 in comparison to P3 (Koenig, Ray et al. 2004).

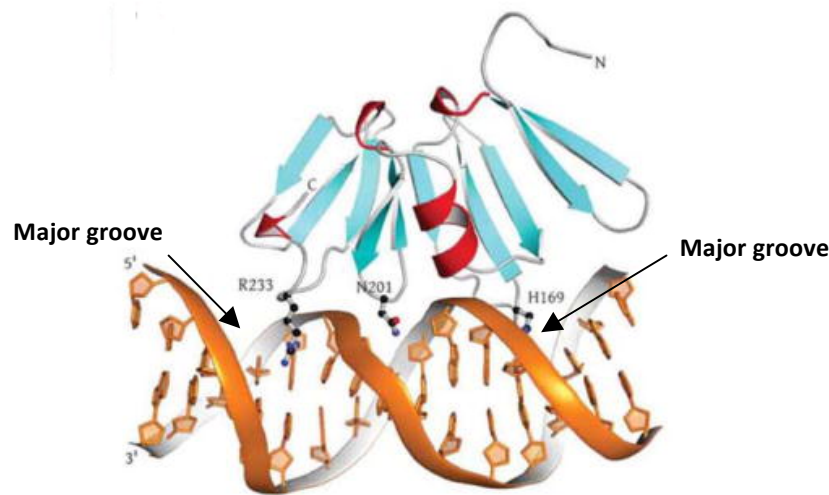


Figure 8: Structure of the AgrA<sub>C</sub> response regulator in complex with DNA.

A stereo ribbon representation of the AgrA<sub>C</sub> LytTR domain in complex with DNA. Helices are coloured in red, loop regions in grey and strands in cyan. The major grooves of the DNA are displayed using an arrow and the residues R233, N201 and H169 making base-specific contact with DNA are displayed in a ball and stick representation. Figure from (Sidote, Barbieri et al. 2008).

The structure of the AgrA LytTR domain (AgrA<sub>C</sub>) has been resolved in complex with DNA (Sidote, Barbieri et al. 2008). The ~105 residue AgrA LytTR domain consists of ten strands arranged in three antiparallel  $\beta$  sheets and a two-turn  $\alpha$  helix. The structure shows that AgrA<sub>C</sub> binds to a single face of the DNA (Figure 8). Three residues, H169, R233 and N201 that protrude from the  $\beta$  sheets make specific contacts with the DNA at 2 adjacent major grooves and the intervening minor groove of the DNA. This binding event leads to the distortion and bending of the DNA, by  $\sim 38^\circ$  as it conforms to the AgrA protein surface. Mutagenesis of the contacting residues H169 and R233 determined that they are both essential for the DNA binding of AgrA as alanine substitution leads to a 40-90 fold increase in  $K_d$ . Alanine substitution of the residue contacting the minor groove, N201, showed that this residue was not essential but its substitution still led to a 10 fold increase in  $K_d$  (Sidote,

Barbieri et al. 2008). Despite these studies the full structure of AgrA containing the phosphorylation site is unresolved. The effect of phosphorylation on the conformation of the LytTR binding domain and DNA remains unknown.

Until recently, the modulation of target genes by the *agr* operon was thought to only occur in an RNAIII dependent mechanism. However a secondary target for AgrA activation has been uncovered. The phenol-soluble modulins (PSMs) contribute to *S. aureus* immune evasion through their leukocidal activity, and studies have demonstrated that AgrA regulates *psm* gene transcription by direct binding to the *psm* promoter regions (Queck, Jameson-Lee et al. 2008). Expression of AgrA from an inducible plasmid in an *agr*-negative *S. aureus* strain demonstrated an AgrA, not RNAIII dependent activation of the PSM genes. This demonstrated an RNAIII independent activation of target genes by AgrA and also highlighted the possibility of further RNAIII independent targets for AgrA.

#### **1.4.5. RNAIII**

The RNAIII regulatory RNA is a 514 nucleotide transcript initiated from the *agr* P3 promoter, which also incorporates the  $\delta$ -haemolysin gene (*hld*) (Janzon and Arvidson 1990; Novick and Geisinger 2008). RNAIII was discovered as the regulatory effector molecule of the *agr* operon in 1993 and was shown to regulate the expression of a number of genes encoding secreted exoproteins and surface associated proteins (Novick, Ross et al. 1993). The secondary structure of RNAIII revealed a complex structure containing 14 hairpin structures (Benito, Kolb et al. 2000).  $\delta$ -haemolysin expression is regulated through conformational changes in the RNAIII secondary structure, allowing translation of the *hld* mRNA encoded in RNAIII (Balaban and Novick 1995). The RNAIII secondary structure includes sequences complementary to the RNA of several pleiotropic regulators and exoproteins. These include *rot* mRNA and the *hla* mRNA leader, identifying the primary function of RNAIII as an antisense RNA involved in, for example, the inhibitory translational regulation of the pleiotropic regulator Rot and the positive regulation of alpha-toxin (*hla*) translation (Morfeldt, Taylor et al. 1995; Geisinger, Adhikari et al. 2006). Recent studies have also recognised the extracellular adherence protein (*map*) as a further translation regulation target for RNAIII as an antisense RNA (Liu, Mu et al. 2011). RNAIII also acts at the transcriptional level, through the repression of the protein A gene (*spa*) through its 3' end

(Novick, Ross et al. 1993; Benito, Kolb et al. 2000). Alignments of RNAlII with other staphylococcal species indicate that the secondary structure of RNAlII, unlike its sequence, is well conserved among several staphylococcal species (Benito, Kolb et al. 2000).

## **1.5. Regulation of transcription initiation in bacteria**

### **1.5.1. Transcription**

The RNA polymerase (RNAP) enzyme is widely regarded as “the engine of gene expression” (Helmann 2009). RNAP is a molecular machine that is essential for the first step in gene expression, transcription. In transcription, RNAP synthesises RNA copies of DNA templates complementary to specific regions of the DNA template *de novo*. This is fuelled by the use of nucleoside ribotriphosphate (NTP) substrates leading to synthesis of RNA at 15-80 nucleotides per second (Wang, Schnitzer et al. 1998; Epshtein and Nudler 2003; Shilatifard, Conaway et al. 2003). Transcription can be split into 3 general phases; transcription initiation, processive transcription elongation and transcription termination. The first phase, transcription initiation, involves the recognition and binding of RNAP to the promoter. The binding of RNAP to promoter DNA leads to the opening of the DNA, where DNA strands are separated. In the second phase, processive transcription elongation, RNA formation is catalysed through the addition of nucleotides to a polyribonucleotide chain in an  $Mg^{2+}$  dependent reaction in the RNAP active centre. One DNA strand acts as a template for the nucleotide linkage to form short abortive products (2-10 nt) (Hsu, Vo et al. 2003) or larger elongated products. The final phase, termed transcription termination, involves the termination of transcription elongation at specific terminator sequences. Each of these phases of transcription are targets for regulation.

### **1.5.2. RNAP**

The DNA-dependent RNAPs are found as both single-subunit RNAP and multisubunit RNAP. Multisubunit RNAP are evolutionary conserved, both through sequence and structure, in eubacteria, archaea and eukarya (Lane and Darst 2010; Opalka, Brown et al. 2010). Both bacteria and archaea contain a single multisubunit RNAP, with bacterial RNAP (bRNAP) containing 5 subunits and archaea containing a further 6 subunits, as displayed in Figure 9. In contrast, eukaryotes contain three multisubunit RNAPs (eRNAP), named Pol I, Pol II and

Pol III. eRNAP Pol I is responsible for the synthesis of ribosomal RNA (rRNA), Pol II for pre-messenger RNA (mRNA) and Pol III for transfer RNA (tRNA) and other small nuclear RNAs (Ebright 2000). The eRNAP are comprised of ten common subunits with 4, 2 and 5 additional subunits for Pol I, Pol II and Pol III respectively (Figure 9).

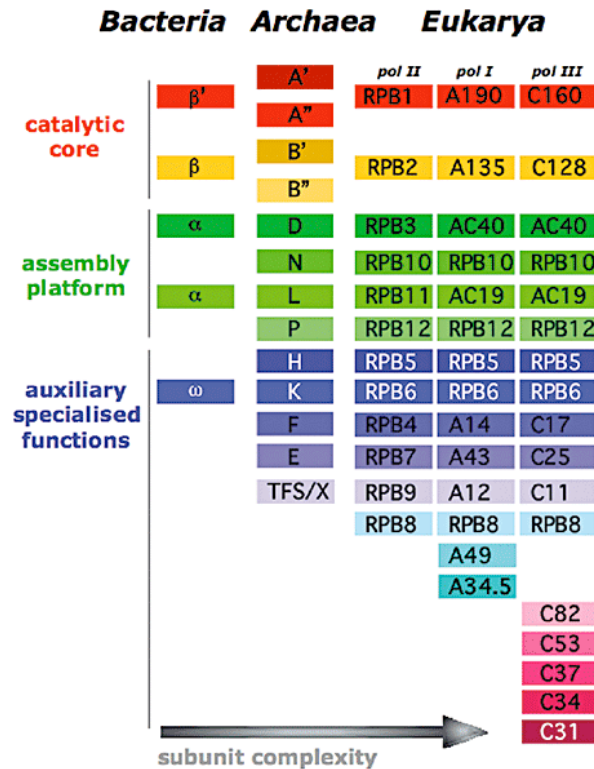


Figure 9: RNAP subunit composition in multisubunit RNAP from bacteria, archaea and eukarya.

Diagrammatic representation of the subunit composition of bacterial RNAP (bRNAP), archaeal RNAP (aRNAP) and eukaryotic RNAP (eRNAP). Homologous subunits are colour coded, with subunits organised due to function not size. The subunit complexity increases towards the right. Figure from (Werner 2007).

The single subunit RNAPs are found in chloroplasts, mitochondria and bacteriophage and share many of the biochemical properties of the multisubunit RNAP. However their sequences are unrelated to the multisubunit RNAP and the architecture of single subunit RNAPs closely resembles that of DNA polymerases (Cheetham and Steitz 2000). Comparison of structures of both single and multisubunit RNAP has revealed the location of some nucleic acids and amino acids to be equivalent, particularly in the active centre (Cramer 2002).

### 1.5.3. Bacterial RNAP

The multisubunit bacterial RNAP (bRNAP) is a large enzyme (~400 kDa) containing five subunits that together form the catalytic core (E);  $\beta'$  (~150 kDa),  $\beta$  (150 kDa),  $\alpha_2$  (2  $\alpha$  subunits, 40 kDa each), and  $\omega$  (10 kDa) (Darst 2001). The two  $\alpha$  subunits form a dimer through the contact of their N-terminal domains ( $\alpha$ NTD) and form the scaffold for the assembly of the  $\beta$  and  $\beta'$  catalytic subunits (Zhang and Darst 1998). The  $\alpha$  C-terminal domains ( $\alpha$ CTD) are involved in the recognition of upstream elements of promoter DNA sequences (Ross, Ernst et al. 2001). The  $\omega$  subunit, although not essential for viability under standard laboratory conditions (Gentry, Xiao et al. 1991), aids the assembly of the  $\beta'$  to the  $\beta \alpha_2$  sub-assembly through the “latching” of the N- and C-terminal of the  $\beta'$  to the  $\beta \alpha_2$  intermediate (Minakhin, Bhagat et al. 2001).

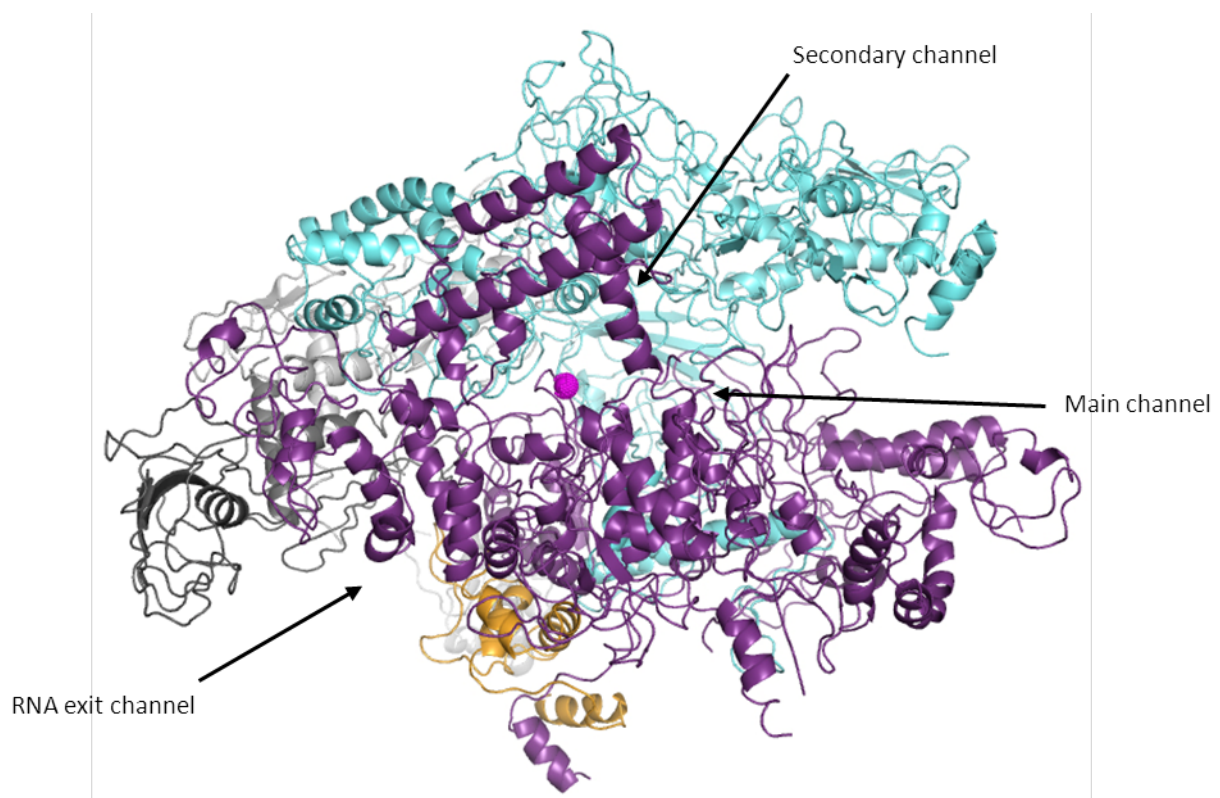


Figure 10: Structure of *Thermophilus aquaticus* RNAP.

Structural model of *Thermophilus aquaticus* RNAP (bRNAP) from (Zhang, Campbell et al. 1999). The subunits are coloured as follows:  $\beta$  in light blue,  $\beta'$  in purple,  $\alpha$  in light grey,  $\omega$  in orange and the other  $\alpha$  in dark grey. The active site metal ion is labelled by a pink sphere. The main channel, secondary channel and RNA exit channel are labelled by arrows. Figure generated using PDB ID code 1HQM using PyMOL version 1.4.

The architecture of bRNAP resembles a crab claw, as conserved in bacteria, archaea and eukarya and displayed in the structure of *Thermophilus aquaticus* (Zhang, Campbell et al. 1999) (Figure 10). The  $\beta$  subunit forms one pincer and the  $\beta'$  subunit forms the other. Although core RNAP (E) is sufficient for the latter stages of transcription, elongation and termination, it requires a sixth dissociable subunit ( $\sigma$ ) to direct the RNAP to promoter DNA. The  $\sigma$  subunit conformation extends upon binding to the core RNAP and its domains primarily contact one face of the RNAP forming the RNAP holoenzyme ( $E\sigma$ ) (Murakami, Masuda et al. 2002).

#### 1.5.4. $\sigma$ factors

There are two families of  $\sigma$  factors,  $\sigma^{70}$  and  $\sigma^{54}$ . The  $\sigma^{70}$  family contain the majority of bacterial  $\sigma$  factors and most are structurally related to the primary housekeeping  $\sigma$  factor  $\sigma^{70}$  in *E. coli*. The  $\sigma^{54}$  family are structurally distinct from the  $\sigma^{70}$  family, whilst maintaining similar function through the recognition of a specialised set of genes involved in specific cellular functions and stress response.

The  $\sigma^{70}$  family of  $\sigma$  factors bind to sites located near to the -35 and -10 promoter positions (numbered in comparison to transcriptional start site +1). For example, the *E. coli*  $\sigma$  factor  $\sigma^{70}$  recognises conserved sequences of 5-6 base-pairs (bp) in length called the -35 (TTGACA) and -10 (TATAAT) promoter elements (Hawley and McClure 1983; Harley and Reynolds 1987). The  $\sigma^{70}$  family also contain 4 domains, named  $\sigma_1$ ,  $\sigma_2$ ,  $\sigma_3$  and  $\sigma_4$ , which are each broken down into subdomains based on promoter interactions (Severinova, Severinov et al. 1996). The domains  $\sigma_2$ ,  $\sigma_3$  and  $\sigma_4$  all contact the core RNAP (E) through an 8000Å interface to form  $E\sigma^{70}$ , with a 45Å linker between  $\sigma$  domains  $\sigma_3$  and  $\sigma_4$  (Campbell, Muzzin et al. 2002). The -10 and -35 conserved promoter elements of  $\sigma^{70}$  dependent promoters are recognised and make contact with  $\sigma$  regions  $\sigma_{2.4}$  and  $\sigma_{4.2}$  respectively (Murakami, Masuda et al. 2002).  $\sigma_{3.0}$  is responsible for the recognition of the TG motif comprising the extended -10 element found at some promoters (Barne, Bown et al. 1997). The  $\sigma^{70}$  family can be broadly divided into 4 phylogenetic groups based on gene structure and function (Table 1) (Lonetto, Gribskov et al. 1992; Paget and Helmann 2003).

| $\sigma^{70}$ family phylogenetic groups | Function                                                                                                                   |
|------------------------------------------|----------------------------------------------------------------------------------------------------------------------------|
| <b>Group 1</b>                           | Essential primary $\sigma$ factors.                                                                                        |
| <b>Group 2</b>                           | Dispensable for bacterial cell growth, closely related to 1.                                                               |
| <b>Group 3</b>                           | Activate regulons in response to specific signals (E.g. heat shock), distantly related to 1.                               |
| <b>Group 4</b>                           | ECF family (Extracytoplasmic function), respond to extracytoplasmic signals, highly divergent from 1, numerically largest. |

Table 1: The function of members of the  $\sigma^{70}$  family of  $\sigma$  factors.

Unlike with  $E\sigma^{70}$ ,  $E\sigma^{54}$  recognises the conserved -24 and -12 promoter elements (numbered in comparison to the transcriptional start site (+1)) and initiate contact with the DNA to form a closed promoter complex (RPC) (Wigneshweraraj, Bose et al. 2008). Furthermore, unlike  $E\sigma^{70}$ , the  $E\sigma^{54}$  RPC does not spontaneously form the open promoter complex (RPO) (Kustu, Santero et al. 1989; Austin and Dixon 1992). The  $E\sigma^{54}$  RPC requires energy through ATP hydrolysis and the binding of a class of activator proteins to enhancer-like sequences at the promoter to form the transcriptionally competent open complex (RPO).

*S. aureus* has four characterised  $\sigma$  factors,  $\sigma^A$ ,  $\sigma^B$ ,  $\sigma^H$  and  $\sigma^S$ , which are all members of the  $\sigma^{70}$  family of  $\sigma$  factors.  $\sigma^A$  is the primary  $\sigma$  factor of *S. aureus*, which is indispensable for *S. aureus* growth (Deora and Misra 1996). *In vivo*  $\sigma^A$  is an unstable protein. In solution, however, it is stabilised by assembly with the core RNAP to form  $E\sigma^A$  (Mondal, Ganguly et al. 2010).

*S. aureus*  $\sigma^B$  is an alternative  $\sigma$  factor, with homology to *Bacillus subtilis*  $\sigma^B$  (Deora, Tseng et al. 1997).  $\sigma^B$  directs RNAP to promoters involved in the regulation of virulence factor production and the stress response. These include the direct binding of  $\sigma^B$  to the *sar* P3 promoter, regulation of capsular polysaccharides involved in protection against opsonophagocytic killing, and the prevention of the expression of many extracellular proteins including proteases lipases and toxins (Deora, Tseng et al. 1997; Ziebandt, Becher et al. 2004; Meier, Goerke et al. 2007).

$\sigma^H$  is also an alternative  $\sigma$  factor of *S. aureus*, which only has 40-90% sequence identity to its homolog  $\sigma^H$  in *B. subtilis*.  $\sigma^H$  was initially shown to be involved in the control of genetic competence of *S. aureus* through directing the core RNAP to the *comG* and *comE* operons (Morikawa, Inose et al. 2003). More recent studies have determined a further role for  $\sigma^H$  through the regulation of the *int* integrase gene from a staphylococcal temperate phage (Tao, Wu et al. 2010). The published results indicate that  $\sigma^H$ , through the transcription regulation of phage integrase could stabilise the lysogeny in the host cell and further influence the prophage life cycle, with potential effects on phage related virulence in *S. aureus*.

Finally, the alternative  $\sigma$  factor of *S. aureus*,  $\sigma^S$ , was recently discovered and characterised as part of the ECF family of  $\sigma$  factors involved in the stress and pathogenic response (Shaw, Lindholm et al. 2008). Comparative studies of wild-type (WT) *S. aureus* SH1000 with a  $\sigma^S$  mutant revealed that  $\sigma^S$  plays an important role in survival during starvation, elevated growth temperatures or under Triton X-100 lysis conditions. The role of  $\sigma^S$  in *S. aureus* pathogenesis was revealed using a murine arthritis model in which a  $\sigma^S$  mutant strain slowed the development of arthritis and increased the survival rate of the mice when compared to the WT *S. aureus* strain.

#### **1.5.5. Transcription initiation**

The process of transcription initiation at  $E\sigma^{70}$  promoters starts with the binding of the  $E\sigma^{70}$  to the promoter DNA to form the initial RPc, with the -35 and -10 hexamers in contact with  $\sigma^{70}$ , shown in Figure 11 and Figure 12. The -35 and -10 hexamers are optimally separated by a  $17\pm 1$  bp spacer length for optimum promoter activity (Harley and Reynolds 1987). Some promoters also contain a ~20 bp A/T-rich “UP element”, upstream of the -35 hexamer, which is recognised by the flexible  $\alpha$ -subunit C-terminal domain ( $\alpha$ CTD) (Gourse, Ross et al. 2000). The  $E\sigma^{70}$  footprint in the RPc stretches from around the -55 to the +1 site on the promoter and remains double stranded (Kovacic 1987).

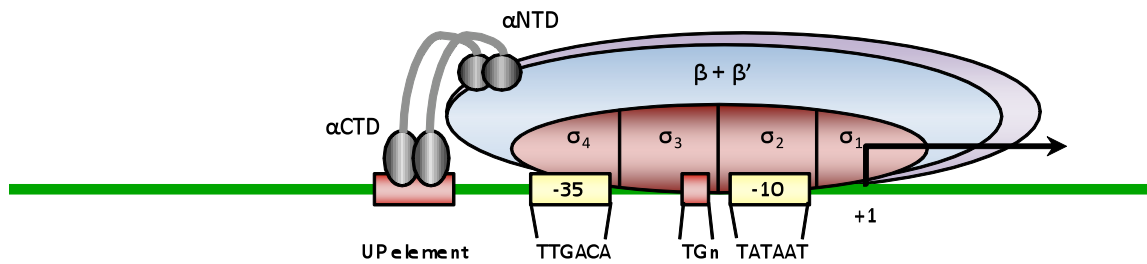


Figure 11: Illustration of  $E\sigma^{70}$  DNA interactions.

A cartoon illustration of a model of  $E\sigma^{70}$  docking to a  $\sigma^{70}$  consensus promoter. The  $E\sigma^{70}$  is shown with the  $\beta$  and  $\beta'$  subunits coloured light blue and purple, respectively. The  $\alpha$ NTDs are grey ovals attached to the spherical  $\alpha$ CTDs also coloured grey. The  $\sigma^{70}$  subunit is coloured red and is sitting on the DNA strands shown in green. The +1 and UP element (red box) are highlighted and the consensus sequences for the -10 (TATAAT) in yellow, extended -10 (TGn) in red and -35 (TTGACA) in yellow are shown. Based on (Browning and Busby 2004).

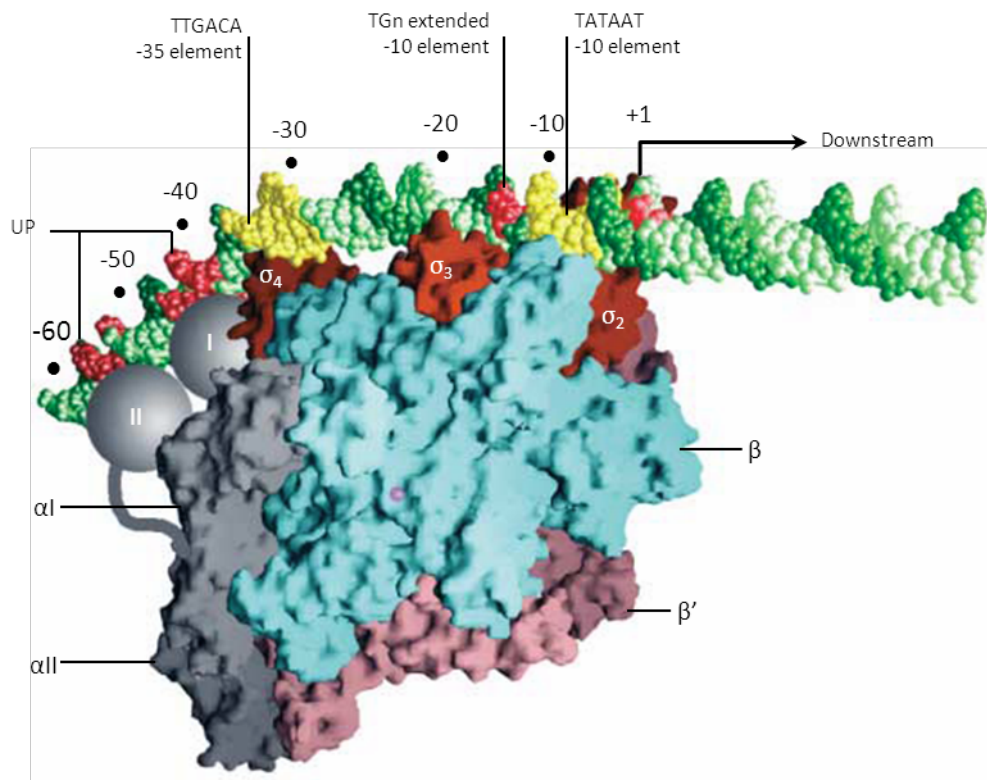


Figure 12: Structure of  $E\sigma^{70}$  and promoter interactions during the RPC.

A model of the initial docking of the  $E\sigma^{70}$  to a promoter, based on crystallographic studies. The  $E\sigma^{70}$  is displayed with the  $\beta$  and  $\beta'$  subunits coloured in light blue and pink, respectively. The  $\alpha$ NTDs are coloured in grey with the  $\alpha$ CTDs represented by grey spheres labelled I and II. The  $\sigma^{70}$  domains are coloured in red and labelled. The  $Mg^{2+}$  ion of the active site is displayed in magenta. The DNA strands are shown in green, with the -35 and -10 promoter elements highlighted in yellow and the extended -10 and the UP elements in red. The consensus sequences for the -35 (TTGACA), extended -10 (TGn) and -10 (TATAAT) promoter elements are displayed. Figure from (Browning and Busby 2004).

The unstable R<sub>Pc</sub> isomerises via several distinct intermediates to form the more stable and transcriptionally active R<sub>Po</sub>. This leads to the opening of the DNA to form an “initiation bubble” of ~13 bp between the -11 and +3 sites just beyond the transcription start site (+1), placing the +1 site of the template strand (T) of the DNA in the active site (Gries, Kontur et al. 2010). The non-template (NT) strand is held in a groove between the  $\beta$  and  $\sigma$  subunits with interactions between  $\sigma_{1.2}$  and the -6 and -5 NT positions (Feklistov, Barinova et al. 2006). The DNA strands then re-anneal at the +4 site (Sasse-Dwight and Gralla 1989). In the R<sub>Po</sub> the double stranded DNA displaces  $\sigma_{1.1}$  from the main DNA channel and the E $\sigma^{70}$  footprint is extended to around the +20 DNA position (Spassky, Kirkegaard et al. 1985; Kontur, Saecker et al. 2006).

Transcription is driven forward by the incorporation of the initiating nucleoside triphosphate NTP (iNTP) to complement the +1 site of the DNA template strand, where covalent bonds are formed between the  $\alpha$  phosphate of the incoming NTP and the 3' OH end of the nascent RNA (Hsu, Vo et al. 2003). This is followed by the addition of further NTPs to either form an abortive transcript or go through the process of elongation (von Hippel 1998). The nascent RNA exits via a channel (RNA exit channel) formed by the  $\beta$  flap,  $\beta'$  zipper,  $\beta'$  Zn finger and  $\beta'$  lid domains (Nudler 2009). For elongation to occur several contacts need to be broken between the core RNAP (E) and  $\sigma$  and between the E $\sigma^{70}$  and the promoter. Energy stored in “scrunching” of promoter DNA, through the intake of further DNA for RNA synthesis, is released when the nascent RNA reaches 11 nucleotides in length (Kapanidis, Margeat et al. 2006; Revyakin, Liu et al. 2006). Breaking the contacts with the promoter allows the RNAP to enter a productive elongation phase, moving down the template and eventually losing the sigma factor (Mooney, Darst et al. 2005).

#### **1.5.6. Transcriptional regulation**

Transcription is a highly regulated process to allow the bacterium to respond quickly to changing requirements and environmental conditions. Transcription is regulated at every step with transcription initiation the first regulatory step (Helmann 2009). The recognition and occupancy of promoters by RNAP is regulated primarily by the promoter sequence and  $\sigma$  factor availability and can also be affected by the folding and structure of the bacterial

chromosome (Azam and Ishihama 1999; Ishihama 2000; Salgado, Santos-Zavaleta et al. 2001). Many other transcription regulators involved at differing steps of transcription, summarised below, play an important role in transcription regulation. These include anti- $\sigma$  factors, small ligands, nucleotide concentration and many transcription factors.

#### **1.5.6.1. Anti- $\sigma$ factors**

One of the simplest mechanisms to modulate occupancy of promoters is by controlling the levels and activities of  $\sigma$  factors within the bacterium. With a spectrum of  $\sigma$  factors found in most bacteria a wide array of mechanisms are used to control sigma factor activity. These can include the use of alternative start codons for  $\sigma$ , signal responsive proteolytic degradation of  $\sigma$  and the processing of sequestered  $\sigma$  factors into an inactive state (Kim, Makino et al. 1995; Kroos, Zhang et al. 1999; Hengge 2009). However, one primary mechanism to prevent the association of specific  $\sigma$  with core RNAP, to form  $E\sigma$ , is by sequestration by an anti- $\sigma$  factor.

One of the best studied examples is the regulation of the ECF  $\sigma$  factor  $\sigma^E$ , involved in the *E. coli* envelope stress response, by the anti- $\sigma$  factor RseA (Ades 2004). The inner membrane protein, RseA, binds to  $\sigma^E$  via its N-terminal cytoplasmic domain, with a 50-100 fold greater affinity than core RNAP, to block  $\sigma^E$  activity. The co-crystal structure of the N-terminal RseA and  $\sigma^E$  indicates that  $\sigma^E$  is sterically occluded from binding to core sites in RNAP, to form  $E\sigma^E$ , by RseA (Campbell, Tupy et al. 2003). To release the negative repression of RseA from  $\sigma^E$ , RseA is degraded by the inner membrane proteases DegS and YaeL in response to the stress response (Ades, Connolly et al. 1999; Ades, Grigorova et al. 2003).

#### **1.5.6.2. Small ligands**

Transcription can be regulated by a number of mechanisms including by small ligands, which allow  $E\sigma$  to adapt rapidly to changing environment conditions. One good example of this is the unusual nucleotides guanosine 5'-diphosphate-3'-diphosphate (ppGpp) and its precursor guanosine 5'-triphosphate-3'-diphosphate (pppGpp), which are collectively referred to as ppGpp. The “alarmone” ppGpp is widely distributed in bacteria and is involved in the global wide-scale control of transcription, with the effect characterised in *B. subtilis* and *E. coli* (Eymann, Homuth et al. 2002; Durfee, Hansen et al. 2008). This occurs

when bacterial cells rapidly growing in a rich environment are exposed to an abrupt amino acid starvation. In the ensuing coordinated response, termed the stringent response (to down-regulate genes involved in rapid growth and up-regulate those involved in amino acid biosynthetic operons) ppGpp is synthesised by the ribosome associated synthetase RelA (Chatterji and Ojha 2001). ppGpp, along with a cofactor DksA, negatively regulate promoters primarily involved in translation, such as the rRNA operons through reducing the half-life of the RPo formed at the promoters (Paul, Barker et al. 2004). As these RPo are already short lived, the ppGpp and DksA move the equilibrium even closer to the dissociation of the RPo therefore reducing transcription initiation (Paul, Berkmen et al. 2005).

#### **1.5.6.3. Transcription factors**

In order for bacterial gene expression to respond to environmental signals, transcription is regulated by many transcription factors. This includes those that bind to promoters to directly target promoters and those that are non-DNA binding. This step also needs regulating, which is achieved through a number of mechanisms that are used to regulate transcription factors. These include the modulation of transcription factors by small ligands, for example by the reduced affinity of the Lac repressor through binding by allolactose (Jobe and Bourgeois 1972). Another includes the covalent modification of transcription factors, such as the phosphorylation of the RR NarL (Walker and DeMoss 1993). A third mechanism involves the sequestration of a transcription factor by another regulatory protein, for example the direct inhibition of  $\sigma^F$  and  $\sigma^G$  in *Bacillus subtilis* by the anti-sigma factor SpoIIAB (Hughes and Mathee 1998). Finally another mechanism involves the regulation of a transcription factor by its cellular concentration, in the case of SoxS where its concentration is controlled by SoxR, which is regulated by oxidising ligands (Nunoshiba, Hidalgo et al. 1992).

Transcription initiation is regulated by a number of transcription regulators that directly contact  $E\sigma$ , but do not directly contact the DNA. One good example includes the ppGpp cofactor DksA, which has been shown to bind to the RNAP secondary channel (Perederina, Svetlov et al. 2004), a channel for incoming NTPs to enter the RNAP active site. DksA enhances the negative regulatory effect of ppGpp by changing the kinetic properties of

promoter complexes formed at promoters that are important in rapid growth during the stringent response (Perederina, Svetlov et al. 2004), see section 1.5.6.2. However, how DksA functions with ppGpp to affect the stability of the RPo formed at these promoters remains unclear. DksA and ppGpp also work in tandem to directly positively regulate transcription from promoters involved in amino acid biosynthesis operons, pathogenicity islands, and the global regulatory factors (Paul, Berkmen et al. 2005; Nakanishi, Abe et al. 2006; Costanzo, Nicoloff et al. 2008). The structure of DksA is reminiscent of the bacterial transcription elongation factors GreA and GreB in *E. coli*, which are involved in facilitating promoter escape and the release from promoter proximal pauses as reviewed in Perederina, Svetlov et al. 2004. DksA, like the Gre factors that also bind the RNAP secondary channel, has a globular domain, a coiled coil and acidic tip. A recent study by Lee et al. (2011) has provided insight into the mechanism of transcription regulation by DksA through the discovery that a single acidic residue in the coiled-coil tip confers DksA-like activity on promoter complexes. The addition of GreA, with a single substitution from glutamic acid to aspartic acid at its coiled-coil tip, in transcription assays showed that GreA gained DksA-like activities, thus demonstrating the importance of the coiled-coil in DksA function (Lee, Lennon et al. 2011).

#### **1.5.6.4. DNA binding Transcription factors**

Unlike non-DNA binding transcription factors, those which bind DNA regulate gene transcription generally by three simple mechanisms named class I, class II and class III activation. In class I activation the  $E\sigma^{70}$  is recruited to the promoter by the direct interaction with the transcription factor (bound upstream of the promoter -35 element, typically between -61 and -91) via the  $\alpha$ CTD of the RNAP, as shown in Figure 14A. The flexibility of the linker between the  $\alpha$ CTD and  $\alpha$ NTD allows the transcription factor to bind at a number of sites upstream of the promoter -35 element and still contact the  $E\sigma^{70}$  (Browning and Busby 2004).

The first step in transcription initiation, where  $E\sigma^{70}$  binds to the promoter DNA to form the RPo, is characterised by the reversible equilibrium binding constant ( $K_b$ ) (Figure 13). As class I activators recruit  $E\sigma^{70}$  to form the RPo it has been established that class I activation mostly affects  $K_b$  (Gaston, Bell et al. 1990). One well characterised example of class I activation is

the activation of the *lac* promoter by the cyclic AMP receptor protein (CRP), where CRP binding is centred at position -61.5 (Ebright 1993). Binding of CRP at the *lac* promoter increases affinity of the  $E\sigma^{70}$  to the promoter and therefore increases the  $K_b$  for the formation of the R<sub>Pc</sub> at the promoter (Malan, Kolb et al. 1984). This results in increased transcription initiation at the *lac* promoter. Interestingly, in class I activation of CRP dependent promoters the CRP binding site can range from -61.5 to -92.5 as long as the CRP binding site is on the same face of the DNA helix as the  $E\sigma^{70}$  (Busby and Ebright 1999).

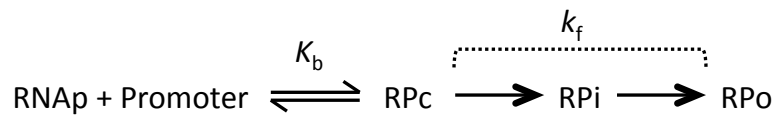


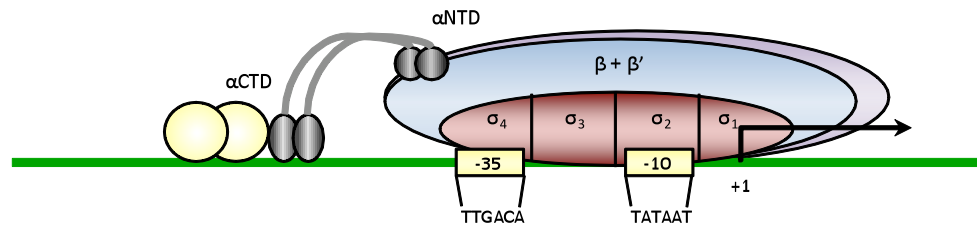
Figure 13: The steps of bacterial transcription initiation.

The first step of bacterial initiation involves the binding of the RNAP to the promoter DNA. This leads to R<sub>Pc</sub> formation and is defined by the equilibrium binding constant ( $K_b$ ). The next step involves the isomerisation of the R<sub>Pc</sub> to the R<sub>Po</sub> via several intermediate RNAP-promoter complexes (R<sub>Pi</sub>) and is defined by the rate constant ( $k_f$ ).

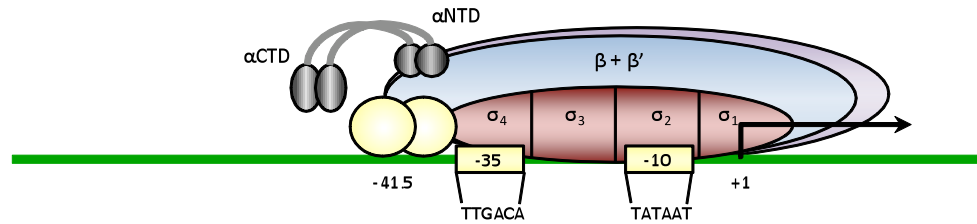
Class II activation also involves the recruitment of the  $E\sigma^{70}$  by a transcription factor to facilitate transcription initiation by increasing  $K_b$ . However class II activators also affect the second step of transcription initiation where the R<sub>Pc</sub> isomerises to the R<sub>Po</sub> (Busby and Ebright 1997). This largely irreversible step is measured by the rate constant ( $k_f$ ) (Figure 13). In class II activation the transcription factor binds to a target overlapping the promoter -35 element, near position -42, and contacts the  $E\sigma^{70}$  generally at  $\sigma_4$ , as shown in Figure 14B (Dove, Darst et al. 2003). The transcription factor can also contact other elements of the  $E\sigma^{70}$  in some cases, including the  $\alpha$ NTD or  $\alpha$ CTD of the  $E\sigma^{70}$ , and this contributes to the lack of flexibility in class II activator binding sites. For example the well-studied CRP transcription factor also activates by a class II mechanism at the *galP1* promoter (Busby and Ebright 1997). Here the CRP dimer binds centred on position -41.5 of the promoter and contacts one  $\alpha$ CTD of  $E\sigma^{70}$ , bound upstream of the CRP binding site, at residue 287 of  $\alpha$ CTD. This contact increases the  $K_b$  for R<sub>Pc</sub> formation at the promoter (Niu, Kim et al. 1996). Whereas, CRP also contacts one  $\alpha$ NTD of  $E\sigma^{70}$  at residues 162-165 of  $\alpha$ NTD resulting in an increase in the  $k_f$  of the isomerisation of the R<sub>Pc</sub> to the R<sub>Po</sub> (Busby and Ebright 1997). Therefore CRP can affect both the  $K_b$  and the  $k_f$ .

The third simple mechanism of activation, class III activation, involves the alteration of the conformation of the promoter DNA by the transcription factor to facilitate the binding of the  $\sigma^{70}$  to the -35 and/or -10 promoter elements, as shown in Figure 14C. The transcription factors bind to or near the promoter elements at most class III promoters. One well studied example of class III activation involves the MerR family of activators and their target promoters. MerR, the first characterised activator of the MerR family, regulates the mercury resistance operons *mer* through binding to a site between the -35 and -10 elements of the *merOP* promoter. In doing so MerR represses transcription from its own *merR* promoter, which overlaps the *merOP* promoter placed divergently on the opposite strand of the DNA. MerR binds to the DNA in a repressed conformation in the absence of mercuric ion ( $\text{Hg(II)}$ ), distorting the promoter by unwinding the DNA (Brown, Stoyanov et al. 2003; Hobman 2007)..  $\sigma^{70}$  is recruited to the promoter, however kept in an unproductive complex with the DNA and MerR. The MerR responds to  $\text{Hg(II)}$ , through conformational change, to distort the DNA at the promoter by a 33° unwinding of the DNA. This re-orientates the sub-optimal 19 nucleotide spacer of the *merOP* promoter to allow productive interaction with the  $\sigma^{70}$

### A Class I activation



### B Class II activation



### C Activation by conformational change

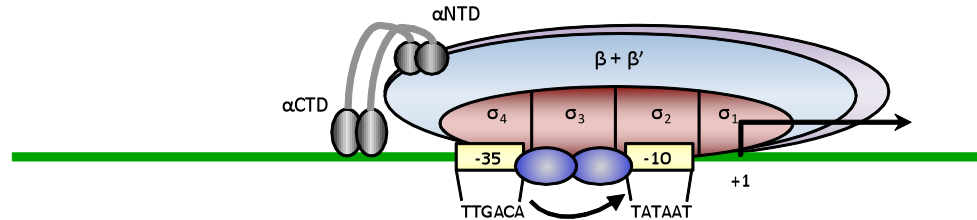
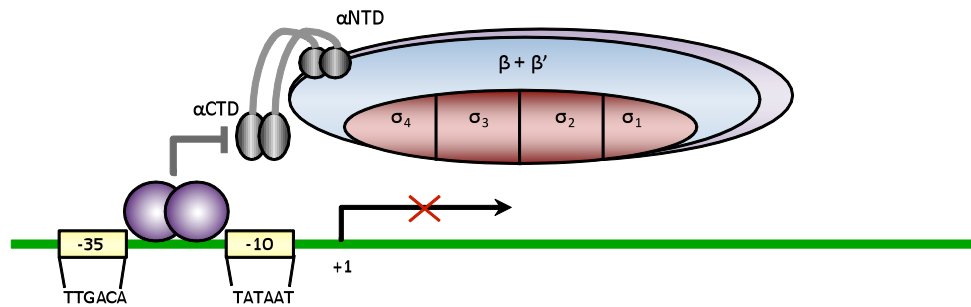


Figure 14: Mechanisms of activation at simple promoters.

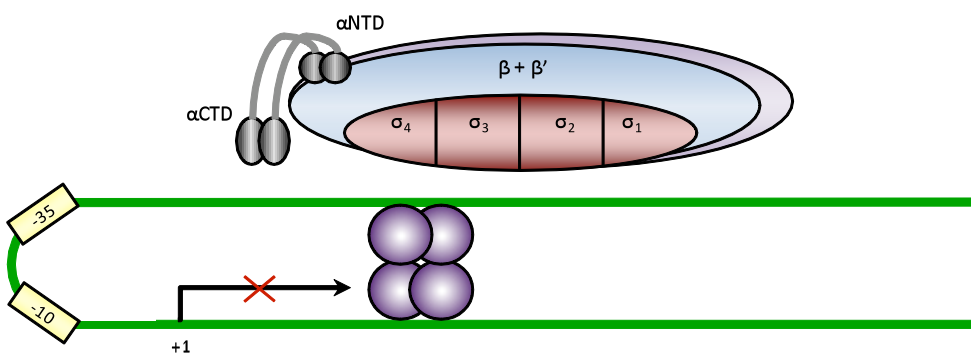
A diagrammatic representation of  $E\sigma^{70}$  and transcription factors (activators) at simple promoters during activation. The interactions of proteins are shown through adjacent placement to the interacting molecule. Activators are displayed as dimers as the majority function as dimers. A. Class I activation. The activator, bound to an upstream site, contacts the  $\alpha$ CTD of  $E\sigma^{70}$ , therefore recruiting the  $E\sigma^{70}$  to the promoter. B. Class II activation. The activator binds to a site adjacent to the -35 element, around position -41.5, and interacts with  $\sigma_4$ . C. Activation by conformational change. The activator, shown in blue, binds at, or near to, the -35 and -10 elements and realigns them enabling the  $E\sigma^{70}$  to bind to the promoter. Based on (Browning and Busby 2004).

Transcription factors that repress gene transcription generally do so at target promoters by simple repression. Three general mechanisms, as shown in Figure 15, are used. The first and simplest mechanism of repression involves steric hindrance by a transcription factor. In steric hindrance the binding of  $E\sigma^{70}$  to a promoter is blocked by the binding of a repressor to a site in or close to the core promoter -35 and -10 elements, as shown in Figure 15A. The most studied example of this mechanism involves the binding of the Lac repressor to the *lac* operator at the *lac* promoter to control lactose utilisation (Lewis 2005). The repression of the Lac repressor is removed through binding of the inducer allolactose, a lactose analog, to the Lac repressor, causing a conformational change and reduced affinity for the operator.

A Repression by steric hindrance



B Repression by looping



C Repression by modulation of an activator

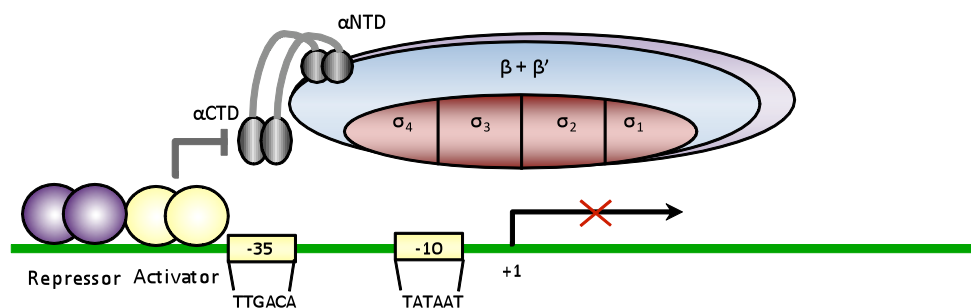


Figure 15: Mechanisms of repression.

A diagrammatic representation of  $E\sigma^{70}$  and transcription factors (repressors) at promoters during repression. Repressors are displayed as dimers or dimers of dimers as the majority function as dimers. A. Repression by steric hindrance. The repressor binds to a site overlapping the -35 and -10 core promoter elements, blocking promoter recognition by the  $E\sigma^{70}$ . B. Repression by looping. Repressors bind to distal sites and interact to loop the DNA, repressing the promoter. C. Repression by modulation of an activator. The repressor, shown in purple, binds to an activator, shown in yellow, to prevent the function of the activator, therefore blocking promoter recognition by  $E\sigma^{70}$ . Based on (Browning and Busby 2004).

A second mechanism of repression, as shown in Figure 15B, involves the binding of multiple repressor molecules to promoter distal sites to repress transcription initiation by DNA looping. This mechanism is used by the GalR transcription factor to repress transcription

initiation from both the galactose operon *galP1* and *galP2* promoters to control galactose metabolism. GalR binds to an operator sequence 60.5 bp upstream of *galP1* to repress transcription initiation at *galP1*. However, in the presence of the histone-like protein HU and supercoiled DNA, GalR forms a tetramer and represses *galP1* and *galP2* through DNA looping that encompasses both promoters (Aki, Choy et al. 1996). The mechanism of GalR repression involves the contact of GalR with the  $\alpha$ -subunit of  $E\sigma^{70}$  to trap a rate-limiting intermediate  $E\sigma^{70}$  complex at the promoter, therefore requiring higher energy to form the RPo (Roy, Semsey et al. 2004).

The third simple mechanism of repression involves the repression of an activator by another transcription factor, as shown in Figure 15C. This is seen at the *E. coli deoP2* promoter where the CRP activator is repressed by the CytR transcription factor to form a regulatory complex that prevents transcription initiation. The *deo* operon promoter *deoP2*, involved in nucleoside catabolism, contains two CRP binding sites centred at -41.5 and -92.5 upstream of the transcription start site. Binding of CytR, as a dimer, occurs between the two CRP-DNA complexes to form a repression complex, with CytR contacting the DNA around position -65 and both CRP complexes (Perini, Doherty et al. 1996). The repression is thought to remove the productive interaction between CRP and  $E\sigma^{70}$  (Shin, Kang et al. 2001). CytR repression is removed in the presence of cytidine to allow transcriptionally active CRP-  $E\sigma^{70}$  complexes to form.

Simple mechanisms of activation and repression of transcription initiation by transcription factors have been well documented. However, in the majority of bacterial promoters, multiple environmental signals are integrated to control promoter transcriptional output. Therefore, many transcription factors can be involved in promoter regulation. A number of mechanisms involving co-dependence on two or more transcription factors have been identified. Examples of these mechanisms are illustrated in Figure 16 and include the independent contacts of two activators with the  $E\sigma^{70}$  or the anti-repression by a second activator.

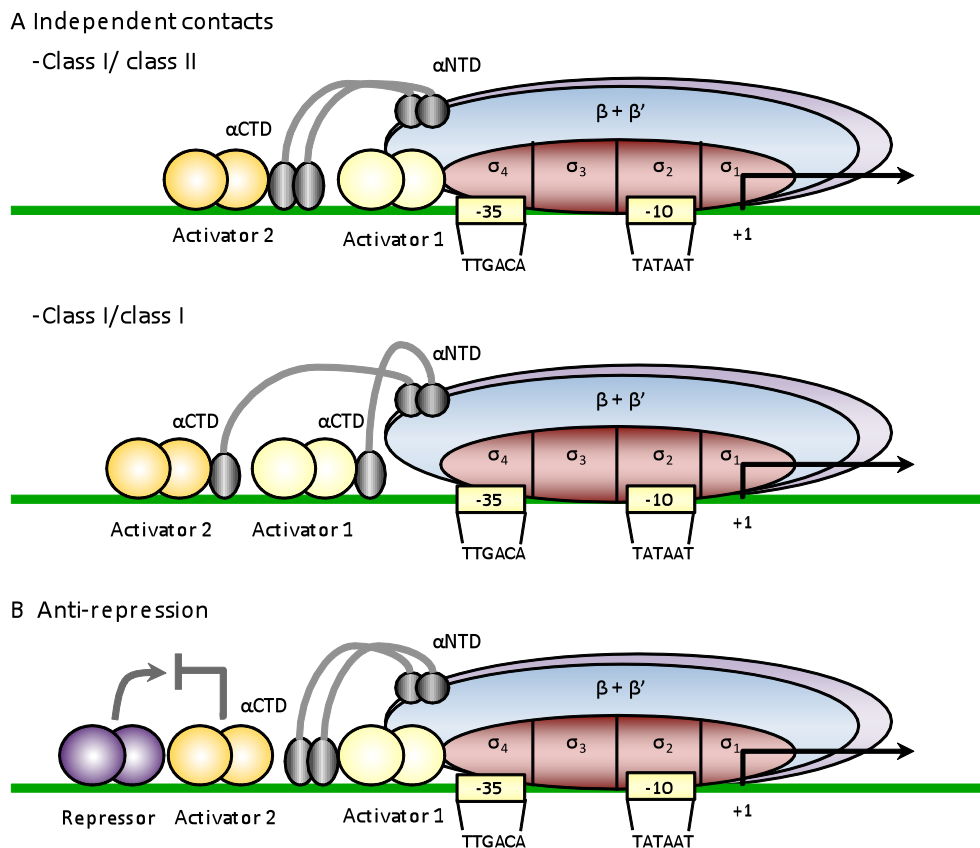


Figure 16: Mechanisms of promoter co-dependence on two or more transcription factors.

A diagrammatic representation of the co-dependence of the  $E\sigma^{70}$  and promoter on multiple transcription factors (repressors and activators). The interactions of proteins are shown through adjacent placement to the interacting molecule. Activators and repressors are displayed as dimers as the majority function as dimers. A. Independent contacts. Class I/class II. One activator functions by a class I mechanism, contacting the  $\alpha$ CTD, whilst a second functions by a class II contacting  $\sigma_4$ . Class I/class I. Both activators function by a class one mechanism with each activator contacting an individual  $\alpha$ CTD. B. Anti-repression. The binding of a second activator is required to remove the inhibitory effects of a repressor, freeing the primary activator to function. Based on (Browning and Busby 2004).

One good example of co-dependence by two activators where both class I and class II mechanisms are involved is the *E. coli proP* P2 promoter. The *proP* P2 promoter controls the expression of an integral membrane transporter of osmoprotecting compounds and contains binding sites for the activator Fis centred at position -41 and for CRP at position -121.5, relative to the transcription start site. The dimeric Fis bound to the -41 site overlaps the promoter -35 element through a class II mechanism and interacts with both an  $\alpha$ CTD ( $\alpha$ CTD<sup>II</sup>) and  $\sigma$  (McLeod, Aiyar et al. 2002). In combination, the CRP dimer bound further upstream at -121.5 contacts the other  $\alpha$ CTD<sup>I</sup>, as illustrated in Figure 17. The two regulators act synergistically to activate transcription initiation at the promoter.

#### Activated transcription at *proPP2*

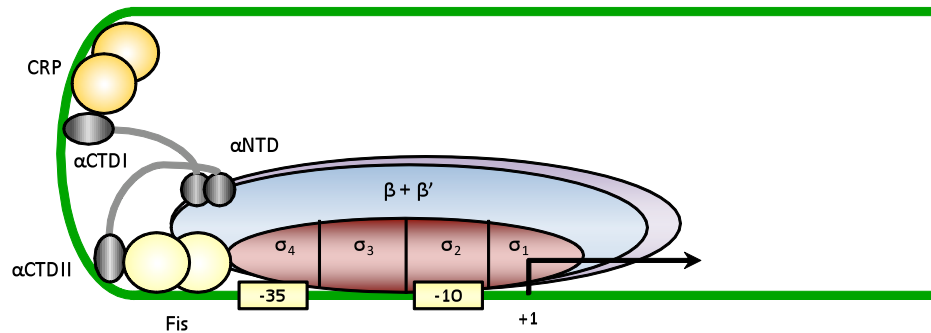


Figure 17: Transcription activation at the *E. coli proP* P2 promoter.

Representation of the co-activation of the *proP* P2 promoter by Fis and CRP. Fis (yellow) bound to a site at -41 interacts with  $\sigma$  and  $\alpha\text{CTD}^{\text{II}}$  to activate transcription in co-operation with CRP (dark yellow). CRP bound at -121.5, interacts with the  $\alpha\text{CTD}^{\text{I}}$  upstream of Fis. Promoter double stranded DNA shown as green line. Based on figure by Mcleod *et al.*, 2002.

#### 1.5.7. Transcriptional regulators of the *agr* operon

The regulation of transcription at the *agr* operon promoters P2 and P3 appears complex as the IR is targeted by multiple transcription factors, as shown in Figure 18. These include those that indirectly regulate *agr* gene expression either positively; *clpP*, *cvfABC*, *Msa* and *CcpA* or negatively; *SarT*, *SarU* or *ArlS/R* (Fournier, Klier *et al.* 2001; Schmidt, Manna *et al.* 2001; Manna and Cheung 2003; Manna and Cheung 2006; Michel, Agerer *et al.* 2006; Sambanthamoorthy, Smeltzer *et al.* 2006; Seidl, Stucki *et al.* 2006; Nagata, Kaito *et al.* 2008). Or, those that directly contact the *agr* operon to regulate gene expression positively; *AgrA*, *SarA*, *SarR*, *SarZ* and *MgrA* or negatively; *SarX* and *SrrA* (Manna and Cheung 2001; Yarwood, McCormick *et al.* 2001; Koenig, Ray *et al.* 2004; Ingavale, van Wamel *et al.* 2005; Kaito, Morishita *et al.* 2006; Manna and Cheung 2006; Cheung, Nishina *et al.* 2008).

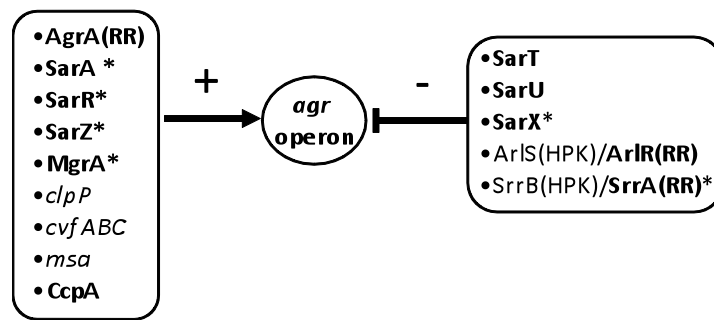


Figure 18: Regulators of the *agr* operon.

Regulatory elements that are to date known to either positively (+) or negatively (-) influence expression of the *agr* operon. DNA binding TRs are shown in bold, with those for which binding to the *agr* IR is demonstrated are marked by an asterisk. The products of the *clpP* (ClpP protease), *cvfABC*, *msa* genes and the CcpA protein have an indirect effect on *agr* operon expression. For references of each regulator see text in section 1.5.7.

One well studied family of regulators that are included in this group are the SarA family of transcriptional factors. The SarA family of transcription factors belongs to the winged-helix family of transcription regulators and includes SarA, SarR, SarS, Rot, SarT, SarU, SarV, MgrA, SarX and SarZ. The winged-helix region is involved in transcriptional regulation through target DNA recognition. Importantly, the SarA family of transcription factors are present throughout most sequenced staphylococcal genomes with at least 9 major SarA paralogues present in the genomes sequenced (Kuroda, Ohta et al. 2001).

SarA, a 124 residue and 14.7 kDa transcriptional regulator, is the first characterised of the SarA family of transcription regulators. SarA was first discovered through its pleiotropic effect on exoprotein expression (Cheung, Koomey et al. 1992). Further study using transcription profiling in a *S. aureus sarA* deletion strain has shown it is involved in the up-regulation of 76 genes and the down-regulation of 44 genes in a cell density and SarA dependent manner (Dunman, Murphy et al. 2001). Furthermore, SarA regulation occurs primarily early on in the growth cycle as its expression peaks during the exponential phase before tapering off during the post-exponential phase (Chien, Manna et al. 1998). SarA is expressed from the *sar* locus, which encodes three overlapping transcripts with a common 3' end, each encoding the 372 bp *sarA* gene. The three transcripts are initiated from three distinct promoters *sar* P1, P2 and P3, highlighting a complex network of regulation. Initial studies determined that SarA binds to a conserved motif at the *agr* operon to regulate the *agr* operon promoters (Chien, Manna et al. 1999). DNase I footprinting assays detected 3

CACATCTCTGTGATCTAGTTAT**ATTA**AAACATGCTAAAAGCATTATTT**TTCCA**ATTTT**CTTAACT**AGTCGT  
 GTGTAGAGACACTAG**A**TCAATATA**TAATTTT**GTACGATTTTCGTAAATA**AAAAGGTT**AAAAAGAATTGATCAGCA  
 +1 ← -10<sub>P3</sub> -35<sub>P3</sub>

**A2** **B1** **B2**  
 TTTTTATT**CTTAACT**GTAAATTTTTTTATGTTAAATATTAAATACAAATTACATTTAA**AGTTAAG**TATTT  
 AAAAATAAGAATTGACATTTAAAAAATACAATTTTATAATTTATGTTTAAATGTAAATTGTCAATTCATAAA

**C1** (18) **C2** → +1  
 ATTTCTCTAC**AGTTAGG**CAATATA**TAAGTA**AAAAGATTGTACTAAATCG**TATAAT**GCAGTG**AGG**GAGAGTGGTG  
 TAAAGGATGTCAATCCGTTATAT**TACTA**TTTTCTAACATGATTTAGCA**ATATTA**CTGTCACT**CCTCTC**ACCAC  
 -35<sub>P2</sub> -10<sub>P2</sub>

The sequence of the *agr* P2 and P3 promoters from *S. aureus* NCTC 8325 are shown with the consensus -10 and -35 hexamers highlighted in bold. The regions protected by SarA in DNase I protection assays are shaded in grey (Rechlin, Gillaspay et al. 1999) and labelled for each dimer bound, for example A1 and A2. The heptad repeats bound by SarA are highlighted by a box (Morfeldt, Tegmark et al. 1996). The region protected by SarR in DNase I protection assays is underlined with a black line (Manna and Cheung 2006). The promoter consensus spacer length and the transcription start site (+1) are labelled. The extended -10 motif at the P3 promoter is boxed in a dashed line.

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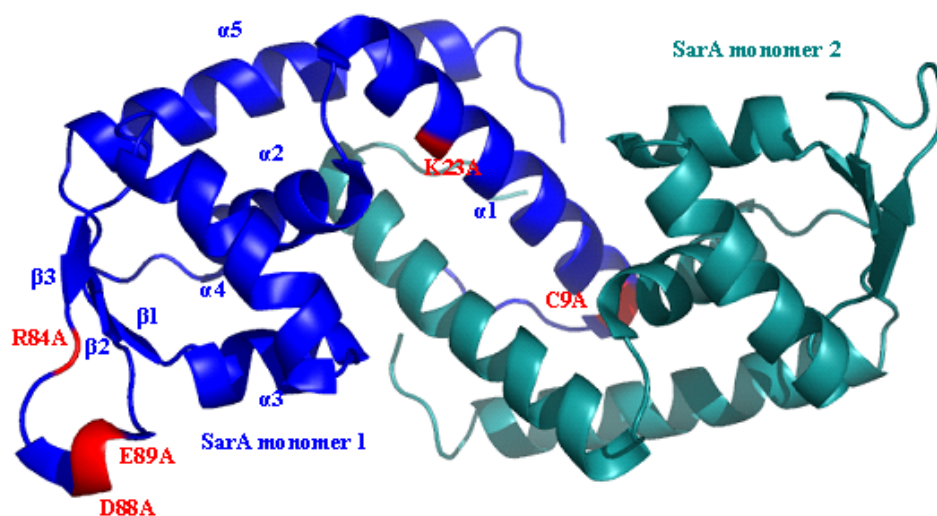


Figure 20: Crystal structure of SarA dimer.

Representation of the crystal structure of a SarA dimer. One SarA monomer is shown in blue and the other monomer in turquoise. SarA point mutations (C9A and K23A in  $\alpha$ 1-helix involved in dimerisation and E89A and D88A of the conserved DER motif and R84A) are highlighted in red (based on structure determined by Liu et al., 2006; Protein Data Bank (PDB: 2FNP) (drawn using PyMOL software; DeLano Scientific: [www.pymol.org](http://www.pymol.org)).

SarR, a 13.6 kDa winged helix transcription regulator, from the SarA family of transcription factors, also directly interacts with the *agr* operon to modulate its gene expression. *In vivo* studies using a *S. aureus sarR* deletion strain has shown that SarR is a positive regulator of the *agr* P2 and P3 promoters (Manna and Cheung 2006). SarR represses SarA expression through binding to the *sar* locus, upstream of the *sar* P1 and P3 promoters, to modulate its expression (Manna and Cheung 2001). DNase I footprinting showed that SarR binds to the *agr* IR at a binding site overlapping the SarA binding site with a higher affinity than SarA, as shown in Figure 19 (Manna and Cheung 2006). As SarR and SarA are both involved in *agr* regulation at the early phase of growth and taking into account the overlapping binding sites at the *agr* IR, there is a possibility for cross-talk between SarA and SarR. The crystal structure of SarR has revealed the similarities between SarA and SarR, compare Figure 20 and Figure 21, and also hints at the possibility of cross-talk through forming a SarA-SarR oligomer (Liu, Manna et al. 2001). The use of EMSAs has identified residues lysine 52 and 80, arginine 82 and 90 and leucine 105 are all necessary for DNA binding (Manna and Cheung 2006).

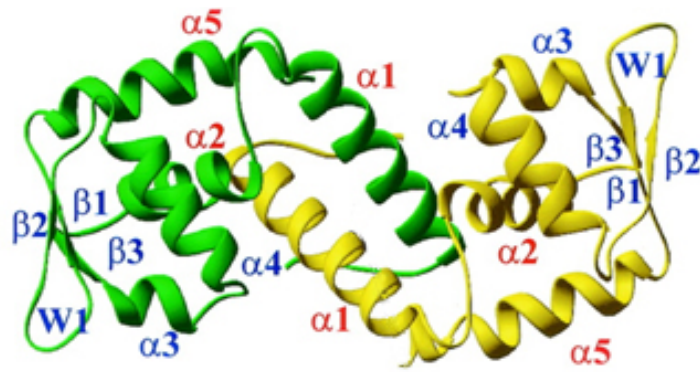


Figure 21: Crystal structure of SarR dimer.

Representation of the structure of a SarR dimer, taken from Liu, Manna *et al.* 2001. One SarR monomer is shown in green and the other monomer in yellow. Subdomain 1 features are labelled in blue ( $\alpha 3$ ,  $\alpha 4$ ,  $\beta 1$ ,  $\beta 2$ ,  $\beta 3$  and W1) and subdomain 2 features are labelled in red ( $\alpha 1$ ,  $\alpha 2$  and  $\alpha 5$ ). Protein Data Bank (PDB: 1HSI) (drawn using PyMOL software; DeLano Scientific: [www.pymol.org](http://www.pymol.org)).

## 1.6. Objectives

How transcription from the P2 and P3 promoters of the *agr* operon is regulated is not known and has not been studied at a molecular level. The chief objective of this thesis is to unravel the mechanisms that govern transcription from the *agr* P2 and P3 promoters at the molecular level *in vitro*. In this regard, the specific aims include:

1. Setting up a fully native *S. aureus in vitro* system to study regulation of transcription from P2 and P3 promoters.
2. Investigating the interaction of  $E\sigma^A$  with the *agr* P2 and P3 promoters.
3. Investigating the basal transcriptional activity of the *agr* P2 and P3 promoters and the effect of AgrA on transcription initiation.
4. Exploring how AgrA activates transcription from the *agr* promoters P2 and P3.
5. Investigating how SarA influences transcription at the *agr* operon.

It is anticipated that results from this thesis will provide the first molecular insights into how transcription initiation is regulated from the *agr* operon promoters P2 and P3 in *S. aureus*.

## CHAPTER TWO

### 2. Materials and Methods

#### 2.1. General methods

##### 2.1.1. Composition of growth media

The composition of media used for bacterial growth is shown below in Table 2. All bacterial growth media were sterilised by autoclaving at 120°C for 30 min.

| Media                                                | Composition (1L)                                                                                                | Use                       |
|------------------------------------------------------|-----------------------------------------------------------------------------------------------------------------|---------------------------|
| Luria-Bertani (LB)                                   | 10 g peptone, 5 g yeast extract, 5 g NaCl                                                                       | General                   |
| Luria-Bertani Agar (LA)                              | 10 g peptone, 5g yeast extract, 5 g NaCl, 20 g agar                                                             | General                   |
| Super Optimal Broth with Catabolite repression (SOC) | 20 g bacto-tryptone, 5 g, yeast extract, 0.584 g NaCl, 0.186 g KCl, 0.952 g MgCl <sub>2</sub> , 3.606 g glucose | Heat-shock transformation |

Table 2: Composition of growth media.

To select for the plasmid or bacterial strain of interest the sterile growth media was supplemented with the appropriate antibiotics. The antibiotics used are shown below in Table 3. All antibiotics were filter sterilised before use. *E. coli* and *S. aureus* agar plates and liquid media were incubated at 37°C, with liquid media shaken at 140 rpm in a temperature controlled water bath, unless otherwise stated.

| Antibiotic      | Stock concentration           | Final concentration | Storage | Abbreviation     |
|-----------------|-------------------------------|---------------------|---------|------------------|
| ampicillin      | 100 mg/ml, in ethanol         | 100 µg/ml           | -20°C   | Amp <sup>R</sup> |
| chloramphenicol | 34 mg/ml, in ethanol          | 34 µg/ml            | -20°C   | Cam <sup>R</sup> |
| kanamycin       | 50 mg/ml, in H <sub>2</sub> O | 50 µg/ml            | 4°C     | Kan <sup>R</sup> |

Table 3: Summary of antibiotics used in this work.

### 2.1.2. General laboratory reagents and solutions

|                                                             |                                                                                         |
|-------------------------------------------------------------|-----------------------------------------------------------------------------------------|
| Acrylamide Proto gel (Solution 1)<br>(National diagnostics) | 30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide stock solution (37.5:1)                 |
| Acrylamide solution II                                      | 1.5 M Tris-HCl pH 8.8, 0.3% (w/v) SDS                                                   |
| Acrylamide solution III                                     | 0.5 M Tris-HCl pH 6.8, 0.4% (w/v) SDS                                                   |
| Native loading dye                                          | Reaction buffer + 50% (v/v) glycerol, 0.05% (w/v) bromophenol blue                      |
| Reaction buffer x5                                          | 40 mM Tris-acetate pH7.9, 100 mM NaCl, 20 mM MgCl <sub>2</sub> and 0.2 mM DTT           |
| Stop buffer x5                                              | 3% (w/v) xylene cyanol, 3% (w/v) bromophenol blue and 20 mM EDTA in deionized formamide |
| Storage buffer (protein)                                    | 10 mM Tris-HCl pH8, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT and 50% (v/v) glycerol            |
| TBE buffer x10<br>(National Diagnostics)                    | 0.89 M Tris base, 0.89 M boric acid (pH 8.3) and 20 mM Na <sub>2</sub> EDTA             |
| Tris Buffered Saline (TBS) x10                              | 50 mM Tris, 150 mM NaCl                                                                 |
| TBSTT x10                                                   | As TBS with 0.3% (v/v) Tween 20                                                         |
| TG buffer x10<br>(National Diagnostics)                     | 0.25 M Tris base and 1.92 M glycine                                                     |
| TGS buffer x10                                              | 0.25 M Tris base, 1.92 M glycine, and 1% (w/v) SDS (National Diagnostics)               |

### 2.1.3. Bacterial strains and plasmids

The bacterial strains used for cloning and protein expression are listed below in Table 4. The background plasmids used in this work are listed below in Table 5, with their antibiotic resistance also included. Constructed vectors are listed in the Appendix, Table 11.

| Strain                        | Genotype                                                                                                                                                                                                                                                   | Reference                |
|-------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------|
| <i>E. coli</i> DH5α           | F <sup>-</sup> endA1 glnV44 thi-1 relA1 gyrA96 deoR nupG lacZ deltaM15 hsdR17                                                                                                                                                                              | Promega                  |
| <i>E. coli</i> BL21 (DE3)     | F <sup>-</sup> ompT gal dcm lon hsdS <sub>B</sub> (r <sub>B</sub> <sup>-</sup> m <sub>B</sub> <sup>-</sup> ) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])                                                                                                 | Stratagene               |
| <i>E. coli</i> BL21 (DE3) C41 | F <sup>-</sup> ompT gal dcm lon hsdS <sub>B</sub> (r <sub>B</sub> <sup>-</sup> m <sub>B</sub> <sup>-</sup> ) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])<br><i>E. coli</i> BL21 (DE3) mutant strain selected for increased expression of toxic proteins. | (Miroux and Walker 1996) |
| <i>E. coli</i> BL21 (DE3) C43 | F <sup>-</sup> ompT gal dcm lon hsdS <sub>B</sub> (r <sub>B</sub> <sup>-</sup> m <sub>B</sub> <sup>-</sup> ) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])<br><i>E. coli</i> BL21 (DE3) mutant strain selected for increased expression of toxic proteins. | (Miroux and Walker 1996) |
| <i>S. aureus</i> NCTC 8325    | Contains temperate bacteriophages ϕ11, ϕ12 and ϕ13.                                                                                                                                                                                                        | (Gillaspy 2006)          |

Table 4: Bacterial strains used in this work.

| Vector      | Resistance | Strain(s) used   | Use                           | Inducer | Reference                |
|-------------|------------|------------------|-------------------------------|---------|--------------------------|
| pGEM-T Easy | ampicillin | DH5α             | Cloning                       | N/A     | Promega                  |
| pET22b(+)   | ampicillin | DH5α, BL21 (DE3) | Protein expression            | IPTG    | Novagen                  |
| pET28b(+)   | kanamycin  | DH5α, BL21 (DE3) | Protein expression            | IPTG    | Novagen                  |
| pMAL-c2X    | ampicillin | DH5α, BL21 (DE3) | Protein expression            | IPTG    | New England Biolabs      |
| pLSR        | ampicillin | DH5α             | <i>in vitro</i> transcription | N/A     | (El-Robh and Busby 2002) |

Table 5: Background plasmids used in this work.

Background plasmids used in this work with their corresponding resistance, bacterial strain used in, use and inducer. See Appendix for vector maps of pGEM-T EASY, pET22b(+), pET28b(+), pMAL-c2x and pLSR.

## 2.2. General DNA methods

### 2.2.1. Purification of chromosomal DNA

*S. aureus* NCTC 8325 chromosomal DNA was purified using the Wizard Genomic DNA Purification kit (Promega). *S. aureus* NCTC 8325 was grown overnight at 37°C in 5 ml LB

media. 1 ml of overnight culture was pelleted and re-suspended in 475 µl of 50 mM EDTA, 20 µl of Lysosyme (50 mg/ml in H<sub>2</sub>O) and 5 µl of Lysostaphin (10 mg/ml in H<sub>2</sub>O). The resuspended cells were then incubated at 37°C for 60 minutes, followed by centrifugation in a microcentrifuge at 14,000 x g for 2 minutes. The supernatant was then removed and the pellet re-suspended in 600 µl of Nuclei Lysis Solution (Promega). The sample was then incubated at 80°C for 5 minutes to lyse the cells and allowed to cool to room temperature. 3 µl of RNase solution (Promega) was then added and mixed into the sample before a further incubation at 37°C for 15 minutes. The sample was again allowed to cool to room temperature. 200 µl of Protein Precipitation Solution (Promega) was then added to the sample and the sample was vortexed vigorously for 20 seconds before being incubated on ice for 5 minutes. The sample was then spun down at 14,000 x g for 3 minutes. The supernatant was then transferred to a clean microcentrifuge tube containing 600 µl of room temperature isopropanol and mixed to precipitate DNA. The sample was then spun down at 14,000 x g for 2 minutes and the supernatant decanted. The DNA pellet was added to 600 µl of room temperature 70% (v/v) ethanol and mixed. This was followed by a further centrifugation at 14,000 x g for 2 minutes and then the ethanol was aspirated and the DNA pellet allowed to air dry for 15 minutes. The DNA pellet was then rehydrated in 100 µl of Rehydration solution (Promega) at 65°C for 1 hour. The DNA solution was then stored at -20°C.

### **2.2.2. Purification of plasmid DNA**

Plasmid DNA was purified from the *E. coli* DH5α cloning strain for transformation into competent cells and *in vitro* transcription assays using the QIAGEN QIAprep miniprep system. 5 ml of bacterial culture containing the plasmid of interest was grown overnight at 37°C to saturation. The cells were then pelleted and the supernatant discarded. The pellets were then treated in accordance with the manufacturer's instructions, which briefly included the re-suspension and lysis of the cells using three different solutions. The lysed cells were then pelleted in a microcentrifuge at 18,000 x g for 10 minutes and the supernatant collected and run through an anion exchange column, by a further centrifugation at 18,000 x g for 1 minute, to trap the plasmid DNA. The column was then washed with a wash solution containing 70% (v/v) ethanol and centrifuged at 18,000 x g for

1 minute. The DNA was then eluted into 50 µl of distilled water by a final spin at 18,000 x g for 1 minute. The DNA was then analysed by agarose gel electrophoresis (section 2.2.4), its concentration measured using a NanoDrop spectrophotometer (Thermo Scientific) and the sample stored at -20°C.

Plasmids with a pLSR background were purified from a larger 500 ml bacterial culture using a Maxi Prep plasmid purification kit (Qiagen). The plasmids were purified following the manufacturer's instructions, following the same steps as above.

### **2.2.3. Restriction analysis and site specific DNA digestion**

Restriction analysis and site specific DNA digestion were carried out in 10 µl reaction volumes and typically contained 1 µl of a x10 reaction buffer (optimal for the restriction enzyme(s)), 7 µl of plasmid DNA (as prepared in section 2.2.2) and 0.5 U of restriction enzyme(s). If required, Bovine Serum Albumin (NEB) was added at a final concentration of 100 µg /ml. The reactions prepared as above were then incubated at 37°C for 1 hour and analysed by agarose gel electrophoresis (section 2.2.4). Digested plasmid DNA to be used for ligations were dephosphorylated at the 5' end to prevent self-ligation through the addition of 2.5 µl of shrimp alkaline phosphatase (SAP) (Promega) and 2.5 µl of x10 SAP buffer (Promega) to the 10 µl digestion reaction. The reaction was incubated at 37°C for 30 minutes and then the digestions purified (section 2.2.5).

### **2.2.4. Agarose gel electrophoresis**

Agarose gels were prepared at either 1% agarose (w/v), to visualise longer DNA, or 2% (w/v) for lengths less than 400 bp, in 1x TBE buffer (National Diagnostics). The agarose and 1x TBE solution was heated until all the agarose was dissolved in a microwave, before being left to cool. Then a 1:10,000 dilution of SYBR® Safe DNA gel stain (Invitrogen) was added and the solution poured into a pre-levelled gel casting tray with a gel comb added to create the wells. 6x DNA loading dye (Fermentas) was added to the samples at 1x final concentration and then the samples were loaded onto the gel alongside a 1kb or 100bp DNA marker (O'Generuler™1kb or 100bp, fermentas), depending on expected DNA size range, to determine DNA length. The gel was run at 100V in 1x TBE for approximately 30 minutes before being removed and visualised using a blue light transilluminator (Invitrogen).

### **2.2.5. DNA purification**

To extract and purify DNA from agarose gels a PCR purification kit (QIAGEN) was used. The DNA was purified according to the manufacturer's instructions. Firstly this involved cutting the DNA band of interest out from the agarose gel using a sterile blade and a blue light transilluminator. The gel band was then placed into a clean microcentrifuge tube and dissolved at 50°C in 300 µl QG binding buffer (QIAGEN) per 100 mg of gel. Isopropanol was added to the gel fragment at a 1:1 (w/v), to the weight of the gel slice, and the solution was bound to the column (QIAGEN) using a microcentrifuge at 14,000 x g for 1 minute. The column was then washed through with a wash solution containing 70% (v/v) ethanol (QIAGEN) and then the DNA eluted in 50 µl of distilled water. DNA samples were stored at -20°C.

PCR reactions were purified as the method above except the PCR reaction was re-suspended in 5 gel volumes (w/v) binding buffer PB (QIAGEN) and then bound to the column. Then the protocol above was followed as described.

### **2.2.6. Polymerase chain reaction (PCR)**

PCR reactions were setup using Pfu Ultra II Fusion HS DNA Polymerase (Stratagene) for high-fidelity amplification of blunt ended DNA fragments. The polymerase was directed by PCR primers (Appendix, Table 9) designed to target the region of interest and insert restriction sites at both the 5' and 3' end for integration into an expression or reporter plasmid (Table 5). A typical reaction volume of 50 µl contained 0.2 µM of each oligonucleotide/primer, 100 ng of template DNA, 25 mM dATP, dCTP, dGTP and dTTP, 5 µl x10 reaction buffer and 1 unit of Pfu DNA polymerase. The reactions were mixed and then temperature cycled in 3 stages, (1) 94°C for 5 minutes, (2) 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 15 seconds per 1kb extension and (3) 72°C for 5 minutes. Stage (2) was cycled x30 with the annealing temperature set (e.g. 55°C) depending on the T<sub>m</sub> of the primers and the extension time (72°C for 15 seconds) altered depending on the length of the product. The products were then purified by PCR purification (section 2.2.5).

To amplify DNA with polyadenylated linear DNA ends for direct ligation into the pGEM-T easy vector, PCR reactions were carried out using Taq DNA polymerase (NEB). A typical 50 µl

Taq PCR reaction contained 0.5  $\mu$ M of each oligonucleotide/primer, 100 ng of template DNA, 25 mM dATP, dCTP, dGTP and dTTP, 5  $\mu$ l x10 reaction buffer and 2.5 units of Taq DNA polymerase. The reactions were cycled as displayed for the high-fidelity amplification of blunt ended DNA fragments with the following alterations to stage (2); 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds per 1kb extension. The product was purified (section 2.2.5) and ligated into pGEM-T easy as in section 2.2.8.

In order to determine whether colonies transformed (section 2.2.12) with plasmid DNA contained the DNA target of interest colony PCRs were setup using Taq DNA polymerase (NEB) to amplify the target for analysis by agarose gel electrophoresis. A typical 50  $\mu$ l colony PCR reaction contained 0.5  $\mu$ M of each oligonucleotide/primer, 25 mM dATP, dCTP, dGTP and dTTP, 5  $\mu$ l x10 reaction buffer and 2.5 units of Taq DNA polymerase. A colony was picked under sterile conditions and placed in a 50  $\mu$ l colony PCR reaction and the reaction cycled as for Taq DNA polymerase with the following alterations to stage (1): 95°C for 5 minutes. The PCR products were identified by agarose gel electrophoresis, as in section 2.2.4.

#### **2.2.7. Site directed mutagenesis**

Site directed mutagenesis was used to alter protein amino acid sequences through single or multiple base changes in the gene sequence on plasmid templates. The high fidelity Pfu Ultra II Fusion HS DNA Polymerase (Stratagene) was used in site directed mutagenesis. Complementary template and non-template primers were designed with up to three consecutive base pair mismatches, with up to a 20 base flanking region on either side for optimal annealing (Appendix, Table 9). A typical 50  $\mu$ l colony PCR reaction contained 0.2  $\mu$ M of each complimentary oligonucleotide/primer, 100 ng template DNA, 25 mM dATP, dCTP, dGTP and dTTP, 5  $\mu$ l x10 reaction buffer and 1 unit of Pfu DNA polymerase. The reaction was cycled in 3 stages, (1) 95°C for 30 seconds, (2) 95°C for 30 seconds, 55°C for 1 minute and 68°C for 15 seconds per 1kb extension and (3) 68°C for 3 minutes. Stage 2 was cycled x15 with the annealing temperature set depending on the  $T_m$  of the primers and the extension time altered depending on the length of the product. The PCR product was then digested with the enzyme *Dpn1* to remove the template DNA by the addition of 6  $\mu$ l x10 buffer (NEB), 3  $\mu$ l distilled water and 1  $\mu$ l of *Dpn1* (NEB) and incubation at 37°C for 1 hour. The *Dpn1*

restriction enzyme targets and digests methylated DNA with the 5'-GmGATC-3' target sequence. The DNA was then purified as in section 2.2.5 and transformed into competent *E. coli* DH5 $\alpha$  as in section 2.2.12. Colonies of transformed cells were then tested for the mutation through antibiotic resistance, colony PCR (section 2.2.6) and DNA sequencing (section 2.2.9).

#### **2.2.8. pGEM-T easy vector ligation**

The ligation of DNA into the linear pGEM-T easy vector (Figure 100) requires purified DNA amplified by PCR using Taq (section 2.2.6). Taq amplifies DNA with polyadenylated linear DNA ends required for direct ligation into the linear pGEM-T easy vector with poly-thiamine tails using T4 DNA ligase (Promega). A typical 10  $\mu$ l ligation reaction consisted of 1  $\mu$ l pGEM-T easy vector, 3  $\mu$ l insert DNA, 5  $\mu$ l x2 ligase buffer and 1  $\mu$ l T4 DNA ligase. The reaction was then incubated at 4°C overnight and then transformed into competent *E. coli* DH5 $\alpha$  (sections 2.2.12 and 2.2.11).

#### **2.2.9. DNA sequencing**

DNA sequences were determined using the Imperial College Core Sequencing Service. 10  $\mu$ l reactions were setup containing 300 ng of DNA per 3 Kb template for sequencing, 3.2  $\mu$ M primer oligonucleotide and distilled water. The samples were then sent to the sequencing service where sequencing was performed using Applied Biosystems BigDye version 3.1. All sequences obtained were then analysed using BioEdit Sequence Alignment Editor software.

#### **2.2.10. Vector ligation**

Ligations were setup using either insert DNA digested from pGEM-T easy plasmids (section 2.2.8) or insert DNA digested from PCR reactions (section 2.2.6) for ligation into the corresponding restriction enzyme digested plasmid DNA. A typical 10  $\mu$ l ligation reaction consisted of 100 ng of plasmid DNA, 25 ng of insert DNA, 1 unit of T4 ligase (Promega) and 1  $\mu$ l of x10 T4 ligase buffer. Ligations were typically setup with a vector to insert ratio of 3:1. The reaction was then incubated at 17°C overnight before transformation into competent *E. coli* DH5 $\alpha$  (section 2.2.12).

### **2.2.11. Competent cell preparation**

Competent cells were prepared using the  $\text{CaCl}_2$  competent cell preparation method for use in the transformation of plasmid DNA into *E. coli* cloning and expression strains. To obtain  $\text{CaCl}_2$  competent cells the *E. coli* strain of interest was streaked out on an LB agar plate, containing corresponding antibiotics, and incubated at  $37^\circ\text{C}$  overnight. A 5 ml LB seed culture was inoculated from this plate and grown at  $37^\circ\text{C}$  until  $\text{OD}_{\lambda 600} = 0.4\text{--}0.45$  in a shaking water bath at 140 rpm, as determined in a spectrophotometer. The cells were then pelleted at  $4000 \times g$  at  $4^\circ\text{C}$  for 10 minutes and the supernatant removed. The cell pellet was re-suspended in 20 ml of 100 mM  $\text{CaCl}_2$  and 15% glycerol pre-chilled to  $<4^\circ\text{C}$  before re-centrifugation at  $4000 \times g$  at  $4^\circ\text{C}$  for 10 minutes. The supernatant was removed and the cells re-suspended in 3 ml of 100 mM  $\text{CaCl}_2$  and 15% glycerol pre-chilled to  $<4^\circ\text{C}$ . 50  $\mu\text{l}$  aliquots of the cell suspension were frozen in liquid nitrogen and the cells stored at  $-80^\circ\text{C}$ .

### **2.2.12. Heat-shock transformation**

To transform purified plasmid DNA (section 2.2.2) into competent *E. coli* strains (section 2.2.11) aliquots of the appropriate cells were taken from  $-80^\circ\text{C}$  and thawed on ice for 5 minutes. 100 ng of plasmid DNA was added to each aliquot and incubated on ice for 30 minutes. The cells were then heat shocked by placing the cell aliquot into a  $42^\circ\text{C}$  water bath for 50 seconds and then promptly placing the aliquot back on ice. 1 ml of SOC media, pre-warmed to  $37^\circ\text{C}$ , was added to the cells under sterile conditions and the cells placed in a shaking water bath at  $37^\circ\text{C}$  for 2 hours at 140 rpm to recover. The cells were then pelleted at  $14,000 \times g$  for 2 minutes and the supernatant removed. The pellet was re-suspended in 50  $\mu\text{l}$  of the original SOC media and spread onto a LB agar plate containing the appropriate antibiotics. The LB agar plate was incubated at  $37^\circ\text{C}$  overnight to allow colony formation.

## **2.3. General protein methods**

### **2.3.1. Protein expression**

Proteins encoded by expression plasmids listed in Table 5 were transformed (section 2.2.12) into *E. coli* expression strains (Table 4) and expressed using their corresponding inducer. The majority of proteins were expressed under the pET system (section 2.3.2). Expression strains were grown in 500 ml of LB media in 2 L flasks, with the corresponding antibiotic added.

Cultures were incubated at 37°C in a shaking water bath at 140 rpm unless otherwise stated. Cultures were grown to  $OD_{\lambda 600} = 0.4-0.45$ , then equilibrated in a 25°C shaking water bath (120 rpm) for 30 minutes before the addition of the inducer (1 mM IPTG for pET vectors). The cultures were then grown for a further 3 hours with 1 ml samples collected at set intervals. Cells were then pelleted at 4,000 x g for 20 minutes and the cell pellet stored at -20°C overnight, ready for protein purification.

To test for protein expression the 1 ml samples collected were spun down to collect the cell pellet and then re-suspended in 100 µl distilled water. 10 µl of x2 laemmli buffer (Sigma) was added to 10 µl of each sample and then pre-heated at 95°C for 5 minutes before running on an SDS-PAGE gel for visualisation, as in section 2.3.4.

### 2.3.2. The pET system

The pET expression system is based on pET expression vectors that contain a T7 promoter region (Studier and Moffatt 1986). The T7 promoter region is specifically recognised by the bacteriophage T7 DNA dependent RNA polymerase (T7 RNAP) (Imburgio, Rong et al. 2000). Genes cloned into the multiple cloning site (MCS) of a pET vector are under direct control of the T7 promoter and therefore the T7 RNAP. The T7 RNAP is under control of a *lac* promoter (*lacUV5*) and is chromosomally located. The addition of the inducer isopropyl β-D-1-thiogalactopyranoside (IPTG), that mimics allolactose, displaces the lac repressor and therefore releases the promoter for the production of T7 RNAP. The T7 RNAP is then able to bind to the T7 promoter and transcribe the target gene encoded in the MCS of the pET vector. Unlike allolactose, IPTG is non-hydrolysable and therefore remains at a constant level. In the absence of the inducer a basal level of transcription is still present due to the dynamic equilibrium between occupied and unoccupied operator sites by the repressor protein. Therefore a low level of target protein is expressed in the absence of the inducer (Dubendorff and Studier 1991). The pET28b<sup>+</sup> vector (Figure 102) encodes a six-histidine tag (6His tag) at the N-terminus of the target protein for nickel affinity chromatography (section 2.3.5.1), whilst pET22b<sup>+</sup> (Figure 101) encodes a six-histidine tag (6His tag) at the C-terminus. If the native stop codon of the gene is maintained in pET22b<sup>+</sup> then the native protein is expressed without a 6His tag. This system was used to express the *S. aureus* proteins AgrA, SarA, SarR, σ<sup>A</sup> and σ<sup>B</sup>. Although [6His]-SarR and [6His]-SigB expression vectors were

constructed, expressed from and the proteins purified to a high purity, they were never used for any of the work presented in this thesis.

### **2.3.3. Maltose binding protein fusion constructs**

The maltose binding protein fusion expression system (New England Biolabs) is based on the pMAL expression vectors that contain the N-terminal *maltE* gene encoding a MBP fusion protein. The gene of interest is cloned into the MCS downstream of the *maltE* gene with a polylinker containing a *Factor Xa* cleavage site. The construct is under control of the  $p_{tac}$  promoter and protein expression is induced upon addition of 0.3 mM IPTG. The pMAL-c2X vector (Figure 103) was used to express the *S. aureus* AgrA protein as a MBP-AgrA fusion protein.

### **2.3.4. SDS denaturing polyacrylamide gel electrophoresis**

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was used to resolve protein samples for visualisation by coomassie staining. SDS gels were prepared at an appropriate percentage for the resolution of differing protein size, with an increased percentage of acrylamide for resolving smaller proteins (Table 6). For example a 10% SDS-PAGE gel was prepared by mixing a separating gel with 3.25 ml Acrylamide Protogel (30% (w/v) acrylamide, 0.8% (w/v) bis-acrylamide) (National diagnostics), 2.5 ml acrylamide solution II (1.5 M Tris-HCl pH 8.8, 0.3% (w/v) SDS), 4.2 ml distilled water, 100  $\mu$ l 10% (w/v) ammonium persulphate (APS) and 10  $\mu$ l TEMED. The solution was then quickly poured into casting plates using the Mini-PROTEAN Tetra Electrophoresis system (Bio-rad), 0.7 mm gel thickness, to just below the well level and isopropanol was placed above to aid the formation of a level gel boundary. After 5 minutes, when the gel was set, the isopropanol was removed and the gel washed with distilled water. The stacking gel was then prepared with 0.6 ml Acrylamide Protogel (National diagnostics), 1 ml acrylamide solution III (0.5 M Tris-HCl pH 6.8, 0.4% (w/v) SDS), 2.4 ml distilled water, 40  $\mu$ l 10% (w/v) APS and 4  $\mu$ l TEMED. The stacking gel solution was then poured on top of the separating gel and the wells inserted. After 5 minutes the set gel was placed into a gel tank (Bio-rad) and pre-warmed at 200 V in 1x Tris-Glycine-SDS-PAGE Buffer (0.025 M Tris base, 0.192 M glycine, and 0.1% (w/v) SDS) (National Diagnostics). 10  $\mu$ l x2 Laemmli buffer (Sigma) was added to 10  $\mu$ l of

each protein sample, pre-heated at 95°C for 5 minutes and then centrifuged briefly. The samples were then loaded into the pre-warmed gel alongside 5 µl of pre-stained protein marker (PageRuler™ Prestained protein ladder, Fermentas) and the gel run at 200 V for 60 minutes or until the loading dye reached the bottom of the gel. The gel was then washed three times in 40 ml distilled water before being placed in 20 ml of Bio-safe coomassie stain (Bio-rad) for 1 hour, then placed in distilled water to remove background stain. The resolved proteins present were then visualised on the gel.

|                         | Separating Gel |        |        |        | Stacking Gel |
|-------------------------|----------------|--------|--------|--------|--------------|
| Component               | 10%            | 12.5%  | 15%    | 20%    | -            |
| Acrylamide Protogel     | 3.25 ml        | 4.1 ml | 5.0 ml | 6.7 ml | 0.6 ml       |
| Acrylamide solution II  | 2.5 ml         | 2.5 ml | 2.5 ml | 2.5 ml | -            |
| Acrylamide solution III | -              | -      | -      | -      | 1 ml         |
| Distilled water         | 4.2 ml         | 3.4 ml | 2.5 ml | 0.8 ml | 2.4 ml       |
| 10 % (w/v) APS          | 100 µl         | 100 µl | 100 µl | 100 µl | 40 µl        |
| TEMED                   | 10 µl          | 10 µl  | 10 µl  | 10 µl  | 4 µl         |

Table 6: Composition of SDS separating and stacking gels used in this study.

### 2.3.5. Protein purification

#### 2.3.5.1. Nickel affinity chromatography

Proteins with a 6His tag were purified from *E. coli* expression strains (Table 2) using Nickel affinity chromatography. Cell pellets containing expressed target proteins (section 2.3.1) stored at -20°C were thawed on ice for 20 minutes and re-suspended in 30 ml nickel buffer A (25 mM NaH<sub>2</sub>PO<sub>4</sub> (pH7), 0.5 M NaCl and 5% (v/v) glycerol) containing 1 tablet of RNase inhibitors (Roche) per 100 ml buffer. 500 mg of lysozyme (Sigma) was sprinkled on top of the cell suspension before the cells were sonicated for 10 minutes (10 seconds on-off cycle) until lysed. The lysed sample was spun in a high speed centrifuge tube at 4°C at 32,000 x g for 30 minutes to separate the soluble from the insoluble protein fraction. The supernatant was removed and stored on ice for loading onto a 5 ml HiTrap™ Chelating HP nickel column (GE Healthcare). The Fast Protein Liquid Chromatography (FPLC) system (ÄKTAprime™, GE healthcare) was prepared by washing the system through with pre-cooled nickel buffer A

and B (nickel buffer A + 1 M imidazole). The nickel column was prepared by loading with 0.1 M NiSO<sub>4</sub> and equilibrating with 3 column volumes of nickel buffer A, B and then A. The nickel column was attached to the FPLC system, with caution taken to reduce air blockages, and the sample loaded into the column at 0.5 ml per minute. The column was then washed at 2 ml per minute with 2 column volumes of buffer A followed by 3% buffer B and then buffer A again. A 40 ml gradient was then started at 1 ml per minute, where the buffer composition was slowly increased from 0% (v/v) buffer B to 100% (v/v) buffer B to elute and collect column bound proteins in 1 ml fractions. Samples were taken throughout the purification steps and the protein present in all fractions was determined through SDS-PAGE electrophoresis (section 2.3.4). Target proteins of >95% purity were dialysed into pre-chilled storage buffer (10 mM Tris-HCl (pH8), 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT and 50% (v/v) glycerol) overnight using Spectra/Por 3 dialysis tubing (Spectrum) and the protein concentration measured (section 2.3.6). Protein samples were aliquoted into microcentrifuge tubes and flash frozen in liquid nitrogen before storage at -80°C.

#### **2.3.5.2. Heparin affinity chromatography**

DNA binding proteins can be purified by heparin affinity chromatography as heparin is highly negatively charged and mimics the negative charge of DNA. Proteins were purified by heparin affinity chromatography using the FPLC system (ÄKTAprime™, GE healthcare Life Sciences) and a 5 ml HiTrap™ Heparin HP column (GE Healthcare), prepared by washing with 1 M NaCl and equilibrating in heparin buffer A (10 mM Tris-HCl (pH8), 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT and 5% (v/v) glycerol). Cell pellets were either re-suspended in heparin buffer A and lysed, as in section 2.3.5.1, or protein samples that has already undertaken one purification step re-dialysed into heparin buffer A for a second purification step. 10 mM MgCl<sub>2</sub> was added to the running buffer (heparin buffer A) and to the sample before loading onto the column. After loading the sample onto the column attached to the FPLC system at 0.5 ml per minute, the column was washed with 3 column volumes of buffer A at 2 ml per minute. Then a 40 ml gradient was started at 1ml per minute, where the buffer composition was slowly increased from 0% (v/v) buffer B (heparin buffer A + 1 M NaCl and 10mM MgCl<sub>2</sub>) to 100% (v/v) buffer B to elute column bound proteins into separate 1 ml fractions. Samples were analysed and stored as in section 2.3.5.1.

#### **2.3.5.3. Amylose affinity chromatography**

Proteins encoding a Maltose Binding Protein (MBP) fusion were purified by amylose affinity chromatography. Cell pellets re-suspended in 30 ml amylose buffer A (20 mM Tris-HCl (pH7.4), 200 mM NaCl and 1mM EDTA) were lysed as in section 2.3.5.1. The soluble protein fraction collected through high speed centrifugation was loaded at 0.5 ml per minute onto an 5 ml MBPTrap<sup>TM</sup> column (GE Healthcare), regenerated in 0.1% (v/v) SDS and equilibrated in excess amylose buffer A. The column was washed at 2 ml per minute with 5 column volumes of amylose buffer A and bound proteins eluted with an increasing gradient of 0% (v/v) to 100% (v/v) amylose buffer B (amylose buffer A + 1 M maltose) at 1 ml per minute. Samples were analysed and stored as in section 2.3.5.1. The MBP fusion can be removed through cleavage using the specific protease Factor Xa at the Factor Xa cleavage site (section 2.3.5.4).

#### **2.3.5.4. Cleavage of Maltose binding protein fusion**

Using the Factor Xa cleavage site, the Maltose binding protein (MBP) was removed from proteins expressed and purified as a MBP fusion (section 2.3.5.3). A typical reaction contained 100 mg fusion protein in amylose buffer A (20 mM Tris-HCl (pH7.4), 200 mM NaCl and 1mM EDTA) and 1 mg of Factor Xa (NEB). The reaction was incubated at 4°C overnight and the sample re-purified using amylose affinity chromatography (section 2.3.5.3) so that the MBP protein bound to the 1 ml MBPTrap<sup>TM</sup> column (GE Healthcare) and the cleaved protein of interest was collected in the flow-through or using heparin affinity chromatography (section 2.3.5.2). The cleaved protein was then dialysed into storage buffer and stored at -80°C as in section 2.3.5.1.

#### **2.3.5.5. Immunoaffinity chromatography**

Immunoaffinity chromatography was used for the extraction of *S. aureus* NCTC 8325 core RNAP using the polyol responsive 8RB13 affinity resin (Neoclone) (Bergendahl, Thompson et al. 2003). A polyol responsive antibody binds to the protein target with high affinity and through the addition of a buffer containing a polyol, such as ethylene glycol, 1,2-propanediol or 2,4-butanediol, and salt, the protein is gently displaced. 4 L of *S. aureus* NCTC 8325 cultures were grown in LB media at 37°C in a shaking water bath at 140 rpm to mid-log phase, OD<sub>λ600</sub> = 0.7. Cells were pelleted by centrifugation at 4°C at 5,000 x g for 20

minutes and the cell pellet re-suspended in 100 ml of RNAP lysis buffer A (10mM Tris-HCl (pH7.5), 1 M KCl, 15 mM MgCl<sub>2</sub>, 5 mM EDTA and 10 mM 2-mercaptoethanol) before re-centrifugation and the pellet stored at -20°C overnight. The cell pellet was thawed on ice and re-suspended in 100 ml of RNAP lysis buffer B (10mM Tris-HCl (pH7.5), 50 mM KCl, 15 mM MgCl<sub>2</sub>, 5 mM EDTA and 10 mM 2-mercaptoethanol) containing 1 tablet of RNase inhibitors (Roche) and 20 mg lysostaphin. The cells were lysed by passing the cell suspension through a cell disrupter ("Basic Z", version 6, Constant systems), pre-chilled to 4°C and pre-washed with RNAP buffer A, at 40,000 psi. The soluble fraction was collected through centrifugation at 4°C at 32,000 x g for 30 minutes. All further steps were carried out at 4°C. The nucleic acid and RNAP were precipitated by the addition of 0.6% (v/v) polyethyleneimine (PEI) (Sigma) from a 10% stock (pH7.9 using HCl), gradually added and mixed over 15 minutes before a further 30 minutes mixing. The precipitate was pelleted in a high speed centrifuge tube at 32,000 x g for 20 minutes and the pellet thoroughly re-suspended in 10 ml RNAP wash buffer (10 mM Tris-HCl (pH7.9), 0.1 mM EDTA, 300 mM NH<sub>4</sub>Cl, 5% (v/v) glycerol and 0.1 mM DTT). The suspension was re-centrifuged at 32,000 x g for 20 minutes and re-suspended in 10 ml RNAP elution buffer (RNAP wash buffer adjusted to pH7 + 1 M NH<sub>4</sub>Cl), before further centrifugation at 32,000 x g for 20 minutes. The supernatant was collected and gently mixed in a glass beaker with 0.4 g (NH<sub>4</sub>)SO<sub>4</sub> (Sigma)/ml supernatant, gradually added over 15 minutes, followed by a further 30 minutes mixing. The precipitate was pelleted at 32,000 x g for 10 minutes and the pellet stored at -20°C overnight.

The (NH<sub>4</sub>)SO<sub>4</sub> pellet was thawed on ice and re-suspended in 10 ml TE buffer (50 mM Tris-HCl (pH7.9) and 0.1 mM EDTA) and centrifuged at 5000 x g for 10 minutes at 4°C to remove insoluble materials. The supernatant was then passed 3 times through a prewashed column, with 10 ml TE buffer, packed with 1 ml 8RB13 polyol responsive antibody resin (Neoclone) to maximise RNAP contact time. The column was then washed with 10 ml TE buffer plus 0.15 M NaCl followed by 10 ml TE buffer plus 0.5 M NaCl and the flow-through was collected. The remaining bound proteins, including RNAP, were eluted with 5 ml TE buffer plus 0.75 M NaCl and 40% propylene glycol (Sigma), with 1 ml elution fractions collected. 20 µl samples taken throughout the purification were analysed through SDS-PAGE analysis

(section 2.3.4) and fractions containing >95% homogeneity of *S. aureus* core RNAP were concentrated through centrifugation at 5,000 x g using Amicon Ultra-4 spin columns (Millipore), with a 5 kDa cut-off, before dialysis and storage at -80°C, as in section 2.3.5.1.

### **2.3.6. Determination of protein concentration by Bradford microassay**

The concentration of proteins stored in storage buffer was determined using a Bio-Rad Bradford microassay. Bradford reagent dye (Quick start Bradford 1x Dye reagent, Bio-Rad) undergoes differential colour change upon addition to protein samples. This binding event shifts the absorbance maximum for an acidic solution of Coomassie G-250 from 465 nm to 595 nm. The absorbance was quantified by a spectrophotometer set to 595 nm. The comparison of absorbance to a standard curve provides a relative measurement of protein concentration. For a microassay, 5 µl of protein samples were loaded into a clear 96 well microtiter plate and 250 µl of Bradford reagent dye added for 15 minutes, before measurement of absorbance at 595 nm in a plate reader. Samples were added in triplicate with a blank buffer control and a Bovine serum albumin (BSA) (NEB) control titration of 0.05 mg/ml to 2 mg/ml diluted in sample buffer for the standard curve. The standard curve compared absorbance at 595 nm against BSA concentration.

### **2.3.7. Western blotting**

Western blotting was performed to identify *S. aureus* RNA polymerase using the monoclonal antibody to the β subunit of RNA polymerase, 8RB13 (Neoclone), diluted to 1:10,000 in 5% Marvel dried milk powder (made in x1 TBS (50 mM Tris, 150 mM NaCl)), followed by the secondary anti-mouse HRP-conjugated antibody, diluted to 1:10,000 in 5% Marvel. The protocol involved the resolving of protein samples through loading onto an appropriate percentage SDS-PAGE gel (section 2.3.4). Once the proteins were sufficiently resolved the gel was incubated in 20 ml x1 transblot buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol) for 10 minutes. In addition eight gel sized pieces of Whatmann paper (Whatmann) were soaked in 60 ml x1 transblot buffer. At the same time a gel sized piece of nitrocellulose membrane was incubated in methanol for 10 seconds, distilled water for 5 minutes and finally equilibrated in 20 ml x1 transblot buffer for 10 minutes. The blot was then assembled with the membrane placed on four layers of whatmann paper, followed by the gel on top and then a further four layers of whatmann paper. The stack was then placed

on a trans-blot semi-dry transfer cell (Bio-rad) and any air pockets removed. The blot was then transferred to the membrane by running the current at a constant 10 volts for 1 hour. After disassembly the membrane was blocked in 40 ml 5% Marvel at room temperature for 1 hour or at 4°C overnight. The membrane was then washed twice in 20 ml x1 TBSTT (x1 TBS + 0.1% (v/v) Tween 20) for 10 minutes, once in 20 ml x1 TBS for 10 minutes and then placed in 20 ml of primary antibody, diluted as required, in 5% Marvel and incubated for 1 hour at room temperature. The membrane was then washed twice again in 20 ml x1 TBSTT for 10 minutes, once in 20 ml x1 TBS for 10 minutes and then placed in 20 ml of secondary antibody, diluted to 1:10,000, in 5% Marvel and incubated for 1 hour at room temperature. The membrane then underwent x5 20 ml x1 TBSTT washes for 10 minutes each. The membrane was then placed down in a pre-mixed solution of 0.5 ml luminal/enhancer solution (ECL) (Pierce) and 0.5 ml of peroxide solution (Pierce) on cling film for 30 seconds. The membrane was then dabbed dry using blue towel, wrapped in cling film and placed in a cassette. The membrane was exposed to x-ray film in a dark room for 5-30 minutes and developed using a Sigma developing machine. The developed film was scanned to create a digital image.

## **2.4. General biochemical methods**

### **2.4.1. Urea denaturing gel electrophoresis**

To resolve DNA and RNA length, urea denaturing gel electrophoresis was performed. Typically, 50 ml of urea sequencing gels were composed of SequaGel concentrate (237.5 g/L Acrylamide, 12.5 g/L methylene bisacrylamide and 7.5M urea, National Diagnostics), SequaGel Diluent (7.5M urea, National Diagnostics) x10 TBE buffer (National diagnostics) and set using 400 µl 10 % w/v APS and 20 µl TEMED. The gel composition was determined based on expected DNA or RNA size (Table 7). Sequencing gels were poured into casting plates of different size depending on the application and number of bands to resolve. For resolution of multiple bands of >100 nucleotides gels were cast using the Sequi-Gen Sequencing cell (Bio-Rad). For resolution of single or shorter bands gels were cast using the Mini-PROTEAN Tetra Electrophoresis system (Bio-Rad). Gels were pre-warmed by running in x1 TBE buffer at 50 W for larger gels or 200 V for smaller gels. Samples obtained from transcription assays were loaded with 4 µl stop dye (3% (w/v) xylene cyanol, 3% (w/v)

bromophenol blue, 20 mM EDTA in 10 mls of deionized formamide) per 10 µl reaction and the gel run under the same conditions as used for gel warming. Electrophoresis was stopped when the bands were sufficiently resolved, based on loading dye position, and the gel dried at 80°C on a piece of Whatmann paper (Whatmann) using a gel dryer (Scie-Plas-GD4534) and pump (KNK lab). Dried gels were exposed on an Imaging plate (Fujifilm) to visualise and quantify radiolabelled nucleotides using a FLA-5000 PhosphorImager (Fujifilm).

| DNA/RNA fragment size (nucleotides) | % Monomer | SequaGel Concentrate | SequaGel Diluent | X10 TBE |
|-------------------------------------|-----------|----------------------|------------------|---------|
| 60-150                              | 6         | 12 ml                | 33 ml            | 5 ml    |
| 40-100                              | 8         | 16 ml                | 29 ml            | 5 ml    |
| 20-80                               | 10        | 20 ml                | 25 ml            | 5 ml    |
| 10-50                               | 12        | 24 ml                | 21 ml            | 5 ml    |
| <20                                 | 20        | 40 ml                | 5 ml             | 5 ml    |

Table 7: Urea Sequencing gel composition.

#### 2.4.2. Native gel electrophoresis

Native gels were poured for use in the study of native protein-protein and protein-DNA interactions. Typically, 20 ml native gels were composed of Acrylamide (Protogel, National Diagnostics), x 10 TG buffer (National Diagnostics), distilled water, APS and TEMED, with the acrylamide composition varied depending on protein size (Table 8). Gel solutions were cast using the Mini-PROTEAN Tetra Electrophoresis system (Bio-Rad) and the gels run in x1 TG buffer (National Diagnostics). Gel tanks were either run on ice using x1 TG buffer pre-chilled to <4°C to study complexes formed at <4°C, or run in a water bath at 37°C with x1 TG buffer pre-warmed to 37°C to study complexes formed at 37°C. Samples were loaded with 2 µl of native loading dye (reaction buffer + 50% (v/v) glycerol, 0.05% (w/v) bromophenol blue) and the gel run at 100 V for 40 minutes or until the dye front had run off the gel. Gels were dried at 80°C on whatmann paper (Whatmann) using a gel dryer (Scie-Plas-GD4534) and pump (KNK lab) and visualised using an imaging plate (Fujifilm) to study radiolabelled protein/ DNA complexes using a FLA-5000 PhosphorImager (Fujifilm).

| Component           | 4.5%        | 4.0%        |
|---------------------|-------------|-------------|
| Acrylamide Protogel | 3 ml        | 2.66 ml     |
| X10 TG buffer       | 2 ml        | 2 ml        |
| Distilled water     | 15 ml       | 15.33 ml    |
| APS                 | 200 $\mu$ l | 200 $\mu$ l |
| TEMED               | 20 $\mu$ l  | 20 $\mu$ l  |

Table 8: Native gel composition.

### 2.4.3. $^{32}\text{P}$ labelling DNA probes

20 pmoles of single stranded oligonucleotides or double stranded DNA PCR products were labelled with 35  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ]-ATP (500 Ci/mmol-PerkinElmer) using Polynucleotide kinase (PNK) (Promega) to form  $^{32}\text{P}$  labelled DNA promoter probes for electrophoretic mobility shift assays (section 2.4.5). PNK catalyses the transfer of a phosphate from ATP to the 5' end of DNA or RNA and therefore catalyses the transfer of the  $^{32}\text{P}$  labelled  $\gamma$ -phosphate from [ $\gamma$ - $^{32}\text{P}$ ]ATP to the 5' end of DNA oligonucleotides. Typically, 20  $\mu\text{l}$  labelling reactions contained 20 pmoles DNA, 35  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ]-ATP, 2  $\mu\text{l}$  x10 PNK ligase buffer A (300 mM Tris-HCl (pH7.8), 100 mM  $\text{MgCl}_2$ , 100 mM DTT and 10 mM ATP) (Promega) and 10 units of PNK (Promega). Reactions were incubated at 37°C for 30 minutes before the kinase was denatured at 70°C for 10 minutes. Duplex DNA was formed in 40  $\mu\text{l}$  reactions with the 20  $\mu\text{l}$  of  $^{32}\text{P}$  labelled single stranded oligonucleotides, 40 pmoles of the complementary single stranded unlabelled oligonucleotide and 4  $\mu\text{l}$  x10 TM buffer (10 mM Tris-HCl (pH10) and 1 mM  $\text{MgCl}_2$ ) made up to 40  $\mu\text{l}$  in distilled water to give a 500 nM final DNA concentration. Reactions were heated in a heat block at 95°C for 5 minutes before the slow cooling of the reactions through placing the heat block at room temperature overnight. The DNA duplex was then stored at -20°C.

### 2.4.4. *In vitro* phosphorylation of response regulators

The response regulator AgrA was phosphorylated using the small molecule phosphodonor acetyl phosphate. 10  $\mu\text{l}$  reactions typically consisted of 2  $\mu\text{l}$  x5 reaction buffer (40 mM Tris-acetate (pH7.9), 100 mM NaCl, 20 mM  $\text{MgCl}_2$  and 0.2 mM DTT), 1-5  $\mu\text{M}$  AgrA and 50 mM acetyl phosphate, diluted in distilled water. The reactions were incubated at 37°C for 30 minutes before being added to electrophoretic mobility shift (section 2.4.5) or transcription

(section 2.4.8) assays for analysis. Control reactions were setup and incubated in the absence of AgrA and/or acetyl phosphate.

#### 2.4.5. Electrophoretic mobility shift assays

Typically, electrophoretic mobility shift assays (EMSAs) were conducted as shown in Figure 22, in 10  $\mu$ l reactions with 2  $\mu$ l x5 reaction buffer (40 mM Tris-acetate (pH7.9), 100 mM NaCl, 20 mM  $MgCl_2$  and 0.2 mM DTT), 100 nM core RNAP, 400 nM  $\sigma^A$ , and 5 nM  $^{32}P$ -labelled promoter DNA (see section 2.4.3). RNAP and  $\sigma^A$  were pre-incubated for 5 minutes to form the holoenzyme before addition to the reactions. In some instances heparin was used at a final concentration of 100  $\mu$ g/ml to study heparin resistant promoter complexes. Reactions were incubated in a heat block for 5 minutes at 37°C to visualise complexes formed at 37°C, or cooled to <4°C and incubated for 5 minutes to visualise complexes formed at <4°C before running on a native gel (see section 2.4.2). 2  $\mu$ l of native loading dye (reaction buffer + 50% (v/v) glycerol, 0.05% (w/v) bromophenol blue) was added to the reactions before loading onto a native gel for native gel electrophoresis (section 2.4.2). Transcriptional regulators were added into the reactions with varying order of addition to study regulator, RNAP and DNA interactions.

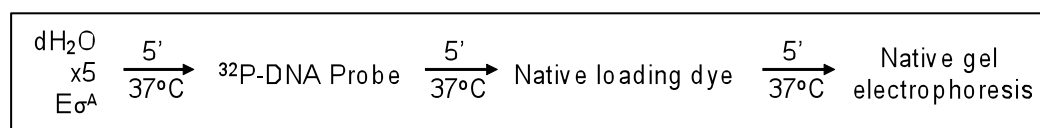


Figure 22: Schematic of typical electrophoretic mobility shift assay. Reaction buffer is labelled as x5.

#### 2.4.6. Short RNA synthesis assays

Short RNA synthesis assays measure the transcriptional output of RNAP promoter complexes. By providing the RNAP with only a short dinucleotide primer complementary to the promoter template strand transcriptional start site and also only the next complimentary nucleotide in radiolabeled form only short RNA synthesis can occur. The reactions followed the schematic in Figure 23, with 10  $\mu$ l binding reactions setup as in section 2.4.5 with the addition of 0.5 mM GpA dinucleotide primer, 3  $\mu$ Ci [ $\alpha$ - $^{32}P$ ]-UTP (800 Ci/mmol-PerkinElmer) (for P3) or [ $\alpha$ - $^{32}P$ ]-GTP (800 Ci/mmol-PerkinElmer) (for P2) and 0.25  $\mu$ l RNasin® Plus RNase Inhibitor (Promega) to allow dinucleotide primed short RNA synthesis to occur. Reactions were incubated at 37°C for a further 5 minutes before the addition of 2  $\mu$ l

of stop dye (3% (w/v) xylene cyanol, 3% (w/v) bromophenol blue and 20 mM EDTA in deionized formamide). Reactions were loaded and resolved on a 20% (w/v) urea denaturing gel and visualised as in section 2.4.1.

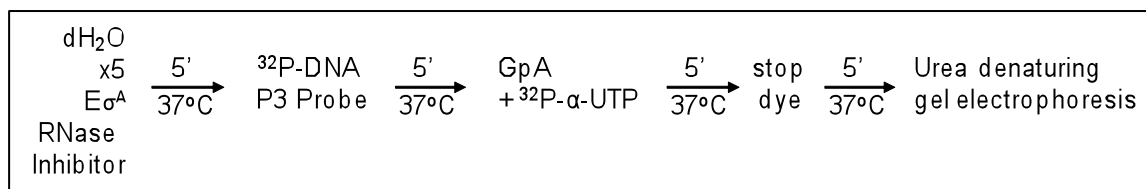


Figure 23: Schematic of typical short RNA synthesis assay using *agr* P3 probe. Reaction buffer is labelled as x5.

#### 2.4.7. RNAP catalytic assays

RNAP catalytic assays were performed using a minimal scaffold (MS) probe as the DNA template for the reactions, as in Figure 24. Minimal scaffold components included 20 pmoles RNA, 20 pmoles template DNA, 20 pmoles non-template DNA and 4 µl x10 TM buffer (10 mM Tris-HCl (pH10) and 1 mM MgCl<sub>2</sub>). The MS components were heated to 95°C for 5 minutes and slowly cooled to room temperature overnight, as in section 2.4.3, to anneal the scaffold. RNAP catalytic assay reactions typically included 2 µl x5 reaction buffer (40 mM Tris-acetate (pH7.9), 100 mM NaCl, 20 mM MgCl<sub>2</sub> and 0.2 mM DTT), 100 nM core RNAP, 100 nM MS scaffold, 0.5 µCi of [α-<sup>32</sup>P]-UTP (800 Ci/mmol-PerkinElmer) and 0.25 µl RNasin® Plus RNase Inhibitor (Promega) made up to 10 µl in distilled water. RNAP was pre-incubated at 37°C in the reaction mix for 5 minutes before the addition of MS DNA for 5 minutes, followed by [α-<sup>32</sup>P]-UTP for a further 5 minutes at 37°C before the reaction was stopped with the addition of 4 µl of stop dye (3% (w/v) xylene cyanol, 3% (w/v) bromophenol blue and 20 mM EDTA in deionized formamide). The reactions were also performed at <4°C to detect RNAP activity at 4°C. 7 µl samples were loaded and resolved on a 20% (w/v) urea denaturing gel and visualised as in section 2.4.1.

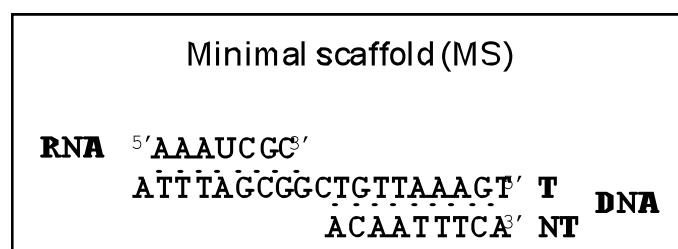


Figure 24: Minimal Scaffold probe used in RNAP catalytic assays.

#### 2.4.8. Full length *in vitro* transcription assays

Full length transcription assays were performed to measure the formation and transcriptional output of RNAP promoter complexes formed on supercoiled templates over time, as shown in the schematic in Figure 25. A 10 µl reaction typically contained 2 µl x5 reaction buffer (40 mM Tris-acetate (pH7.9), 100 mM NaCl, 20 mM MgCl<sub>2</sub> and 0.2 mM DTT), 100 nM RNAP, 400 nM σ<sup>A</sup>, 20 nM supercoiled promoter template and 0.25 µl RNasin® Plus RNase Inhibitor (Promega). The reaction components were pre-heated to 37°C for 5 minutes before the addition of the promoter template and a further 37°C incubation for 5 minutes. Then 0.575 µl of elongation mixture was added containing a final concentration of 0.5 mM ATP, CTP and GTP, 0.25 mM UTP and 0.75 µCi of [α-<sup>32</sup>P]-UTP (800 Ci/mmol-PerkinElmer). 1µl of 100 µg/ml heparin per reaction was added to the elongation mix for single-round transcription reactions. The reaction was then incubated for 10 minutes at 37°C to allow transcription to occur before being stopped with 4 µl of stop dye (3% (w/v) xylene cyanol, 3% (w/v) bromophenol blue and 20 mM EDTA in deionized formamide). 7 µl of each reaction was run on a 10% sequencing gel (as made in section 2.4.1) in x10 TBE buffer (National diagnostics) for 2 hours at 50W. Gels were dried and transcripts visualised using a Fuji FLA-5000 PhosphorImager as in section 2.4.1.

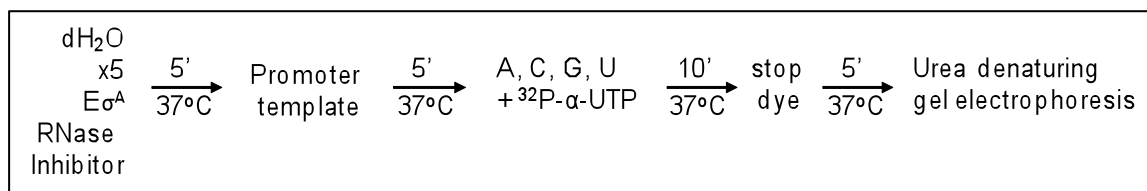


Figure 25: Schematic of typical full length *in vitro* transcription assay using a reporter plasmid as the promoter template. Reaction buffer is labelled as x5.

## CHAPTER THREE

### 3. Establishing a fully native *in vitro* transcription system to study transcription initiation at the *agr* operon

#### 3.1. Introduction

The *agr* operon has been well studied *in vivo* using a wide range of *S. aureus* infection models (Abdelnour, Arvidson et al. 1993; Gillaspay, Hickmon et al. 1995). However, due to the strain to strain differences and variation between experimental conditions in these *in vivo* studies, our understanding of the molecular and mechanistic events involved in the regulation of transcription initiation at the *agr* operon is still unclear. In order to study transcription initiation at the *agr* operon in a more controlled environment a fully native *in vitro* transcription system was setup, with all the components cloned, expressed or extracted from one *S. aureus* strain NCTC 8325. *S. aureus* NCTC 8325 was chosen as its genome has been fully sequenced and phylogenetic analyses have revealed it is closely related to clinically significant *S. aureus* strains including MRSA252 and N315 (Holden, Feil et al. 2004).

It was important to obtain native *S. aureus* core RNAP (E) to study the regulatory mechanisms that govern transcription initiation at the *agr* P2 and P3 promoters. Although *E. coli* core RNAP can form  $E\sigma^A$  with *S. aureus*  $\sigma^A$  and has structural conservation with *S. aureus* core RNAP, any small differences in the amino acid sequence could affect RNAP, DNA or regulator interactions. Challenges in *S. aureus* core RNAP purification include the inability to tag the core RNAP on the *S. aureus* genome with a poly histidine tag. Also, previous attempts to purify were complex and involved two to three chromatography steps, thereby increasing the handling and therefore decreasing core RNAP activity (Deora and Misra 1995). Therefore previous purification methods were not viable. Consequently an immunoaffinity chromatography approach was attempted, using a polyol-responsive antibody to reduce the use of harsh conditions to preserve core RNAP activity. The cross-reactive polyol-responsive monoclonal antibody (MAb) 8RB13 has previously been used to purify core RNAP from a number of bacteria including *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Streptomyces coelicolor* and *Shewalla oneidensis* (Bergendahl,

Thompson et al. 2003; Probasco, Thompson et al. 2007). The 8RB13 MAb specifically detects the  $\beta$ -flap domain of RNAP and recently the epitope was shown to be the amino acid sequence 5'-PEEKLLRAIFGEKAS-3' in the flap-tip helix of the  $\beta$ -flap domain. A polyol responsive antibody is advantageous for the purification of large complex proteins such as core RNAP as it binds with high affinity and eluting the core RNAP from the MAb only requires a polyol containing buffer, such as ethylene glycol, 1,2-propanediol or 2,4-butanediol, and salt, which gently displaces the core RNAP. This method should also result in extracting core RNAP without any contaminating  $\sigma$  factors, which is important for downstream activity assays, as in section 4.2. Purified *S. aureus* core RNA polymerase has previously been demonstrated to be active when reconstituted with  $\sigma$  in *in vitro* transcription of *S. aureus* genes (Rao, Karls et al. 1995).

In this study, the *S. aureus*  $\sigma$  factors  $\sigma^A$  and  $\sigma^B$  as well as the transcriptional regulators of the SarA family, SarA and SarR, were expressed with an N-terminal 6 histidine (6His) tag using the pET expression system for purification by simple nickel affinity purification, as in section 2.3.5.1. A wide range of approaches were used to express and purify the *agr* response regulator AgrA in a soluble form. Previous studies have found that AgrA is a challenge to purify as the majority is expressed in an insoluble form in *E. coli* (Sidote, Barbieri et al. 2008).

To study transcription initiation at the *agr* operon P2 and P3 promoters *in vitro* a number of templates were required. These included *agr* reporter plasmids with just one or both of the *agr* P2 and P3 promoters present. These reporter plasmids allowed the study of transcription initiation both independently of and simultaneously from the divergent promoter. Therefore, the effect of one promoter on the occupation of  $E\sigma^A$  at the divergent promoter was studied. The reporter plasmids were also used to investigate the effect of transcription regulators on the *agr* P2 and P3 promoters. Linear probes were also designed to study the initial DNA binding step of transcription initiation and for short RNA synthesis assays.

### 3.1.1. Objectives

The overall objective of this part of the project was to obtain the protein and nucleic acid components to study transcription initiation at the *agr* operon *in vitro*. In this regard, the specific aims include:

- I. Obtain pure core RNA polymerase from *S. aureus* NCTC 8325.
- II. Express and purify the *S. aureus*  $\sigma$  factors  $\sigma^A$  and  $\sigma^B$ .
- III. Express and purify the transcription regulators SarA and SarR.
- IV. Express AgrA in a soluble form, available for purification by single step affinity chromatography.
- V. Create reporter plasmids to measure transcription initiation from the *agr* P2 and P3 promoters.
- VI. Design linear DNA probes for the study of binding and transcription initiation at the *agr* P2 and P3 promoters.

## 3.2. Protein components

### 3.2.1. *S. aureus* core RNAP purification

The MAb 8RB13 was used, for the first time, to purify *S. aureus* NCTC 8325 core RNAP extracted from exponentially growing cells. However, first it was determined whether the 8RB13 MAb cross-reacts with *S. aureus* core RNAP using western blot analysis as in section 2.3.7.

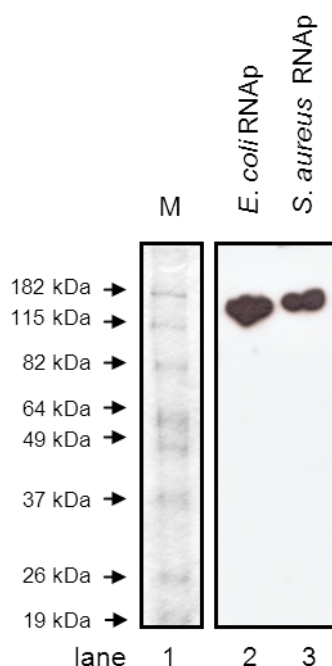


Figure 26: Image of a western blot showing the *E. coli* and *S. aureus* cell extracts probed using 8RB13 antibody.

Western blot analysis of 10% (w/v) SDS-PAGE gel using MAb 8RB13 containing whole cell extracts from *E. coli* and *S. aureus*. M = marker, overlaid onto film from SDS-PAGE gel.

As shown in Figure 26, the MAb 8RB13 was able to cross-react with the  $\beta$ -flap domain of *S. aureus* core RNAP, see lane 3. A control with *E. coli* core RNAP showed the specificity of the MAb to the  $\beta$ -flap of core RNAP from both bacteria, see lane 2. Once it was determined that the MAb 8RB13 cross-reacts with *S. aureus* core RNAP, *S. aureus* core RNAP was purified by cell lysis and extraction using Polymyxin P and ammonium sulphate precipitation steps, following section 2.3.5.5, as originally devised in Burgess and Jendrisak 1975. The preparation was then purified by immunoaffinity chromatography using a pre-packed 8RB13 resin column.

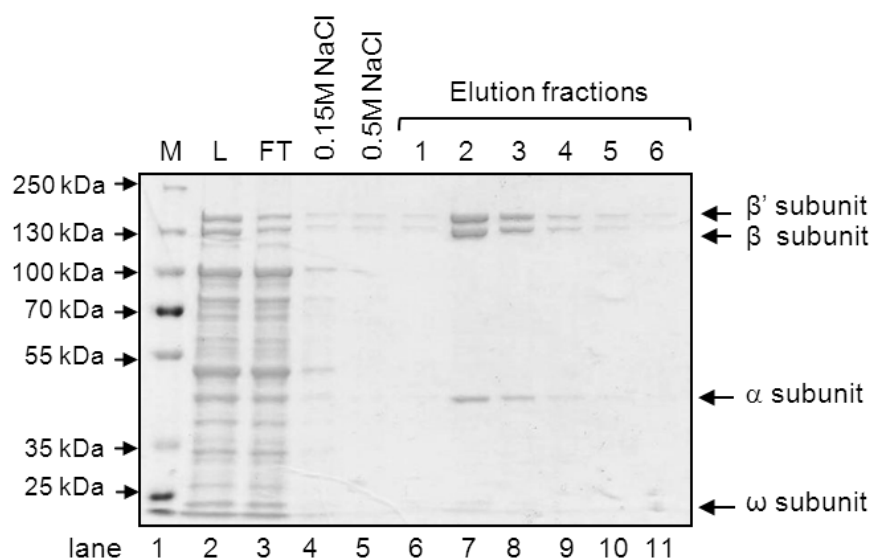


Figure 27: Image of a Coomassie stained 10% (w/v) SDS-PAGE gel showing the immunoaffinity chromatography of *S. aureus* core RNAP.

*S. aureus* core RNAP was extracted using the 8RB13 polyol responsive resin. The elution fractions of core RNAP are shown in lanes 6-11. The migration positions of *S. aureus* RNAP  $\beta$ ,  $\beta'$ ,  $\alpha$  and  $\omega$  subunits are labelled. M = Marker, L = Load, FT = Flow-through, 0.15 M NaCl = 0.15 M NaCl wash and 0.5 M NaCl = 0.5 M NaCl wash.

Results showed that *S. aureus* core RNAP was purified using the 8RB13 resin to a high purity, >95%, with an absence of contaminating  $\sigma$  factors, lanes 7-11 (Figure 27). The RNAP  $\beta$ ,  $\beta'$ ,  $\alpha$  and  $\omega$  subunits were all identified in the elution fractions, indicating that the core RNAP preparation had been assembled correctly. The preparation loaded onto the column (Figure 27, lane 2) still contained many contaminating proteins as well as the core RNAP after the Polymin P and ammonium sulphate precipitation steps however they either do not bind or are clearly removed by the washing of the 8RB13 column with increasing salt concentrations lanes 3-5.

### 3.2.2. *S. aureus* $\sigma^A$ and $\sigma^B$ expression and purification

The *S. aureus*  $\sigma$  factors  $\sigma^A$  and  $\sigma^B$  were both expressed under control of the pET expression system, as in section 2.3.2, where the genes were ligated into the pET28b+ vector to encode an N-terminal 6His tag. The proteins were expressed in the *E. coli* expression strain BL21 under normal growth conditions (section 2.3.1) with induction at mid-log phase using IPTG.

### 3.2.2.1. *S. aureus* [6His]- $\sigma^A$ purification

*S. aureus* [6His]- $\sigma^A$  was purified by nickel affinity chromatography following the methodology in section 2.3.5.1. Elution fractions contained soluble [6His]- $\sigma^A$  of ~55 kDa, lanes 6-10, however contaminating *E. coli* RNAP was present, as displayed in the SDS-PAGE gel (Figure 28).

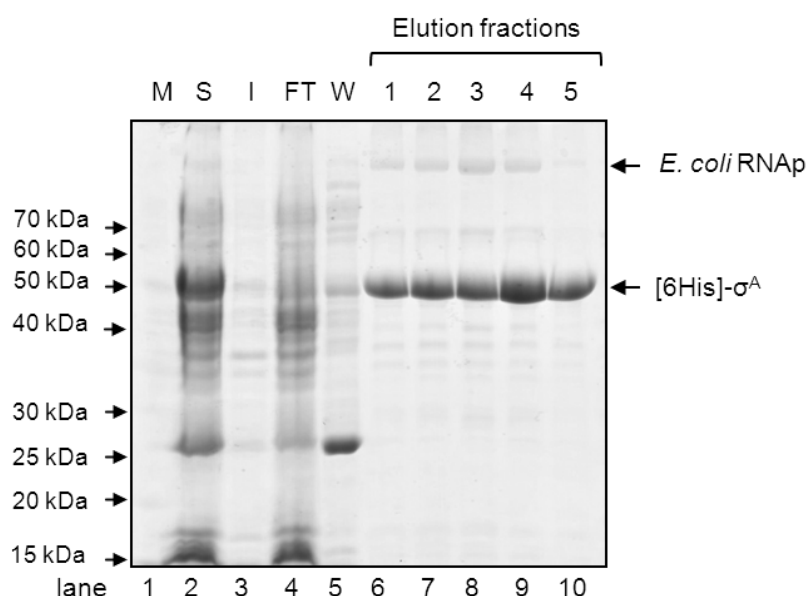


Figure 28: Image of a Coomassie stained 12.5% (w/v) SDS-PAGE gel showing the nickel affinity chromatography of [6His]- $\sigma^A$ .

The [6His]- $\sigma^A$  peak elution fractions are shown in lanes 6-10. Contaminating *E. coli* RNAP is marked by an arrow. M = Marker, S = Soluble fraction, I = Insoluble fraction, FT = Flow-through and W = Wash.

As [6His]- $\sigma^A$  was bound to the column independently or in complex with core RNAP, as  $E\sigma^A$ , the contaminating *E. coli* RNAP was removed using heparin affinity chromatography as in section 2.3.5.2. Elution fractions from the heparin column show that [6His]- $\sigma^A$  was eluted from the column earlier, lanes 8 and 9, in the NaCl gradient than the *E. coli* RNAP, lanes 10-12, therefore removing the contamination (Figure 29).

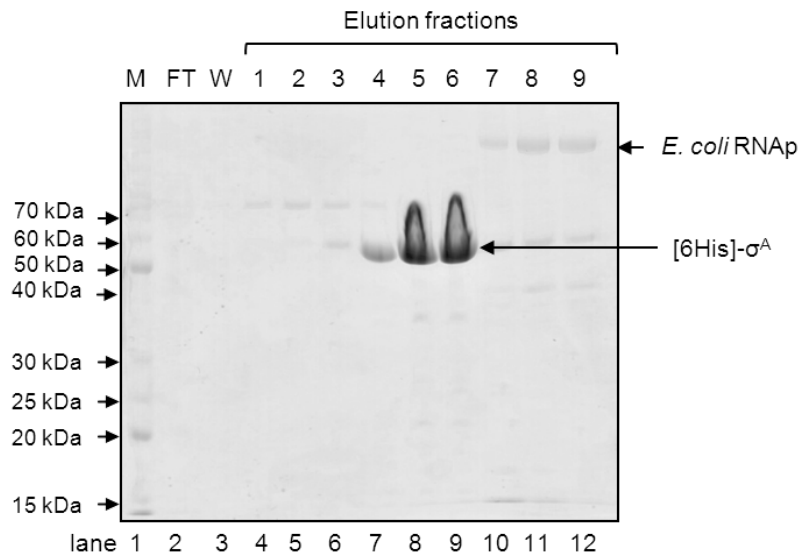


Figure 29: Image of a Coomassie stained 12.5% (w/v) SDS-PAGE gel showing the heparin affinity chromatography of [6His]- $\sigma^A$

The [6His]- $\sigma^A$  elution fraction are shown in lanes 5 and 6. The contaminating *E. coli* RNAP is marked by an arrow in elution fractions 7-9. M = Marker, FT = Flow-through and W = Wash.

### 3.2.2.2. *S. aureus* [6His]- $\sigma^B$ purification

*S. aureus* [6His]- $\sigma^B$  was purified by nickel affinity chromatography following the methodology in section 2.3.5.1. Elution fractions contained soluble [6His]- $\sigma^B$ , however protein contaminants were present, as displayed in the SDS-PAGE gel (Figure 30, lanes 8-12).

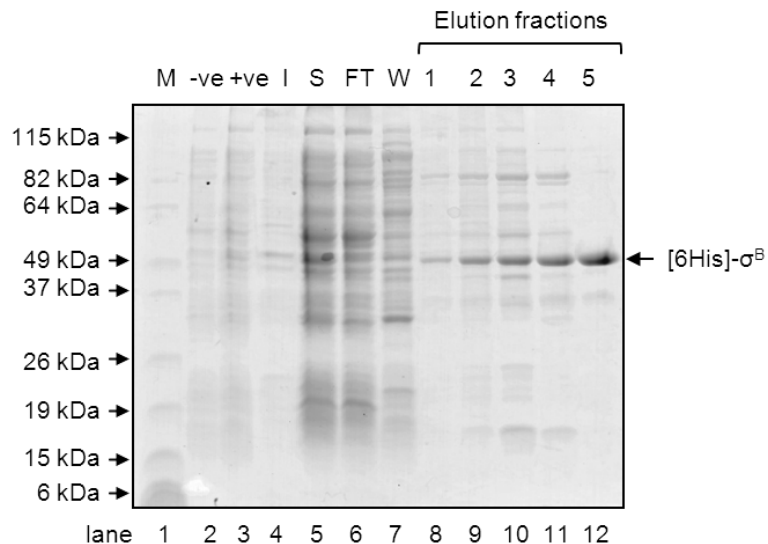


Figure 30: Image of a Coomassie stained 12.5% (w/v) SDS-PAGE gel showing the nickel affinity chromatography of [6His]- $\sigma^B$ .

The [6His]- $\sigma^B$  peak elution fractions are shown in lanes 8-12. M = Marker, -ve = un-induced, +ve = induced, I = Insoluble fraction, S = Soluble fraction, FT = Flow-through and W = Wash.

Protein contaminants were removed using heparin affinity chromatography as in section 2.3.5.2. Elution fractions from the heparin column showed that [6His]- $\sigma^B$  was eluted using a NaCl gradient at >90% purity, lanes 2-6, removing the contamination (Figure 31). Although a [6His]- $\sigma^B$  expression vector was constructed, expressed from and [6His]- $\sigma^B$  purified to a high purity it was never used for any of the work presented in this thesis.

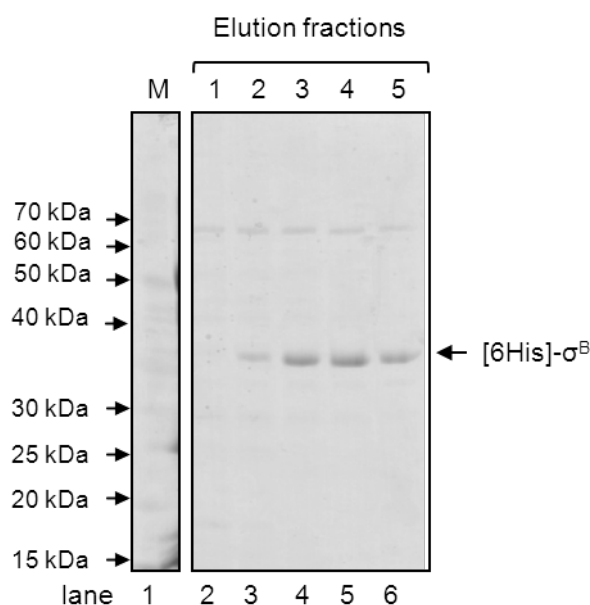


Figure 31: Image of a Coomassie stained 12.5% (w/v) SDS-PAGE gel showing the heparin affinity chromatography of [6His]- $\sigma^B$ .

The [6His]- $\sigma^B$  peak elution fractions are shown in lanes 2-6. M = Marker.

### 3.2.3. *S. aureus* SarA and SarR expression and purification

The transcriptional regulators SarA and SarR were both expressed under control of the pET expression system, as in section 2.3.2. The proteins were expressed in the *E. coli* expression strain BL21 under normal growth conditions as in section 2.3.1, with induction at mid-log phase using IPTG.

#### 3.2.3.1. *S. aureus* SarA purification

*S. aureus* [6His]-SarA was purified by a single step approach using nickel affinity chromatography following the methodology in section 2.3.5.1. Elution fractions contained soluble [6His]-SarA to a high purity, as displayed in the SDS-PAGE gel (Figure 32, lanes 11-13).

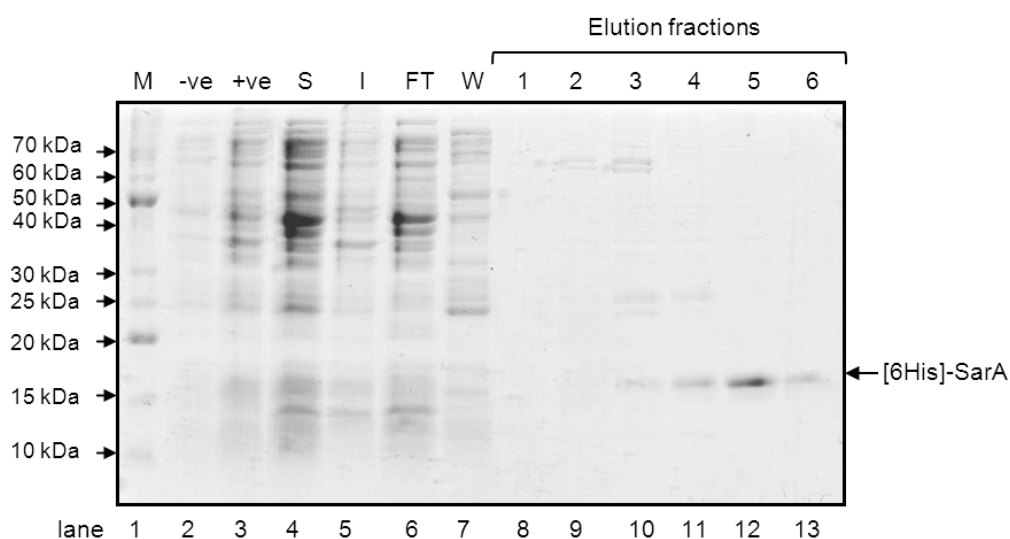


Figure 32: Image of a Coomassie stained 15% (w/v) SDS-PAGE gel showing the nickel affinity chromatography of [6His]-SarA.

The [6His]-SarA peak elution fractions are shown in lanes 11-13. M = Marker, -ve = un-induced, +ve = induced, S = Soluble fraction, I = Insoluble fraction, FT = Flow-through and W = Wash.

### 3.2.3.2. *S. aureus* SarR purification

*S. aureus* [6His]-SarR was also purified by a single step approach using nickel affinity chromatography following the methodology in section 2.3.5.1. Elution fractions contained soluble [6His]-SarR to a high purity, as displayed in the SDS-PAGE gel (Figure 33, lanes 11-13).

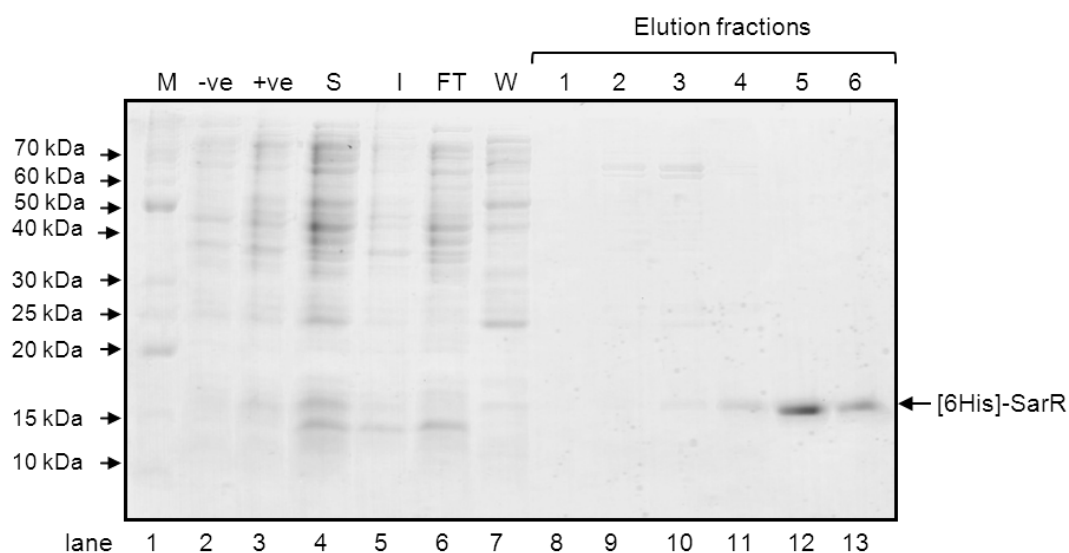


Figure 33: Image of a Coomassie stained 15% (w/v) SDS-PAGE gel showing the nickel affinity chromatography of [6His]-SarR.

The [6His]-SarR peak elution fractions are shown in lanes 11-13. M = Marker, -ve = Un-induced, +ve = Induced, S = Soluble fraction, I = Insoluble fraction, FT = Flow-through and W = Wash.

### 3.2.4. *S. aureus* AgrA expression and purification

AgrA was expressed under control of the pET expression system, as in section 2.3.2, in the *E. coli* BL21 (DE3) expression strain through induction with IPTG. Due to the problems expected in the solubility and purification of AgrA encountered in previous studies, the *agrA* gene was ligated into the pET28b+ vector to encode a N-terminal 6His tag, into the pET22b+ vector to encode a C-terminal 6His tag and into pET22b+ with a C-terminal stop codon inserted for expression in its native state. The expression of AgrA in these three forms increased the chance of successful expression and purification through reduced conformational changes and maximising the 6His availability.

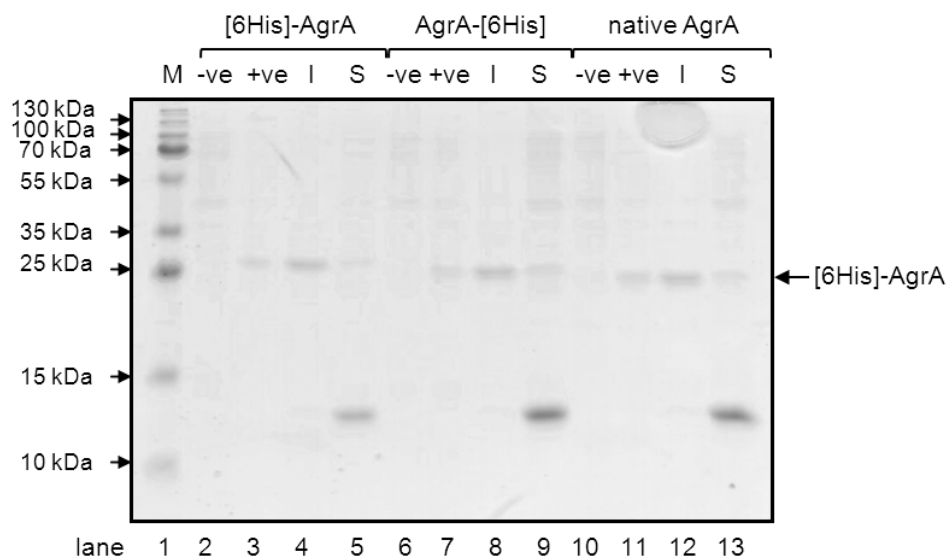


Figure 34: Image of a Coomassie stained 12.5% (w/v) SDS-PAGE gel showing the expression of *S. aureus* [6His]-AgrA, AgrA-[6His] and native AgrA.

M = Marker, -ve = Un-induced, +ve = Induced, I = Insoluble fraction and S = Soluble fraction.

As shown in Figure 34, AgrA was clearly expressed with an N-terminal 6His tag (compare lanes 2 and 3), a C-terminal 6His tag (compare lanes 6 and 7) and in its native form (compare lanes 10 and 11). However when the lysate was separated into insoluble and soluble fractions through centrifugation it was evident that all three forms of AgrA were mainly present in an insoluble form, compare lanes 4, 8 and 12 to 5, 9 and 13. As there was little AgrA expressed in a soluble form for purification, expression tests were performed using a selection of *E. coli* expression strains (Table 4) derived for the expression of toxic proteins and membrane proteins (Figure 35).

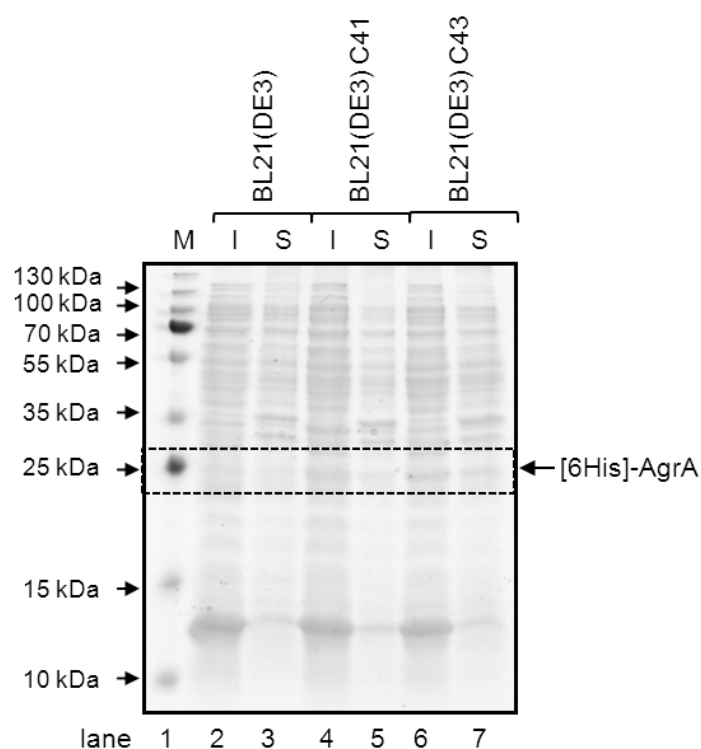


Figure 35: Image of a Coomassie stained 12.5% (w/v) SDS-PAGE gel showing the expression of [6His]-AgrA using a number of *E. coli* expression strains.

*S. aureus* [6His]-AgrA expressed from *E. coli* BL21 (DE3), BL21 (DE3) C41 and BL21 (DE3) C43 expression strains. [6His]-AgrA expression is boxed with a dashed line. M = Marker, I = Insoluble fraction and S = Soluble fraction.

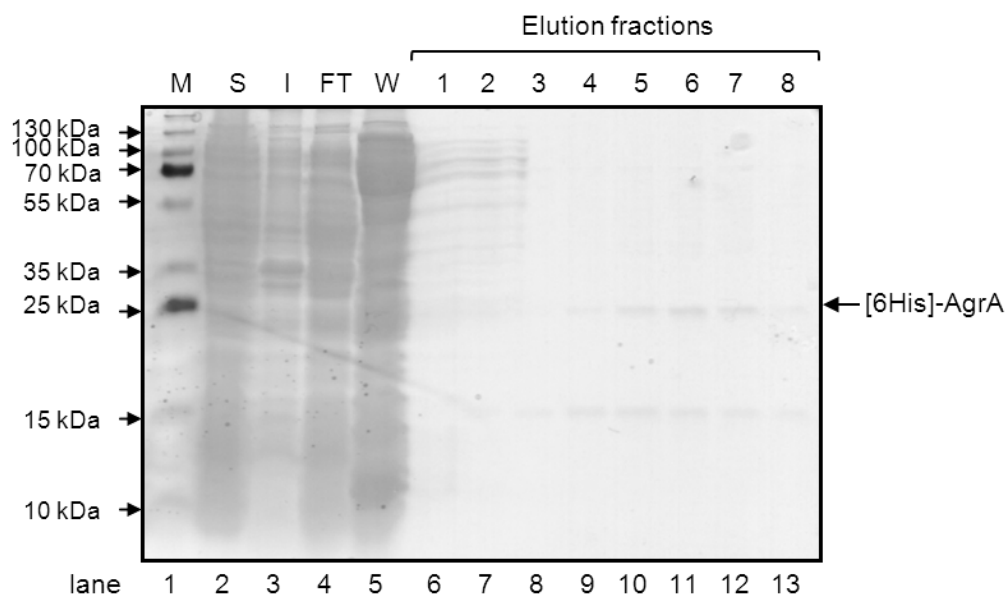


Figure 36: Image of a Coomassie stained 12.5% (w/v) SDS-PAGE gel showing the nickel affinity chromatography of [6His]-AgrA expressed using the *E. coli* BL21 (DE3) C41 expression strain.

The [6His]-AgrA peak elution fractions are shown in lanes 10-13. M = Marker, S = Soluble fraction, I = Insoluble fraction, FT = Flow-through and W = Wash.

Comparison of the expression of [6His]-AgrA in the *E. coli* expression strains BL21 (DE3), BL21 (DE3) C41 and BL21 (DE3) C43 identified a small increase in solubility when expressed in *E. coli* BL21 (DE3) C41, (Figure 35, compare lanes 3, 5 and 7). Therefore, the [6His]-AgrA expression in *E. coli* BL21 (DE3) C41 was scaled-up to a 1 L culture volume. The [6His]-AgrA was expressed with IPTG induction as described in section 2.3.1 and the cells lysed before purification by nickel affinity chromatography, as in section 2.3.5.1. The SDS-PAGE gel shown in Figure 36 showed that the 6His tag at the N-terminal of AgrA was accessible as [6His]-AgrA was able to bind to the Nickel column as it was eluted in the elution fractions, lanes 9-13. However [6His]-AgrA collected was at a low concentration and contained significant contaminants. This yield was too low for a further purification step so a new approach was required.

#### 3.2.4.1. Maltose Binding Protein AgrA fusion protein expression and purification

To attempt to increase the solubility of AgrA, it was expressed with a soluble N-terminal MBP fusion in the pMAL-c2x expression vector using the *E. coli* BL21 (DE3) expression strain and IPTG for induction, following section 2.3.1. The cell pellets were then lysed and the soluble fraction purified by amylose affinity chromatography as in section 2.3.5.3.

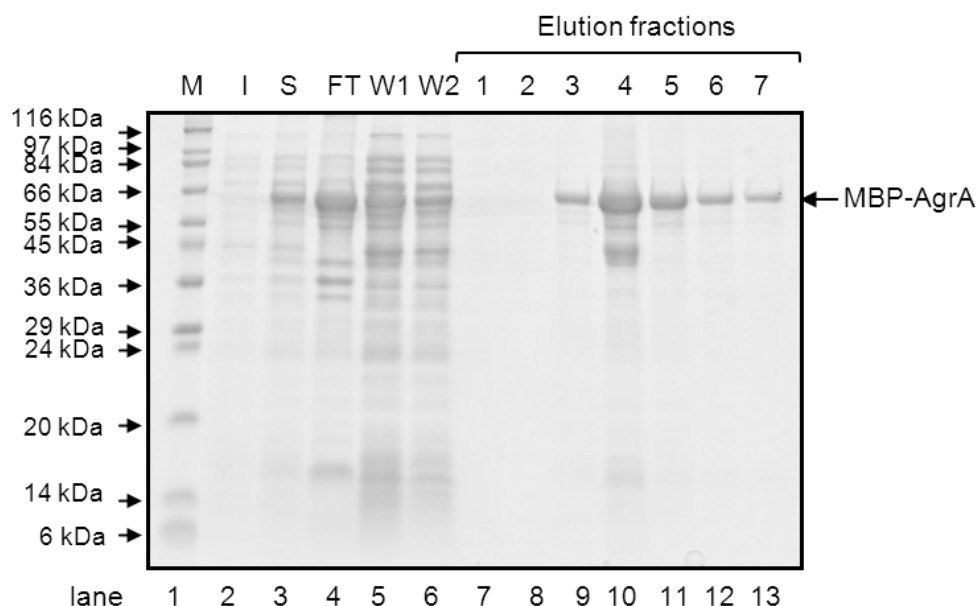


Figure 37: Image of a Coomassie stained 12.5% (w/v) SDS-PAGE gel showing the amylose affinity chromatography of MBP-AgrA

The MBP-AgrA peak elution fractions are shown in lanes 9-13. M = Marker, I = Insoluble fraction, S = Soluble fraction, FT = Flow-through and W1-2 = Wash 1 and 2.

The MBP-AgrA fusion protein was expressed in a soluble form and bound to the amylose column before being eluted at a high purity in the elution fraction (Figure 37, lanes 9-13). However, in order to ensure the highest sample purity the MBP-AgrA collected from the elution fractions was re-purified using a heparin affinity chromatography second purification step, as in section 2.3.5.2. The sample was loaded onto the heparin column and collected in a number of elution fractions (Figure 38, lanes 7-19). Fractions collected were of very high purity and therefore they were amassed together for further processing.

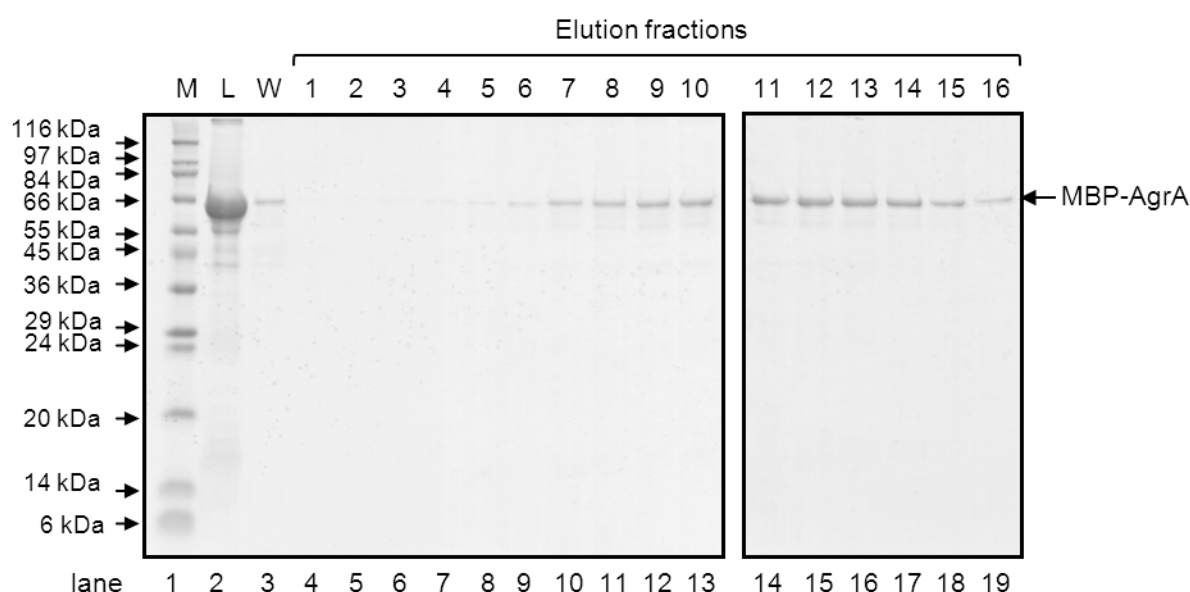


Figure 38: Image of a Coomassie stained 12.5% (w/v) SDS-PAGE gel showing the heparin affinity chromatography of MBP-AgrA.

The MBP-AgrA peak elution fractions are shown in lanes 7-19. M = Marker, L = Load, and W = Wash.

#### 3.2.4.2. Maltose Binding Protein AgrA fusion protein cleavage and purification

Once the MBP-AgrA fusion protein was successfully purified in a soluble form cleavage tests were setup to separate the MBP from the AgrA protein. As the MBP-AgrA fusion contains a linker with a Factor Xa cleavage site, cleavage tests were setup at 4°C and 23°C over a range of Factor Xa concentrations to optimise cleavage and maintain AgrA function. Cleavage samples run on a SDS-PAGE gel clearly showed that the cleavage is equally efficient at 4°C and 23°C (Figure 39, compare lanes 3-7 with lanes 9-13), therefore 4°C was chosen for further cleavage to maintain protein activity. The highest Factor Xa concentration of 2 units Factor Xa per µg MBP-AgrA was chosen to maximise MBP-AgrA cleavage (Figure 39, lane 3).

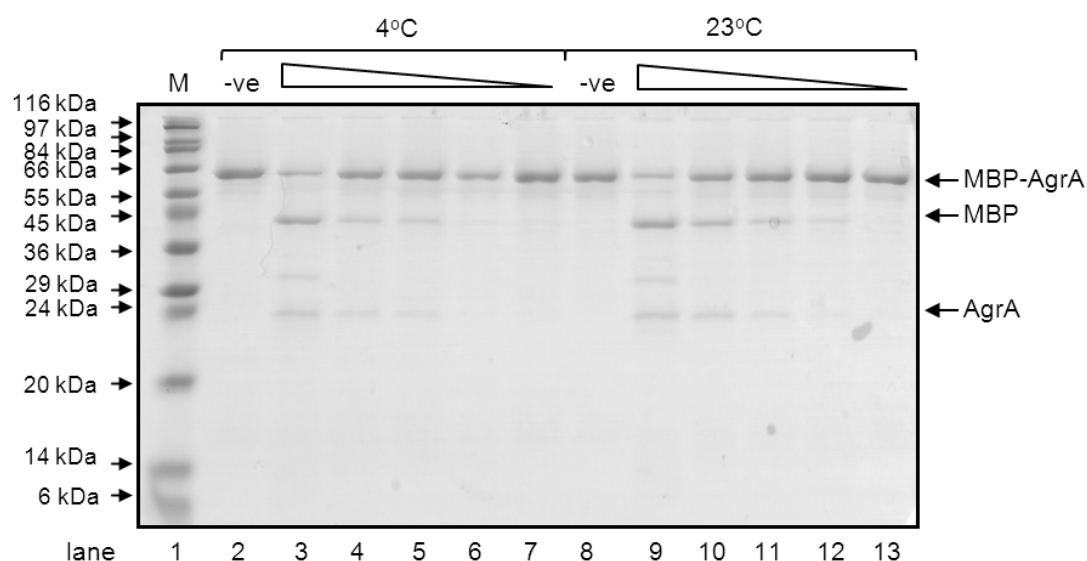


Figure 39: Image of a Coomassie stained 12.5% (w/v) SDS-PAGE gel showing the Factor Xa cleavage of MBP-AgrA under test conditions.

The MBP-AgrA cleavage was performed at 4°C overnight, lanes 2-7, and 23°C overnight, lanes 8-13 with Factor Xa concentration at 0.25, 0.5, 0.7, 1 and 2 units per µg MBP-AgrA (indicated by the gradient above the gel). M = Marker and -ve = Uncleaved.

The optimised cleavage reaction was then scaled up to cleave the whole MBP-AgrA preparation at 4°C overnight. Samples were run on a SDS-PAGE gel to check sample cleavage (Figure 40). The SDS-PAGE gel identified cleavage as expected, when comparing the un-cleaved sample in lane 2 to the cleaved sample in lane 3, however the yield of cleaved AgrA was low.

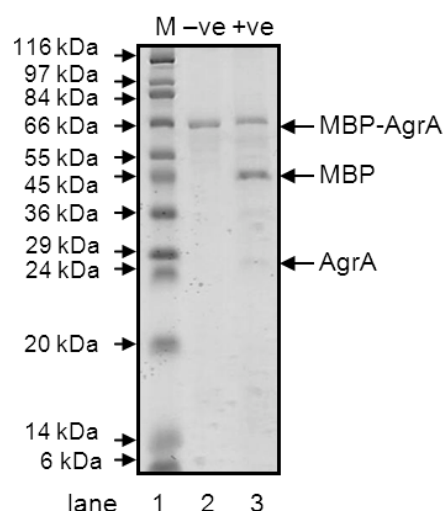


Figure 40: Image of a Coomassie stained 12.5% (w/v) SDS-PAGE gel showing the Factor Xa cleavage of MBP-AgrA.

The MBP-AgrA cleavage was performed at 4°C overnight, lane 3, with Factor Xa concentration at 2 units per µg MBP-AgrA. M = Marker, -ve = Un-cleaved and +ve = Cleaved.

The cleaved MBP-AgrA preparation was then re-purified by amylose affinity chromatography to separate the cleaved AgrA from the MBP, as in section 2.3.5.3.

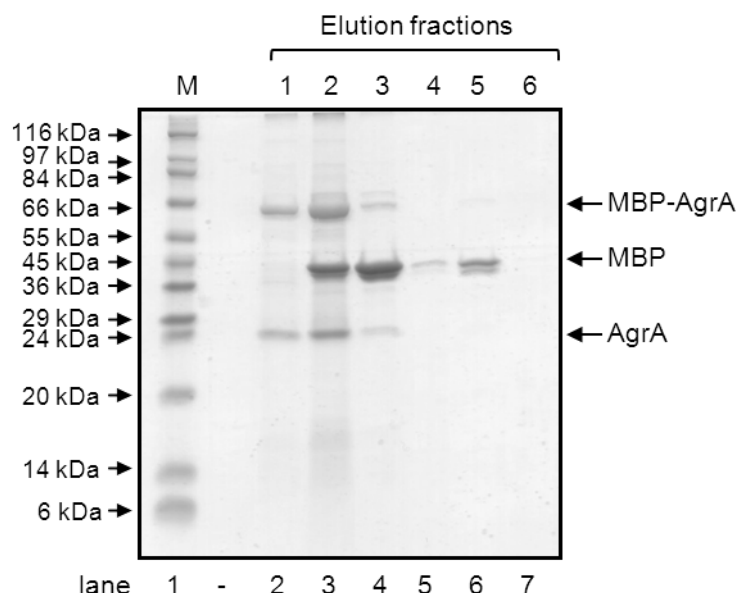


Figure 41: Image of a Coomassie stained 12.5% (w/v) SDS-PAGE gel showing the amylose affinity chromatography of Factor Xa cleaved MBP-AgrA.

The peak elution fractions are shown in lanes 2-7. M = Marker.

The results, shown in Figure 41, demonstrated that the cleaved AgrA was eluted in the elution fraction alongside the MBP-AgrA and the MBP proteins, see lanes 2-6. Therefore, cleaved AgrA was not successfully separated from the cleaved MBP.

After unsuccessful purification of the cleaved MBP-AgrA preparation using amylose affinity chromatography to try and separate the MBP and MBP-AgrA from the cleaved AgrA, as in section 2.3.5.3, another cleaved MBP-AgrA preparation was purified by heparin affinity chromatography, as in section 2.3.5.2. As shown in Figure 42, the purification of the cleaved preparation, shown in lane 3, was separated through binding and elution from the heparin column using a NaCl gradient. The cleaved MBP was collected in the flow-through as MBP does not bind to the heparin column, lane 4. The cleaved AgrA was collected in the elution fractions, lanes 5-14, however the fractions were contaminated with un-cleaved MBP-AgrA. Also the AgrA collected was at a very low concentration and barely visible on the SDS-PAGE gel. To obtain a soluble preparation of AgrA at a viable concentration a new approach was required.

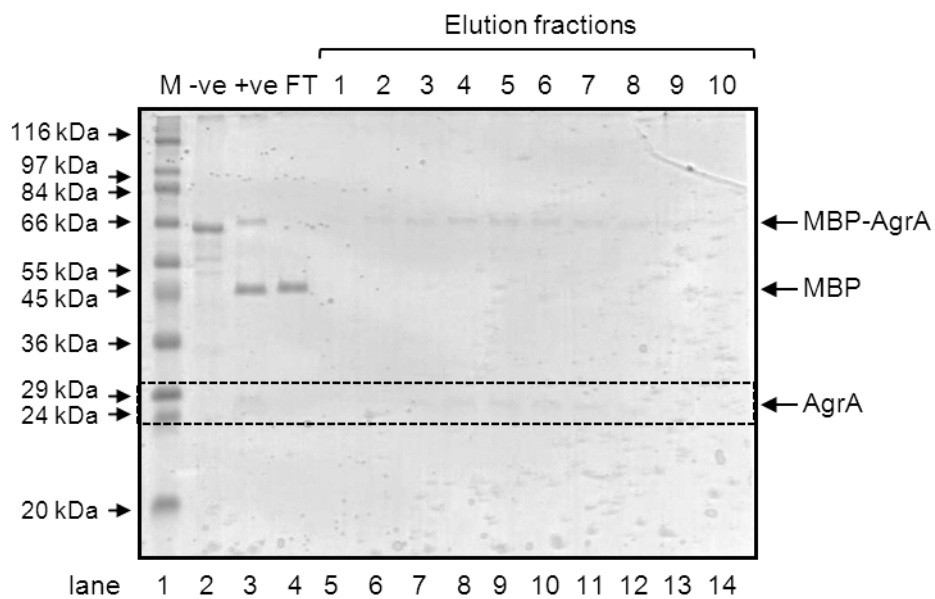


Figure 42: Image of a Coomassie stained 12.5% (w/v) SDS-PAGE gel showing the heparin affinity chromatography of Factor Xa cleaved MBP-AgrA.

The peak elution fractions are shown in lanes 5-14. M = Marker, -ve = Un-cleaved, +ve = Cleaved and FT = flow-through.

#### 3.2.4.3. [6His]-AgrA un-induced expression and purification

Previous studies using the pET vector system where the gene of interest is under control of a inducible T7 promoter have shown that a low basal level of target gene expression is achieved without induction (Dubendorff and Studier 1991). Therefore [6His]-AgrA was expressed in a 2 L culture volume of *E. coli* BL21 (DE3) as in section 2.3.1 except that the culture was grown un-induced for 3 hours after the exponential phase and the cell pellet collected. The [6His]-AgrA was then purified by a single step nickel affinity chromatography, as in section 2.3.5.1.

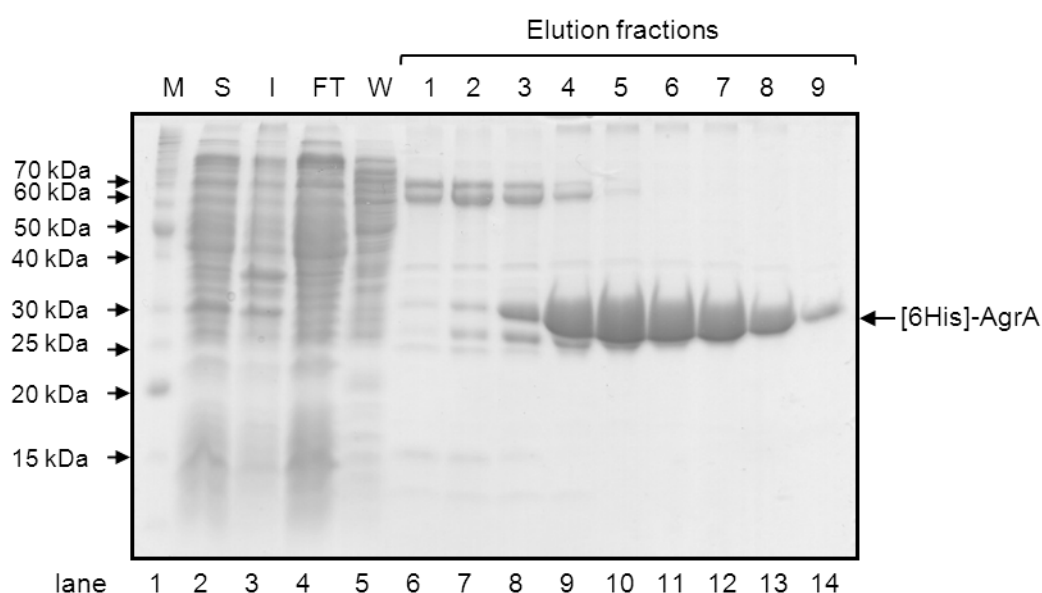


Figure 43: Image of a Coomassie stained 12.5% (w/v) SDS-PAGE gel showing the nickel affinity chromatography of un-induced [6His]-AgrA.

[6His]-AgrA was expressed un-induced in *E. coli* BL21 cells. The [6His]-AgrA peak elution fractions are shown in lanes 9-14. M = Marker, S = Soluble fraction, I = Insoluble fraction, FT = Flow-through and W = Wash.

Samples taken from the nickel affinity chromatography purification of [6His]-AgrA show that the [6His]-AgrA was eluted from the nickel column at a high concentration (Figure 43, lanes 9-14). [6His]-AgrA eluted in lanes 10-14 (Figure 43) was of high purity ( $\geq 95\%$ ) and was collected and dialysed into storage buffer for analysis. The [6His]-AgrA concentration from preparations was from 20-40  $\mu\text{M}$ , a concentration viable for transcriptional initiation analysis. Therefore the un-induced “leaky” expression of [6His]-AgrA in pET28b+ has allowed for a simple single step purification of [6His]-AgrA despite the problems associated with the usually insoluble response regulator.

### 3.3. DNA components

#### 3.3.1. *In vitro* transcription reporter plasmids

To try and determine the complex regulation at divergent promoters, the promoters had to be studied when both were present as well as independently. Therefore, the *agr* divergent promoters P2 and P3 were amplified from *S. aureus* NCTC 8325 with primers inserting restriction sites for digestion and ligation into the pLSR background reporter plasmid (Table 5).

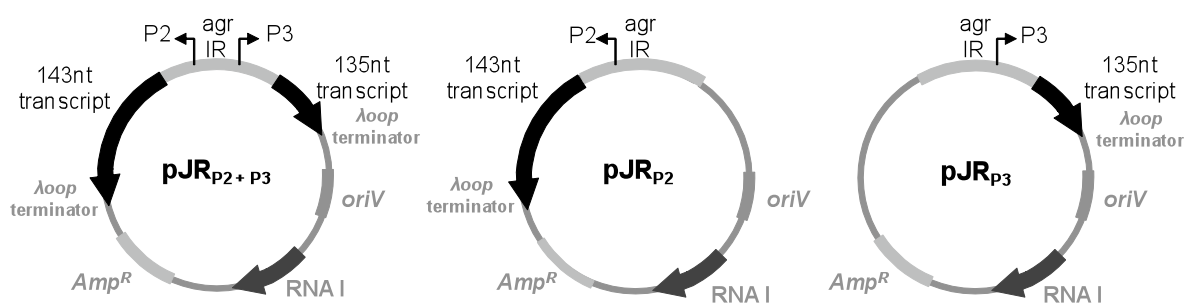


Figure 44: Schematic of the reporter plasmids used as templates for the *in vitro* transcription assays.

The supercoiled pJR<sub>p2+p3</sub> reporter plasmid reports transcription initiation from the divergent promoters through the transcription of discrete sized transcripts, 143 and 135 nt for P2 and P3 respectively. These transcripts are visualised through urea denaturing gel electrophoresis, as in section 2.4.1. The promoter region was also amplified with either the P2 or P3 promoter removed, however the entire *agr* IR containing the transcription regulator binding sites was maintained (Table 11). All the constructs were inserted into the pLSR background reporter plasmid and named pJR<sub>p2+p3</sub> for both promoters, pJR<sub>p2</sub> for only the P2 promoter and pJR<sub>p3</sub> for only the P3 promoter, as displayed in Figure 44. These reporter plasmids were used for the study of transcription initiation at the *agr* P2 and P3 promoters in both single round and multiple round transcription assays.

### 3.3.2. Short DNA probes

Linear DNA probes were designed to study the binding of RNAP and transcriptional regulators to the *agr* P2 and P3 promoters. The DNA probes were also used in short RNA synthesis assays to study transcription initiation from a linear DNA template, as opposed to from a supercoiled template, as in section 3.3.1. DNA probes were either amplified using primers, as in Table 9 (Appendix), to contain both *agr* P2 and P3 promoters as found in the native context, or duplex DNA formed from single stranded DNA for each promoter independently, as in Table 10 (section 2.4.3). DNA probes extended at least 10 base pairs beyond the transcription start site as shown in Figure 45, so that promoter length did not interfere with RNAP binding. DNA probes were <sup>32</sup>P labelled when used for visualisation in native gel electrophoresis, as in section 2.4.3.

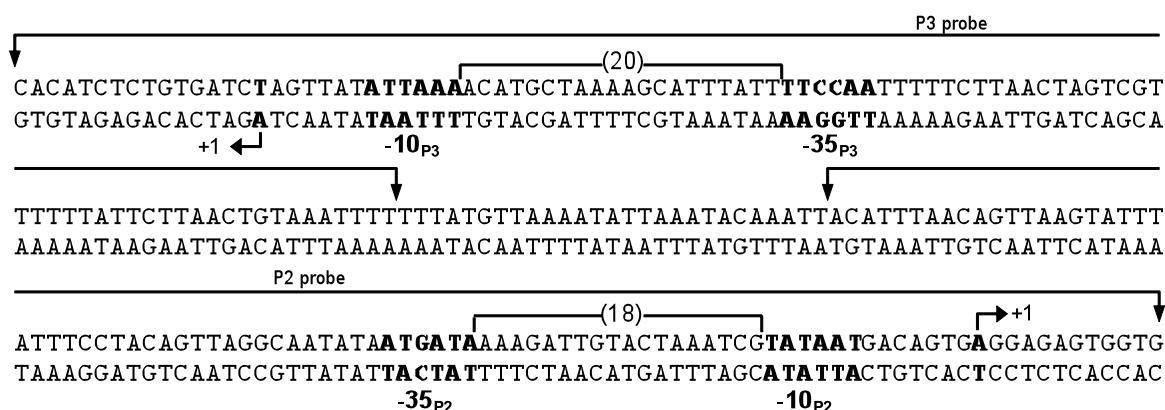


Figure 45: The sequence of the *agr* intergenic region from *S. aureus* NCTC 8325.

The sequence of the *agr* P2 and P3 promoters are shown with the consensus -10 and -35 hexamers highlighted in bold. The promoter consensus spacer length and the transcription start site (+1) are labelled. The sequence for the *agr* P2 and P3 probes is indicated.

### 3.4. Conclusions

All the components required to study transcription initiation *in vitro* at the *agr* operon were obtained from one relevant *S. aureus* strain (NCTC 8325). Therefore, removing obstacles such as inhibition or cross-activation by differing specificity groups has provided a useful tool for comparison with *in vivo* studies from the same strain. The components successfully obtained include the *S. aureus* NCTC 8325 core RNAP, the  $\sigma$  factors  $\sigma^A$  and  $\sigma^B$ , the transcriptional regulators AgrA, SarA and SarR and the DNA reporters and probes containing the *agr* P2 and P3 promoters.

This chapter describes a new method for the extraction and purification of *S. aureus* core RNAP using a polyol responsive resin. The core RNAP extracted had good activity, as shown in chapters 4 and 5, and could transcribe from the *agr* promoters when associated with the relevant  $\sigma$  factor. The chapter also describes a reproducible method for the expression and purification of the *agr* response regulator AgrA, despite the obstacles presented in expression (Sidote, Barbieri et al. 2008). This method involves the uninduced expression of [6His]-AgrA at a low basal level using the pET vector system. Previous studies have found a higher yield of target gene expression in the absence of the inducer in comparison to cells grown in the presence of the inducer, when both cultures are allowed to enter the stationary phase (Kelley, Huestis et al. 1995). This “leaky” expression could be relevant to

AgrA expression in a pET vector, as either higher yield would increase the amount of soluble protein collected or low yield could result in fewer higher oligomeric forms of AgrA that could lead to increased protein precipitation. Methods are also described for the simple and reproducible purification of the *S. aureus* regulators SarA and SarR and the  $\sigma$  factors  $\sigma^A$  and  $\sigma^B$ , which is invaluable for the efficient production of large numbers of mutants to study transcriptional regulation.

The *in vitro* transcription reporter plasmids generated provide an excellent tool for the study of transcription initiation at the *agr* divergent promoters simultaneously as well as independently. The generation of DNA probes combined with the reporter plasmids provide tools for the study of complex binding and transcription initiation events at the *agr* operon involving multiple regulators. Overall, the fully native *in vitro* transcription system that has been established is fully functional, as shown in section 5.2, allowing for the study of transcription initiation at the *agr* operon at the molecular and mechanistic level.

## CHAPTER FOUR

### 4. Investigating the interaction of $E\sigma^A$ with the *agr* P2 and P3 promoters

#### 4.1. Introduction

Transcription initiation at bacterial promoters is a multi-step process and the first step is the specific binding of the  $E\sigma$  to the promoter DNA. The  $E\sigma$  is directed to the promoters by the  $\sigma$  factor. The  $\sigma^{70}$ -type  $\sigma$  factors (E.g.  $\sigma^A$  found in *S. aureus*) direct the  $E\sigma$  to the hexameric recognition sequences at the -10 and -35 positions in relation to the transcription start site. This forms an initial  $E\sigma$ -DNA complex termed the closed promoter complex (RPc) where the DNA is in the double stranded form.

At many bacterial promoters, such as *E. coli*  $\lambda P_R$  and *lac* UV5, the RPc formed by  $E\sigma^{70}$  is short lived as it rapidly isomerises to the transcription-competent open complex (RPo) via a series of conformational changes through several intermediate  $E\sigma$ -promoter complexes (Saecker, Tsodikov et al. 2002; Gries, Kontur et al. 2010). These conformational changes include the opening of around 13 bp between the -10 and just after the transcription start site (+3) to place the transcription start site (+1) on the template strand in the RNAP catalytic cleft, reviewed in Gries et al. (2010). The RPc can only be identified at experimental conditions of  $<4^\circ\text{C}$  and can also be characterised by its sensitivity to the highly negatively charged compound heparin (Kovacic 1987). At  $37^\circ\text{C}$  the predominant RNAP-complex is the RPo, which is experimentally characterised by the opened DNA and its relatively more resistance to heparin than the RPc (Mangel and Chamberlin 1974). Transcriptional regulators, such as Agra, could help to form the RPc and/or RPo through several mechanisms of activation, see section 1.5.6.4.

When comparing the sequence of the *agr* P2 and P3 promoters it was noted that they had similar conservation to the -10 and -35 consensus promoter elements (5/6 and 4/6, and 6/6 and 4/6 for P2 and P3 respectively). However, the length of the P3 promoter spacer region (i.e. the distance separating the -10 and -35 elements) deviates significantly by three nucleotides (20) from the optimal  $17\pm 1$  nucleotides, whilst the P2 promoter spacer region length is optimal (18). The optimal  $17\pm 1$  nucleotide promoter spacer region is required to align the -10 and -35 promoter elements with  $\sigma^{70}$  regions  $\sigma_{2.4}$  and  $\sigma_{4.2}$  respectively, for

transcription initiation (Murakami, Masuda et al. 2002). Since P3 has a sub-optimal spacer, it should be a weaker promoter than P2, and  $E\sigma^A$  could potentially have a higher affinity for the P2 promoter than for the P3 promoter.

To investigate the interaction of  $E\sigma^A$  with the *agr* P2 and P3 promoters, EMSAs were performed at both  $<4^\circ\text{C}$ , to visualise the R<sub>Pc</sub>, and  $37^\circ\text{C}$ , where the R<sub>Po</sub> is expected to be the predominant  $E\sigma^A$ -DNA complex. The stability of any  $E\sigma^A$ -DNA complexes formed were tested by a heparin challenge to distinguish between R<sub>Pc</sub> and R<sub>Po</sub> formation. Furthermore, to determine whether any  $E\sigma^A$ -DNA complexes formed at  $37^\circ\text{C}$  contained the R<sub>Po</sub>, short RNA synthesis assays were also used. Short RNA synthesis assays test the output of promoter complexes by providing the components needed to transcribe the first few nucleotides from the transcription start site, as in section 2.4.6. The assays were all performed in the absence of AgrA, to determine if  $E\sigma^A$  can (productively) interact with the *agr* P2 and P3 promoters in the absence of any regulators.

#### **4.1.1. Objectives**

The overall objective of this part of the project was to investigate the interaction of  $E\sigma^A$  with the *agr* P2 and P3 promoters in the absence of AgrA. In this regard, the specific aims include:

- I. Determine whether  $E\sigma^A$  preferably binds to one *agr* promoter over the other or binds to the *agr* P2 and P3 promoters equally well at  $<4^\circ\text{C}$  and  $37^\circ\text{C}$ .
- II. Ascertain whether  $E\sigma^A$ -DNA complexes formed are transcriptionally productive at  $<4^\circ\text{C}$  and  $37^\circ\text{C}$ .

## 4.2. Eo<sup>A</sup> binding to the *agr* P2 and P3 promoters

### 4.2.1. <4°C Electrophoretic mobility shift assays

EMSAs were performed with Eo<sup>A</sup> at <4°C to quantify the initial Eo<sup>A</sup>-DNA complexes formed at the *agr* P2 and P3 promoters, following the protocol in section 2.4.5 with the following alteration: Eo<sup>A</sup> was titrated at 25, 50, 100 and 200 nM final concentrations into reactions containing the <sup>32</sup>P-labelled P2 and P3 probes at a final concentration of 5 nM. The <sup>32</sup>P labelled P2 and P3 probes (see Figure 45) contain the P2 and P3 promoter sequences and spans the -81 to +12 positions and the -80 to +16 positions for the P2 and P3 probe respectively.

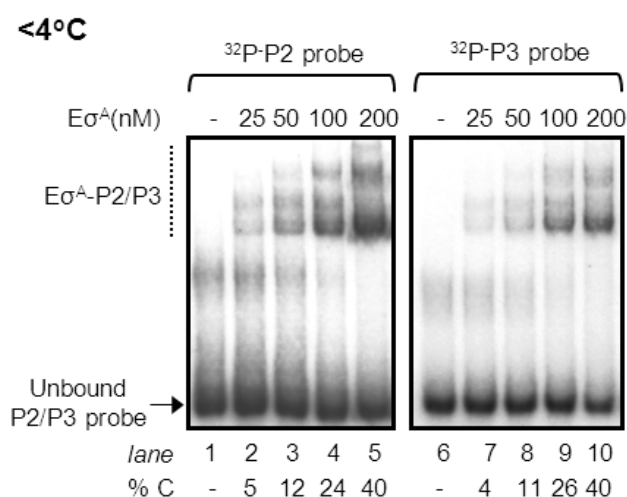


Figure 46: Image of a 4.5% (w/v) native-PAGE gel showing Eo<sup>A</sup> binding to the *agr* P2 and P3 promoters at <4°C.

The EMSA was performed as a function of Eo<sup>A</sup> concentration with 5 nM of promoter probe. The migration positions of the Eo<sup>A</sup>-P2/P3 complexes and the unbound P2 and P3 promoter probes are indicated. The percentage of the total DNA probe present in the reaction bound by Eo<sup>A</sup> is labelled as %C. In at least two independent experiments, the data range for each point fell within 15% of the %C value shown.

Results, as shown in Figure 46, demonstrate that Eo<sup>A</sup> was able to bind both *agr* promoters at <4°C. Eo<sup>A</sup> was able to bind to both the *agr* P2 and P3 promoter probes equally well, despite the potentially weaker P3 promoter sequence, for example, 40% of each promoter probe was bound by the highest concentration of Eo<sup>A</sup> (Figure 46, lanes 5 and 10). The multiple higher bands present may be explained by the additional non-specific binding of Eo<sup>A</sup> to the probes already bound by Eo<sup>A</sup>.

#### 4.2.1.1. Sensitivity of Eo<sup>A</sup>-promoter complexes formed at <4°C to heparin

To determine whether the complexes which were formed at <4°C were, as expected, heparin sensitive, and therefore represented the R<sub>Pc</sub> or an intermediate Eo<sup>A</sup>-promoter complex that has not reached the R<sub>Po</sub> status, EMSAs were performed as in section 2.4.5. Reactions contained 200 nM Eo<sup>A</sup> incubated with 5 nM of P2 or P3 promoter probes, which were subsequently challenged with 100 µg/ml heparin. Results, as shown in Figure 47, indicate that initial Eo<sup>A</sup>-DNA complexes formed were heparin sensitive at both the P2 and P3 promoters, compare lanes 1 and 3 with 2 and 4 respectively, with only 3-4% of complexes remaining. Therefore the Eo<sup>A</sup>-DNA complexes present at <4°C are most likely to be the early non-transcriptionally competent R<sub>Pc</sub> since the R<sub>Po</sub> are more heparin resistant.

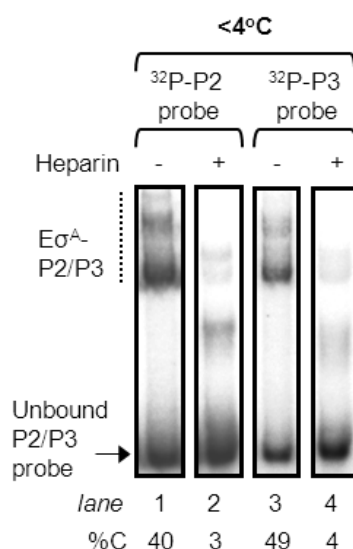


Figure 47: Image of a 4.5% (w/v) native-PAGE gel showing the heparin sensitivity of Eo<sup>A</sup>-DNA complexes formed at the *agr* P2 and P3 promoters at <4°C.

The EMSA was performed with 200 nM Eo<sup>A</sup> and 5 nM of either P2 or P3 promoter probes in the absence and presence of 100 µg/ml heparin. The migration positions of the Eo<sup>A</sup>-P2/P3 complexes and the unbound P2 and P3 promoter probes are indicated. The percentage of the total DNA probe present in the reaction bound by Eo<sup>A</sup> is labelled as %C. In at least two independent experiments, the data range for each point fell within 15% of the %C value shown.

#### 4.2.2. 37°C Electrophoretic mobility shift assays

At many bacterial promoters, at 37°C (which represents the physiologically relevant temperature for *S. aureus*) the R<sub>Pc</sub> is short lived and rapidly isomerises to the R<sub>Po</sub>, see section 4.1. To characterise the Eo<sup>A</sup>-DNA complexes formed at 37°C at the *S. aureus agr* promoters EMSAs were performed, as in Figure 46, as a function of Eo<sup>A</sup> concentration using

the *agr* P2 and P3 promoter probes, with the reactions performed at 37°C. As seen at <4°C, E $\sigma^A$  was able to bind the *agr* P2 and P3 promoters equally well at 37°C with 47% and 49% of the P2 and P3 promoter probes bound respectively at the highest E $\sigma^A$  concentration (200 nM) (Figure 48, lanes 5 and 10).

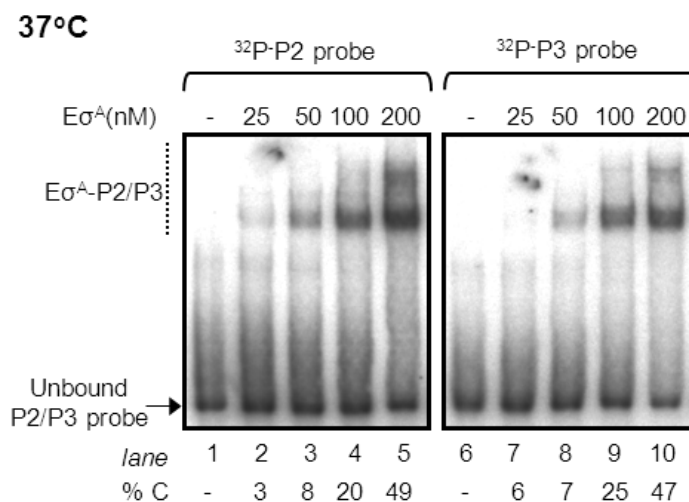


Figure 48: Image of a 4.5% (w/v) native-PAGE gel showing the binding of E $\sigma^A$  to the *agr* P2 and P3 promoters at 37°C.

The EMSA was performed as a function of E $\sigma^A$  concentration with 5 nM of promoter probe. The migration positions of the E $\sigma^A$ -P2/P3 complexes and the unbound P2 and P3 promoter probes are indicated. The percentage of the total DNA probe present in the reaction bound by E $\sigma^A$  is labelled as %C. In at least two independent experiments, the data range for each point fell within 15% of the %C value shown.

To determine whether the E $\sigma^A$ -DNA complexes formed at the *agr* P2 and P3 promoter probes at 37°C were [6His]- $\sigma^A$  dependent, EMSAs were performed using the conditions in Figure 48 with a E $\sigma^A$  concentration of 100 nM core RNAP (E). The experiment was carried out in the presence and absence of [6His]- $\sigma^A$  at a ratio of 4:1 (E: $\sigma^A$ ). Results indicated that the E $\sigma^A$ -DNA complexes formed at 37°C were [6His]- $\sigma^A$  dependent, as shown in Figure 49, with a decrease from 20% and 17% E $\sigma^A$ -DNA complexes at P2 and P3 respectively to 1% in the absence of [6His]- $\sigma^A$ , compare lanes 1 and 2 to 3 and 4.

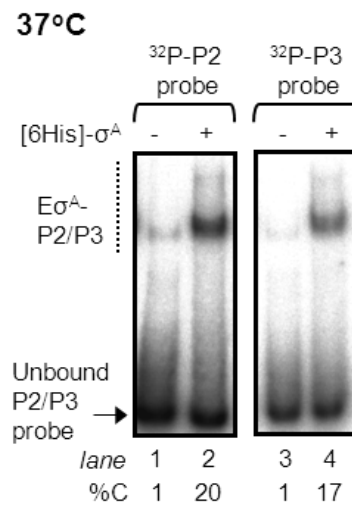


Figure 49: Image of a 4.5% (w/v) native-PAGE gel showing the  $\sigma^A$  dependent binding of  $E\sigma^A$  to the *agr* P2 and P3 promoters.

The EMSA was performed with 100 nM core RNAP and 5 nM of either P2 or P3 promoter probes in the absence and presence of 400 nM  $\sigma^A$ . The migration positions of the  $E\sigma^A$ -P2/P3 complexes and the unbound P2 and P3 promoter probes are indicated. The percentage of the total DNA probe present in the reaction bound by  $E\sigma^A$  is labelled as %C. In at least two independent experiments, the data range for each point fell within 15% of the %C value shown.

#### 4.2.2.1. Competition EMSAs

As the *agr* P2 and P3 promoters are both present, and therefore potentially compete in the native context,  $E\sigma^A$ -DNA complexes formed on the P2 and P3 promoter probes were challenged with the second unlabelled promoter probe at a 10 fold excess. The decrease in  $^{32}\text{P}$ -labelled DNA promoter probe complexes at P2 and P3 were compared to determine if  $E\sigma^A$  preferentially binds one promoter over the other when both promoters are available to  $E\sigma^A$ .

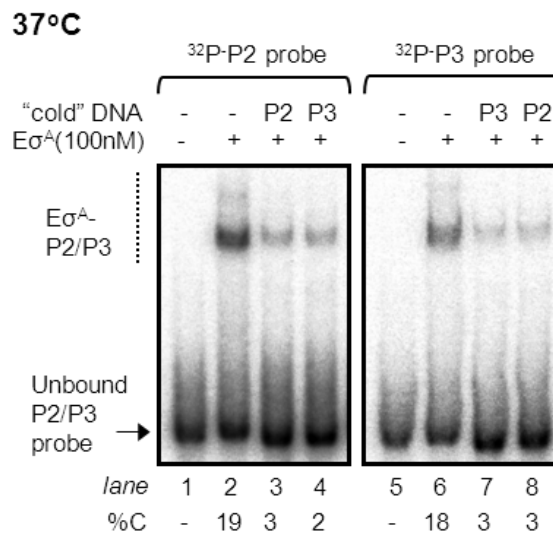


Figure 50: Image of a 4.5% (w/v) native-PAGE gel showing an Eσ<sup>A</sup> competition assay at the *agr* P2 and P3 promoters.

The Eσ<sup>A</sup> competition assay was performed with 100 nM Eσ<sup>A</sup> and 5 nM of either P2 or P3 promoter probes. The Eσ<sup>A</sup>-P2/P3 promoter complexes, lanes 2 and 6, were challenged with 10 fold excess unlabelled P2, lane 3 and 7, or P3, lane 4 and 8, promoter probes. The migration positions of the Eσ<sup>A</sup>-P2/P3 complexes and the unbound P2 and P3 promoter probes are indicated. In at least two independent experiments, the data range for each point fell within 15% of the %C value shown.

As shown in Figure 50, Eσ<sup>A</sup>-<sup>32</sup>P-P2 complexes formed at 37°C were challenged with a 10 fold excess of either P2 or P3 unlabelled promoter probes, both of which resulted in a decrease of initial Eσ<sup>A</sup>-<sup>32</sup>P-P2 complexes (by 85-90%, lanes 2-4). Likewise, when Eσ<sup>A</sup>-<sup>32</sup>P-P3 complexes were challenged with a 10 fold excess of unlabelled P3 or P2 promoter probes there was a similar decrease of initial Eσ<sup>A</sup>-<sup>32</sup>P-P3 complexes (by 85-90%, lanes 6-8). Taking into account these findings and the <4°C (Figure 46) and 37°C (Figure 48) EMSAs it was established that Eσ<sup>A</sup> does not have an initial preference for either the *agr* P2 or P3 promoter in the absence of AgrA and under these experimental conditions.

#### 4.2.2.2. Sensitivity of Eσ<sup>A</sup>-promoter complexes formed at 37°C to heparin

To determine whether the complexes formed at 37°C and in the absence of AgrA contained the heparin resistant RPo; EMSAs were performed, as in section 2.4.5, with 200 nM Eσ<sup>A</sup> incubated with 5 nM of P2 or P3 promoter probes in the presence or absence of 100 µg/ml heparin. Surprisingly, results, as shown in Figure 51, indicate that the Eσ<sup>A</sup>-DNA complexes formed at 37°C were heparin sensitive at both the P2 and P3 promoters with only 2-3% of

complexes surviving a heparin challenge, compare lanes 1 and 2 with 3 and 4 respectively. This implies that either the complexes formed on P2 and P3 at 37°C are not the RPo when AgrA is absent or the RPo formed at these promoters are more unstable than other characterised promoters e.g. *E. coli* lacUV5 (Buc and McClure 1985). Therefore, Eo<sup>A</sup>-promoter complexes formed at 37°C were tested for transcriptional competence in section 4.3.2.

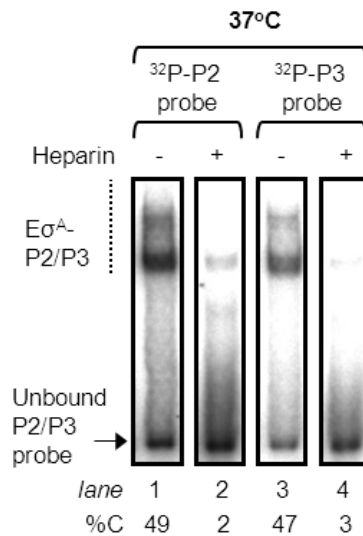


Figure 51: Image of a 4.5% (w/v) native-PAGE gel showing the heparin sensitivity of Eo<sup>A</sup>-DNA complexes formed at the *agr* P2 and P3 promoters at 37°C.

The stability assay was performed with 200 nM Eo<sup>A</sup> and 5 nM of either P2 or P3 promoter probes in the absence and presence of 100 µg/ml heparin. The migration positions of the Eo<sup>A</sup>-P2/P3 complexes and the unbound P2 and P3 promoter probes are indicated. The percentage of the total DNA probe present in the reaction bound by Eo<sup>A</sup> is labelled as %C. In at least two independent experiments, the data range for each point fell within 15% of the %C value shown.

### 4.3. Transcriptional competence of Eo<sup>A</sup>-P2/P3 complexes formed at <4°C and 37°C in the absence of AgrA

#### 4.3.1. RNAP catalytic assay

To determine whether the *S. aureus* core RNAP (E), extracted in section 3.2.1, was catalytically active at <4°C and 37°C (i.e. under conditions which the EMSAs were conducted), the catalytic activity of the *S. aureus* core RNAP (E) was initially measured at <4°C and 37°C, as in section 2.4.7. The RNAP catalytic assay uses a minimal nucleic acid scaffold (MS probe), as shown in Figure 24, and measures whether core RNAP can extend

the seven nucleotide RNA in the MS probe by a further one nucleotide using  $\alpha^{32}\text{P}$ -UTP. This assay does not require an  $\sigma$  factor as the MS probe does not contain the consensus promoter sequences. The  $\alpha^{32}\text{P}$ -labelled RNA can then be analysed by denaturing gel electrophoresis, as in section 2.4.1.

RNAp catalytic assays were performed as a function of *S. aureus* core RNAp concentration using 200 nM and 400 nM core RNAp with 100 nM MS probe. Results from the  $<4^\circ\text{C}$  RNAp catalytic assay, shown in Figure 52, demonstrate that core RNAp is able to extend the seven nucleotide RNA by one position at both RNAp concentrations and therefore indicates that the core RNAp is catalytically active at  $<4^\circ\text{C}$ , see lanes 1-2.

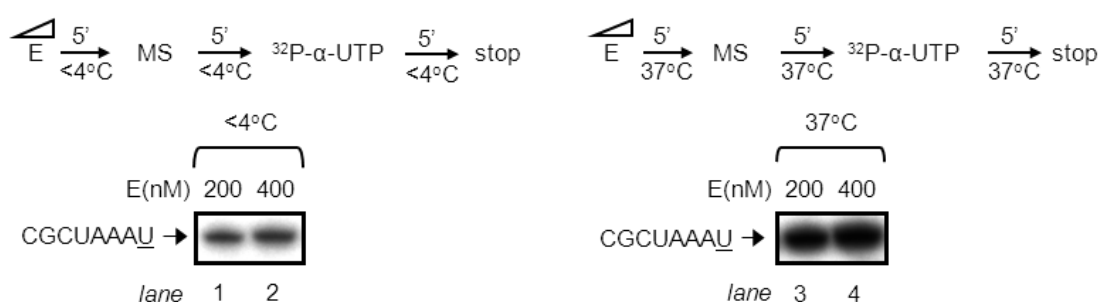


Figure 52: Image of a 20% (w/v) urea-denaturing-PAGE gel showing the ability of *S. aureus* core RNAp to extend the MS probe by one nucleotide at  $<4^\circ\text{C}$  and  $37^\circ\text{C}$ .

An RNAp catalytic assay showing the ability of *S. aureus* core RNAp to extend the RNA from the MS probe by one nucleotide at  $<4^\circ\text{C}$  and  $37^\circ\text{C}$  (RNA sequence indicated by the arrow, underlined nucleotide is  $^{32}\text{P}$ -labelled  $\alpha$ -UTP). The reactions were performed as a function of core RNAp (E). A schematic of the assay is shown above. MS = MS probe. In at least two independent experiments, the observed increasing trend between each data point for the quantity of MS probe extended at either  $<4^\circ\text{C}$  or  $37^\circ\text{C}$  was comparable.

The extension of the RNAp catalytic assays to  $37^\circ\text{C}$ , shown in Figure 52, as a function of core RNAp concentration using 200 nM and 400 nM RNAp with 100 nM MS probe, resulted as expected in an increased synthesis of the eight nucleotide RNA in comparison to the  $<4^\circ\text{C}$  assay, compare lanes 3 and 4 with lanes 1 and 2 respectively. Extension of the seven nucleotide RNA increased as a function of core RNAp concentration in both the  $<4^\circ\text{C}$  and  $37^\circ\text{C}$  assays (Figure 52, lanes 1-2 and 3-4). It was concluded that the *S. aureus* core RNAp is catalytically active at both  $<4^\circ\text{C}$  and  $37^\circ\text{C}$ .

### 4.3.2. Short RNA synthesis assays

Having determined that the *S. aureus* core RNAP was catalytically active at  $<4^{\circ}\text{C}$  and  $37^{\circ}\text{C}$  (Figure 52), short RNA synthesis assays were performed, as described in section 2.4.6, to assess whether the  $\text{E}\sigma^{\text{A}}$ -DNA complexes formed at  $<4^{\circ}\text{C}$  and  $37^{\circ}\text{C}$  at the *agr* P2 and P3 promoters were the transcriptionally competent RPo. In short RNA synthesis assays the transcriptional output from the  $\text{E}\sigma^{\text{A}}$ -DNA complexes can be quantitatively measured. Reactions were established as in previously described EMSAs, sections 4.2.1 and 4.2.2, using the same linear DNA probes as the DNA template. A short dinucleotide primer GpA, that complements the positions -1 and +1 of the P2 template strand and +2 and +3 of the P3 template strand, was then provided along with the following nucleotide,  $[\alpha\text{-}^{32}\text{P}]\text{-GTP}$  complementing the +2 position of the P2 template strand or  $[\alpha\text{-}^{32}\text{P}]\text{-UTP}$  complementing the +4 position of the P3 template strand. This results in the synthesis of the short RNA product GpAGG from the  $\text{E}\sigma^{\text{A}}$ -P2 promoter complexes and GpAU from the  $\text{E}\sigma^{\text{A}}$ -P3 promoter complexes, which was visualised by urea denaturing gel electrophoresis, as in section 2.4.1.

Results from the  $<4^{\circ}\text{C}$  short RNA synthesis assay, shown in Figure 53A, revealed that no short RNA products were synthesised from the P2 and P3 promoter probes at  $<4^{\circ}\text{C}$ , as shown in lane 1. This indicated that under these conditions the initial  $\text{E}\sigma^{\text{A}}$ -DNA complexes formed at  $<4^{\circ}\text{C}$  were not predominantly the RPo, since any  $\text{E}\sigma^{\text{A}}$ -DNA complexes present were not transcriptionally competent. Further control reactions were conducted in the absence of the promoter probe, GpA dinucleotide primer or  $\sigma^{\text{A}}$ , to indicate that any promoter-specific short RNA synthesis at the P2 or P3 promoters depends upon all the components being present and is therefore highly specific (Figure 53A, lanes 2-4).

A short RNA synthesis assay was then performed to determine whether the  $\text{E}\sigma^{\text{A}}$ -DNA complexes formed at  $37^{\circ}\text{C}$  were transcriptionally competent and therefore were predominantly the RPo. Results, as shown in Figure 53B, show that short RNA products were synthesised from both the P2 and P3 promoter probes at  $37^{\circ}\text{C}$ , lane 1. Control reactions omitting the promoter probe, GpA dinucleotide primer or  $\sigma^{\text{A}}$  were performed to show that the promoter-specific short RNA synthesis at the P2 or P3 promoters depends upon all the components being present and is therefore highly specific, as shown in lanes 2-4. This indicates that the  $\text{E}\sigma^{\text{A}}$ -DNA complexes formed at  $37^{\circ}\text{C}$  were predominantly the

transcriptionally competent RPo, despite  $E\sigma^A$ -DNA complexes formed at 37°C being heparin sensitive (Figure 51). Therefore, as the core RNAP is catalytically active at <4°C and 37°C (Figure 52),  $E\sigma^A$  can only form promoter specific *bona fide* RPo at the *agr* P2 and P3 promoters at 37°C.

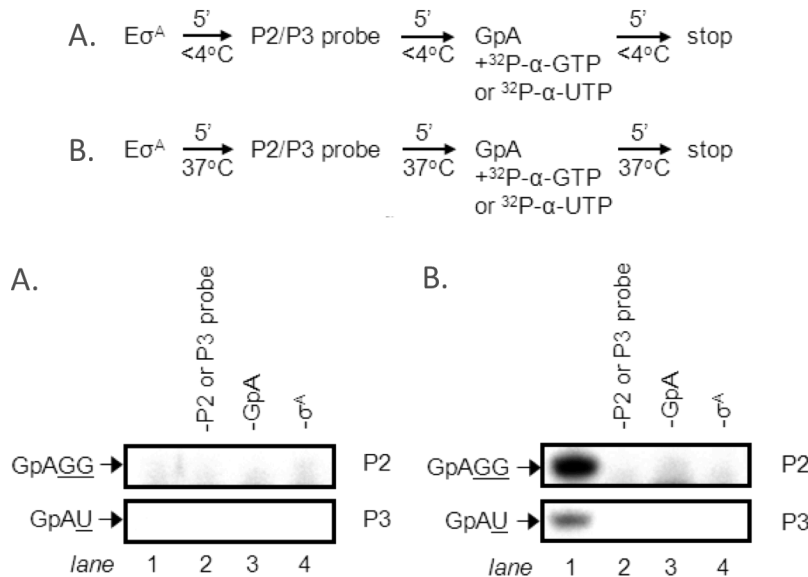


Figure 53: Image of a 20% (w/v) urea-denaturing-PAGE gel showing the synthesis of short RNA products from P2 and P3 by  $E\sigma^A$  at <4°C and 37°C.

**A.** A short RNA synthesis assay showing the synthesis of the short RNA products GpAGG (from P2) and GpAU (from P3) at <4°C by  $E\sigma^A$ , lane 1 (indicated by the arrow, underlined nucleotides are  $^{32}\text{P}$ -labelled). The reactions were performed in the absence of the P2 or P3 probes, lane 2, the GpA dinucleotide, lane 3 or  $\sigma^A$ , lane 4. A schematic of the assay is shown above. **B.** A short RNA synthesis assay showing the synthesis of the short RNA products GpAGG (from P2) and GpAU (from P3) at 37°C by  $E\sigma^A$ , lane 1 (indicated by the arrow, underlined nucleotides are  $^{32}\text{P}$ -labelled). The reactions were performed in the absence of the P2 or P3 probes, lane 2, the GpA dinucleotide, lane 3 or  $\sigma^A$ , lane 4. A schematic of the assay is shown above. In at least two independent experiments, the observed trend between each data point for the quantity of short RNA products synthesised from either the P2 or P3 promoter probe at <4°C and 37°C was comparable.

#### 4.4. Conclusions

Surprisingly, results showed that despite the atypical spacer length at the *agr* P3 promoter when compared to the *agr* P2 promoter, initial  $E\sigma^A$ -DNA promoter complexes formed equally well at <4°C (Figure 46) and 37°C (Figure 48) in the absence of AgrA.  $E\sigma^A$ -DNA complexes formed were shown to be  $\sigma^A$  dependent (Figure 49). Competition assays, where

both *agr* promoters were available for  $E\sigma^A$  as in the native context, also indicated that  $E\sigma^A$  does not have an initial preference to either the *agr* P2 or P3 promoter under these experimental conditions (Figure 50). Therefore, in the absence of AgrA or any regulators, the *agr* P2 and P3 promoters appear to compete equally well for  $E\sigma^A$  under the conditions tested.

Heparin stability assays revealed that  $E\sigma^A$ -DNA complexes formed at the *agr* P2 and P3 promoters were equally heparin sensitive at  $<4^\circ\text{C}$  (Figure 47) and  $37^\circ\text{C}$  (Figure 51). This could indicate that the  $E\sigma^A$  promoter complexes present at  $37^\circ\text{C}$  were not the more heparin resistant RPo, or that RPo accumulate slowly in these experimental conditions, or that the RPo are unstable and short lived in the presence of heparin.

To test whether any of these hypotheses were correct, short RNA synthesis assays were performed. They indicated that  $E\sigma^A$ -DNA complexes formed at both the *agr* P2 and P3 promoters at  $37^\circ\text{C}$  were predominantly the RPo, as  $E\sigma^A$ -DNA complexes were transcriptionally competent (Figure 53B). However, this assay alone cannot be used to compare transcriptional output and therefore transcription initiation between the P2 and P3 promoters as the short RNA products incorporate a differing number of  $^{32}\text{P}$ -labelled nucleotides and (GG for P2 and U for P3) and different  $^{32}\text{P}$ -labelled nucleotides are used for each promoter. Short RNA synthesis assays performed at  $<4^\circ\text{C}$  were also used to demonstrate that initial  $E\sigma^A$ -DNA complexes formed at the *agr* P2 and P3 promoters were not transcriptionally competent and therefore were not predominantly the RPo (Figure 53A).

The RNAP catalytic assays performed at  $<4^\circ\text{C}$  (Figure 52) were able to satisfy that the *S. aureus* core RNAP extracted in section 3.2.1 was catalytically active at  $<4^\circ\text{C}$  and therefore could be used to determine  $E\sigma^A$ -DNA complexes formed at  $<4^\circ\text{C}$ . As presented in this chapter,  $E\sigma^A$  does not have a preference for either *agr* promoter under the conditions tested, however  $E\sigma^A$  can form promoter specific *bona fide* RPo at either the *agr* P2 or P3 promoters at  $37^\circ\text{C}$  in the absence of AgrA. Further analysis is required with both promoters present, as in the native context, and in the presence of regulators, to compare transcriptional initiation from the two *agr* promoters P2 and P3, as AgrA could help to form the R<sub>Pc</sub> and/or R<sub>Po</sub>, see section 1.4.4 and 1.5.6.4.



## CHAPTER FIVE

### 5. Investigating AgrA-dependent and independent transcription initiation by Eo<sup>A</sup> at the *agr* P2 and P3 promoters

#### 5.1. Introduction

It is widely regarded that the basal transcriptional activity from the P2 promoter of the *agr* operon is important to allow for the constant presence of the TCS partners, AgrA and AgrC, at low levels (Novick, Projan et al. 1995). This is to ensure that the *agr* operon is ready to mount a quorum response.

The *agr* operon promoters, P2 and P3, are separated by a ~180 bp intergenic region (*agr* IR) in a back-to-back arrangement, and transcribe in the opposite direction (divergent) to each other (Figure 7). In the previous chapter (Chapter 4) it was established that Eo<sup>A</sup> can interact and form the transcriptionally competent RPo equally well at 37°C at the *agr* P2 and P3 promoters, in the absence of any regulators. This was despite the atypical length of the *agr* P3 promoter spacer. However, as the short RNA synthesis assays cannot be used to compare the basal transcription from the P2 and P3 promoters simultaneously (as in the native context) or independently, a full length *in vitro* transcription assay needed to be established using a reporter plasmid containing both the *agr* P2 and P3 promoters. The reporter plasmid pJR<sub>P2+P3</sub>, constructed in section 3.3.1, containing both the *agr* promoters, allows for the monitoring of transcription initiation when both promoters are present (Figure 44). Whereas, the reporter plasmids pJR<sub>P2</sub> and pJR<sub>P3</sub>, containing either the P2 or P3 promoter, allow the monitoring of transcription initiation when only one promoter is present (Figure 44). Full length *in vitro* transcription assays using either pJR<sub>P2+P3</sub>, pJR<sub>P2</sub> or pJR<sub>P3</sub> reporter plasmids were used to compare the effect of transcription initiation from P2 and P3 when present in the back-to-back orientation (as in the native context) using plasmid pJR<sub>P2+P3</sub> and when present separated from the divergent promoter using plasmids pJR<sub>P2</sub> and pJR<sub>P3</sub> (Figure 44).

The occupation of a promoter by Eo and its regulation by transcription regulators have been shown to have an effect on its divergent promoter (if present). One study of the *E. coli* cAMP receptor protein (CAP) at two Class II CRP-dependent promoters demonstrated,

through the use of permanganate footprinting, that  $E\sigma$  was not able to occupy two divergent promoter's simultaneously, even though *in vivo* studies indicated one promoter had no effect on the divergent promoters activation (El-Robh and Busby 2002). The effect of promoter occupation on a divergent promoter is also demonstrated by the *E. coli gcd* and *hpt* genes involved in sugar metabolism and base and nucleotide utilisation (Izu, Ito et al. 2002). Here  $E\sigma$  bound to the *hpt* promoter had a negative effect on the *gcd* promoter by interfering with  $E\sigma$  interactions at the *gcd* promoter.

A number of mechanisms may be involved in divergent promoter transcription. Firstly short distances between promoters could lead to steric hindrance, where one promoter is physically blocked from binding to  $E\sigma$  by the occupation of the divergent promoter by  $E\sigma$ , leading to a negative effect on transcription initiation. Another mechanism takes into account a change in DNA topology by  $E\sigma$  binding to one promoter. These topological changes of the DNA influence the binding of another  $E\sigma$  molecule to the divergent promoter with either a negative or positive outcome (Borowiec and Gralla 1986). Thirdly, the binding of a regulator protein to sites located in the promoter region may affect  $E\sigma$  binding to either promoter. This reinforces the need to study divergent promoters at the *in vitro* level to fully understand transcriptional initiation.

In the *agr* operon the response regulator AgrA, of the AgrC/AgrA TCS, is a major transcriptional regulator with binding sites at both the *agr* P2 and P3 promoters, see section 1.4.4 (Koenig, Ray et al. 2004). The importance of AgrA in *S. aureus* virulence has been demonstrated in the *S. aureus* strain RN4220, which is non-haemolytic due to a slipped-mispairing mutation in AgrA leading to delayed *agr* activation (Traber and Novick 2006). AgrA is thought to be required for activation of the P2 promoter through auto-activation to maintain a basal transcription level to mount a quorum response. However, the understanding of AgrA activation on transcription from the *agr* P2 and P3 promoters and the level of transcription initiation in the presence of AgrA is relatively unknown.

To investigate the basal level, i.e. AgrA independent, of transcription initiation by  $E\sigma^A$  at the *agr* P2 and P3 promoters, full length *in vitro* transcription assays were performed at 37°C using the reporter plasmids containing one or both of the *agr* P2 and P3 promoters. Heparin

stability assays were performed, using the same reporter plasmids, to compare the stability, i.e. longevity, of the RPo formed at the P2 or P3 promoter. EMSAs and full length *in vitro* transcription assays were performed to determine how AgrA affects Eo<sup>A</sup> occupation and transcription initiation at the *agr* P2 and P3 promoters. Heparin stability assays were also used, alongside order of addition assays, to further unravel the mechanism of AgrA transcription regulation.

#### **5.1.1. Objectives**

The overall objective of this part of the project was to investigate the basal transcriptional activity of the *agr* P2 and P3 promoters and the effect of AgrA on transcription initiation at the *agr* operon *in vitro*. In this regard, the specific aims include:

- I. Determine the basal transcription initiation of Eo<sup>A</sup> from the *agr* P2 and P3 promoters in the absence of any regulators.
- II. Determine how AgrA affects Eo<sup>A</sup> binding to the *agr* P2 and P3 promoters.
- III. Determine how AgrA affects transcription initiation from the *agr* P2 and P3 promoters.

## 5.2. Determining *agr* P2 and P3 basal transcription activity

### 5.2.1. Single round full length *in vitro* transcription assays

Once it was established that  $E\sigma^A$  interacts equally well with the *agr* P2 and P3 promoters, and can form RPo at both the *agr* promoters in the absence of AgrA (Chapter 4), transcriptional initiation from the *agr* P2 and P3 promoters was measured when the promoters were available to  $E\sigma^A$  in their native divergent setting. A fully native *in vitro* transcription assay was performed using the supercoiled reporter plasmids, constructed as in section 3.3.1, as promoter templates to replicate the *in vivo* organisation of the *agr* P2 and P3 promoters. The assay, following the protocol in section 2.4.8 and the schematic in Figure 54, was performed as a function of  $E\sigma^A$  concentration, where  $E\sigma^A$  was pre-incubated with the pJR<sub>P2+P3</sub> reporter plasmid at 37°C to form the RPo before the addition of ribonucleotides including [ $\alpha$ -<sup>32</sup>P] labelled UTP. The assay was performed where only a single round of transcription can occur through the addition of the DNA competitor heparin.

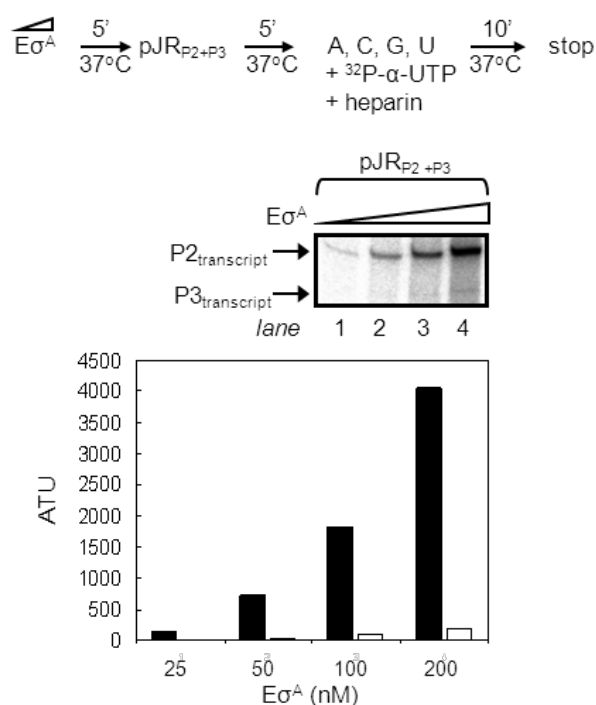


Figure 54: Image of a 10% (w/v) urea-denaturing-PAGE gel showing Eσ<sup>A</sup> single round *in vitro* transcription initiation from pJR<sub>P2+P3</sub>.

Transcripts were synthesised from the pJR<sub>P2+P3</sub> reporter plasmid at 37°C by Eσ<sup>A</sup> (indicated by the arrows). The reactions were performed as a function of Eσ<sup>A</sup> concentration under single round conditions. A schematic of the assay is shown above. The amount of transcripts synthesised from P2 (black bars) and P3 (white bars) are expressed as arbitrary transcription units (ATU), derived from the band intensity calculated from the gel imaged by phosphorimager analysis (see section 2.4.1). In at least three independent experiments, the observed increasing trend between each data point for the quantity of transcripts synthesised from either the P2 or P3 promoters was comparable.

RNA transcripts visualised on a urea-denaturing-PAGE gel showed that under these experimental conditions transcripts originating from the P2 promoter were ~15-20 fold higher at all concentrations of Eσ<sup>A</sup> when compared to transcripts originating from P3 (Figure 54) in the absence of AgrA. Interestingly, when the assay was conducted under the same experimental conditions with only the P2 or P3 promoter present (using either pJR<sub>P2</sub> or pJR<sub>P3</sub>) similar results were obtained (Figure 55). Transcripts originating from the P2 promoter were ~5-10 fold higher than those originating from P3, with the same trend in transcriptional output.

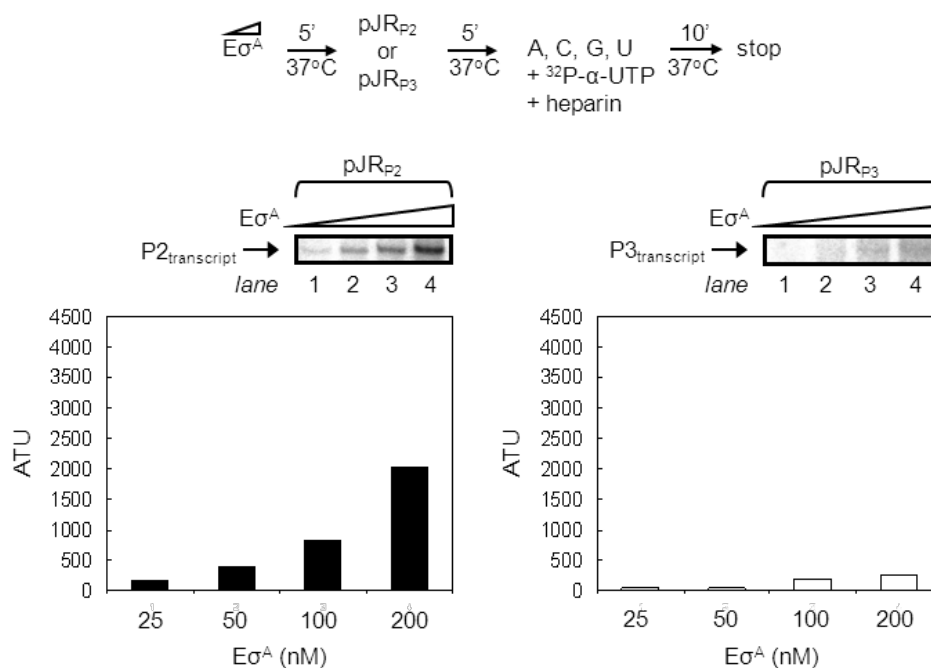


Figure 55: Image of a 10% (w/v) urea-denaturing-PAGE gel showing Eσ<sup>A</sup> single round *in vitro* transcription initiation from pJR<sub>P2</sub> and pJR<sub>P3</sub>.

Transcripts were synthesised from the pJR<sub>P2</sub> and pJR<sub>P3</sub> reporter plasmids at 37°C by Eσ<sup>A</sup> (indicated by the arrows). The reactions were performed as a function of Eσ<sup>A</sup> concentration under single round conditions. A schematic of the assay is shown above. The amount of transcripts synthesised from P2 (black bars) and P3 (white bars) are expressed as arbitrary transcription units (ATU), derived from the band intensity calculated from the gel imaged by phosphorimager analysis (see section 2.4.1). In at least three independent experiments, the observed increasing trend between each data point for the quantity of transcripts synthesised from the P2 or P3 promoters was comparable.

As it was previously shown that Eσ<sup>A</sup> can initially interact equally well with P2 and P3 to form the R<sub>Pc</sub> and R<sub>Po</sub>, the results from the single round *in vitro* transcription assay appear to indicate that R<sub>Po</sub> formation at the P3 promoter could occur more slowly than at P2 in the absence of regulators. Alternatively, as shown by the heparin sensitivity of the Eσ<sup>A</sup> promoter complexes in Figure 47 and Figure 51, the R<sub>Po</sub> formed at the P3 promoter may be more unstable and dissociate more rapidly upon heparin exposure than the P2 promoter under these single-round experimental conditions.

### 5.2.2. Multiple round full length *in vitro* transcription assays

To better reflect the native *agr* operon promoter conditions and address the questions posed by the reduced transcription from P3 in previous experiments, multiple round *in vitro* transcription assays were performed as a function of time in the absence of the competitor

heparin, and in the absence of AgrA. Under these experimental conditions, following the schematic in Figure 56, multiple rounds of transcription were allowed to occur with  $E\sigma^A$  from the  $pJR_{P2+P3}$  reporter plasmid with the reaction stopped at set time intervals. The 100 nM  $E\sigma^A$  concentration was chosen as this provides a good occupancy of both the P2 and P3 promoters under these experimental conditions, as seen in Figure 48.

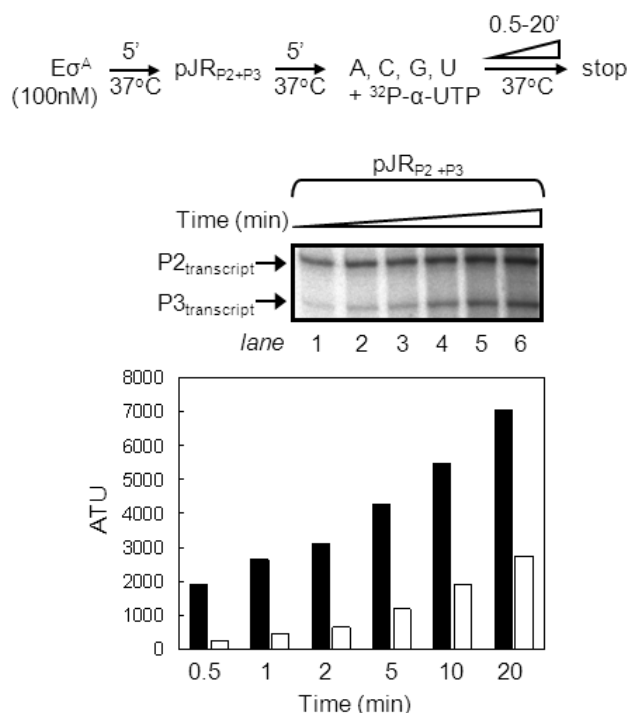


Figure 56: Image of a 10% (w/v) urea-denaturing-PAGE gel showing 100 nM  $E\sigma^A$  multiple round *in vitro* transcription initiation from  $pJR_{P2+P3}$ .

Transcripts were synthesised from the  $pJR_{P2+P3}$  reporter plasmid at 37°C by  $E\sigma^A$  (indicated by the arrows). The reactions were performed as a function of time under multiple round conditions. A schematic of the assay is shown above. The amount of transcripts synthesised from P2 (black bars) and P3 (white bars) are expressed as arbitrary transcription units (ATU), derived from the band intensity calculated from the gel imaged by phosphorimager analysis (see section 2.4.1). In at least three independent experiments, the observed increasing trend between each data point for the quantity of transcripts synthesised from either the P2 or P3 promoters was comparable.

Results, as shown in Figure 56, demonstrate that transcripts originating from the P3 promoter accumulated slower over time than from the P2 promoter. Reactions using the  $pJR_{P2}$  or  $pJR_{P3}$  templates, encoding just the P2 or P3 promoter, under the same conditions indicated that the time-dependent accumulation from P2 or P3 occurs at the same rate as from the same promoter when the divergent promoter is absent, compare Figure 57 with Figure 56. This result also implies that the RPo formed at the P3 promoter may be less stable

than the RPo formed at the P2 promoter, or that the RPo accumulate slower at the P3 promoter, in the absence of AgrA.

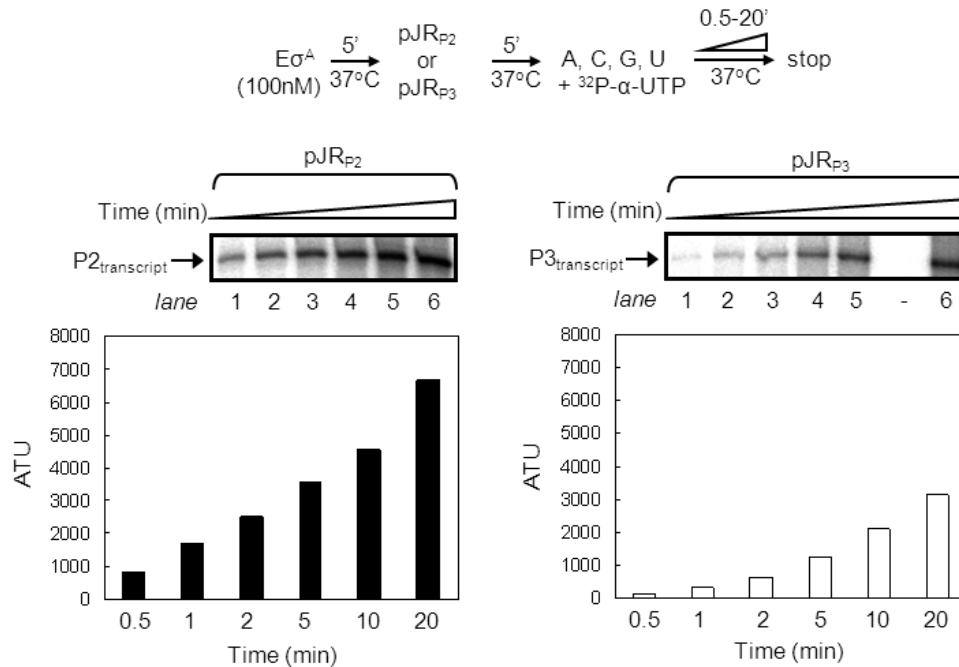


Figure 57: Image of a 10% (w/v) urea-denaturing-PAGE gel showing E $\sigma^A$  multiple round *in vitro* transcription initiation from pJR<sub>P2</sub> and pJR<sub>P3</sub>.

Transcripts were synthesised from the pJR<sub>P2</sub> and pJR<sub>P3</sub> reporter plasmids at 37°C by E $\sigma^A$  (indicated by the arrows). The reactions were performed as a function of time under multiple round conditions. A schematic of the assay is shown above. The amount of transcripts synthesised from P2 (black bars) and P3 (white bars) are expressed as arbitrary transcription units (ATU), derived from the band intensity calculated from the gel imaged by phosphorimager analysis (see section 2.4.1). In at least three independent experiments, the observed increasing trend between each data point for the quantity of transcripts synthesised from either the P2 or P3 promoters was comparable.

### 5.2.3. Stability of the *agr* P2 and P3 complexes challenged with heparin

To address whether the RPo formed at the P3 promoter, in the absence of AgrA, are more unstable and dissociate more rapidly upon heparin exposure than the P2 promoter, a heparin stability assay was performed. Experimentally this involved the accumulation of RPo for 5 minutes at 37°C through the addition of E $\sigma^A$  and the pJR<sub>P2+P3</sub> template. The reactions were then exposed to heparin for 0, 5, 10 and 20 minute time points before the addition of the elongation mix (EM) to measure the RPo present at each time point through single round transcription. The 0 minute time point represents 100% of the RPo present before the addition of heparin.

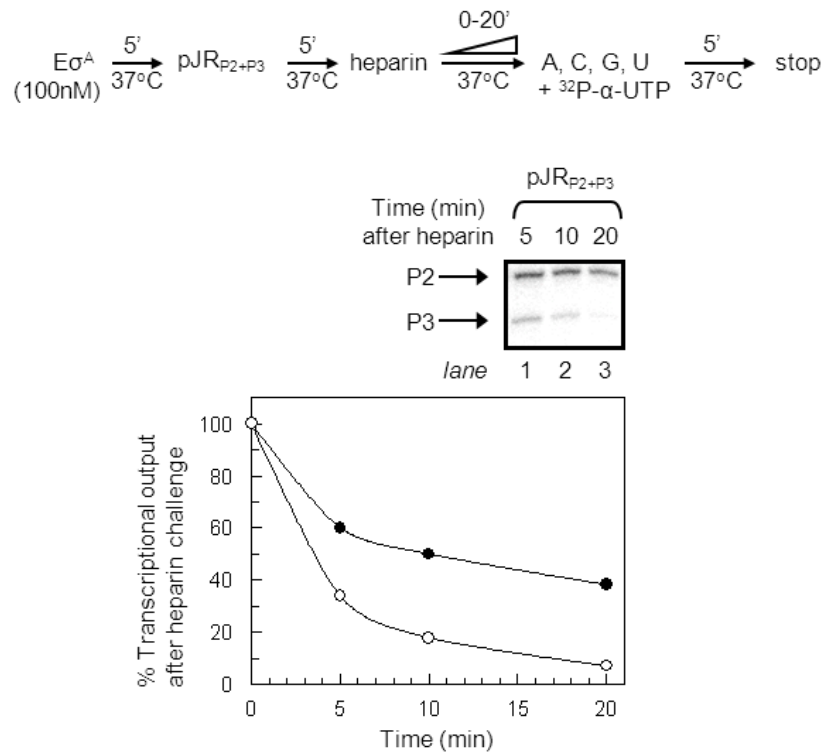


Figure 58: Image of a 10% (w/v) urea-denaturing-PAGE gel showing the stability of  $\text{E}\sigma^A$ -P2/P3 complexes using pJR<sub>P2+P3</sub>.

Transcriptional output from  $\text{E}\sigma^A$ -P2/P3 promoter complexes challenged with 100  $\mu\text{g/ml}$  heparin (indicated by the arrows). The reactions were performed at 37°C from the pJR<sub>P2+P3</sub> reporter plasmid as a function of time, under single round conditions. A schematic of the assay is shown above. The amount of transcripts synthesised at P2 (black dots) and P3 (white dots) are expressed as percentage of transcriptional output after the heparin challenge. In at least three independent experiments, the observed decreasing trend between each data point for the quantity of transcripts synthesised from either the P2 or P3 promoters was comparable.

Results, as shown in Figure 58, indicated that in the absence of AgrA the RPo formed at P3 were less stable than those formed at P2, due to the drop in transcriptional output over time after heparin challenge. The reactions were also carried out using pJR<sub>P2</sub> or pJR<sub>P3</sub> as the template, thus measuring heparin stability at P2 or P3 independently of the divergent promoter. The results reproduce those seen from the pJR<sub>P2+P3</sub> template, with the RPo formed at the P3 promoter less stable than those formed at P2, as shown in Figure 59.

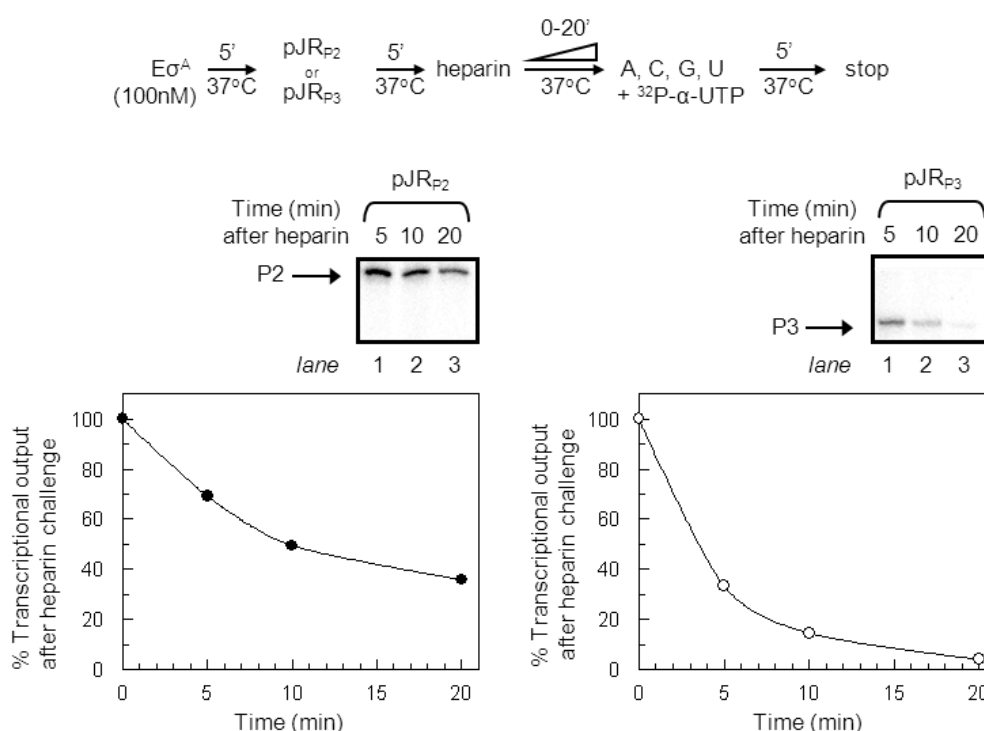


Figure 59: Image of a 10% (w/v) urea-denaturing-PAGE gel showing the stability of  $E\sigma^A$ -P2/P3 complexes using pJR<sub>P2</sub> and pJR<sub>P3</sub>.

Transcriptional output from  $E\sigma^A$ -P2/P3 promoter complexes challenged with 100  $\mu$ g/ml heparin (indicated by the arrows). The reactions were performed at 37°C from the pJR<sub>P2</sub> and pJR<sub>P3</sub> reporter plasmid as a function of time, under single round conditions. A schematic of the assay is shown above. The amount of transcripts synthesised at P2 (black dots) and P3 (white dots) are expressed as percentage of transcriptional output after the heparin challenge. In at least three independent experiments, the observed decreasing trend between each data point for the quantity of transcripts synthesised from either the P2 or P3 promoters was comparable.

The *in vitro* multiple round transcription assays presented here (Figure 56 and Figure 57) support the idea that the lower transcription initiation rate from the P3 promoter compared to the P2 promoter indicate that RPo formation at the P3 promoter occurs more slowly than at P2 in the absence of AgrA. Results also seem to indicate that the RPo formed at the P3 promoter were less stable than those formed at the P2 promoter (Figure 58 and Figure 59). *In vitro* transcription assays using pJR<sub>P2</sub> and pJR<sub>P3</sub> as the templates also indicated that RPo formation at the P3 promoter by  $E\sigma^A$  is not detectably influenced by RPo formation at the P2 promoter. Overall, the results presented by the full length *in vitro* transcription assays support, and are consistent with, the findings from the short RNA synthesis assays (Figure 53) i.e. that  $E\sigma^A$  is able to form the RPo at both the P2 and P3 promoters. However RPo

formation appears to be more efficient from the P2 promoter than from the P3 promoter in the absence of AgrA.

### **5.3. What is the effect of [6His]-AgrA on transcription initiation from the *agr* P2 and P3 promoters?**

#### **5.3.1. Effect of acetyl phosphate on [6His]-AgrA activation**

Having determined the basal level of transcription from the *agr* P2 and P3 promoters in the absence of any regulators (section 5.2), the effect of AgrA on *agr* transcription initiation was investigated using [6His]-AgrA purified in section 3.2.4.3. To phosphorylate RRs such as AgrA *in vitro*, small phosphodonors such as acetyl phosphate are sometimes used (Koenig, Ray et al. 2004). To determine the effect of [6His]-AgrA, in the presence and absence of acetyl phosphate, on transcription initiation from the *agr* P2 and P3 promoters, short RNA synthesis assays were performed. Assays were performed, as in section 2.4.6., with E $\sigma^A$  as a function of [6His]-AgrA concentration following the schematic in Figure 60, where [6His]-AgrA was pre-incubated with acetyl phosphate for 30 minutes before the addition of E $\sigma^A$ , as in section 2.4.4. Acetyl phosphate was used at a 50 mM concentration as used in previously reported *in vitro* assays to phosphorylate AgrA (Koenig, Ray et al. 2004).

Surprisingly, results indicated that [6His]-AgrA~P, phosphorylated by acetyl phosphate, had an antagonistic effect on E $\sigma^A$  transcription initiation at the *agr* P2 and P3 promoters (Figure 60, lanes 4-5 and 10-11). Control reactions performed with acetyl phosphate in the absence of [6His]-AgrA indicated that acetyl phosphate alone was able to antagonise E $\sigma^A$  transcription initiation at the *agr* P2 and P3 promoters under these experimental conditions (Figure 60, compare lanes 3 and 9 to 6 and 12).

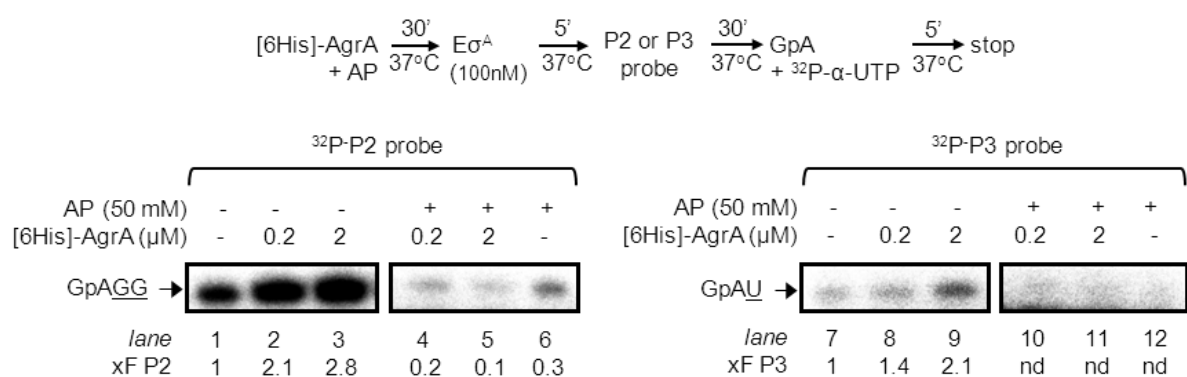


Figure 60: Image of a 20% (w/v) urea-denaturing-PAGE gel showing the effect of acetyl phosphate on [6His]-AgrA activation in a short RNA synthesis assay.

A short RNA synthesis assay showing the synthesis of the short RNA products GpAGG (from P2) and GpAU (from P3) at 37°C by Eσ<sup>A</sup> (indicated by the arrow, underlined nucleotides are <sup>32</sup>P-labelled). The reactions are performed as a function of [6His]-AgrA in the presence and absence of 50 mM acetyl phosphate (AP). A schematic of the assay is shown above. The amount of transcripts synthesised are expressed as fold increase in transcription initiation when compared to basal transcription initiation (lane 1) of P2 (xF P2) and (lane 1) of P3 (xF P3). In at least two independent experiments, the observed trend between each data point for the quantity of short RNA products synthesised from either the P2 or P3 promoter probes was comparable.

To further test the antagonistic effect of acetyl phosphate on Eσ<sup>A</sup> transcription initiation at the *agr* P2 and P3 promoters a multiple round full length *in vitro* transcription assay was performed, using pJR<sub>P2+P3</sub> as the template. The assay was conducted in the presence and absence of [6His]-AgrA and acetyl phosphate under the experimental conditions set out in section 2.4.8 and shown in the schematic at the top of Figure 61. The results, shown in Figure 61, show a decrease in transcription initiation from both the *agr* P2 and P3 promoters in the presence of acetyl phosphate alone, when compared to the Eσ<sup>A</sup> alone reaction, compare lanes 1 and 4. This demonstrated that acetyl phosphate had an antagonistic effect on Eσ<sup>A</sup> transcription initiation at the *agr* P2 and P3 promoters in the full length *in vitro* transcription assay (Figure 61), equivalent to that seen in the short RNA synthesis assay (Figure 60).

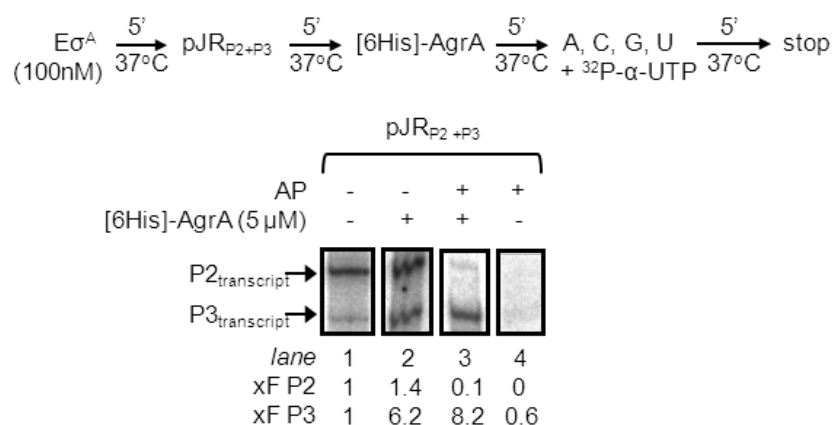


Figure 61: Image of a 10% (w/v) urea-denaturing-PAGE gel showing the effect of acetyl phosphate on [6His]-AgrA activation in a full length *in vitro* transcription assay.

Transcripts were synthesised from the pJR<sub>P2+P3</sub> reporter plasmid at 37°C by Eσ<sup>A</sup> (indicated by arrows). The reactions were performed under multiple round conditions in the presence and absence of 5 μM [6His]-AgrA and 50 mM acetyl phosphate (AP). A schematic of the assay is shown above. The amount of transcripts synthesised are expressed as fold increase in transcription initiation when compared to basal transcription initiation (lane 1) of P2 (xF P2) and P3 (xF P3). In at least three independent experiments, the observed trend between each data point for the quantity of transcripts synthesised from either the P2 or P3 promoters was comparable.

As acetyl phosphate alone appeared to antagonise transcription initiation from the *agr* P2 and P3 promoters under these experimental conditions, the effect of unphosphorylated [6His]-AgrA on transcription initiation from the *agr* P2 and P3 promoters was investigated. There is an increasing body of evidence demonstrating that the unphosphorylated state of a two-component RR exists in a dynamic equilibrium between the phosphorylated (active) and unphosphorylated conformations (inactive) (Dyer and Dahlquist 2006; Barbieri, Mack et al. 2010). For example the crystal structure of CheY demonstrates that the unphosphorylated CheY can achieve an active like arrangement in the absence of phosphorylation and that CheY does not exist in just two states, see section 1.4.4.

### 5.3.2. [6His]-AgrA binding to the *agr* P2 and P3 promoters

Firstly, to check the binding ability of the unphosphorylated [6His]-AgrA preparation, EMSAs were performed, following the protocol in section 2.4.5, using either the *agr* P2 or P3 <sup>32</sup>P-labelled promoter probes highlighted in Figure 45. Both *agr* P2 and P3 <sup>32</sup>P-labelled promoter probes contain a high affinity AgrA binding site.

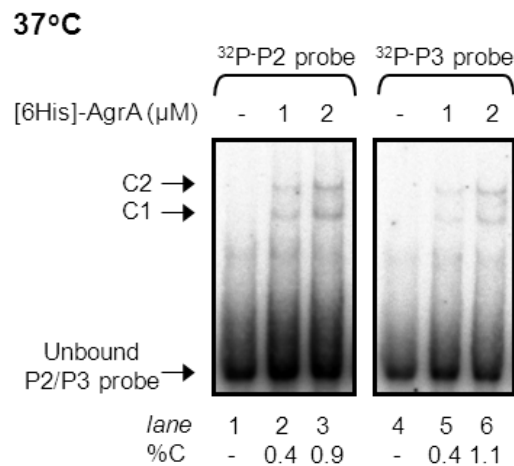


Figure 62: Image of a 4.5% (w/v) native-PAGE gel showing the binding of [6His]-AgrA to the *agr* P2 and P3 promoters at 37°C.

The assay was performed as a function of AgrA concentration with 20 nM of promoter probe. The migration positions of the [6His]-AgrA-P2/P3 complexes (C1 and C2) and the unbound P2 and P3 promoter probes are indicated. The percentage of the total DNA probe present in the reaction bound by [6His]-AgrA is labelled as %C. In at least two independent experiments, the data range for each point fell within 15% of the %C value shown.

Results, as shown in Figure 62, show a pattern of migration including two bands (C1 and C2) upon the addition of [6His]-AgrA. The two bands, originally seen by Koenig et al. 2004, could represent two conformers of unphosphorylated [6His]-AgrA in the active conformation (Koenig, Ray et al. 2004).

### 5.3.3. Full length transcription assays

To determine the effect of unphosphorylated [6His]-AgrA on Eo<sup>A</sup> transcription initiation from the *agr* P2 and P3 promoters, multiple round full length *in vitro* transcription assays were performed as function of [6His]-AgrA concentration using pJR<sub>P2+P3</sub> as the template. Control reactions were also performed using a mutant AgrA with a histidine to alanine mutation at residue 169 (H169A), which is shown to be essential for the DNA binding ability of AgrA (Sidote, Barbieri et al. 2008). Results, as shown in Figure 63, indicated that [6His]-AgrA stimulates Eo<sup>A</sup> transcription initiation by ~9-fold from the *agr* P3 promoter, lanes 1-3. However, no stimulation was observed at the *agr* P2 promoter under these experimental conditions. The control reaction using the [6His]-AgrA DNA binding mutant ([6His]-AgrA<sub>H169A</sub>) demonstrated that the stimulation of transcription initiation from *agr* P3 promoter was specific to [6His]-AgrA (Figure 63, lane 4).

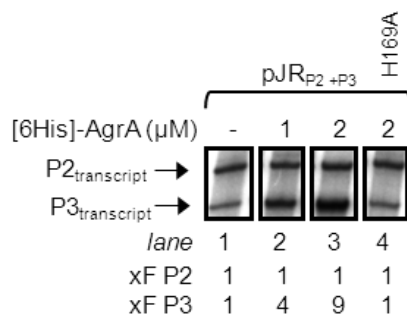
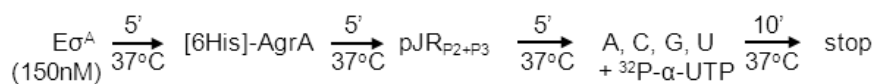


Figure 63: Image of a 10% (w/v) urea-denaturing-PAGE gel showing the effect of [6His]-AgrA on transcription initiation from  $\text{pJR}_{\text{P2+P3}}$ .

Transcripts were synthesised from the  $\text{pJR}_{\text{P2+P3}}$  reporter plasmid at  $37^\circ\text{C}$  by  $\text{E}\sigma^A$  (indicated by arrows) in the presence and absence of [6His]-AgrA. The reactions were performed as a function of [6His]-AgrA concentration under multiple round conditions. A schematic of the assay is shown above. The amount of transcripts synthesised are expressed as fold increase in transcripts synthesised from P2 (xF P2) and P3 (xF P3) (lanes 2 and 3) with respect to the corresponding no [6His]-AgrA reaction (lane 1). A H169A [6His]-AgrA DNA-binding mutant is used as a control (lane 4). In at least three independent experiments, the observed trend between each data point for the quantity of transcripts synthesised from either the P2 or P3 promoters was comparable.

The assays were repeated using either the  $\text{pJR}_{\text{P2}}$  or  $\text{pJR}_{\text{P3}}$  templates so that the effect of [6His]-AgrA on  $\text{E}\sigma^A$  transcription initiation at the *agr* P2 or P3 promoters could be measured independently. Results, as shown in Figure 64, were comparable with the previous assay using  $\text{pJR}_{\text{P2+P3}}$  (Figure 63), as [6His]-AgrA stimulated  $\text{E}\sigma^A$  transcription initiation by ~14-fold from the *agr* P3 promoter, compare lanes 3 and 4. As in Figure 63, no significant increase in P2 transcription initiation was observed under these experimental conditions. Overall these results demonstrate that [6His]-AgrA, in an unphosphorylated form is able to stimulate transcription initiation from the *agr* P3 promoter and has little observable effect on the P2 promoter under these experimental conditions.

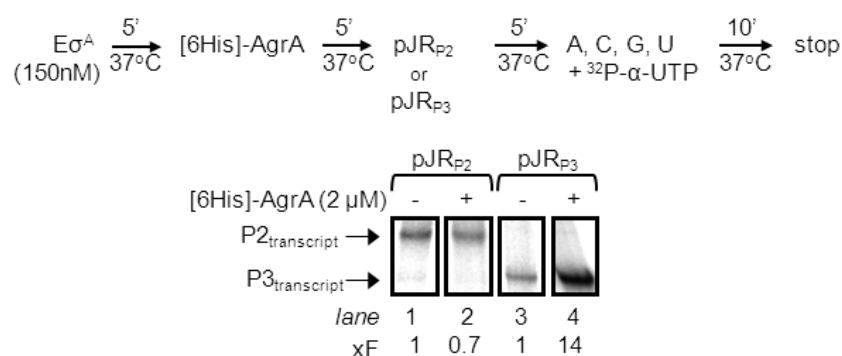


Figure 64: Image of a 10% (w/v) urea-denaturing-PAGE gel showing the effect of [6His]-AgrA on transcription initiation from pJR<sub>P2</sub> and pJR<sub>P3</sub>.

Transcripts were synthesised from the pJR<sub>P2</sub> and pJR<sub>P3</sub> reporter plasmids at 37°C by Eσ<sup>A</sup> (indicated by the arrows) in the presence and absence of [6His]-AgrA under multiple round conditions. A schematic of the assay is shown above. The amount of transcripts synthesised are expressed as fold increase in transcripts synthesised from P2 (xF) and P3 (xF) (lanes 2 and 4) with respect to the corresponding no [6His]-AgrA reaction (lane 1 and 3). In at least three independent experiments, the observed trend between each data point for the quantity of transcripts synthesised from either the P2 or P3 promoters was comparable.

#### 5.3.4. Effect of [6His]-AgrA binding on Eσ<sup>A</sup> promoter occupation

To determine the mechanism of activation of Eσ<sup>A</sup> transcription initiation by [6His]-AgrA at the *agr* P2 and P3 promoters, EMSAs were performed following section 2.4.5. The possible mechanism of recruitment of Eσ<sup>A</sup> by AgrA is highlighted by the overlapping binding sites of Eσ<sup>A</sup> and AgrA at the *agr* P2 and P3 promoter regions (Figure 7). Therefore, assays were performed to measure the binding of Eσ<sup>A</sup> to the <sup>32</sup>P-labelled *agr* P2 or P3 promoter probes in the presence and absence of [6His]-AgrA.

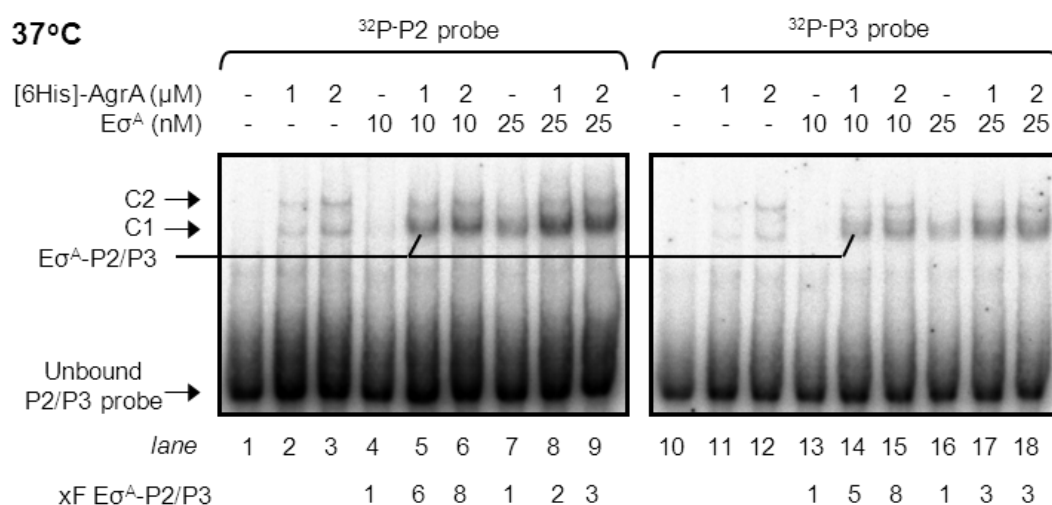


Figure 65: Image of a 4.5% (w/v) native-PAGE gel showing the Influence of [6His]-AgrA on the binding of Eσ<sup>A</sup> to the *agr* P2 and P3 promoters.

EMSA showing the binding of Eσ<sup>A</sup> to the *agr* P2 and P3 promoter probes at 37°C in the presence and absence of [6His]-AgrA. The assay was performed as a function of Eσ<sup>A</sup> and [6His]-AgrA concentration with 20 nM of promoter probe. The migration positions of the [6His]-AgrA-P2/P3 complexes (C1 and C2), the Eσ<sup>A</sup>-P2/P3 complexes and the unbound P2 and P3 promoter probes are indicated. The fold increase in Eσ<sup>A</sup>-P2 (xF - lanes 5, 6, 8 and 9) and Eσ<sup>A</sup>-P3 (xF - lanes 14, 15, 17 and 18) complexes with respect to the corresponding reactions with no [6His]-AgrA (lanes 4, 7, 13 and 16) are indicated. In at least three independent experiments, the data range for each point fell within 15% of the %C value shown.

Results, shown in Figure 65, demonstrate a sharp contrast in the binding of Eσ<sup>A</sup> to the *agr* P2 and P3 promoters in the presence and absence of [6His]-AgrA, compare lanes 4 and 7 to 5-6 and 8-9 for P2 and 13 and 16 to 14-15 and 17-18 for P3 respectively. Surprisingly, a ~2-8 fold increase in Eσ<sup>A</sup> occupancy was observed in the presence of [6His]-AgrA at both the *agr* P2 and P3 promoters, despite the lack of stimulation of transcription initiation at the *agr* P2 promoters in earlier transcription assays (Figure 63 and Figure 64). Stimulation of Eσ<sup>A</sup> occupancy by [6His]-AgrA was highest at conditions where Eσ<sup>A</sup> concentration was limited, as in lanes 4-6 and 13-15. Therefore, considering the increased occupation of the *agr* P2 promoter by Eσ<sup>A</sup> in the presence of [6His]-AgrA, it was considered whether [6His]-AgrA has an effect on transcription initiation at the *agr* P2 promoter under conditions where Eσ<sup>A</sup> is limited (i.e. 150 nM is normally used). To investigate this, a multiple round full length *in vitro* transcription assays was performed as a function of Eσ<sup>A</sup> concentration in the presence and absence of [6His]-AgrA.

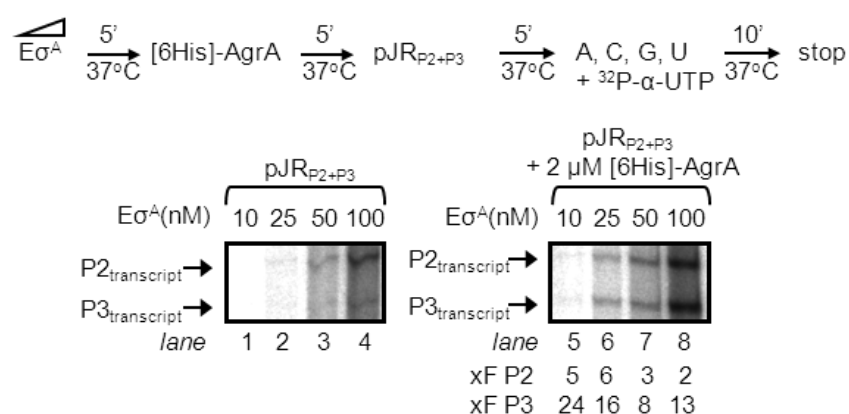


Figure 66: Image of a 10% (w/v) urea-denaturing-PAGE gel showing the effect of [6His]-AgrA on transcription initiation from pJR<sub>P2+P3</sub> as a function of Eσ<sup>A</sup> concentration.

The reactions were performed as a function of Eσ<sup>A</sup> concentration, under multiple round conditions, in the presence and absence of [6His]-AgrA, at 37°C. A schematic of the assay is shown above. The amount of transcripts synthesised are expressed as fold increase in transcripts synthesised from P2 (xF P2) and P3 (xF P3) (lanes 5-8) with respect to the corresponding no [6His]-AgrA reactions (lanes 1-4). In at least three independent experiments, the observed trend between each data point for the quantity of transcripts synthesised from either the P2 or P3 promoters was comparable.

Results, shown in Figure 66, indicated that up to a ~6 fold stimulation of transcription initiation from the *agr* P2 promoter was observed in the presence of [6His]-AgrA, compare lanes 1-4 with 5-8. Stimulation of transcription initiation from the *agr* P3 promoter by [6His]-AgrA was observed at similar levels, a ~13-24 fold increase, to previous multiple round full length transcription assays (compare Figure 66 with Figure 63). It was concluded that [6His]-AgrA increases Eσ<sup>A</sup> occupancy at both the *agr* P2 and P3 promoters, leading to increased transcription initiation at both the promoters. However [6His]-AgrA seems have a more pronounced positive effect on transcription initiation at the *agr* P3 promoter.

### 5.3.5. Determining the [6His]-AgrA activation order of addition effect

Having revealed that [6His]-AgrA has a more pronounced effect on the *agr* P3 promoter, it was next important to further the understanding of AgrA activation by determining the step of transcription initiation that is affected by [6His]-AgrA. Therefore an order of addition assay was performed where [6His]-AgrA was added to single-round full length *in vitro* transcription reactions at various stages of the reactions, indicated in the schematic in Figure 67. The reactions were performed as in section 2.4.8, in the presence of the

competitor heparin, so that the  $E\sigma^A$  promoter complexes formed could only produce a single transcript from the  $pJR_{P2+P3}$  promoter template.

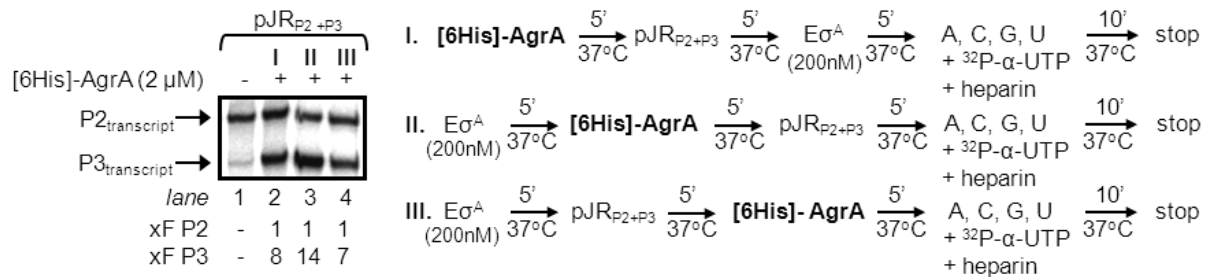


Figure 67: Image of a 10% (w/v) urea-denaturing-PAGE gel showing the [6His]-AgrA order of addition effect.

A full length *in vitro* transcription assay showing the transcripts synthesised from the  $pJR_{P2+P3}$  reporter plasmid at  $37^\circ\text{C}$  by  $E\sigma^A$  (indicated by the arrows) in the presence and absence of [6His]-AgrA. [6His]-AgrA was added to the reaction at three points (I-III). The reactions were performed under single round conditions. A schematic of the assay is shown on the right. The amount of transcripts synthesised are expressed as fold increase in transcripts synthesised from P2 (xF P2) and P3 (xF P3) (lanes 2-4) with respect to the corresponding no [6His]-AgrA reaction (lane 1). In at least three independent experiments, the observed trend between each data point for the quantity of transcripts synthesised from either the P2 or P3 promoters was comparable.

Results, as shown in Figure 67, indicated that the presence of [6His]-AgrA has a positive effect on  $E\sigma^A$  transcription initiation at the *agr* P3 promoter, by ~7-14 fold, irrespective of the order of addition. As expected, under these experimental conditions no stimulation of transcription initiation and no order of addition effect was seen at the *agr* P2 promoter. However, the [6His]-AgrA effect on the *agr* P3 promoter was highest under condition (II) where [6His]-AgrA was pre-incubated with  $E\sigma^A$  before the addition of the promoter template, suggesting a potential role of AgrA in recruiting  $E\sigma^A$  to the promoter. It is possible that complexes formed under conditions (I) and (III) could revert to scenario (II) through the dissociation of  $E\sigma^A$ -promoter complexes and [6His]-AgrA-promoter complexes, therefore leading to enhanced P3 transcription initiation in all scenarios.

### 5.3.6. Effect of [6His]-AgrA on RPo stability at the *agr* P2 and P3 promoters

Subsequently, it was questioned whether the [6His]-AgrA effect is restricted to steps leading to RPo formation, or whether [6His]-AgrA has an influence on the stability of the RPo formed at the *agr* P2 or P3 promoters. To answer this RPo stability assays were performed in the presence and absence of [6His]-AgrA through the formation of the RPo at the *agr* P2

and P3 promoters at 37°C. The RPo were then challenged with heparin for set time points before the addition of ribonucleoside triphosphates to allow a single round of transcription, to measure transcription initiation from the remaining RPo present in the reactions. Reactions were conducted following the schematic in Figure 68, where Eσ<sup>A</sup> was always pre-incubated with [6His]-AgrA before to the addition of the pJR<sub>P2+P3</sub> promoter template.

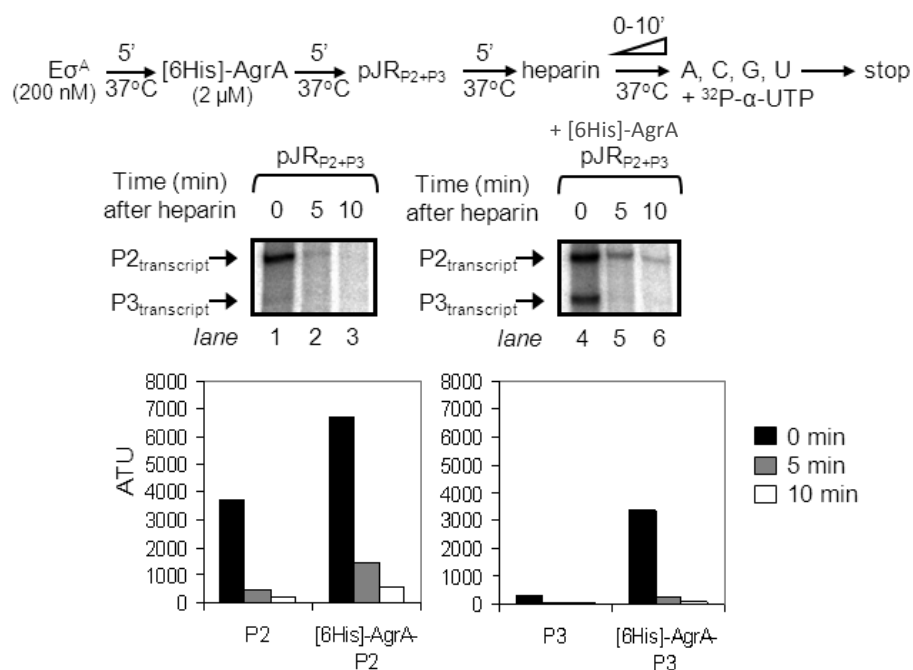


Figure 68: Image of a 10% (w/v) urea-denaturing-PAGE gel showing the effect of [6His]-AgrA on RPo stability at the *agr* P2 and P3 promoters.

The transcripts were synthesised from the pJR<sub>P2+P3</sub> reporter plasmid at 37°C by Eσ<sup>A</sup> (indicated by the arrows) as a function of time after heparin challenge. Reactions were performed in the presence (lanes 1-3) and absence (lanes 4-6) of [6His]-AgrA under single round conditions. A schematic of the assay is shown above. The amount of transcripts synthesised are expressed as arbitrary transcription units (ATU), derived from the band intensity calculated from the gel imaged by phosphorimager analysis (see section 2.4.1). In at least three independent experiments, the observed trend between each data point for the quantity of transcripts synthesised from either the P2 or P3 promoters was comparable.

The results, shown in Figure 68, identify the 0 minute time point as the amount of heparin-resistant RPo present at the time of addition of heparin. After 5 minutes of exposure to heparin the RPo present at the P2 promoter resulted in a clear decrease in transcripts synthesised. Transcripts initiated from the P3 promoter were barely detectable at the 0 minute time point, as shown previously (Figure 54), and therefore after 5 minutes were absent. However, both conditions were in the absence of [6His]-AgrA, shown in lanes 1-3. The presence of [6His]-AgrA led to an increase in transcripts synthesised from both the P2

and P3 promoters at the 0 minute time point, however no meaningful effect was observed on P2 or P3 RPo stability, compare lanes 2 and 3 with lanes 5 and 6. Therefore, it appears that under these experimental conditions, [6His]-AgrA has no effect on the longevity of the RPo once formed at the *agr* P2 and P3 promoters.

#### 5.4. Conclusions

Following on from the discovery that  $E\sigma^A$  can interact with, and form the RPo, at both the *agr* P2 and P3 promoters in the absence of AgrA, results from this chapter have identified that in the absence of AgrA transcription initiation is more efficient from the *agr* P2 than the P3 promoter. *In vitro* transcription assays, both under single and multiple round conditions and where the promoters are available in their native divergent form, have demonstrated that the number of RPo formed at the P2 promoter is significantly higher than those formed at the P3 promoter (Figure 54 and Figure 55). Therefore it can be concluded that there is higher basal transcription initiation from the *agr* P2 promoter when compared to the *agr* P3 promoter, independently of AgrA.

It also appears that the RPo accumulate at a slower rate at the P3 promoter than the P2 promoter (Figure 56 and Figure 57). The RPo formed at the P2 promoter were also more stable than those formed at the P3 promoter, which rapidly dissociated when exposed to heparin (Figure 58 and Figure 59). Overall the results confirm the findings from the short RNA synthesis assays (Figure 53) that  $E\sigma^A$  can form the RPo at both the *agr* operon promoters, albeit more efficiently from the P2 promoter, in the absence of AgrA.

The similarity between the results obtained from transcription assays when the promoters were present in a native back-to-back arrangement, using pJR<sub>P2+P3</sub>, in comparison to either promoter present independently, using pJR<sub>P2</sub> or pJR<sub>P3</sub>, indicate that  $E\sigma^A$  activity at one promoter does not affect activity at the divergent promoter (compare Figure 56 and Figure 57). This leads to the possibility that divergent transcription initiation can occur from the *agr* P2 and P3 promoters simultaneously.

The effect of AgrA on transcription initiation from the *agr* P2 and P3 promoters was examined using EMSAs and *in vitro* transcription assays in the presence of [6His]-AgrA. Despite the inability to phosphorylate [6His]-AgrA *in vitro*, the use of excess unphosphorylated [6His]-AgrA, representing a small population of AgrA in a phosphorylated-like conformation, was sufficient to stimulate transcription initiation from both the *agr* P2 and P3 promoters in *in vitro* transcription assays (Figure 63 and Figure 66). The activation by [6His]-AgrA was exaggerated from the P3 promoter with a 9-14 fold increase in transcription initiation. Binding assays, where [6His]-AgrA was pre-incubated with  $E\sigma^A$ , demonstrated that despite the stark contrast in activation at the P2 and P3 promoters, [6His]-AgrA equally increased occupation of  $E\sigma^A$  at the *agr* P2 and P3 promoters (Figure 65). The results demonstrate that AgrA could recruit  $E\sigma^A$  to the *agr* P2 and P3 promoters, however the differential activation of transcription initiation at the *agr* P2 and P3 promoters suggests two possible mechanisms of activation, discussed in section 8.2.

Single round *in vitro* transcription assays, where [6His]-AgrA was added at various stages of the reactions, was used to target the step of transcription initiation that is affected by AgrA at the *agr* P2 and P3 promoters. The results demonstrated that the highest activation by [6His]-AgrA was when [6His]-AgrA was pre-incubated with  $E\sigma^A$  before the addition of the promoter template (Figure 67). RPo stability assays performed in the presence of [6His]-AgrA, indicated that [6His]-AgrA has no effect on the longevity of the RPo once formed at the *agr* P2 and P3 promoters (Figure 68). Taking into account these and earlier observations it appears that transcription initiation at the *agr* P2 and P3 promoters is affected by [6His]-AgrA in the steps leading to RPo formation.

## CHAPTER SIX

### 6. Exploring how AgrA activates transcription from the *agr* promoters

#### 6.1. Introduction

In the previous chapter (Chapter 5) it was demonstrated that the AgrA mediated positive effect on transcription initiation from the *agr* P2 and P3 promoters is restricted to the steps leading to RPo formation, as [6His]-AgrA had no effect on the longevity of the RPo once formed at the *agr* P2 and P3 promoters. However, the mechanism of AgrA activation still needs to be further unveiled. The effect of [6His]-AgrA was greatest at the *agr* P3 promoter.

There are a number of possible mechanisms of activation of transcription initiation by AgrA at the *agr* promoters. As the AgrA binding sites are located close to the -35 promoter element at the *agr* P2 and P3 promoters and the region protected by AgrA in DNase I protection assays overlaps the -35 elements (Koenig, Ray et al. 2004), shown in Figure 7, the promoters resemble class II promoters. At a class II promoter the AgrA mechanism of activation could include E $\sigma^A$  recruitment by interactions with  $\sigma_4$  and the  $\alpha$ CTD or  $\alpha$ NTD of E $\sigma^A$ . Consistent with this view, [6His]-AgrA significantly increased E $\sigma^A$  binding to both *agr* promoters (Figure 65). However, the level of activation of transcription initiation by [6His]-AgrA at the *agr* P2 and P3 promoters is not equal, shown by the increased activation of P3 under all conditions tested (Figure 63, Figure 64 and Figure 66). Therefore further mechanisms of activation of transcription initiation may be present at the *agr* P3 promoter.

The 17 $\pm$ 1 nucleotide spacing between the -10 and -35 promoter elements is considered optimal for the efficient and productive use of the promoter by the RNAP (Hawley and McClure 1983). At the *agr* P3 promoter the -10 and -35 spacer is sub-optimal, at 20 nucleotides in length (Figure 7). It is thought that AgrA could potentially compensate for the sub-optimal 20 nucleotide promoter element spacer at the P3 promoter by altering the promoter DNA conformation to properly re-align the promoter elements, as demonstrated by the higher activation from the P3 promoter (8-24 fold) compared to the P2 promoter (2-6 fold). This is in agreement with the structure of AgrA<sub>C</sub> in complex with DNA, where the DNA bends by  $\sim 38^\circ$  as it conforms to the AgrA protein surface, shown in Figure 8 (Sidote, Barbieri

et al. 2008). Therefore AgrA might activate transcription by facilitating the formation of the R<sub>P</sub>c and/or R<sub>P</sub>o at the *agr* P3 promoter.

The *agr* P3 promoter also contains an extended -10 like motif, where the characteristic TG sequence is separated by one nucleotide from the -10 element, highlighted in Figure 7. The extended -10 like motif is recognised by residues in  $\sigma_{3.0}$  at extended -10 promoters (Barne, Bown et al. 1997). The extended -10 like motif, found in 20% of *E. coli* promoters (Burr, Mitchell et al. 2000), can compensate for the lack of a -35 element as at the *galP1* promoter. At *galP1* the R<sub>P</sub>o footprint covers DNA further upstream (-55) than promoters where the -35 element is present (-45). This suggests a mechanism where the  $E\sigma^{70}$  makes additional compensatory contacts with the extended -10 motif, involving significant bending and distortion of the DNA template (Chan, Spassky et al. 1990). Studies of R<sub>P</sub>o formation at low temperatures indicate that extended -10 promoters form R<sub>P</sub>o more readily than promoters without (Burns, Belyaeva et al. 1996; Agarwal and Tyagi 2003). This implies that the extended -10 motif reduces the thermal energy requirement for R<sub>P</sub>o formation, therefore favouring R<sub>P</sub>o formation. The activity from the *galP1* promoter is stimulated in the presence of the transcription factor CRP (Attey, Belyaeva et al. 1994), just as *agr* P3 promoter activity is stimulated in the presence of its activator AgrA (Figure 63). However, unlike the *galP1* promoter, the *agr* P3 promoter contains a near consensus -35 promoter element. It could therefore be questioned whether AgrA binding near to and overlapping the -35 element influences the contacts made by the  $E\sigma^A$  to optimise transcription initiation. The role of the extended -10 like motif in transcription initiation at the *agr* P3 promoter in the presence and absence of AgrA is not known. One possibility is that the extended -10 like motif could compensate for the sub-optimally located -35 promoter element at P3.

To investigate the activation of transcription initiation by AgrA at the *agr* P3 promoter, promoter probes were constructed with the removal of the sub-optimal P3 spacer length, extended -10 like motif, and the P3 -35 promoter element to assess the role of the promoter elements on AgrA activation. EMSAs were performed to assess the ability of  $E\sigma^A$  to form  $E\sigma^A$ -promoter complexes at the various promoter probes. Short RNA synthesis assays were also performed to compare the levels of R<sub>P</sub>o formation and R<sub>P</sub>o stability at

each promoter probe. Assays were performed in the presence and absence of [6His]-AgrA to identify the possible mechanisms of AgrA activation at the *agr* P3 promoter.

#### **6.1.1. Objectives**

The overall objective of this part of the project was to explore how AgrA activates transcription from the *agr* promoters. In this regard, the specific aims include:

- I. Determine the importance of the extended -10 like motif on AgrA mediated transcription initiation at the *agr* P3 promoter.
- II. Determine the effect of optimising the promoter element spacers on AgrA mediated transcription initiation at the *agr* P3 promoter.
- III. Determine the effect of removing (i.e. scrambling) the -35 promoter element on AgrA mediated transcription initiation at the *agr* P3 promoter.

## 6.2. Characterisation of the role of the extended -10 like motif on transcription initiation at the P3 promoter

### 6.2.1. The role of the extended -10 like motif on Eo<sup>A</sup> binding to the *agr* P3 promoter

To establish the role of the extended -10 like motif at the *agr* P3 promoter and to determine if it plays any role in the activation of transcription initiation by AgrA a linear template was constructed where the TGn motif was replaced by ACn (P3-AC) as in section 3.3.2 (Appendix, Table 10). The TGn motif was replaced by ACn as studies of  $\beta$ -galactosidase assays with cells containing *lacZ* fusions of a number of extended -10 promoters revealed that replacing the TGn motif with the ACn motif significantly diminished the effect of the extended -10 motif on promoter activity (Mitchell, Zheng et al. 2003). EMSAs were performed with Eo<sup>A</sup> at <4°C to quantify the initial Eo<sup>A</sup>-DNA complexes formed at the *agr* P3 and P3-AC promoters in the absence of AgrA, following the protocol in section 2.4.5 with the following alterations. Eo<sup>A</sup> was added at a 100 nM final concentration into reactions containing the <sup>32</sup>P-labelled P3 and P3-AC probes at a final concentration of 5 nM. The P3 probe contains the P3 promoter sequence and spans the -80 to +16 positions (Figure 45).

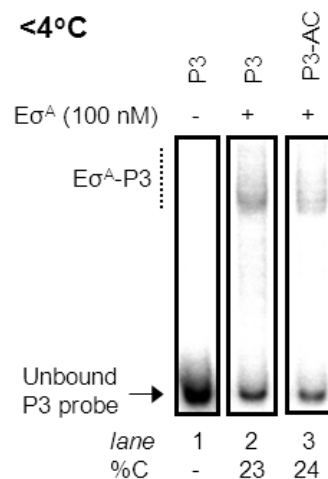


Figure 69: Image of a 4.5% (w/v) native-PAGE gel showing the binding of Eo<sup>A</sup> to the *agr* P3 and P3-AC promoters at <4°C.

The assay was performed using 100 nM Eo<sup>A</sup> with 5 nM of promoter probe. The migration positions of the Eo<sup>A</sup>-P3 complexes and the unbound P3 promoter probes are indicated. The percentage of the total DNA probe present in the reaction bound by Eo<sup>A</sup> is labelled as %C. In at least three independent experiments, the data range for each point fell within 15% of the %C value shown.

Results, as shown in Figure 69, demonstrated that  $E\sigma^A$  was able to bind both P3 and P3-AC promoters equally well at  $<4^\circ\text{C}$ , despite the mutation of the extended -10 like motif in the P3-AC probe, with 23% and 24% of P3 and P3-AC respectively, bound by 100 nM  $E\sigma^A$  (Figure 69, lanes 2-3). To characterise the  $E\sigma^A$ -DNA complexes formed at  $37^\circ\text{C}$  EMSAs were setup, as in Figure 69, as a function of  $E\sigma^A$  concentration using the *agr* P3 and P3-AC promoter probes, with the reactions incubated at  $37^\circ\text{C}$  in the absence of AgrA. As seen at  $<4^\circ\text{C}$ , results demonstrate that  $E\sigma^A$  was able to bind the *agr* P3 and P3-AC promoters equally well at  $37^\circ\text{C}$ , with 44% and 40% of the P3 and P3-AC promoter probes respectively, bound by  $E\sigma^A$  at the highest concentration (100 nM) (Figure 70, compare lanes 4 and 7).

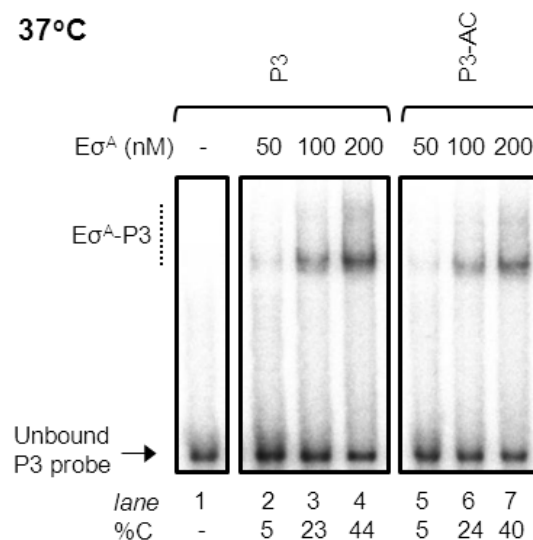


Figure 70: Image of a 4.5% (w/v) native-PAGE gel showing the binding of  $E\sigma^A$  to the *agr* P3 and P3-AC promoters at  $37^\circ\text{C}$ .

The assay was performed as a function of  $E\sigma^A$  concentration with 5 nM of promoter probe. The migration positions of the  $E\sigma^A$ -P3 complexes and the unbound P3 promoter probes are indicated. The percentage of the total DNA probe present in the reaction bound by  $E\sigma^A$  is labelled as %C. In at least three independent experiments, the data range for each point fell within 15% of the %C value shown.

Overall, results demonstrated that the mutation of the extended -10 like motif from the P3 promoter had no effect on  $E\sigma^A$  binding to the promoter under the experimental conditions used, in the absence of AgrA. However the extended -10 like motif could potentially affect transcriptional output at the P3 promoter as previous studies have identified that it reduces the thermal energy requirement for RPo formation (Burns, Belyaeva et al. 1996; Agarwal and Tyagi 2003).

### 6.2.2. The role of the extended -10 like motif on Eo<sup>A</sup> activity at the *agr* P3 promoter

As Eo<sup>A</sup> was demonstrated to bind the P3 and P3-AC promoters equally well in the absence of AgrA, a short RNA synthesis assay was performed to assess whether the Eo<sup>A</sup>-DNA complexes formed at 37°C at the *agr* P3-AC promoter were the transcriptionally competent RPo. The assay was also used to determine whether the extended -10 like motif has a role in the rate of transcription initiation at the P3 promoter. Reactions were setup as in previously described EMSAs, section 6.2.1, using the same linear DNA probes as the DNA templates. In short RNA synthesis assays the transcriptional output from the Eo<sup>A</sup>-DNA complexes is measured. A short dinucleotide primer GpA, that complements the positions +2 and +3 of the P3 template strand, were then provided along with the following nucleotide, [ $\alpha$ -<sup>32</sup>P]-UTP complementing the +4 position of the P3 template strand. This results in the synthesis of the short RNA product GpAU from the Eo<sup>A</sup>-P3 promoter complexes, which can be visualised by urea denaturing gel electrophoresis, as in section 2.4.1.

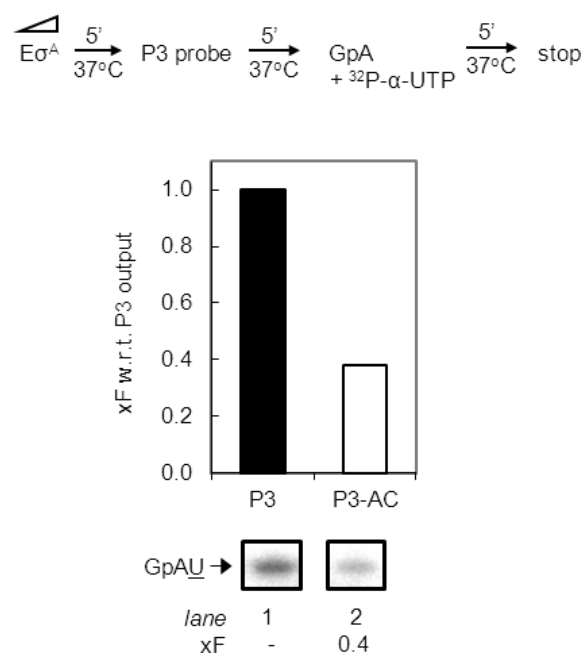


Figure 71: Image of a 20% (w/v) urea-denaturing-PAGE gel showing the short RNA synthesis from P3 and P3-AC at 37°C.

A short RNA synthesis assay showing the synthesis of the short RNA products GpAU (from P3 or P3-AC) at 37°C by 100 nM Eo<sup>A</sup> (indicated by the arrow, underlined nucleotides are <sup>32</sup>P-labelled). A schematic of the assay is shown above. The amount of short RNA products synthesised are expressed as fold increase in short RNA products synthesised from P3-AC (xF) (lane 2) with respect to the corresponding P3 reaction (lane 1). In at least two independent experiments, the observed trend between each data point for the quantity of short RNA products synthesised from the P3 promoter was comparable.

Results from the short RNA synthesis assay, shown in Figure 71, demonstrated that despite the mutation of the extended -10 like motif having no effect on  $E\sigma^A$  binding, there was a ~2 fold decrease in  $E\sigma^A$  transcription initiation at the *agr* P3-AC promoter probe when compared to the wild-type P3 promoter, compare lanes 1 and 2. Therefore it can be concluded that the extended -10 like motif is required for optimal promoter activity at the *agr* P3 promoter in the absence of AgrA. Thus, it seems that the TGN motif at positions -14 and -15 at the P3 promoter is a bona fide extended -10 motif.

### 6.2.3. The role of the extended -10 motif on the activation of transcription by AgrA at the *agr* P3 promoter

To continue to develop our understanding of the mechanism of AgrA activation at the *agr* P3 promoter, it was asked whether the extended -10 motif also plays a role in AgrA activation. Therefore the short RNA synthesis assay performed in section 6.2.2 was extended to include the pre-incubation of  $E\sigma^A$  with [6His]-AgrA. The assay was setup as a function of [6His]-AgrA at 2  $\mu$ M using 100 nM  $E\sigma^A$  and 5 nM of the *agr* P3 or P3-AC probe.

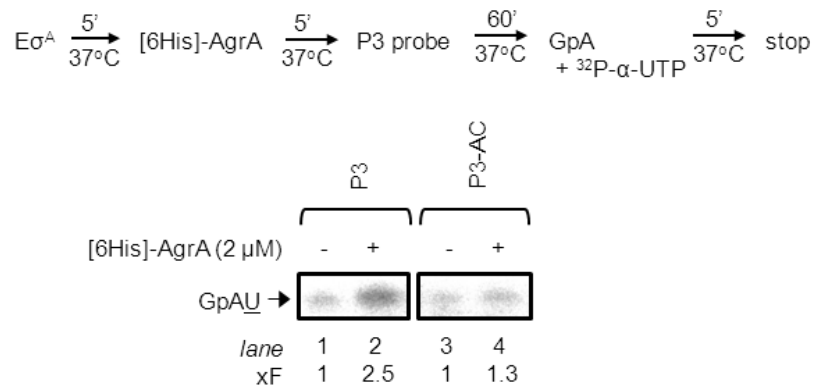


Figure 72: Image of a 20% (w/v) urea-denaturing-PAGE gel showing the effect of [6His]-AgrA on short RNA synthesis from P3 and P3-AC at 37°C.

A short RNA synthesis assay showing the synthesis of the short RNA products GpAU (from P3 or P3-AC) at 37°C by 100 nM  $E\sigma^A$  (indicated by the arrow, underlined nucleotides are  $^{32}$ P-labelled). Reactions are conducted in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of 2  $\mu$ M [6His]-AgrA, pre-incubated with  $E\sigma^A$  before the addition of the DNA probe. A schematic of the assay is shown above. The amount of short RNA products synthesised are expressed as fold increase in short RNA products synthesised from P3 (xF) and P3-AC (xF) (lanes 2 and 4) with respect to the corresponding no [6His]-AgrA reaction (lane 1 and 3). In at least two independent experiments, the observed trend between each data point for the quantity of short RNA products synthesised from the P3 promoter was comparable.

Results, shown in Figure 72, demonstrated a 2.5 fold increase in transcription initiation from the *agr* P3 promoter in the presence of [6His]-AgrA under these experimental conditions. This is in accordance with the activation of P3 by unphosphorylated [6His]-AgrA in full length transcription assays using supercoiled DNA (Figure 64). In contrast, the mutation of the extended -10 motif in the P3-AC promoter probe severely reduced/removed all activation by [6His]-AgrA, with only a 1.3 fold increase in transcription initiation (Figure 72, compare lanes 3 and 4). Therefore it is apparent that AgrA activates transcription from the P3 promoter in an extended -10 motif dependent manner.

#### **6.2.4. The effect of optimising the promoter spacer length on $E\sigma^A$ activity at the *agr* P3 promoter**

##### **6.2.4.1. $E\sigma^A$ activity at the P2-17 and P3-17 promoter**

As previously mentioned, the structure of the AgrA LytTR binding domain bound to DNA reveals a mode of binding where AgrA<sub>C</sub> induces a substantial bend in the DNA (Sidote, Barbieri et al. 2008). It is therefore regarded that one mechanism of activation by AgrA involves AgrA compensating for the sub-optimal spacer (20 nucleotides) at the *agr* P3 promoter via this distortion in the DNA. To investigate the possible molecular basis of this AgrA activation *in vitro*, a P3 promoter probe was constructed where the 20 nucleotide -10 and -35 promoter element spacer was reduced to 17 nucleotides in length (hereafter called P3-17). The optimal P2 spacer (18 nucleotides) was reduced to 17 nucleotides in length as a control (hereafter called P2-17). To determine whether the optimal 17 nucleotide spacers at the P2 and P3 promoters were more active than the wild-type promoters, short RNA synthesis assays were performed, as in section 6.2.2, using the P2, P2-17, P3 and P3-17 promoter probes. This results in the synthesis of the short RNA product GpAGG from the  $E\sigma^A$ -P2 promoter complexes and GpAU from the  $E\sigma^A$ -P3 promoter complexes, which can be visualised by urea denaturing gel electrophoresis, as in section 2.4.1.

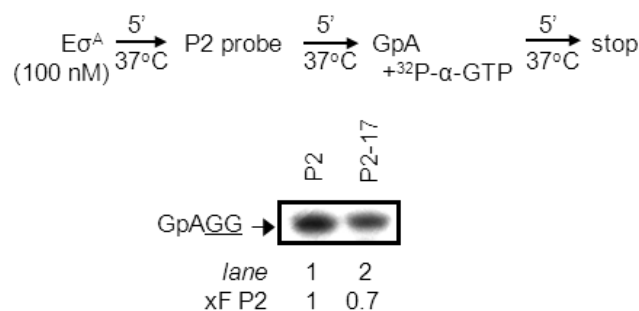


Figure 73: Image of a 20% (w/v) urea-denaturing-PAGE gel showing the short RNA synthesis from P2 and P2-17 at 37°C in the absence of [6His]-AgrA.

A short RNA synthesis assay showing the synthesis of the short RNA products GpAGG (from P2 or P2-17) at 37°C by 100 nM  $\text{E}\sigma^A$  (indicated by the arrow, underlined nucleotides are  $^{32}\text{P}$ -labelled). A schematic of the assay is shown above. The amount of short RNA products synthesised are expressed as fold increase in short RNA products synthesised from P2-17 (xF) (lane 2) with respect to the corresponding P2 reaction (lane 1). In at least two independent experiments, the observed trend between each data point for the quantity of short RNA products synthesised from the P2 promoter was comparable.

Results from the short RNA synthesis assay comparing the P2 and P2-17 promoter probes at a set  $\text{E}\sigma^A$  concentration (100 nM) revealed that reducing the P2 promoter spacer length to the optimal 17 nucleotides did not lead to a detectable increase in GpAGG synthesis (Figure 73). In fact a small decrease in transcription initiation was observed, by ~30%, when using the P2-17 template. Next a short RNA synthesis assay was setup as a function of  $\text{E}\sigma^A$  concentration to measure GpAU synthesis from the P3 and P3-17 promoter probes.

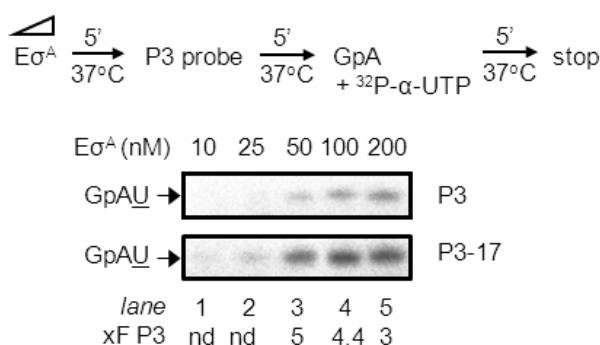


Figure 74: Image of a 20% (w/v) urea-denaturing-PAGE gel showing the short RNA synthesis from P3 and P3-17 at 37°C in the absence of [6His]-AgrA.

A short RNA synthesis assay showing synthesis of the short RNA products GpAU (from P3 or P3-17) at 37°C as a function of  $\text{E}\sigma^A$  concentration (indicated by the arrow, underlined nucleotides are  $^{32}\text{P}$ -labelled). A schematic of the assay is shown above. The amount of short RNA products synthesised are expressed as fold increase in short RNA products synthesised from P3-17 (xF) (lanes 1-5) with respect to the corresponding no P3 reaction (lanes 1-5). In at least two independent experiments, the observed trend between each data point for the quantity of short RNA products synthesised from the P3 promoter was comparable.

Results unambiguously demonstrated that activity from the P3-17 promoter was 3-5 fold higher than from the wild-type P3 promoter (Figure 74, lanes 3-5). The optimal P3 spacer region in P3-17, mimicking the potential AgrA compensation for the sub-optimal spacer, therefore increases transcription initiation from the P3 promoter.

#### 6.2.5. The effect of the 17 nucleotide spacer length on E $\sigma^A$ binding to the *agr* P3 promoter

To test if the increased promoter activity from the P3-17 promoter was due to increased E $\sigma^A$  occupancy, and therefore increased R<sub>Pc</sub> formation at <4°C, EMSAs were performed with E $\sigma^A$  at <4°C using the P3 and P3-17 promoters probes. EMSAs were performed following the protocol in section 2.4.5. E $\sigma^A$  was added at a 100 nM final concentration into reactions containing the <sup>32</sup>P-labelled P3 and P3-17 probes at a final concentration of 5 nM.

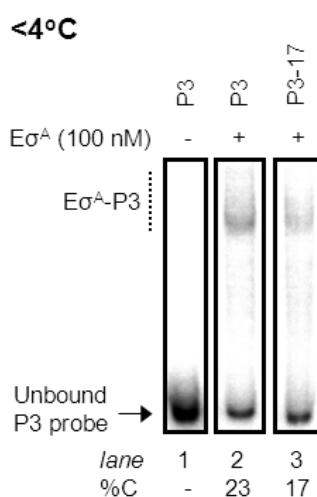


Figure 75: Image of a 4.5% (w/v) native-PAGE gel showing the binding of E $\sigma^A$  to the *agr* P3 and P3-17 promoters at <4°C.

The assay was performed using 100 nM E $\sigma^A$  with 5 nM of promoter probe. The migration positions of the E $\sigma^A$ -P3 complexes and the unbound P3 promoter probes are indicated. The percentage of the total DNA probe present in the reaction bound by E $\sigma^A$  is labelled as %C. Lanes 1 and 2 are reproduced from Figure 69 and are presented for comparison. In at least three independent experiments, the data range for each point fell within 15% of the %C value shown.

Results, shown in Figure 75, demonstrated that E $\sigma^A$  binds equally well to the P3 and P3-17 promoters, despite the significant increase in transcription initiation from the P3-17 promoter (Figure 74). Therefore, indicating that the increased transcription initiation from

the optimal spacer region promoter (P3-17) was not due to increased occupation at the P3 promoter.

#### **6.2.5.1. The effect of the 17 nucleotide spacer length on RPo stability at the P3 promoter**

As  $E\sigma^A$  formed  $E\sigma^A$ -DNA complexes equally well at the P3 and P3-17 promoter probes, subsequent steps of transcription initiation were explored, including RPo stability and the rate of RPo formation, to determine the basis of higher activity from the P3-17 promoter probe when compared to the wild-type P3 promoter.

Firstly, the short RNA synthesis assay using the P3 and P3-17 promoter probes was extended to determine if RPo formed at the P3-17 promoter were more stable than RPo formed at the wild-type P3 promoter. Reactions were setup with  $E\sigma^A$  and either the P3 or P3-17 promoter probes to form RPo at 37°C. The RPo were challenged with heparin and the surviving RPo were measured over time as GpAU synthesis, through the addition of the short dinucleotide primer GpA, that complements the positions +2 and +3 of the P3 template strand and [ $\alpha$ - $^{32}$ P]-UTP complementing the +4 position of the P3 template strand, which can be visualised by urea denaturing gel electrophoresis, as in section 2.4.1. Results, shown in Figure 76, indicated that RPo formed at the P3-17 promoter probe were only slightly more stable than those formed at the wild-type P3 probe, compare lanes 1-5 with 6-10. After 10 minutes the RPo remaining at both the P3 and P3-17 promoter probes were reduced by 0.4 fold (Figure 76, lanes 5 and 10).

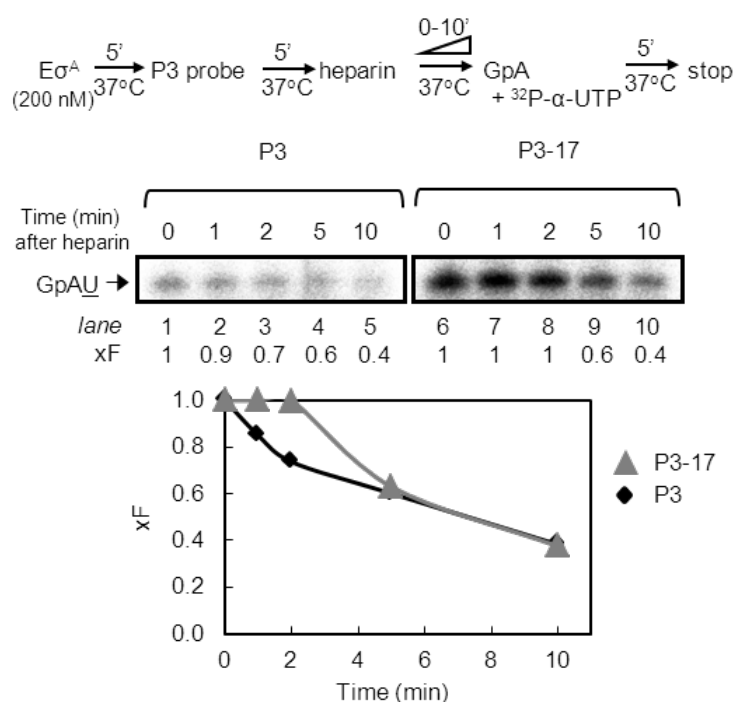


Figure 76: Image of a 10% (w/v) urea-denaturing-PAGE gel showing the stability of  $E\sigma^A$ -P3/P3-17 complexes challenged by heparin.

Stability assay showing the synthesis of the short RNA products GpAU from  $E\sigma^A$ -P3/P3-17 promoter complexes challenged with 1 mg/ml heparin (indicated by the arrows). The reactions were performed at 37°C using the P3 and P3-17 promoter probes as a function of time, under single round conditions. A schematic of the assay is shown above. The amount of short RNA products synthesised are expressed as fold increase in short RNA products synthesised after the heparin challenge from P3 ( $x_F$ ) (lanes 1-5) and P3-17 ( $x_F$ ) (lanes 6-10) with respect to the corresponding 0 minute reaction (lane 1 and 6). The results are shown in a line chart below comparing fold increase in short RNA products synthesised after the heparin challenge against time (minutes) from the P3 (black diamonds) and P3-17 (grey triangles). In at least two independent experiments, the observed decreasing trend between each data point for the quantity of short RNA products synthesised from the P3 promoter was comparable.

#### 6.2.5.2. The effect of the 17 nucleotide spacer length on the rate of RPo formation at the P3 promoter

Next the rate of RPo formation at the P3 and P3-17 promoter probes was investigated using a short RNA synthesis assay, following section 2.4.6 with the following changes. The time dependent accumulation of the GpAU transcript synthesised by  $E\sigma^A$  was measured through the addition of the short dinucleotide primer that complements the positions +2 and +3 of the P3 template strand, along with the following nucleotide [ $\alpha$ - $^{32}P$ ]-UTP complementing the +4 position of the P3 template strand and the RNAP competitor heparin to measure a single round of GpAU synthesis upon addition.

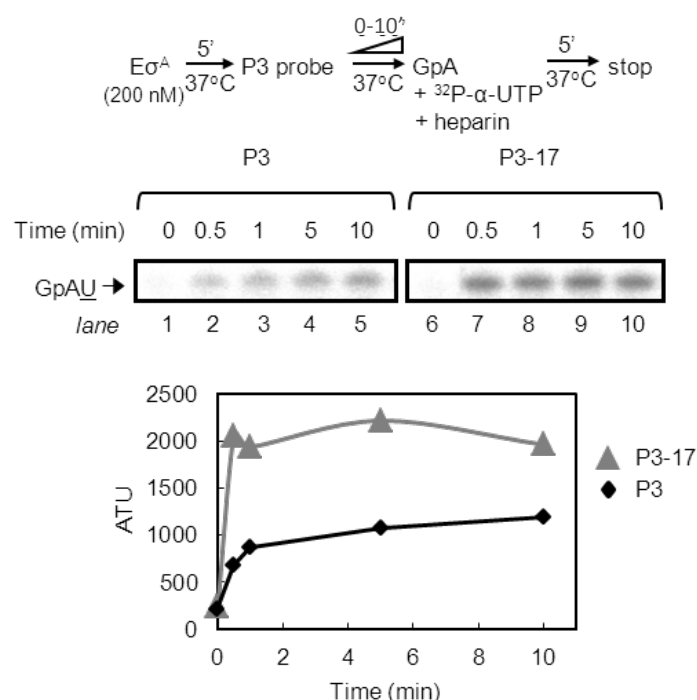


Figure 77: Image of a 10% (w/v) urea-denaturing-PAGE gel showing the rate of RPo formation at P3 and P3-17.

RPo formation assay showing the synthesis of the short RNA products GpAU from  $E\sigma^A$ -P3/P3-17 promoter complexes over time (indicated by the arrows). The reactions were performed at 37°C using the P3 and P3-17 promoter probes as a function of time, under single round conditions. A schematic of the assay is shown above. The amount of short RNA products synthesised are expressed as arbitrary transcription units (ATU) from P3 (lanes 1-5) and P3-17 (lanes 6-10). The results are shown in a line chart below comparing short RNA products synthesised as ATU against time (minutes) from the P3 (black diamonds) and P3-17 (grey triangles). ATU are derived from the band intensity calculated from the gel imaged by phosphorimager analysis (see section 2.4.1). In at least two independent experiments, the observed increasing trend between each data point for the quantity of short RNA products synthesised from the P3 promoter was comparable.

The results, shown in Figure 77, implied that the rate of RPo formation is initially faster at the P3-17 promoter than the wild-type P3 promoter, compare lanes 1-3 with 6-8. However RPo formation reaches equilibrium at either promoter at the same time. Results from the RPo stability and RPo formation assays suggest that reducing the sub-optimal 20 nucleotides spacer length to the optimal 17 nucleotides length clearly improves the ability of the initial  $E\sigma^A$ -promoter complexes, formed at the P3 promoter, to rapidly isomerise to the RPo, when compared to those formed at the wild-type P3 promoter. Therefore AgrA could increase the rate of RPo formation (in an extended -10 motif dependent manner, see section 6.2) by bending the DNA upon binding and thereby compensating for the sub-optimal spacer length.

### 6.2.6. The effect of the 17 nucleotide spacer length on activation of transcription by AgrA at the *agr* P3 promoter

To test the role of the sub-optimal spacer in the proposed AgrA mechanism of activation, where AgrA activates transcription from the P3 promoter by compensating for the sub-optimal spacer length, the optimal P3-17 promoter probe was used in a short RNA synthesis assay in the presence of [6His]-AgrA and compared to activation by the wild-type P3 promoter probe. The assay was performed to determine if AgrA can no longer activate transcription from P3 when the promoter element spacer is optimised. The assay was performed with Eo<sup>A</sup> as in section 6.2.3 using the P3 and P-17 promoter probes in the presence and absence of [6His]-AgrA, following the basic protocol in section 2.4.6. The activity of each promoter was measured as GpAU synthesised and visualised by urea denaturing gel electrophoresis, as in section 2.4.1.

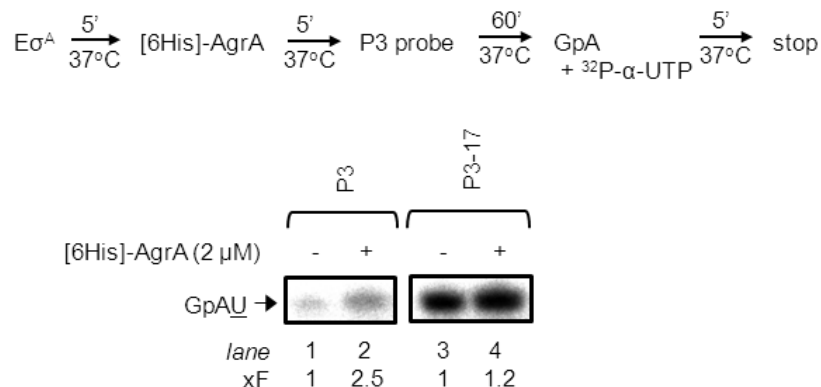


Figure 78: Image of a 20% (w/v) urea-denaturing-PAGE gel showing the effect of [6His]-AgrA on short RNA synthesis from P3 and P3-17 at 37°C.

A short RNA synthesis assay showing the synthesis of the short RNA products GpAU (from P3 or P3-17) at 37°C by 100 nM Eo<sup>A</sup> (indicated by the arrow, underlined nucleotides are <sup>32</sup>P-labelled). Reactions are conducted in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of 2 μM [6His]-AgrA, pre-incubated with Eo<sup>A</sup> before the addition of the DNA probe. A schematic of the assay is shown above. The amount of short RNA products synthesised are expressed as fold increase in short RNA products synthesised from P3 (xF) and P3-17 (xF) (lanes 2 and 4) with respect to the corresponding no [6His]-AgrA reaction (lane 1 and 3). Lanes 1 and 2 are reproduced from Figure 72 and are presented for comparison. In at least three independent experiments, the observed trend between each data point for the quantity of short RNA products synthesised from the P3 promoter was comparable.

The results, shown in Figure 78, clearly indicated that [6His]-AgrA is unable to detectably activate transcription from the P3-17 promoter when compared to the 2.5 fold increase in activation using the wild-type P3 promoter, compare lanes 2 and 4. As [6His]-AgrA is unable

to activate transcription initiation at a pre-optimised P3 promoter (P3-17); this confirms the proposed mechanism where AgrA possibly activates transcription from the P3 promoter by compensating for the sub-optimal spacer length.

Another short RNA synthesis assay was performed to further determine the importance of the extended -10 motif (TGn), in the context of the P3 promoter with an optimised spacer element (P3-17AC). The P3-17AC probe was constructed through the shortening of the P3 consensus element spacer to 17 nucleotides and by replacing the TGn motif with ACn, as in section 3.3.2. The assay was performed as in section 6.2.2 in the absence of AgrA, using the P3, P3-17, P3-17AC and P3-AC promoter probes. The activity of the promoters were measured through synthesis of the short RNA product GpAU from the  $E\sigma^A$ -P3 promoter complexes, visualised by urea denaturing gel electrophoresis, as in section 2.4.1.

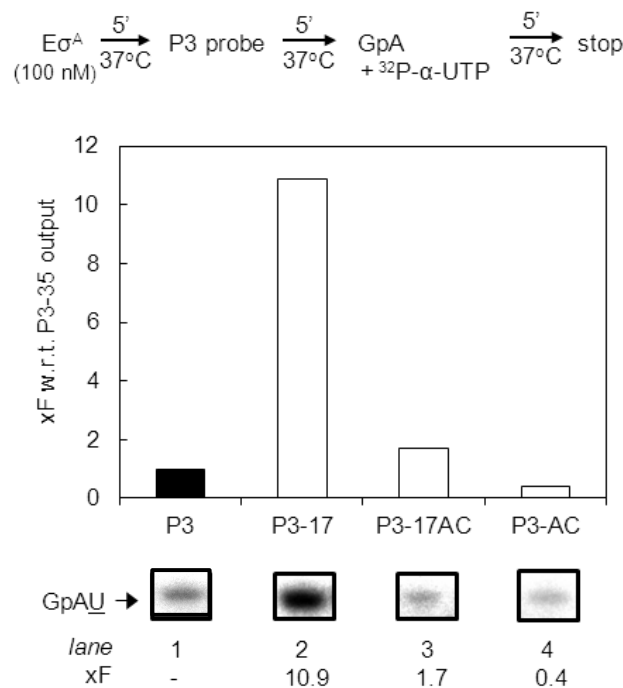


Figure 79: Image of a 20% (w/v) urea-denaturing-PAGE gel showing the short RNA synthesis from P3, P3-17, P3-17AC and P3-AC at 37°C

A short RNA synthesis assay showing the synthesis of the short RNA products GpAU (from P3, P3-17, P3-17AC and P3-AC) at 37°C by 100 nM  $E\sigma^A$  (indicated by the arrow, underlined nucleotides are  ${}^{32}P$ -labelled). A schematic of the assay is shown above. The amount of short RNA products synthesised is expressed as fold increase in short RNA products synthesised from P3-17 (xF) (lane 2) and P3-17AC (lane 3) with respect to the corresponding P3 reaction (lane 1). Lanes 1 and 4 are reproduced from Figure 71 and are presented for comparison. In at least two independent experiments, the observed trend between each data point for the quantity of short RNA products synthesised from the P3 promoter was comparable.

The results, as shown in Figure 79, agreed with previous results (Figure 74) showing a significant increase in activity from the P3-17 promoter probe when compared to the WT P3 promoter probe, compare lanes 1 and 2. The result also demonstrated that the extended -10 motif is required for the increased activity from the P3-17 promoter (Figure 79, compare lanes 2 and 3). Therefore, this result further supports the view that AgrA activated RPo formation at the P3 promoter occurs via AgrA compensating for the sub-optimal spacer length in an extended -10 motif dependent manner.

### **6.3. The effect of “scrambling” the -35 promoter element at the P3 promoter**

#### **6.3.1. The effect of scrambling the -35 promoter element on E $\sigma^A$ binding to the P3 promoter**

As extended -10 promoter elements generally compensate for the lack of a -35 element, as at the *galP1* promoter (Burr, Mitchell et al. 2000), it is possible to question the importance of the -35 promoter element at the *agr* P3 promoter, since the *agr* P3 promoter contains an extended -10 motif. Therefore a P3 promoter probe was constructed with the -35 promoter element scrambled (in order to prevent or at least compromise E $\sigma^A$  from efficiently contacting the -35 element; hereafter called the P3-35 probe) (Appendix, Table 10), following section 3.3.2. To determine the effect of scrambling the -35 element interactions on the E $\sigma^A$ -promoter complexes formed at <4°C (RPc) an EMSA was performed. Reactions were carried out at <4°C using the P3 and P3-35 promoter probes, following the protocol in section 2.4.5.

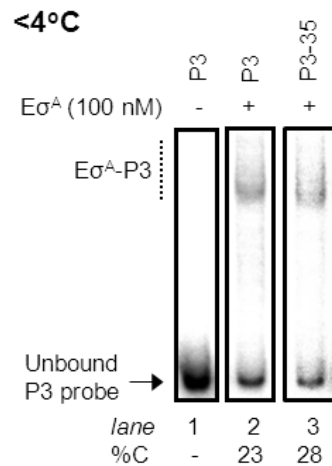


Figure 80: Image of a 4.5% (w/v) native-PAGE gel showing the binding of E $\sigma^A$  to the *agr* P3 and P3-35 promoters at <4°C.

The assay was performed using 100 nM E $\sigma^A$  with 5 nM of promoter probe. The migration positions of the E $\sigma^A$ -P3 complexes and the unbound P3 promoter probes are indicated. The percentage of the total DNA probe present in the reaction bound by E $\sigma^A$  is labelled as %C. Lanes 1 and 2 are reproduced from Figure 69 and are presented for comparison. In at least three independent experiments, the data range for each point fell within 15% of the %C value shown.

Surprisingly, results, shown in Figure 80, demonstrated that E $\sigma^A$  was able to bind both P3 and P3-35 promoters equally well at <4°C to form R<sub>Pc</sub>, despite the scrambling of the -35 promoter element in the P3-35 probe. 23% and 28% of P3 and P3-35 respectively, were bound by 100 nM E $\sigma^A$  (Figure 80, lanes 2-3). To further these findings the reactions were repeated as a function of E $\sigma^A$  concentration at 37°C to determine whether this effect is limited to E $\sigma^A$ -promoter complexes formed at <4°C or whether the scrambling of the -35 promoter element had any effect on E $\sigma^A$ -promoter complexes formed at 37°C.

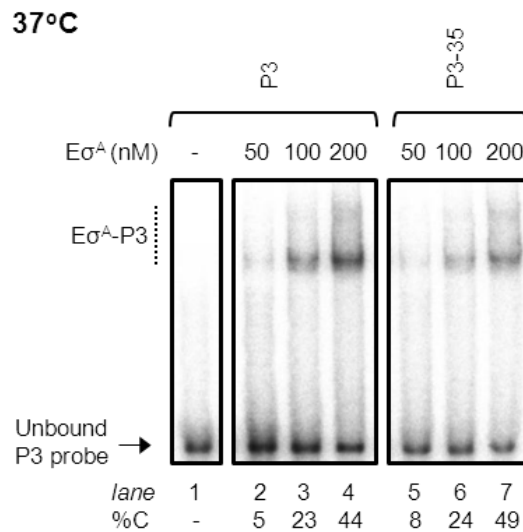


Figure 81: Image of a 4.5% (w/v) native-PAGE gel showing the binding of Eo<sup>A</sup> to the *agr* P3 and P3-35 promoters at 37°C.

The assay was performed as a function of Eo<sup>A</sup> concentration with 5 nM of promoter probe. The migration positions of the Eo<sup>A</sup>-P3 complexes and the unbound P3 promoter probes are indicated. The percentage of the total DNA probe present in the reaction bound by Eo<sup>A</sup> is labelled as %C. Lanes 1-4 are reproduced from Figure 70 and are presented for comparison. In at least three independent experiments, the data range for each point fell within 15% of the %C value shown.

Results, shown in Figure 81, demonstrated that Eo<sup>A</sup> was also able to bind both P3 and P3-35 promoters equally well at 37°C despite the scrambling of the -35 promoter element in the P3-35 probe. 23% and 24% of P3 and P3-35 respectively, were bound by 100 nM Eo<sup>A</sup> (Figure 81, lanes 3 and 6). Therefore Eo<sup>A</sup> was able to bind the P3-35 promoter and form Eo<sup>A</sup>-promoter complexes equally well at both <4°C and 37°C, in comparison to the wild-type P3 promoter. It hence appears that Eo<sup>A</sup> does not require the -35 element for efficient Eo<sup>A</sup>-promoter complex formation at the *agr* P3 promoter.

### 6.3.2. The effect of scrambling the -35 promoter element on Eo<sup>A</sup> activity at the P3 promoter

#### 6.3.2.1. Eo<sup>A</sup> activity at the P3-35 promoter

To directly establish RPo formation at the P3-35 promoter in comparison to the wild-type P3 promoter and therefore the level of transcription initiation from the P3-35 promoter, a short RNA synthesis assay was performed. The assay was performed as in section 6.2.2, with Eo<sup>A</sup> using the P3 and P3-35 promoter probes. The activity of the promoters was measured

through synthesis of the short RNA product GpAU from the  $E\sigma^A$ -P3 promoter complexes, visualised by urea denaturing gel electrophoresis, as in section 2.4.1.

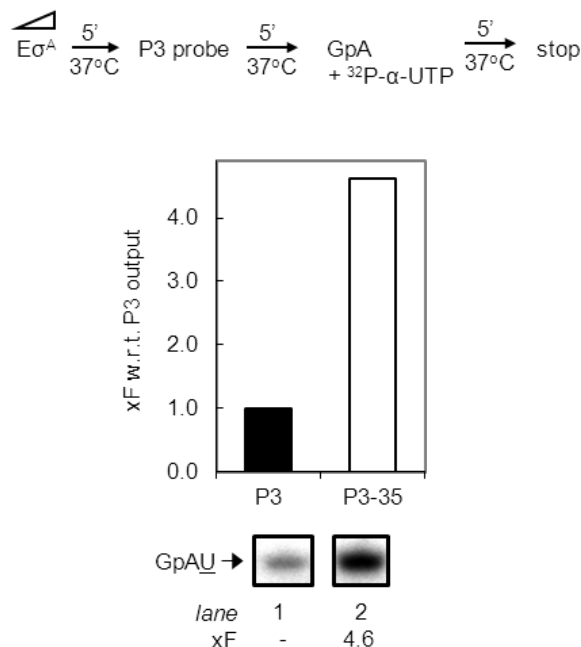


Figure 82: Image of a 20% (w/v) urea-denaturing-PAGE gel showing short RNA synthesis from P3 and P3-35.

A short RNA synthesis assay showing the synthesis of the short RNA products GpAU (from P3 or P3-35) at 37°C by 100 nM  $E\sigma^A$  (indicated by the arrow, underlined nucleotides are  $^{32}P$ -labelled). A schematic of the assay is shown above. The amount of short RNA products synthesised is expressed as fold increase in short RNA products synthesised from P3-35 (x F) (lane 2) with respect to the corresponding P3 reaction (lane 1). Lane 1 is reproduced from Figure 71 and is presented for comparison. In at least two independent experiments, the observed trend between each data point for the quantity of short RNA products synthesised from the P3 promoter was comparable.

Results, shown in Figure 82, revealed that RPo formation was 4.6 fold higher from the P3-35 promoter than from the wild-type P3 promoter, compare lanes 1 and 2. As  $E\sigma^A$  formed the RPo equally well at the P3 and P3-35 promoter probes (Figure 80), subsequent steps of transcription initiation were explored including RPo stability and the rate of RPo accumulation to determine the basis of higher activity from the P3-35 promoter probe.

### 6.3.2.2. The stability of the RPo at the P3-35 promoter

First, the short RNA synthesis assay using the P3 and P3-35 promoter probes was extended to determine if the RPo formed at the P3-35 promoter were more stable than the RPo formed at the wild-type P3 promoter. Reactions were setup with  $E\sigma^A$  and either the P3 or P3-35 promoter probes to form RPo at 37°C. The RPo were challenged with heparin and the

RPo surviving were measured over time as GpAU synthesis, through the addition of the short dinucleotide primer GpA, that complements the positions +2 and +3 of the P3 template strand and [ $\alpha$ - $^{32}$ P]-UTP complementing the +4 position of the P3 template strand, which can be visualised by urea denaturing gel electrophoresis, as in section 2.4.1.

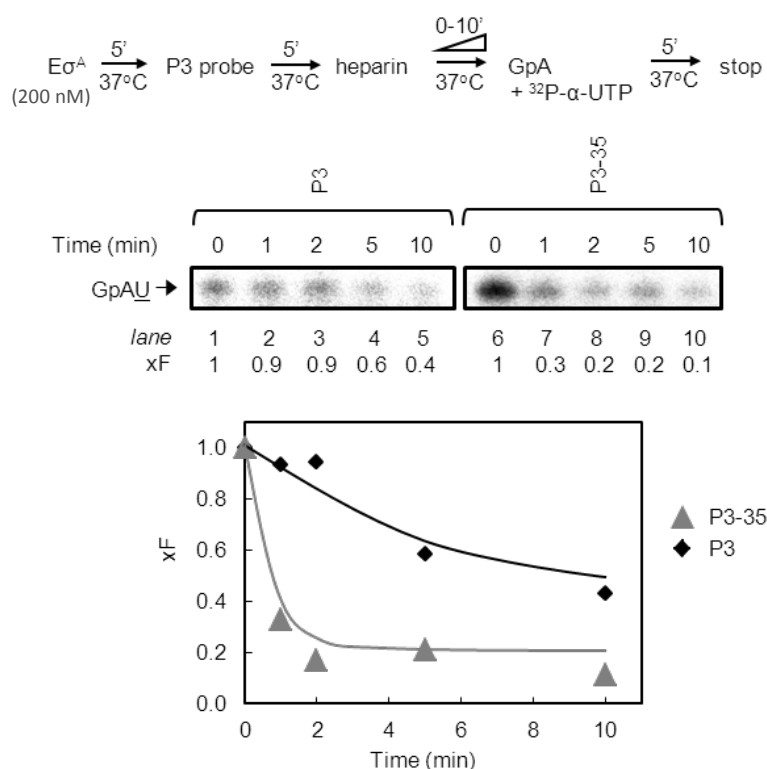


Figure 83: Image of a 10% (w/v) urea-denaturing-PAGE gel showing the stability of Eo<sup>A</sup>-P3/P3-35 complexes challenged by heparin.

A short RNA synthesis assay showing the synthesis of the short RNA products GpAU from Eo<sup>A</sup>-P3/P3-35 promoter complexes challenged with 100  $\mu$ g/ml heparin (indicated by the arrows). The reactions were performed at 37°C using the P3 and P3-35 promoter probes as a function of time, under single round conditions. A schematic of the assay is shown above. The amount of short RNA products synthesised are expressed as fold increase in short RNA products synthesised after the heparin challenge from P3 (xF) (lanes 1-5) and P3-35 (xF) (lanes 6-10) with respect to the corresponding 0 minute reaction (lane 1 and 6). The results are shown in a line chart below comparing fold increase in short RNA products synthesised after the heparin challenge against time (minutes) from the P3 (black diamonds) and P3-35 (grey triangles). In at least two independent experiments, the observed decreasing trend between each data point for the quantity of short RNA products synthesised from the P3 promoter was comparable.

Results, shown in Figure 83, indicated that RPo formed at the P3-35 promoter probe were less stable than those formed at the wild-type P3 probe, compare lanes 1-5 with 6-10. After just 1 minute the RPo remaining at the P3-35 promoter probes were reduced to 0.3 fold compared to only 0.9 fold with the wild-type P3 promoter probe (Figure 83, lanes 2 and 7). Therefore despite the increased RPo formation at the P3-35 promoter, shown in Figure 82,

the -35 promoter element at the P3 promoter plays an important role in RPo stability at the P3 promoter, possibly due to compromised interactions between the RNAP and the promoter within the RPo formed at the mutant P3-35 promoter.

### 6.3.2.3. The rate of RPo formation at the P3-35 promoter

Next the rate of RPo formation at the P3 and P3-35 promoter probes was investigated using a short RNA synthesis assay, following the assay performed in section 6.2.5.2. The accumulation of the GpAU transcript synthesised by  $E\sigma^A$  was measured from the P3 and P3-35 promoter probes as a function of time under single round conditions.

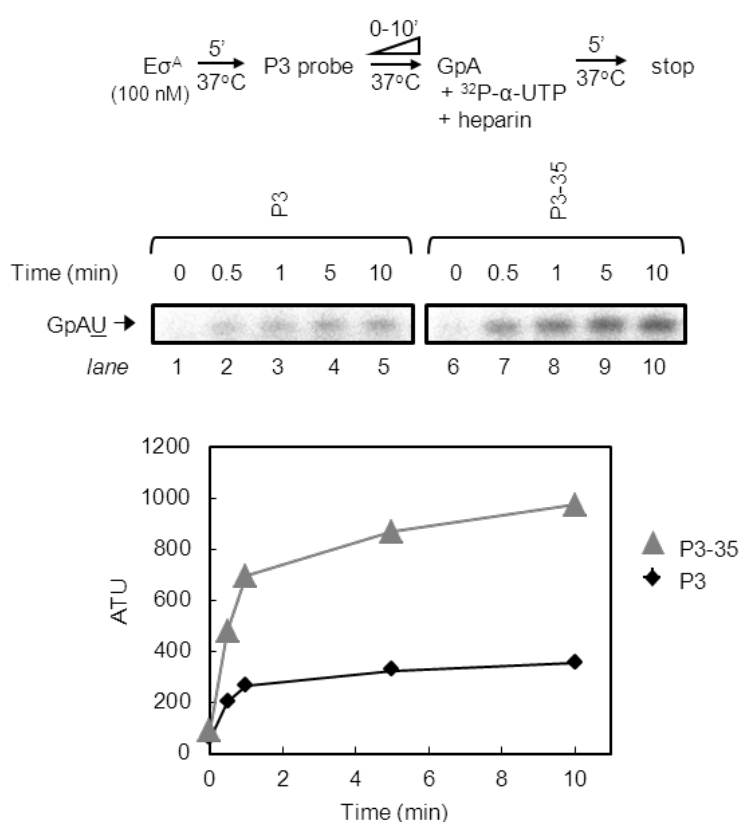


Figure 84: Image of a 10% (w/v) urea-denaturing-PAGE gel showing the rate of RPo formation at P3 and P3-35.

A short RNA synthesis assay showing the synthesis of the short RNA products GpAU from  $E\sigma^A$ -P3/P3-35 promoter complexes over time (indicated by the arrows). The reactions were performed at 37°C using the P3 and P3-35 promoter probes as a function of time, under single round conditions. A schematic of the assay is shown above. The amount of short RNA products synthesised are expressed as actual transcription units (ATU) from P3 (lanes 1-5) and P3-35 (lanes 6-10). The results are shown in a line chart below comparing short RNA products synthesised as ATU against time (minutes) from the P3 (black diamonds) and P3-35 (grey triangles). ATU is derived from the band intensity calculated from the gel imaged by phosphorimager analysis (see section 2.4.1). In at least two independent experiments, the observed increasing trend between each data point for the quantity of short RNA products synthesised from the P3 promoter was comparable.

The results, shown in Figure 84, implied that the rate of RPo formation is initially faster at the P3-35 promoter than the wild-type P3 promoter, compare lanes 1-3 with 6-8. However the rate of RPo formation reaches equilibrium at either promoter at the same time. Results from the RPo stability and rate of RPo formation assays suggest that scrambling the -35 promoter element decreases the stability of the Eo<sup>A</sup>-promoter complexes formed, however this leads to an increased rate of isomerisation ( $k_f$ ) from the RPc to the RPo at the P3 promoter. This observation is very similar to that seen on the P3-17 promoter (see section 6.2.5.2). Therefore it can be suggested that removing the -35 interaction *ipso facto* compensates for the sub-optimal spacer length, by potentially forcing Eo<sup>A</sup> to make additional contacts with the extended -10 motif.

### **6.3.3. The effect of scrambling the -35 promoter element on the activation of transcription by AgrA at the *agr* P3 promoter**

As AgrA has been shown to bind to direct repeats near to the -35 element, with DNaseI footprinting showing that AgrA protects a region just overlapping the -35 element (Koenig, Ray et al. 2004), the -35 element may be involved in AgrA activation at the P3 promoter. To test the importance of the -35 element in AgrA activation the P3-35 promoter probe was used in a short RNA synthesis assay in the presence of [6His]-AgrA and compared to activation by the wild-type P3 promoter probe. Please note that the major AgrA binding sites were retained in the P3-35 promoter probe (see Figure 7 and Table 10). The assay was constructed to determine if AgrA can activate transcription from P3 when the -35 promoter element is scrambled. The assay was performed with Eo<sup>A</sup>, as in section 6.2.3, using the P3 and P3-35 promoter probes in the presence and absence of [6His]-AgrA, following the basic protocol in section 2.4.6. The activity of each promoter was measured as GpAU synthesised and visualised by urea denaturing gel electrophoresis, as in section 2.4.1.

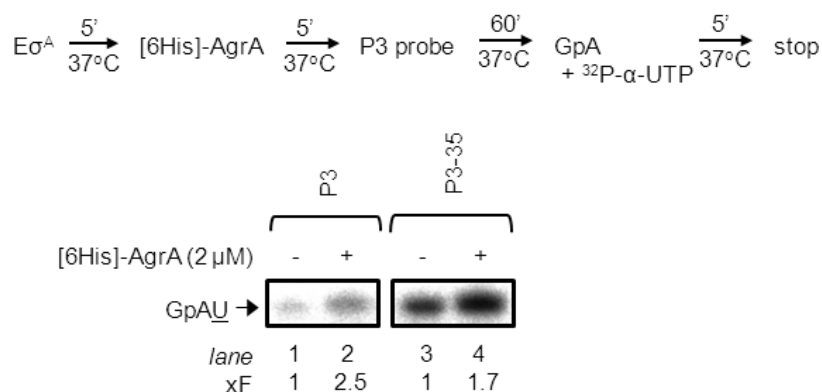


Figure 85: Image of a 20% (w/v) urea-denaturing-PAGE gel showing the effect of [6His]-AgrA on the short RNA synthesis at P3 and P3-35.

A short RNA synthesis assay showing the synthesis of the short RNA products GpAU (from P3 or P3-35) at 37°C by 100 nM  $\text{E}\sigma^A$  (indicated by the arrow, underlined nucleotides are  $^{32}\text{P}$ -labelled). Reactions are conducted in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of 2 μM [6His]-AgrA, pre-incubated with  $\text{E}\sigma^A$  before the addition of the DNA probe. A schematic of the assay is shown above. The amount of short RNA products synthesised are expressed as fold increase in short RNA products synthesised from P3 (xF) and P3-35 (xF) (lanes 2 and 4) with respect to the corresponding no [6His]-AgrA reaction (lane 1 and 3). Lanes 1 and 2 are reproduced from Figure 72 and are presented for comparison. In at least two independent experiments, the observed trend between each data point for the quantity of short RNA products synthesised from the P3 promoter was comparable.

Results, shown in Figure 85, indicated that activation by [6His]-AgrA was reduced from the P3-35 promoter to just 1.7 fold, when compared to the 2.5 fold increase in activation using the wild-type P3 promoter, compare lanes 2 and 4. The results suggest that AgrA activation could involve antagonising the interactions  $\text{E}\sigma^A$  makes with the -35 element, which are mimicked by the scrambling of the -35 promoter element at P3. Thus, the proposed mechanism of transcription activation by AgrA at the P3 promoter could involve (in addition to compensating for the sub-optimal spacer length, see section 6.2.4) the binding of AgrA near to the -35 promoter element thereby compromising or blocking  $\text{E}\sigma^A$  from contacting the -35 promoter element and causing it to make additional contacts at the extended -10 like motif (see above and below), and so increasing the rate of RPo formation at the P3 promoter. Consistent with this suggestion, results from the RPo stability (see section 6.3.2.2) and the rate of RPo formation assays (see section 6.3.2.3) suggest that scrambling the -35 promoter element decreases the stability of the  $\text{E}\sigma^A$ -promoter complexes formed, however this leads to an increased rate of isomerisation ( $k_i$ ) from the R<sub>Pc</sub> to the R<sub>Po</sub> at the P3 promoter.

#### 6.4. The effect of scrambling the -35 promoter element and mutating the extended -10 motif on Eo<sup>A</sup> activity at the P3 promoter

To provide further experimental evidence for the proposed mechanism of how AgrA might activate transcription from the P3 promoter (see above) a new P3 promoter probe was constructed with the -35 promoter element scrambled and the extended -10 motif TG changed to AC (P3-35AC) (Table 10) following section 3.3.2. To determine the effect of scrambling the -35 element and mutating the extended -10 motif on the initial Eo<sup>A</sup>-promoter complex formation (RPC) an EMSA was established at <4°C. Reactions were performed at <4°C using the P3 and P3-35AC promoter probes, following the protocol in section 2.4.5.

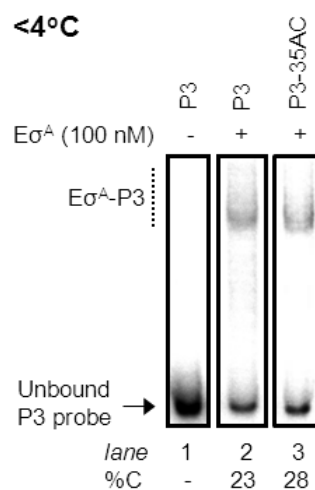


Figure 86: Image of a 4.5% (w/v) native-PAGE gel showing the binding of Eo<sup>A</sup> to the *agr* P3 and P3-35AC promoters at <4°C.

The assay was performed using 100 nM Eo<sup>A</sup> with 5 nM of promoter probe. The migration positions of the Eo<sup>A</sup>-P3 complexes and the unbound P3 promoter probes are indicated. The percentage of the total DNA probe present in the reaction bound by Eo<sup>A</sup> is labelled as %C. Lanes 1 and 2 are reproduced from Figure 69 and are presented for comparison. In at least three independent experiments, the data range for each point fell within 15% of the %C value shown.

Results, shown in Figure 86, demonstrated that Eo<sup>A</sup> was able to bind both P3 and P3-35AC promoters equally well at <4°C despite the scrambling of the -35 promoter element and the mutation of the extended -10 motif in the P3-35AC probe. 23% and 28% of P3 and P3-35AC respectively were bound by 100 nM Eo<sup>A</sup> (Figure 86, lanes 2-3). To further these findings the reactions were repeated as a function of Eo<sup>A</sup> concentration at 37°C to determine whether

this effect is limited to Eo<sup>A</sup>-promoter complexes formed at <4°C or whether the scrambling of the -35 promoter element has any effect on Eo<sup>A</sup>-promoter complexes formed at 37°C.

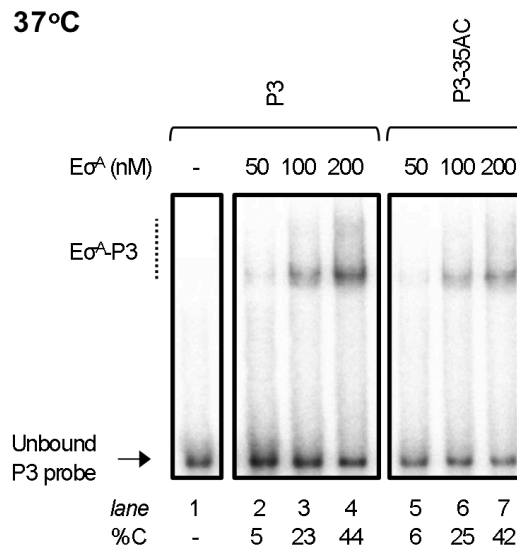


Figure 87: Image of a 4.5% (w/v) native-PAGE gel showing the binding of Eo<sup>A</sup> to the *agr* P3 and P3-35AC promoters at 37°C.

The assay was performed as a function of Eo<sup>A</sup> concentration with 5 nM of promoter probe. The migration positions of the Eo<sup>A</sup>-P3 complexes and the unbound P3 promoter probes are indicated. The percentage of the total DNA probe present in the reaction bound by Eo<sup>A</sup> is labelled as %C. Lanes 1-4 are reproduced from Figure 70 and are presented for comparison. In at least three independent experiments, the data range for each point fell within 15% of the %C value shown.

The results, shown in Figure 87, demonstrated that Eo<sup>A</sup> was also able to bind both P3 and P3-35AC promoters equally well at 37°C despite the scrambling of the -35 promoter element and the mutation of the extended -10 motif in the P3-35AC probe. 23% and 25% of P3 and P3-35AC respectively were bound by 100 nM Eo<sup>A</sup> (Figure 87, lanes 3 and 6). Therefore Eo<sup>A</sup> is able to bind the P3-35AC promoter and form Eo<sup>A</sup>-promoter complexes equally well in comparison to the wild-type P3 promoter, even when both the -35 promoter element is scrambled and the extended -10 motif is mutated.

#### 6.4.1. The effect of scrambling the -35 promoter element and mutating the extended -10 motif on Eo<sup>A</sup> activity at the P3 promoter

To directly establish the RPo formation and therefore the productivity of Eo<sup>A</sup> at the P3-35AC promoter, in comparison to the wild-type P3 promoter, and the scrambling/mutating of each element independently, a short RNA synthesis assay was performed. The assay was performed as in section 6.2.2, with Eo<sup>A</sup> using the P3, P3-35, P3-AC and P3-35AC promoter

probes. The activity of the promoters were measured through synthesis of the short RNA product GpAU from the Eo<sup>A</sup>-P3 promoter complexes, visualised by urea denaturing gel electrophoresis, as in section 2.4.1.

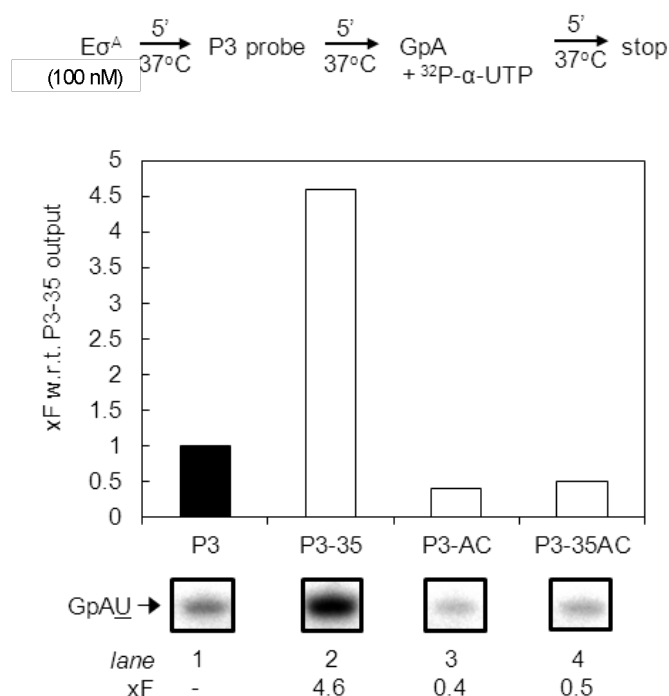


Figure 88: Image of an autoradiograph of a 20% (w/v) urea-denaturing-PAGE gel showing the synthesis of short RNA from P3, P3-35, P3-AC and P3-35AC.

A short RNA synthesis assay showing the synthesis of the short RNA products GpAU (from P3, P3-35, P3-AC or P3-35AC) at 37°C by 100 nM Eo<sup>A</sup> (indicated by the arrow, underlined nucleotides are <sup>32</sup>P-labelled). A schematic of the assay is shown above. The amount of short RNA products synthesised is expressed as fold increase in short RNA products synthesised from P3-35 (xF) (lane 2), P3-AC (lane 3) and P3-35AC (lane 4) with respect to the corresponding P3 reaction (lane 1). Lanes 1 and 3, reproduced from Figure 71 and lane 2 reproduced from Figure 82 are presented for comparison. In at least two independent experiments, the observed trend between each data point for the quantity of short RNA products synthesised from the P3 promoter was comparable.

The results, shown in Figure 88, revealed that RPo formation was 2 fold lower whether the extended -10 motif was mutated or the both the -35 promoter element was scrambled and the extended -10 motif was mutated, compare lanes 3 and 4. Mutating the extended -10 motif alone led to a ~9 fold decrease in RPo formation when the -35 element is already scrambled (Figure 88, lanes 2 and 4). Therefore, based on results so far, it can be concluded that the extended -10 motif is a major determinant for AgrA dependent and independent transcription from the *agr* P3 promoter. The extended -10 motif is required for optimum

productivity of the *agr* P3 promoter and that scrambling of the -35 element leads to increased productivity of the P3 promoter.

#### 6.4.2. The effect of scrambling the -35 promoter element and mutating the extended -10 motif on the activation of transcription by AgrA at the *agr* P3 promoter

To test the importance of the -35 promoter element in addition to the extended -10 motif on the proposed mechanism of AgrA activation, a short RNA synthesis assay was performed with E $\sigma^A$ . The assay was conducted in the presence of [6His]-AgrA with the P3-35AC promoter probe and the wild-type P3 promoter probe, as in section 6.2.3 following the basic protocol in section 2.4.6. The activity of each promoter was measured as GpAU synthesised and visualised by urea denaturing gel electrophoresis, as in section 2.4.1.

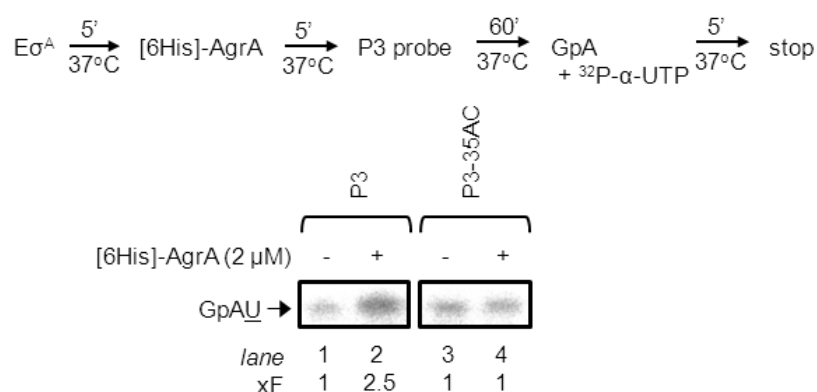


Figure 89: Image of a 20% (w/v) urea-denaturing-PAGE gel showing the effect of [6His]-AgrA on short RNA synthesis at P3 and P3-35AC.

A short RNA synthesis assay showing the synthesis of the short RNA products GpAU (from P3 or P3-35AC) at 37°C by 100 nM E $\sigma^A$  (indicated by the arrow, underlined nucleotides are <sup>32</sup>P-labelled). Reactions are conducted in the absence (lanes 1 and 3) and presence (lanes 2 and 4) of 2 μM [6His]-AgrA, pre-incubated with E $\sigma^A$  before the addition of the DNA probe. A schematic of the assay is shown above. The amount of short RNA products synthesised are expressed as fold increase in short RNA products synthesised from P3 (xF) and P3-35AC (xF) (lanes 2 and 4) with respect to the corresponding no [6His]-AgrA reaction (lane 1 and 3). Lanes 1 and 2 are reproduced from Figure 72 and are presented for comparison. In at least two independent experiments, the observed trend between each data point for the quantity of short RNA products synthesised from the P3 promoter was comparable.

The results, shown in Figure 89, indicated that [6His]-AgrA cannot activate transcription from the P3-35AC promoter when compared to the wild-type P3 promoter probe, compare lanes 2 and 4. As [6His]-AgrA is unable to activate transcription initiation at the P3 promoter with a scrambled -35 promoter element and extended -10 motif, this confirms that both elements play an important role in the mechanism of AgrA activation. The results therefore

support the proposed mechanism of AgrA activating transcription from the P3 promoter by compensating for the sub-optimal spacer length in an extended -10 motif dependent manner.

## 6.5. Conclusions

Experiments to unravel the possible mechanism of AgrA dependent activation at the *agr* P3 promoter have focussed on the extended -10 motif, the sub-optimal promoter element spacer and the -35 promoter element.

Short RNA synthesis assays demonstrated that the extended -10 motif is required for optimal promoter activity at the *agr* P3 promoter (Figure 71). This is highlighted by the extended -10 motif being critical for transcription initiation when the -35 element was scrambled (Figure 88). Like other extended -10 promoters the extended -10 motif at the *agr* P3 promoter could potentially be contacted by  $\sigma_{3.0}$  of  $E\sigma^A$  (Sanderson, Mitchell et al. 2003). As the removal of the extended -10 motif had no effect on initial  $E\sigma^A$  binding (Figure 69) the role of the extended -10 motif appears to be in steps after R<sub>Pc</sub> formation. It is also apparent that the extended -10 motif plays a role in the mechanism of activation by AgrA as the mutation of the extended -10 motif reduces/removes all activation by [6His]-AgrA (Figure 72). Therefore it is apparent that AgrA activates transcription from the P3 promoter in an extended -10 motif dependent manner.

The structure of the AgrA LytTR binding domain bound to DNA reveals a mode of binding where AgrA<sub>c</sub> induces a substantial bend in the DNA (Sidote, Barbieri et al. 2008). It is therefore regarded that one mechanism of activation by AgrA involves AgrA compensating for the sub-optimal spacer (20 nucleotides) at the *agr* P3 promoter via this distortion in the DNA. Shortening the optimal P2 spacer had, as expected little, if any, effect on transcription initiation at the P2 promoter (Figure 73), whereas shortening the sub-optimal P3 spacer to 17 nucleotides in length had a dramatic effect on the P3 promoter (Figure 74). Transcription initiation from the optimised P3 spacer promoter (P3-17) was 3-5 fold higher than from the wild-type P3 promoter (Figure 74). Results from the R<sub>Po</sub> stability (Figure 76) and R<sub>Po</sub> formation assays (Figure 77) suggest that optimising the P3 spacer length to the optimal 17 nucleotides length clearly improves the ability of the initial  $E\sigma^A$ -promoter complexes formed

at the P3 promoter to isomerise to the RPo. Therefore AgrA could increase the rate of RPo formation by bending the DNA upon binding and thereby compensating for the sub-optimal spacer length. Furthermore, short RNA synthesis assays indicate that [6His]-AgrA is unable to activate transcription at the optimised spacer P3 promoter (Figure 78). Therefore, this result supports the view that AgrA activated RPo formation at the P3 promoter occurs via AgrA compensating for the sub-optimal spacer length and that this occurs in an extended -10 motif dependent manner.

The -35 promoter element also seems to play an important role in AgrA activation at the *agr* P3 promoter. The scrambling of the P3 -35 element led to a 4.6 fold increase in transcription initiation (Figure 82). Taking into account that scrambling of the -35 element had no effect on RPo formation by  $E\sigma^A$  at the P3 promoter (Figure 80), the RPo stability (Figure 83) and RPo formation assays (Figure 84) suggest that scrambling the -35 promoter element decreases the stability of initial  $E\sigma^A$ -promoter complexes formed and increases the rate of isomerisation ( $k_f$ ) from the RPo to the RPo at the P3 promoter. Therefore the proposed mechanism of AgrA activation at the P3 promoter could involve the binding of AgrA near to the -35 promoter element, thereby antagonising or directly blocking  $E\sigma^A$  from contacting the -35 promoter element and causing it to make additional contacts at the extended -10 motif, which increases the rate of RPo formation at the P3 promoter. This view is supported by the reduced activation by [6His]-AgrA when the P3 -35 element is scrambled (Figure 85). Both mechanisms proposed, involving either AgrA distorting the DNA or hiding the -35 element, indicate that AgrA possibly activates transcription from the P3 promoter by compensating for the sub-optimal spacer length in an extended -10 motif dependent manner. The possible mechanisms of activation by AgrA at the P3 promoter are further discussed in chapter 8.

## CHAPTER SEVEN

### 7. Investigating how SarA influences transcription initiation at the *agr* operon

#### 7.1. Introduction

SarA, a member of the staphylococcal accessory regulator A (SarA) family of regulators is a global regulator of *S. aureus* virulence factors, like the *agr* operon. SarA was first characterised as an 'anti-*agr*' regulatory element by phenotypical characterisation of a *S. aureus sarA* deletion strain (Cheung, Koomey et al. 1992). However further *in vivo* characterisation of SarA in the commonly studied *S. aureus* NCTC 8325-4 strain identified, through Northern blot analysis with a *sarA* deletion strain, that SarA has a role in enhancing *agr* function rather than antagonising it (Chien, Manna et al. 1998). In a different *in vivo* study using a *sarA* deletion strain, it was discovered that SarA is required for wild type levels of transcription of the *agr* RNAII and RNAPIII transcripts, with a threefold decrease in the *sarA* deletion strain (Dunman, Murphy et al. 2001).

Further characterisation of SarA *in vitro*, using DNase I footprinting, demonstrated that SarA binds to the *agr* promoter intergenic region (*agr* IR) as dimers at three binding sites with two sites overlapping elements of the *agr* P2 and P3 promoters (Rechlin, Gillaspay et al. 1999). The structure of SarA is known and contains a helix-turn-helix (HTH) DNA binding domain that contacts the DNA (Liu, Manna et al. 2001). Two residues in the recognition helix of SarA, arginine 84 (R84) and 90 (R90), have been identified as critical for DNA binding.

In contrast to the *in vivo* characterisation, an earlier *in vitro* study implied that SarA inhibits transcription from both the *agr* P2 and P3 promoters (Chakrabarti and Misra 2000). However, this study was not done with a fully homologous *S. aureus* transcription system and not under conditions where the *agr* P2 and P3 were simultaneously present on the same DNA fragment (as in the native context). Furthermore it has also been suggested that SarA may regulate *agr* operon expression indirectly, through the regulation of factors involved in the activation of *agr* operon expression (Chakrabarti and Misra 2000).

Now that a fully native *S. aureus in vitro* transcription system has been established (Chapter 3) it was used to further study the effect of SarA on transcription initiation at the *agr* operon

promoters. Experiments to unravel the mechanism of SarA regulation were carried out using SarA expressed and purified with a 6His tag from the pET28b+ expression vector in section 3.2.3. Both EMSAs and full length *in vitro* transcription assays were performed to assess the effect of SarA on transcription initiation at the *agr* P2 and P3 promoters. Order of addition assays were also performed to determine the step of transcription initiation which is affected by SarA at the *agr* operon.

#### **7.1.1. Objectives**

The overall objective of this part of the project was to investigate the effect of SarA on transcription initiation at the *agr* operon. In this regard, the specific aims include:

- I. Determine the binding activity of SarA at the *agr* P2 and P3 promoters.
- II. Determine how SarA affects transcription output from the *agr* P2 and P3 promoters.
- III. Unravel the mechanism of SarA regulation at the *agr* operon.

## 7.2. SarA binding to the *agr* P2 and P3 promoters

EMSA were performed with [6His]-SarA at 37°C to determine the activity of the [6His]-SarA preparation, purified in section 3.2.3.1 and to demonstrate the ability of SarA to bind to the *agr* P2 and P3 promoters. [6His]-SarA was titrated at 0.5, 1, 2, 3, 4 and 5 µM final concentrations into reactions containing the <sup>32</sup>P-labelled P2 and P3 probes at a final concentration of 5 nM, following the protocol in section 2.4.5. The P2 and P3 probes contain the P2 and P3 promoter sequences and each contains a SarA binding site (Figure 19).

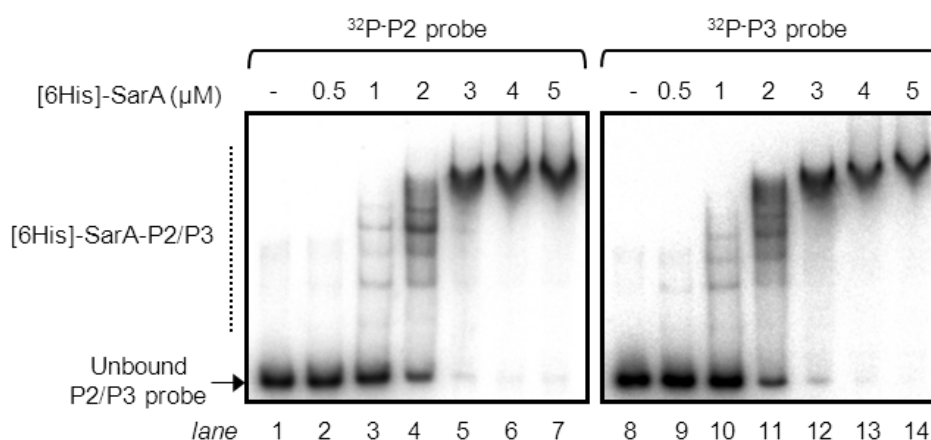


Figure 90: Image of an autoradiograph of a 4.5% (w/v) native-PAGE gel showing the binding of [6His]-SarA to the *agr* P2 and P3 promoters at 37°C.

The assay was performed as a function of [6His]-SarA concentration with 5 nM of promoter probe. The migration positions of the [6His]-SarA-P2/P3 complexes and the unbound P2 and P3 promoter probes are indicated. In at least three independent experiments, the observed increasing trend between each data point for the quantity of SarA bound to either the P2 or P3 promoter probe was comparable.

The results, shown in Figure 90, identified that the [6His]-SarA preparation was able to bind to the *agr* P2 and P3 probes containing SarA binding sites equally well, demonstrating the activity of the [6His]-SarA preparation. This concurs with the initial characterisation of SarA binding sites at the *agr* operon, where SarA bound to sites overlapping the *agr* P2 and P3 promoters equally well. The binding of SarA created an unusual laddering pattern possibly due to multiple SarA binding to half-sites in the *agr* IR. EMSA reactions were also performed as above in the presence of a SarA DNA binding mutant [6His]-SarA<sub>R84A</sub>, to be used as a control for SarA in full length *in vitro* transcription assays (Rechtin, Gillaspay et al. 1999). The reactions were performed to show that the SarA DNA binding mutant was unable to bind

DNA and therefore were tested with the binding of 250 nM wild-type SarA and [6His]-SarA<sub>R84A</sub> to 2 nM of the <sup>32</sup>P-P2 promoter probe.

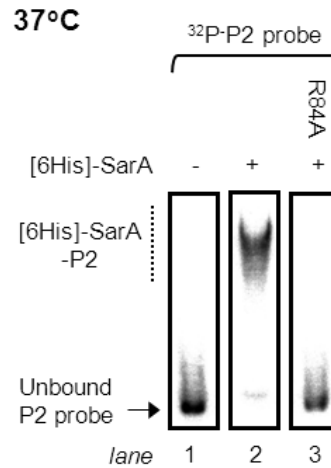


Figure 91: Image of an autoradiograph of a 4.5% (w/v) native-PAGE gel showing the binding of [6His]-SarA and [6His]-SarA<sub>R84A</sub> to the *agr* P2 promoter at 37°C.

The assay was performed in the presence and absence of 250 nM [6His]-SarA and [6His]-SarA<sub>R84A</sub> with 2 nM of <sup>32</sup>P-P2 promoter probe. The migration positions of the [6His]-SarA-P2 complexes and the unbound P2 promoter probes are indicated. In at least three independent experiments, the observed trend between each data point for the quantity of SarA bound to the P2 promoter probe was comparable.

The results, presented in Figure 91, show, as previously identified, that [6His]-SarA<sub>R84A</sub> does not bind to the *agr* P2 promoter due to the mutation of arginine at residue 84 (R84A), shown to be essential for the DNA binding ability of SarA (Rechlin, Gillaspay et al. 1999), to alanine, compare lanes 1 and 3. The wild-type SarA control lane demonstrates that the non-DNA binding ability of [6His]-SarA<sub>R84A</sub> is due to the SarA R84A mutation (Figure 91, lane 2 and 3).

### 7.3. Influence of SarA on Eσ<sup>A</sup> transcription initiation from the *agr* P2 and P3 promoters

To determine the effect of [6His]-SarA on Eσ<sup>A</sup> transcription initiation at the *agr* P2 and P3 promoters, a multiple round full length *in vitro* transcription assay was performed with Eσ<sup>A</sup>, as in section 2.4.8. The assay was performed as function of [6His]-SarA concentration using pJR<sub>P2+P3</sub> as the template. Control reactions were also performed using [6His]-SarA<sub>R84A</sub>, with an arginine to alanine mutation at residue 84 (R84A), which has been shown to be essential for the DNA binding ability of SarA (Rechlin, Gillaspay et al. 1999).

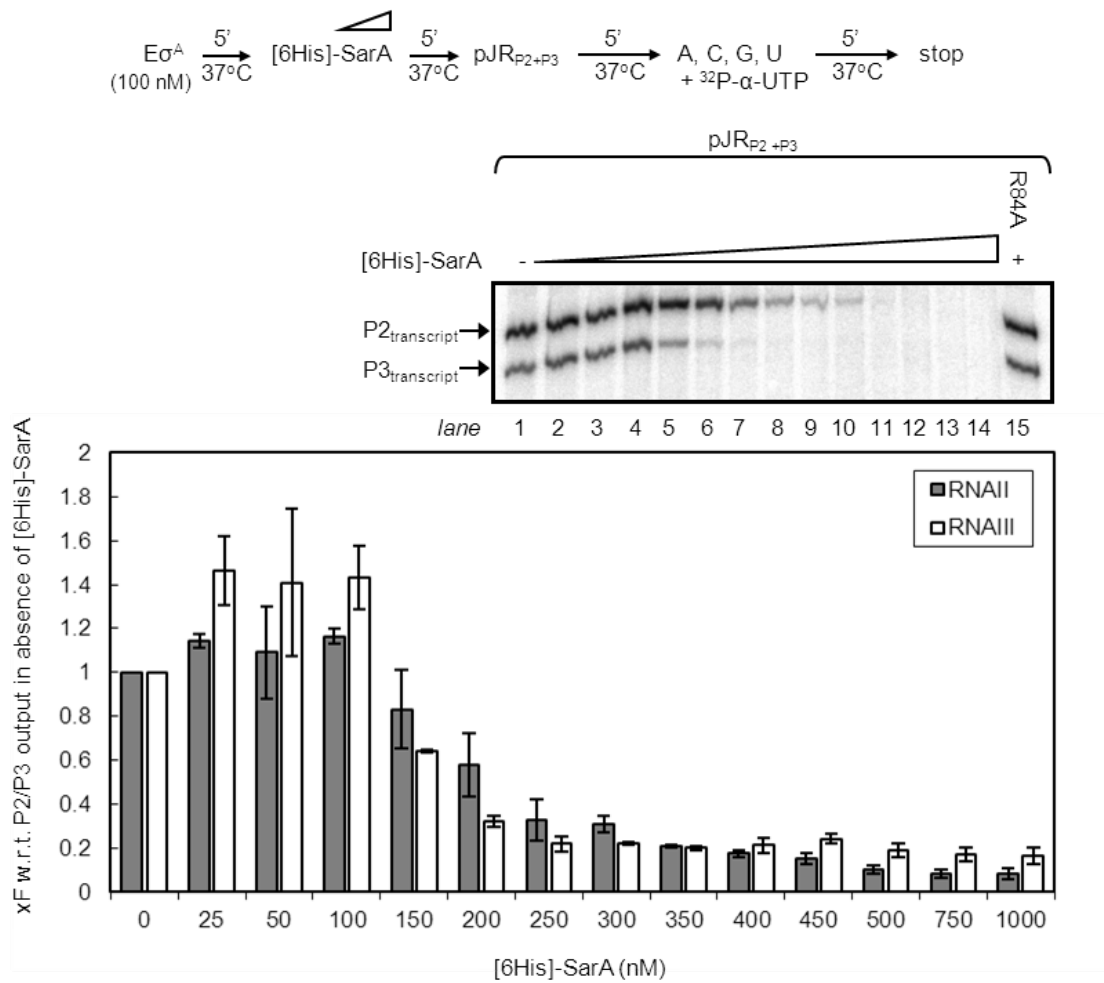


Figure 92: Image of a 10% (w/v) urea-denaturing-PAGE gel showing the effect of [6His]-SarA on transcription initiation from pJR<sub>P2+P3</sub>.

A full length *in vitro* transcription assay showing the transcripts synthesised from the pJR<sub>P2+P3</sub> reporter plasmid at 37°C by Eo<sup>A</sup> (indicated by the arrows) in the presence and absence of [6His]-SarA. A R84A [6His]-SarA DNA-binding mutant is used as a control (lane 15). The reactions were performed as a function of [6His]-SarA concentration under multiple round conditions. A schematic of the assay is shown above. A bar chart of the reactions is shown below, with the amount of transcripts synthesised expressed as fold output of transcripts synthesised (xF) from P2 and P3 (lanes 2-14) with respect to the corresponding no [6His]-SarA reaction (lane 1), against [6His]-SarA concentration. The range is calculated for each data point from three independent experiments and is indicated by bars in the bar chart.

Results, as shown in Figure 92, indicated that [6His]-SarA represses Eo<sup>A</sup> transcription initiation at both the *agr* P2 and P3 promoters with a similar trend under these experimental conditions, lanes 1-14. The control reaction using the [6His]-SarA DNA binding mutant ([6His]-SarA<sub>R84A</sub>) indicates that the repression of transcription initiation from the *agr* P2 and P3 promoters was specific to [6His]-SarA, lane 15. Interestingly, it is noted that [6His]-SarA may have a small stimulatory effect on transcription initiation from P2 and P3 at

lower concentrations, compare lanes 1-4; however this observation was not investigated further due to time restrictions.

Multiple round full length *in vitro* transcription assays with  $E\sigma^A$  were also performed using the pJR<sub>P2</sub> and pJR<sub>P3</sub> templates, to measure whether the SarA repression occurs at each *agr* promoter independently of the divergent promoter. The assay was performed as in Figure 92 as function of [6His]-SarA concentration using the SarA non-DNA binding mutant R84A as a control.

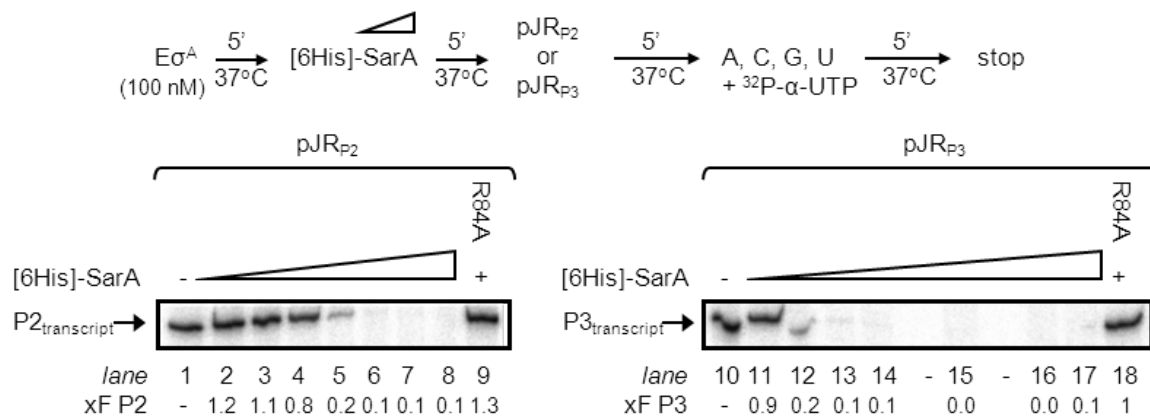


Figure 93: Image of a 10% (w/v) urea-denaturing-PAGE gel showing the effect of [6His]-SarA on transcription initiation from pJR<sub>P2</sub> and pJR<sub>P3</sub>.

A full length *in vitro* transcription assay showing the transcripts synthesised from the pJR<sub>P2</sub> and pJR<sub>P3</sub> reporter plasmids at 37°C by  $E\sigma^A$  (indicated by the arrows) in the presence and absence of [6His]-SarA. A R84A [6His]-SarA DNA-binding mutant is used as a control (lane 9 and 18). The reactions were performed as a function of [6His]-SarA concentration, with 0, 100, 200, 300, 400, 500, 750 and 1000 nM of [6His]-SarA, under multiple round conditions. A schematic of the assay is shown above. The amount of transcripts synthesised are expressed as fold increase in transcripts synthesised from P2 (xF P2) and P3 (xF P3) (lanes 2-9 and 11-18) with respect to the corresponding no [6His]-SarA reaction (lane 1 and 10). In at least two independent experiments, the observed trend between each data point for the quantity of transcripts synthesised from either the P2 or P3 promoters was comparable.

The results, shown in Figure 93, replicated the [6His]-SarA repression when both *agr* P2 and P3 promoters are present, shown in Figure 92. SarA represses transcription initiation at the *agr* P2 and P3 promoters to a similar degree when either promoter is present independently, with a slightly increased level of repression at the P3 promoter, compare lanes 1-8 with 10 to 17. The control reactions using the [6His]-SarA DNA binding mutant ([6His]-SarA<sub>R84A</sub>) indicate that the repression of transcription initiation from the *agr* P2 and

P3 promoters was specific to [6His]-SarA, lanes 9 and 18. Therefore it was concluded that SarA repression at either *agr* promoter occurs independently of the divergent promoter.

#### 7.4. Mechanism of SarA regulation at the *agr* operon

To unravel the mechanism of SarA repression at the *agr* operon an order of addition assay was performed, where [6His]-SarA was added to single-round full length *in vitro* transcription reactions at various stages of the reactions, indicated in the schematic in Figure 94. The reactions were performed, as in section 2.4.8, in the presence of the competitor heparin, so that the  $E\sigma^A$ -promoter complexes formed could only produce a single transcript from the pJR<sub>P2+P3</sub> reporter plasmid. Reactions were also performed using the [6His]-SarA DNA binding mutant [6His]-SarA<sub>R84A</sub> as a control.

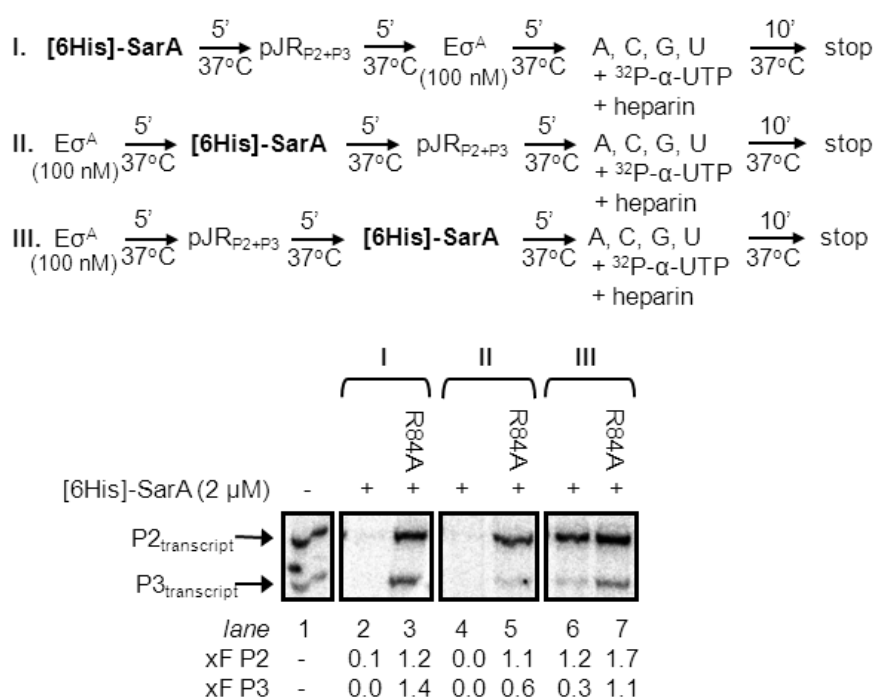


Figure 94: Image of a 10% (w/v) urea-denaturing-PAGE gel showing the order of addition effect of [6His]-SarA on transcription initiation from the *agr* P2 and P3 promoters.

A full length *in vitro* transcription assay showing the transcripts synthesised from the pJR<sub>P2+P3</sub> reporter plasmid at 37°C by  $E\sigma^A$  (indicated by the arrows) in the presence and absence of [6His]-SarA and the non-DNA binding [6His]-SarA mutant R84A. [6His]-SarA was added to the reaction at three points (I-III). The reactions were performed under single round conditions. A schematic of the assay is shown at the top. The amount of transcripts synthesised are expressed as fold increase in transcripts synthesised from P2 (xF P2) and P3 (xF P3) (lanes 2-7) with respect to the corresponding no [6His]-SarA reaction (lane 1). In at least three independent experiments, the observed trend between each data point for the quantity of transcripts synthesised from either the P2 or P3 promoters was comparable.

Results, shown in Figure 94, indicated that the presence of [6His]-SarA has a negative effect on Eo<sup>A</sup> transcription initiation at the *agr* P2 and P3 promoters, see lanes 2 and 4, in an order of addition dependent manner. The [6His]-SarA repression on the *agr* P2 and P3 promoters was highest under conditions I, lane 2, and II, lane 4, where [6His]-SarA was pre-incubated with Eo<sup>A</sup> before the addition of the promoter template. The [6His]-SarA repression was significantly reduced under condition III, lane 6, where [6His]-SarA was added after Eo<sup>A</sup> and the promoter template, suggesting the role of SarA in the regulation of transcription initiation at the *agr* P2 and P3 promoters is before RPo formation.

## 7.5. Conclusions

Overall, the results are consistent with earlier *in vitro* results (Chakrabarti and Misra 2000) and reveal that SarA binds to the *agr* P2 and P3 promoters to repress transcription initiation (Figure 90 and Figure 92). *In vitro* transcription assays performed at either *agr* promoter independently have demonstrated that repression by SarA at one *agr* promoter can occur independently of the divergent promoter (Figure 93).

The lack of repression of transcription initiation by SarA after RPo formation indicates that SarA repression occurs before RPo formation at the *agr* P2 and P3 promoters (Figure 94). The mechanism of repression by SarA therefore most likely involves steric hindrance, where the RNAP is physically blocked from binding to the promoter. This could be explained by the SarA binding sites overlapping the *agr* P2 and P3 promoter elements, with one SarA dimer binding site overlapping both the *agr* P2 -10 and -35 elements (Rechlin, Gillaspay et al. 1999). However the contrasting findings from *in vivo* and *in vitro* assays demonstrate that many regulatory factors may be involved to regulate *agr* expression alongside SarA and this is currently being investigated in our laboratory and briefly discussed in section 8.2.

Furthermore, recent studies which have revealed that SarA is subjected to post translational modification through phosphorylation, and that SarA responds to changes in redox potential and pH (Fujimoto, Higginbotham et al. 2009; Didier, Cozzone et al. 2010). Studies have found that SarA can be modified at threonine and serine residues by threonine kinase, SA0077, and serine kinase, Stk1. For example, phosphorylation of SarA by Stk1 (*in vitro*)

leads to a 4 fold increase in transcription from the *agr* P2 promoter (Didier, Cozzone et al. 2010). Further studies have revealed another potential mode of SarA regulation involving the effect of redox potential and pH on DNA binding (Fujimoto, Higginbotham et al. 2009). Binding of SarA to the *agr* promoter region was enhanced by changing the reaction conditions to a low pH or by the addition of a reducing agent such as DTT. Interestingly, mutation of the cysteine residue 9 (C9) to alanine eliminated the redox response. The position of residue C9, near the dimerisation region, questions whether the changes in redox potential play a role in DNA binding through altering the ability of SarA to form a functional dimer. It is important to consider that at the physiologically relevant (=intracellular) pH of 7-7.5 no change in SarA binding was seen. However, the oxidative state encountered by *S. aureus* may vary *in vivo*. A further role of SarA involving the stimulation of DNA repair enzymes in response to DNA damage by reactive oxygen species has recently been uncovered (Fujimoto, Higginbotham et al. 2009). These studies underline the pleiotropic effect and multiple modes of regulation of SarA on target gene transcription and could potentially explain the differences in SarA regulation at the *agr* operon found in *in vivo* and *in vitro* studies.

## CHAPTER EIGHT

### 8. Final conclusions and overview

#### 8.1. Introduction

*S. aureus* is a major virulent human pathogen that is responsible for both hospital acquired and community acquired infections. The ability and success of *S. aureus* as a pathogen is facilitated by a vast array of surface-associated and secreted virulence factors. One major determinant of *S. aureus* virulence is the *agr* operon, which encodes a quorum sensing system (Figure 1).

The quorum sensing system includes the transmembrane protein AgrB, which processes and secretes the quorum signalling molecule, AIP. The AIP is detected by the sensor histidine kinase AgrC, which upon autophosphorylation phosphorylates the master regulator AgrA. AgrA then binds to the *agr* IR to upregulate transcription from both the P2 and P3 promoters. Transcription from P3 encodes the regulatory RNA molecule RNAIII, which is the effector molecule of the *agr* operon. This allows *S. aureus* to respond to cell density at the site of infection, as the *agr* operon acts as a major regulatory switch for the cell density dependent regulation of virulence factors in *S. aureus*.

The *agr* operon is itself regulated by a self-encoded response regulator AgrA and several global transcriptional regulators (Figure 18). Therefore, obtaining molecular insights into the regulation of the *agr* operon at the transcriptional level is of importance to understanding the regulation of virulence gene expression in this major human pathogen. Previous *in vivo* studies have highlighted the importance of the *agr* operon for *S. aureus* virulence since the removal of the *agr* operon, or single mutations in regulatory elements (E.g. AgrA or *agr* IR), results in an significantly attenuated *S. aureus* strain (Abdelnour, Arvidson et al. 1993; Gillasp, Hickmon et al. 1995). Earlier *in vitro* studies have identified a number of transcriptional regulators that directly interact with the *agr* IR, the regulatory region which separates the P2 and P3 promoters, and have provided an initial outlook of the regulation by individual regulators (Rechlin, Gillasp et al. 1999; Koenig, Ray et al. 2004; Manna and Cheung 2006). The complexity of *agr* regulation has created many contrasting findings

between *in vivo* and *in vitro* regulation of the *agr* operon. What has remained unknown are the (i) basal level of transcription at the *agr* P2 and P3 promoters and the importance of this for *S. aureus* virulence; (ii) the mechanism of activation of transcription by AgrA at the P3 promoter and (iii) how SarA influences transcription at the *agr* P2 and P3 promoters.

## 8.2. Summary of findings

This study aimed to provide the first molecular insights into how transcription initiation is regulated from the *agr* operon promoters P2 and P3 in *S. aureus*. To do this a fully native *S. aureus in vitro* transcription system has been developed using protein and nucleic acid components from one clinically *S. aureus* strain NCTC 8325. Thus  $\sigma^A$ , AgrA and SarA were purified by various affinity chromatography methods from *E. coli*. The tools obtained include the transcription machinery (*S. aureus* core RNAP and the  $\sigma^A$  promoter specificity factor). Importantly, a simple protocol was established to produce high yields of recombinant AgrA (Figure 43).

Initially, the *in vitro* transcription system was used to determine the basal level of transcription at the *agr* P2 and P3 promoters through first identifying the ability of the RNAP holoenzyme ( $E\sigma^A$ ) to bind to the *agr* P2 and P3 promoters. Since the *agr* P3 promoter contains a sub-optimal 20 nucleotide spacer length it was expected to be a weaker promoter than the *agr* P2 promoter for  $E\sigma^A$  binding, and strictly dependent upon AgrA for activity. However, results obtained show that despite the sub-optimal P3 spacer,  $E\sigma^A$  binds equally well at the P2 and P3 promoters to form the RPc (Figure 46), and the RPo (Figure 48). Competition assays confirmed this finding as radiolabelled  $E\sigma^A$ -promoter complexes challenged with the divergent cold-promoter probe led to a similar decrease in visible complexes in both combinations of promoters (Figure 50). Interestingly at both P2 and P3 promoters the RPo formed at 37°C appeared to be heparin sensitive and therefore relatively unstable (Figure 51). However, short RNA synthesis assays demonstrated that the complexes formed at 37°C were still productive and therefore consist of the RPo (Figure 53B).

Alongside the short RNA synthesis assays, full length *in vitro* transcription assays were also used to measure RPo formation at the *agr* P2 and P3 promoters. Multiple round and single round transcription assays demonstrated that Eo<sup>A</sup> can form transcriptionally competent complexes at both the *agr* P2 and P3 promoters in the absence of AgrA (Figure 54 and Figure 56). Although, it seems that RPo formation is more efficient at the *agr* P2 promoter than at the *agr* P3 promoter. Thus, it is possible that the atypical organisation of the P3 promoter may restrict efficient RPo formation at the P3 promoter, as RPo accumulation occurs at a slower rate from P3 than from P2. The sub-optimal spacer length at P3 may also play a role in RPo stability at the P3 promoter as the stability of the RPo formed at the P3 promoter appears to be lower than from the P2 promoter (Figure 58).

Transcription initiation at one promoter having a negative effect on transcription initiation at the divergent promoter is regularly seen at back-to-back divergent promoters, as in the case of the *E. coli gcd* and *hpt* genes involved in sugar metabolism and base and nucleotide utilisation (Izu, Ito et al. 2002). To assess the possibility of an antagonistic effect on one of the promoters by the divergent promoter, transcription assays were performed both independently of the opposite promoter, using pJR<sub>P2</sub> or pJR<sub>P3</sub>, and in the presence of both promoters, using the reporter plasmid pJR<sub>P2+P3</sub>. The results appear to suggest that transcription from one *agr* promoter has no detectable effect on the ability of the Eo<sup>A</sup> to initiate transcription from the other divergent promoter. Thus, it would appear that Eo<sup>A</sup> can initiate transcription simultaneously at the *agr* promoters.

The basal level of transcription initiation determined at both *agr* P2 and P3 promoters could play an important role in regulation in *S. aureus*. The higher level of transcription initiation seen at the *agr* P2 promoter supports the idea proposed by Novick *et al.* that a higher basal level of activity at the P2 promoter ensures the quorum sensing system is available and ready to respond to changes in cell density. To enable *S. aureus* to rapidly upregulate the transcription of secreted virulence factors and downregulate the transcription of surface associated virulence factors when the cell density at the site of infection reaches a certain threshold. The basal level of transcription seen at the P2 promoter may also be relevant for the availability of AgrA in RNAIII independent regulation, such as at the recently discovered binding of AgrA to regulate the leucocidal phenol-soluble modulins (Queck, Jameson-Lee et

al. 2008). In that study genome wide expression analyses also revealed further potential RNAIII independent regulation targets by AgrA, including several metabolic genes.

Furthermore, the results presented here may also help to provide a molecular explanation of previous *in vivo* findings, which observed a RNAII independent activation of RNAIII, albeit at lower levels, in a rabbit endocarditis infection model (Xiong, Van Wamel et al. 2002). In this study an *S. aureus* AgrA deletion strain was compared to a wild-type strain to compare the activation of RNAII and RNAIII *in vivo* in the absence of the activator AgrA. Therefore basal transcription initiation from both the *agr* P2 and P3 promoters is of regulatory significance and provides a potential RNAIII independent route for *S. aureus* virulence gene expression to adapt to the changing environmental and metabolic signals.

The fully native *S. aureus in vitro* transcription system has also been used to determine the possible mechanism of activation of transcription by AgrA at the P3 promoter. The addition of AgrA, albeit in an unphosphorylated state, to *in vitro* transcription assays clearly activates transcription initiation from both the *agr* P2 and P3 promoters (Figure 63 and Figure 66). Transcription initiation from the P3 promoter was found to be significantly higher from the *agr* P3 than the P2 promoter in the presence of AgrA, thereby demonstrating RNAIII activation in response to an increased quorum via AgrA. However, it is formally possible that phosphorylation of AgrA may lead to a more pronounced effect at the P2 and P3 promoters.

Order of addition experiments gave a first indication of the mechanism of activation of transcription by AgrA at the *agr* P2 and P3 promoters. A result where AgrA was pre-incubated with  $E\sigma^A$  seems to suggest that AgrA recruits  $E\sigma^A$  to the *agr* promoters (Figure 67). Stability assays also show that AgrA had no effect on the longevity of the RPo once formed at the *agr* P2 and P3 promoters, therefore it appears that AgrA affects transcription initiation at the *agr* P2 and P3 promoters in the steps leading to RPo formation. The binding sites for AgrA at the *agr* IR are close to, with regions overlapping, the -35 promoter elements at both the P2 and P3 promoters (Koenig, Ray et al. 2004), suggesting a possible class II mechanism of activation (Figure 14B). In the class II mechanism of activation  $E\sigma$  is recruited by an activator binding to a site centred at position -41.5. The activator contacts  $E\sigma$  generally at  $\sigma_4$  to increase RPo formation by increasing the binding constant of  $E\sigma$  to the

promoter DNA (Busby and Ebright 1997). Results displayed here indicate that AgrA increases the occupancy of both the *agr* P2 and P3 promoters (Figure 65) and therefore that AgrA increases the  $K_b$  of  $E\sigma^A$  and promoter interactions at both the *agr* P2 and P3 promoters. The conversion of the RPc to the RPo is also facilitated in the class II mechanism of activation (Busby and Ebright 1997). As AgrA increases  $E\sigma^A$  promoter occupation and also increases RPo formation at the P3 promoter (Figure 66), it can be suggested that AgrA also increases the  $k_f$  for the isomerisation of the RPc to the RPo at the P3 promoter. However, despite the equal P2 and P3 promoter occupation in the presence of AgrA the RPc at the P2 promoter does not isomerise to the RPo at the same rate, leading to a relatively lower level of transcription initiation from the P2 promoter than the P3 promoter in the presence of AgrA.

A possible mechanism by which activation of transcription by AgrA occurs at the P3 promoter could be derived by considering the overall organisation of the P3 promoter and the AgrA binding sites proximal to the P3 promoter. The sub-optimal 20 nucleotide promoter element spacer length, once optimised to 17 nucleotides led to a significant increase in transcription initiation at the P3 promoter (Figure 77 and displayed in Figure 95).

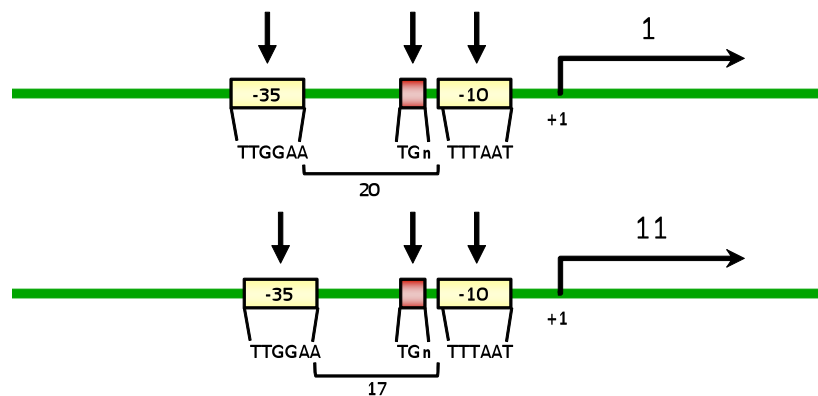


Figure 95: Optimising the sub-optimal promoter element spacer of the *agr* P3 promoter.

The figure is a diagrammatic representation of the effect of shortening the sub-optimal promoter element spacer at the *agr* P3 promoter from 20 to 17 nucleotides. Shortening the sub-optimal promoter element spacer leads to an increase in transcription initiation.  $E\sigma^A$ -DNA contacts highlighted by arrows and transcription initiation displayed as fold increase compared to wild-type P3 promoter.

The crystal structure of AgrA<sub>C</sub> bound to DNA suggests that AgrA could compensate for the sub-optimal spacing at the P3 promoter, through AgrA binding to distort the DNA and re-organise the -10 and -35 promoter elements, therefore facilitating RPo formation at the P3

promoter (Figure 95). Results obtained here support this hypothesis as the optimisation of the P3 promoter element spacer to 17 nucleotides removed the ability of AgrA to activate transcription initiation at the *agr* P3 promoter (Figure 78). The use of the optimised P3-17 promoter in RPo stability (Figure 76) and RPo formation assays (Figure 77) suggest that optimising the P3 spacer length through AgrA binding would increase the ability of the initial  $E\sigma^A$ -promoter complexes formed at the P3 promoter to isomerise to the RPo. This potential mechanism of activation by AgrA at the P3 promoter is seen with other regulators involving a sub-optimal promoter element spacer. One example is the *B. subtilis* Spo0A regulator from the AraC family of regulators, where promoters activated by Spo0A have a 20 to 22 nucleotide spacer (Kenney, York et al. 1989). The current model of activation by Spo0A proposes that the RNAP holoenzyme pre-bound to the -35 element is repositioned, through contact of Spo0A with  $\sigma_4$ , to contact the -10 promoter element to facilitate RPo formation (Kumar and Moran 2008). Thus, based on the results presented, it could be suggested that AgrA potentially also activates transcription from the P3 promoter by compensating for the sub-optimal spacer length.

Another feature of the P3 promoter is the extended -10 motif made up of a TGn motif immediately upstream of the -10 promoter element. The role of extended -10 elements at bacterial promoters has been widely studied. Unlike in *S. aureus*, the percentage of *E. coli* promoters with extended -10 elements has been identified as ~20% (Mitchell, Zheng et al. 2003). The -10 promoter element is contacted by  $\sigma_{3.0}$  of  $E\sigma$ . In extended -10 promoters crystallographic studies have shown that the  $\alpha$  helix of  $\sigma_{2.4}$  to the helix of  $\sigma_{3.0}$  with the connecting linker wrap around the -10 and extended -10 promoter elements upon binding by  $E\sigma$  (Murakami, Masuda et al. 2002). The principle residues contacting the -10 element are H455 and E458 in the first helix of  $\sigma_{3.0}$  (Sanderson, Mitchell et al. 2003). The extra contacts of  $E\sigma$  through  $\sigma_{3.0}$  with the -10 element can compensate for a weak -10 promoter element, as in the bacteriophage T4  $P_{minor}$  promoter, or a non-consensus -35 promoter element, as in the case of the *galP1* promoter. Unlike at the  $P_{minor}$  or *galP1* promoters, the *agr* P3 promoter contains near consensus -10 (5/6) and -35 (4/6) elements.

The results presented in this study show that the extended -10 motif is required for optimal promoter activity at the *agr* P3 promoter (Figure 71). However, the mutation of the

extended -10 motif had no effect on initial  $E\sigma^A$  binding (Figure 69), indicating the extended -10 motif has a role in facilitating the isomerisation of the R<sub>Pc</sub> to the R<sub>Po</sub> at the P3 promoter. The extended -10 motif also seems to be required for AgrA activation as the mutation of the extended -10 motif reduces/removes all activation by [6His]-AgrA (see later) (Figure 72). Therefore it is apparent that AgrA activates transcription from the P3 promoter in an extended -10 motif dependent manner.

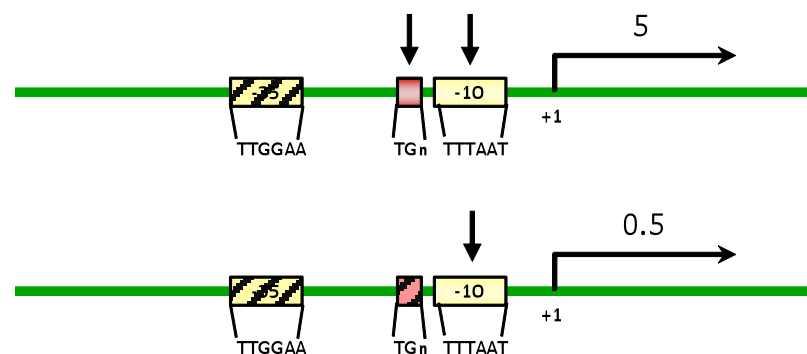


Figure 96: The mutation of the extended -10 like element and scrambling of the -35 element from the *agr* P3 promoter

The figure is a diagrammatic representation of the effect of mutating the extended -10 motif as well as scrambling the -35 element at the *agr* P3 promoter. Mutation of the extended -10 motif led to a decrease in transcription initiation.  $E\sigma^A$ -DNA contacts highlighted by arrows and transcription initiation displayed as fold increase compared to wild-type P3 promoter.

However, unlike other bacterial promoters with extended -10 motifs, the P3 promoter also contains a near consensus -35 element as well as the extended -10 motif. Given that AgrA binding to the P3 promoter protects a region overlapping the -35 element in DNA footprinting assays (Koenig, Ray et al. 2004), the role of the -35 element in the mechanism through which activation of transcription at the P3 promoter by AgrA occurs was experimentally addressed. Results using a P3 promoter probe containing a “scrambled” -35 sequence (in order to prevent or at least compromise  $E\sigma^A$  from efficiently contacting the -35 element; hereafter called the P3-35 probe) showed that transcription from the P3-35 probe was in fact ~5 fold higher than transcription from the wild-type P3 promoter (Figure 82 and displayed in Figure 97). Therefore the -35 element at the P3 promoter seems to antagonise promoter activity to a certain degree.

$E\sigma^A$  forms the R<sub>Pc</sub> equally well at the P<sub>3</sub> and P<sub>3</sub>-35 promoters (Figure 80). However, the stability of the R<sub>Po</sub> formed at the P<sub>3</sub>-35 promoter is detectably reduced when compared with the R<sub>Po</sub> formed at the wild-type P<sub>3</sub> promoter (Figure 83), despite the increased transcriptional output from P<sub>3</sub>-35 (Figure 97). Results also suggest that the extended -10 motif at the P<sub>3</sub> promoter is required for the increased activity at the P<sub>3</sub>-35 promoter. Thus, it would seem that removing/compromising the -35 interaction made by  $E\sigma^A$  *ipso facto* compensates for the sub-optimal spacer length, by potentially forcing  $E\sigma^A$  to make additional contacts with the extended -10 motif, and thereby leading to increased R<sub>Po</sub> formation (summarised in Figure 97). Consistent with this view, [6His]-AgrA was no longer able to activate, i.e. increase transcription, from the P<sub>3</sub>-35 promoter when compared to the situation at the wild-type P<sub>3</sub> promoter (Figure 85).

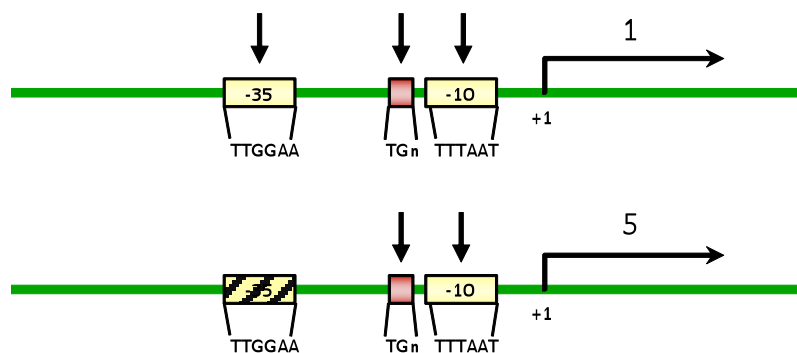


Figure 97: The “scrambling” of the -35 element from the *agr* P<sub>3</sub> promoter

The figure is a diagrammatic representation of the effect of scrambling the -35 element at the *agr* P<sub>3</sub> promoter. Scrambling of the -35 element forces  $E\sigma^A$  to rely on the extended -10 and -10 contacts. This leads to an increase in transcription initiation.  $E\sigma^A$ -DNA contacts highlighted by arrows and transcription initiation displayed as fold increase compared to wild-type P<sub>3</sub> promoter.

Overall, the results above suggest two possible mutually inclusive mechanisms by which AgrA activates transcription at the *agr* operon promoters. One involves a simple class II like mechanism of activation where AgrA could recruit  $E\sigma^A$  to the *agr* P<sub>2</sub> and P<sub>3</sub> promoters to enhance  $E\sigma^A$  occupation at both promoters and therefore increase transcription initiation by increasing  $K_b$  for R<sub>Pc</sub> formation, and subsequently the  $k_f$  for R<sub>Po</sub> formation (Figure 14B). The second mechanism of activation could involve a two pronged strategy of activation and seems to be only applicable at the P<sub>3</sub> promoter (Figure 98). Firstly, AgrA binds to a binding site near to, with regions overlapping, the -35 promoter element to remove or antagonise

the interaction  $E\sigma^A$  makes with the -35 element. This consequently forces  $E\sigma^A$  to rely on the extended -10 element, which here has been shown to facilitate RPo formation by possibly increasing the  $k_f$  of the isomerisation reaction during RPo formation (Figure 98). This has been demonstrated through increased transcription initiation when the -35 promoter element was scrambled (Figure 82). Secondly, AgrA binding to the P3 promoter *per se* could result in DNA bending and thereby compensate for the sub-optimal spacer length.

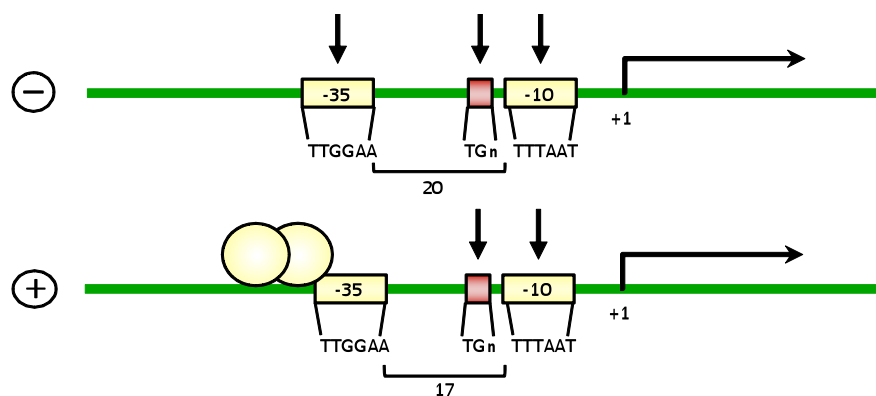


Figure 98: Model of AgrA activation at the *agr* P3 promoter

The figure is a diagrammatic representation of activation by AgrA at the *agr* P3 promoter. The interactions of proteins are shown through adjacent placement to the interacting molecule. AgrA (yellow) binds to a site adjacent to the -35 element and interacts with  $\sigma^{70}_4$  to displace  $E\sigma^A$  from the -35 element, forcing  $E\sigma^A$  to rely on the extended -10 and -10 contacts. Binding of AgrA also reduces the P3 promoter element spacer length through distorting the DNA.

An example of another bacterial promoter that has a dispensable -35 promoter element is the  $P_{\text{excSC}}$  promoter of *Pseudomonas aeruginosa* involved in the control of a type III secretion system (T3SS) (Vakulskas, Brutinel et al. 2010). The  $P_{\text{excSC}}$  promoter, like *agr* P3, contains conserved -35 and -10 elements separated by a sub-optimal 23 nucleotide spacer. Also, like *agr* P3, the  $P_{\text{excSC}}$  promoter also requires an extended -10 promoter element. The transcriptional activator of the  $P_{\text{excSC}}$  promoter is ExsA, a member of the AraC family of transcriptional activators. ExsA, like AgrA, binds at sites upstream of the -35 element (~50 bp upstream of +1) and contacts  $\sigma^{70}_4$  (Vakulskas, Brutinel et al. 2010). The current model suggests this interaction compensates for the -35 promoter to recruit the  $E\sigma$  in a class II mechanism of activation (Figure 14B). Unlike at the *agr* P3 promoter, the shortening of the spacer to an optimal  $17 \pm 1$  nucleotides does not increase transcription initiation but instead

decreases it, therefore shortening the sub-optimal spacer is not the mechanism of ExsA activation, and thus could be a unique feature of AgrA.

In the final part of this thesis, the *in vitro* transcription system has been used to gain insights into how SarA potentially influences transcription at the *agr* P2 and P3 promoters. *In vitro* characterisation of SarA has revealed that SarA binds to the *agr* P2 and P3 promoters to repress transcription initiation (Figure 90 and Figure 92). It also appears that repression by SarA occurs before RPo formation and repression at one *agr* promoter can occur independently of the divergent promoter (Figure 93 and Figure 94). As a SarA binding site overlaps both the *agr* P2 -10 and -35 promoter elements it appears the one possible mechanism of repression by SarA involves steric hindrance (Rechlin, Gillaspay et al. 1999). In steric hindrance  $\sigma$  is physically blocked from binding to the promoter, as in Figure 15A. At the *agr* P3 promoter a SarA binding site also overlaps the -35 element but not the -10 element, therefore the mechanism of repression by steric hindrance is also possible.

Another model of SarA repression, first proposed by Morfeldt *et al.*, (1996) questions whether SarA can induce changes in the DNA superhelicity at the P3 promoter. Although the structure of SarA binding to DNA has not been resolved, the model of the structurally similar SarR regulator bound to DNA indicates a 90° bend in the DNA (Liu, Manna et al. 2001). If SarA binds DNA with a similar effect, it could overwind the P3 promoter through binding to sites overlapping the P3 -35 promoter element. The presence of further SarA binding sites upstream of the *agr* P2 and P3 promoters suggests the binding of additional SarA dimers to the upstream sites could potentially repress activation in the presence of AgrA, as in Figure 15C. In this scenario SarA could disrupt AgrA binding to sites adjacent to the *agr* P2 promoter -35 element. This possible mechanism requires further characterisation through studies in the presence of both transcription factors.

### **8.3. Future directions**

This study has provided some molecular insights into the control of transcription initiation at the *agr* operon. The resources generated and the results obtained provide a solid basis to further extend our understanding of the molecular mechanisms that govern transcription

initiation at the *agr* operon in *S. aureus*. Some suggestions for future studies are discussed below.

The model presented in Figure 98 suggests that AgrA possibly removes or antagonises the interaction  $E\sigma^A$  makes with the -35 element via  $\sigma_4$  to activate transcription at the P3 promoter. This can be experimentally addressed by conjugating the chemical nuclease FeBABE ((p-bromoacetamidobenzyl)-EDTA·Fe) to  $\sigma_4$  and observing the presence or absence of the promoter DNA cleavage at the -35 promoter element in the absence and presence of ArgA. The absence of promoter DNA cleavage at the -35 promoter element in the presence of AgrA and *vice versa* would provide data in support of the model proposed above. The FeBABE nuclease, tethered to single cysteines, cleaves sites proximal to its location by generating free radicals (Murakami, Kimura et al. 1997; Owens, Chmura et al. 1998). Therefore the presence of AgrA at the -35 element would increase this distance and reduce cleavage of the promoter, demonstrating the mechanism whereby AgrA displaces  $E\sigma^A$  from the -35 element.

To confirm that the  $\sigma_{3.0}$  interacts with the extended -10 motif of the P3 promoter, site directed mutagenesis of the interacting  $\sigma$  residues could be performed. The principle residues that have previously been shown to interact with the extended -10 TGN motif at *E. coli* promoters are the histidine and glutamic acid residues, at positions 455 and 458 respectively, in the first helix of  $\sigma_{3.0}$  (Murakami, Masuda et al. 2002; Sanderson, Mitchell et al. 2003). Mutagenesis of the corresponding H455 and E458 residues in  $\sigma^A$  to remove the  $E\sigma^A$  extended -10 interactions would allow further confirmation of the importance of the extended -10 motif. Their mutagenesis would decrease the binding of  $E\sigma^A$  to the P3 promoter in EMSAs and potentially decrease transcription activity in full length *in vitro* transcription assays.

AgrA is likely to physically interact with components of  $E\sigma^A$  to activate transcription from the *agr* promoters. One method to demonstrate the potential physical interactions between AgrA and  $E\sigma^A$  is by using the bacterial adenylate cyclase two hybrid system (BACTH). The BACTH system detects the *in vivo* interaction of two proteins of interest fused to the T25 and T18 complementary fragments of adenylate cyclase from *Bordetella pertussis* (Figure

99). When the two proteins of interest heterodimerise, this results in functional complementation of the T25 and T18 complementary fragments of adenylate cyclase, leading to cyclic AMP (cAMP) synthesis (Karimova, Pidoux et al. 1998). When the two domains are physically separated they are not active. The resulting cyclic AMP which is produced is able to bind to the catabolite activator protein (CAP) and form a cAMP/CAP complex that in turn is a pleiotropic regulator of gene transcription in *E. coli*. This includes the positive regulation of genes involved in lactose and maltose catabolism (*lac* and *mal*), so that bacteria are able to be selected through their use of lactose or maltose as the unique carbon source. The BACTH assay is performed in the *E. coli* strain BTH101, which has a *cya* deletion that encodes adenylate cyclase.

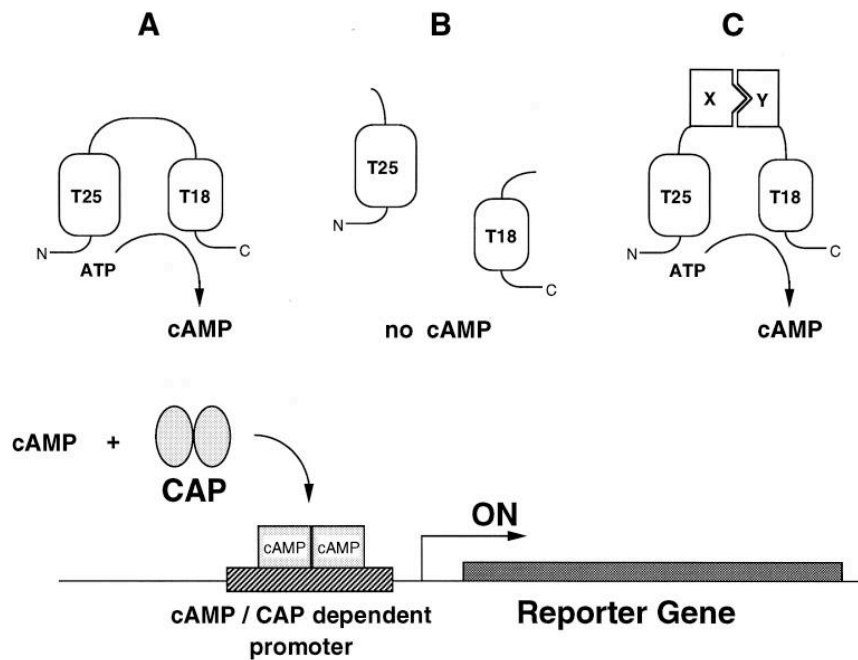


Figure 99: Principles of the *E. coli* BACTH system.

When two proteins of interest, fused to the T25 and T18 complementary fragments of adenylate cyclase from *Bordetella pertussis*, heterodimerise in *E. coli*, this results in functional complementation of the T25 and T18 fragments, leading to cyclic AMP (cAMP) synthesis (C). When the two domains are physically separated they are not active (B). The resulting cyclic AMP which is produced is able to bind to the catabolite activator protein (CAP) and form a cAMP/CAP complex that in turn recognises specific promoters and switches on the transcription of the corresponding reporter genes. This could include the genes involved in lactose and maltose catabolism (*lac* and *mal*). Figure from (Karimova, Pidoux et al. 1998).

Thus, to detect the potential interactions of AgrA and  $E\sigma^A$ , AgrA could be fused to one complementary fragment whilst the possible interacting regions, including  $\sigma_4$ ,  $\alpha$ CTD and

$\alpha$ NTD could be fused to the other complementary fragment. If interactions were detected then site directed mutagenesis of possible interacting residues could be used to determine the exact interface. Mutant proteins could be used in further studies of the molecular mechanisms that govern transcription regulation at the *agr* P2 and P3 promoters in the context of the assays developed in this thesis.

The ability to *in vitro* phosphorylate AgrA would also allow further insight into the levels of transcription initiation at the *agr* P2 and P3 promoters in the presence of AgrA~P. One potential approach to phosphorylate AgrA is by using its native two component partner, AgrC, which in its full form phosphorylates AgrA *in vivo*. As the full AgrC protein contains a trans-membrane domain and therefore would be difficult to purify as a recombinant protein in *E. coli*, the AgrC histidine kinase domain could potentially be used *in vitro* to phosphorylate AgrA (displayed in Figure 4). The discovery of amino acid residues in the AgrC histidine kinase domain that can be mutated to form a constitutively active protein could allow for the phosphorylation of AgrA *in vitro* for biochemical analyses (Figure 4) (Geisinger, Muir et al. 2009).

The fully native *in vitro* transcription system established in this study can also be extended to further study the regulation that occurs at the *agr* operon in the presence of SarA (see Chapter 7), SarR and AgrA. Such studies will help to discern the complex transcription regulatory mechanism that seems to govern transcription at the *agr* operon. Furthermore, the changing conditions in *in vivo* *S. aureus* infection could also be modelled to explore the regulation at the *agr* operon with particular focus on regulation by SarA (Fujimoto, Higginbotham et al. 2009; Didier, Cozzone et al. 2010).

The transcription from the *agr* promoters *in vivo* can also be monitored using bioluminescent imaging methods. One approach is to integrate a second copy of the *agr* IR with *agr* P2 and P3 promoters fused to two different bioluminescent promoters onto the *S. aureus* chromosome. The differential transcriptional activity from the P2 and P3 promoters under various growth conditions or in the context of mutant regulators (e.g. AgrA or SarA/SarR) can then be directly monitored by measuring bioluminescence directly from the cell.

Finally, the recent finding that AgrA regulates *psm* gene transcription by directly binding to the *psm* promoter regions has highlighted the possibility that AgrA could regulate the transcription of genes outside of the *agr* operon (Queck, Jameson-Lee et al. 2008). Therefore a genomic approach should be undertaken to determine further AgrA binding sites. In particular the chromatin immunoprecipitation followed by sequencing (ChIP-seq) technique used for genome-wide profiling of DNA-binding proteins should allow for the precise mapping of AgrA sites (Park 2009).

## APPENDICES

### 9. Appendices

#### 9.1. Vector maps

##### 9.1.1. pGEM-T Easy – used for PCR product cloning

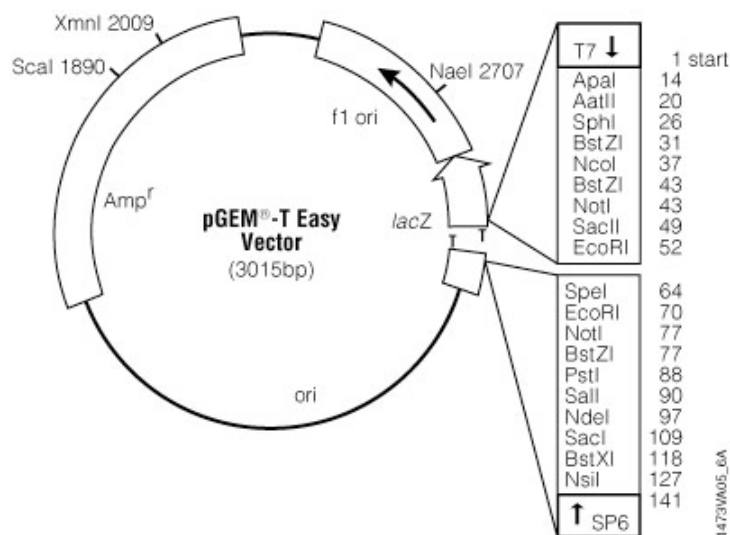


Figure 100: Vector map of pGEM-T Easy Vector from Promega.

##### 9.1.2. pET22b+ - used for protein expression

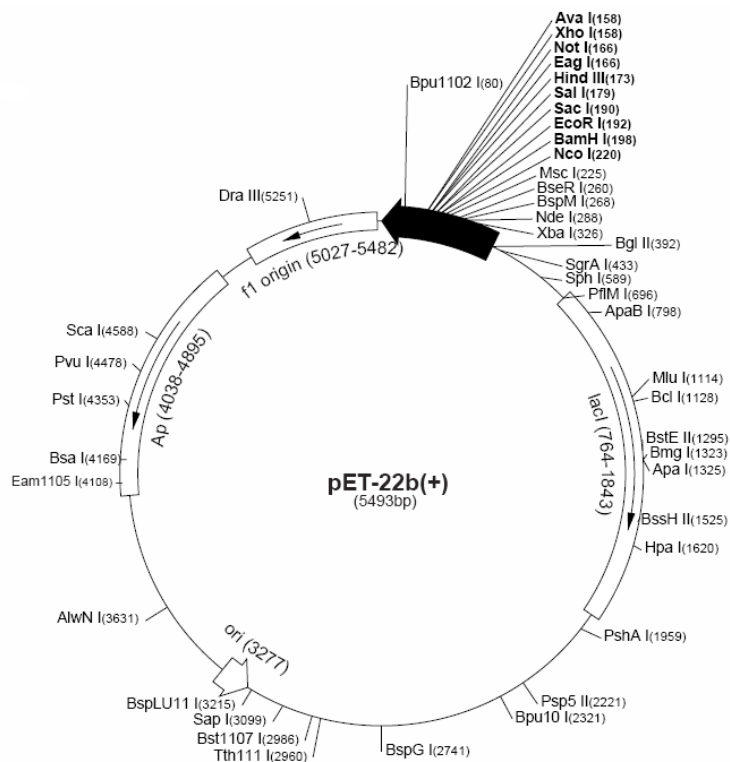


Figure 101: Vector map of pET22b+ from Novagen.

### 9.1.3. pET28b+ - used for protein expression

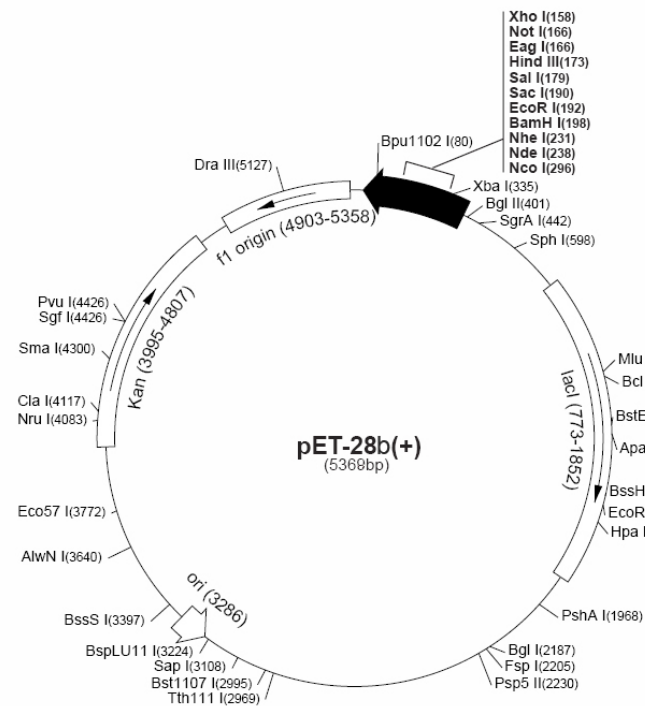


Figure 102: Vector map of pET28b+ from Novagen.

### 9.1.4. pMAL-c2X- used for protein expression with MBP fusion

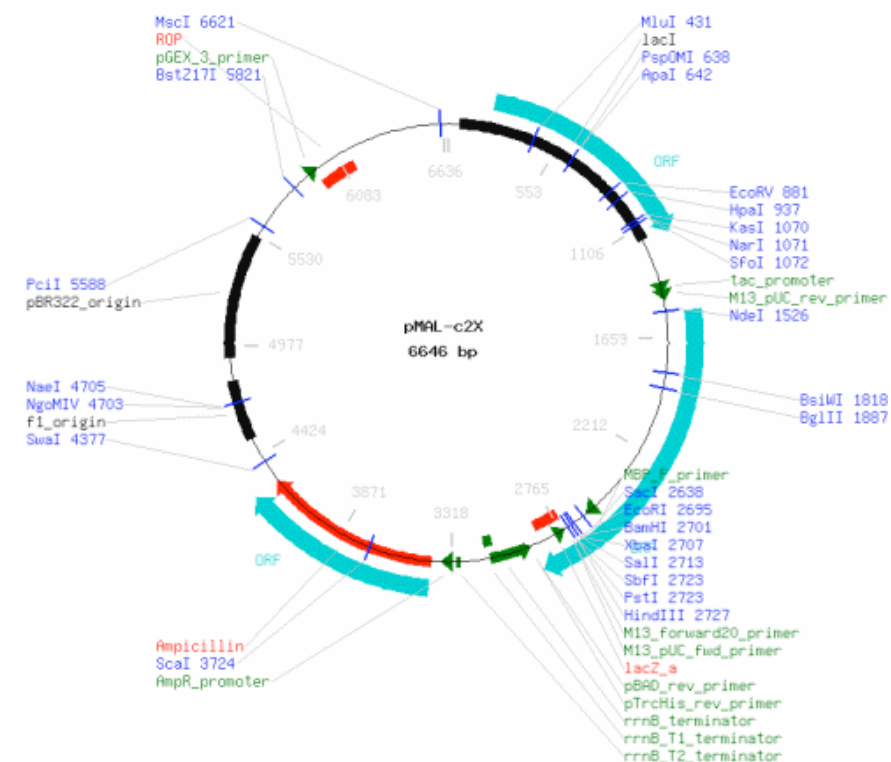


Figure 103: Vector map of pMAL-c2X from New England Biolabs.

From [http://www.lablife.org/p?a=vdb\\_view&id=g2.EHBOxirdwxdkziWnuZpJgRxA.hE-](http://www.lablife.org/p?a=vdb_view&id=g2.EHBOxirdwxdkziWnuZpJgRxA.hE-).

### pMAL-c2X Polylinker:

5' *ma* *l*E...TCG AGC TCG (AAC) SacI AAT AAC AAT (AAC) CTC GGG ATC GAG GGA AGG ATT TCA XmnI  
 Ile Glu Gly Arg

EcoRI BamHI XbaI SalI PstI HindIII  
 GAA TTC GGA TCC TCT AGA GTC GAC CTG CAG GCA AGC TTG ... *lacZ* $\alpha$

Figure 104: Polylinker sequence of pMAL-c2X from New England Biolabs.

### 9.1.5. pLSR- used for reporter plasmid construction

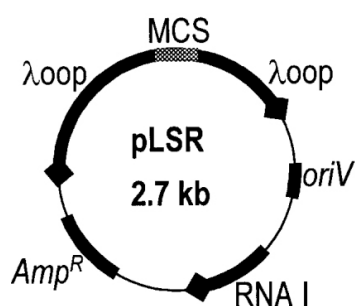


Figure 105: Diagram of pLSR vector from (El-Robh and Busby 2002).

## 9.2. Protein ladders

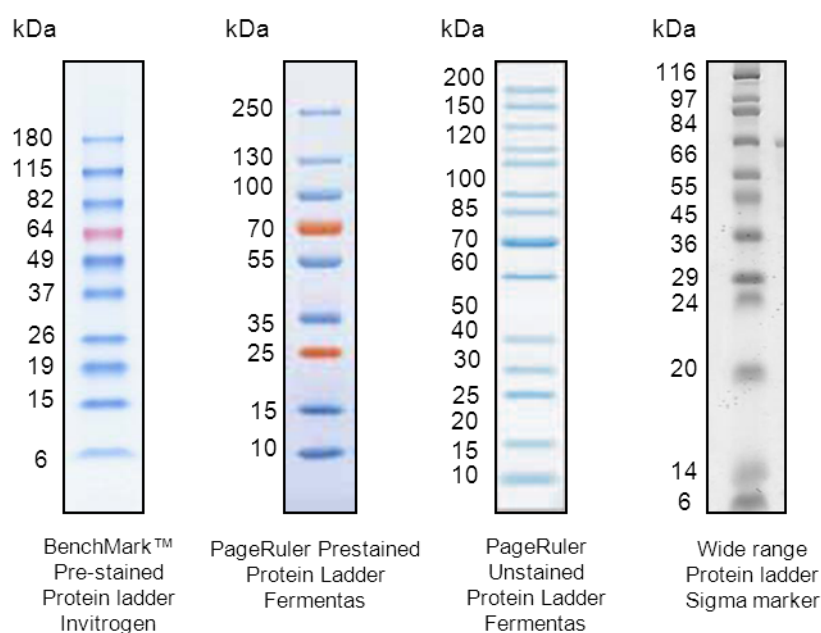


Figure 106: Protein markers.

Protein markers used for SDS-PAGE. BenchMark Pre-stained Protein Ladder used as a marker in Western Blots.

### 9.3. Oligonucleotides

#### 9.3.1. Oligonucleotides - cloning and mutagenesis

| Vector construct(s)        | Sequences 5'-3'                                                                                               | Vector construct(s)                                    | Sequences 5'-3'                                                                                  |
|----------------------------|---------------------------------------------------------------------------------------------------------------|--------------------------------------------------------|--------------------------------------------------------------------------------------------------|
| <b>pJRsarA</b>             | <i>NdeI</i><br>CCCATATGGCAATTACAAAATCAATGATTG<br><i>HindIII</i><br>CCCAAGCTTTTATAGTTCAATTCGTTGTTG             | <b>pJRagrA-N (pET28b+)</b><br>N-6His tag               | <i>NdeI</i><br>CCCATATGAAAATTTTCATTTGCGAAGACG<br><i>HindIII</i><br>CCAAAGCTTTATATTTTTTAACGTTCTC  |
| <b>pJRsarR</b>             | <i>NdeI</i><br>CCCATATGAGTAAAATTAATGACATTAATG<br><i>BamHI</i><br>CGGGATCCCTAATTTTAATGTATCTTC                  | <b>pJRagrA-nat (pET22b+)</b><br>native                 | <i>NdeI</i><br>CCCATATGAAAATTTTCATTTGCGAAGACG<br><i>HindIII</i><br>CCAAAGCTTTATATTTTTTAACGTTCTC  |
| <b>pJRsigA</b>             | <i>NdeI</i><br>GGAGGCCGTCAATGCTCTGATAAC<br><i>HindIII</i><br>CATTAAATGGAAGCTTTAATCCATAAAG                     | <b>pJRagrA-C (pET22b+)</b><br>C-6His tag               | <i>NdeI</i><br>CCCATATGAAAATTTTCATTTGCGAAGACG<br><i>HindIII</i><br>CCAAAGCTTAGCTATTTTTTAACGTTCTC |
| <b>pJRsigB</b>             | <i>NdeI</i><br>CCCATATGGCGAAAGAGTCGAAATCAG<br><i>BamHI</i><br>CGGGATCCCTATTGATGTGCTGCTCTTC                    | <b>pJRagrA (pMAL-c2X)</b>                              | <i>EcoRI</i><br>CGGAATTCATGAAAATTTTCATTTGCGAAG<br><i>HindIII</i><br>CCAAAGCTTTATATTTTTTAACGTTCTC |
| <b>pJR<sub>P2+P3</sub></b> | <i>EcoRI</i><br>CGGAATTCATCACATCTCTGTGATCTAG<br><i>HindIII</i><br>CCAAAGCTTAACTGGTCAATTTTATTATC               | <b>pJRagrA<sub>H169A</sub></b><br>Used in SDM reaction | GAATCATCAACAAAATCTGCTAGACTCATT<br>GCCATTTA<br>TAAATGGGCAATGAGTCTAGCAGATTTTG<br>TTGATGATTC        |
| <b>pJR<sub>P2</sub></b>    | <i>NdeI</i><br>CGGAATTCGCAATTTATTTCCAATTTTCTTA<br>ACTAGTCG<br><i>HindIII</i><br>CCAAAGCTTCGATTAGTACAATCTTTATC | <b>pJRsarA<sub>R84A</sub></b><br>Used in SDM reaction  | GATTACTTCGATAAAAAAGCTAATGAGCA<br>TGATGAAAG<br>CTTTCATCATGCTCATTAGCTTTTTTATCGA<br>AGTAATC         |
| <b>pJR<sub>P3</sub></b>    | <i>NdeI</i><br>CGGAATTCATCACATCTCTGTGATCTAG<br><i>HindIII</i><br>CCAAAGCTTCGATTAGTACAATCTTTATC                |                                                        |                                                                                                  |

Table 9: List of oligonucleotides used for cloning and mutagenesis in this study.

Oligonucleotides were synthesised by Invitrogen.

### 9.3.2. Oligonucleotides - promoter probes

| Promoter probe construct(s) | Sequences 5'-3'                                                                                                                                                                                                                                     |
|-----------------------------|-----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>P2</b>                   | Non-template strand<br>ACATTTAACAGTTAAGTATTTATTTCTACAGTTAGGCAATATAATGATAAAAGATTGCTAAATCGTATAAT<br>GACAGTGAGGAGAGTGGTG<br>Template strand<br>CACCACTCTCCTCACTGTCATTATACGATTTAGTACAATCTTTATCATTATATTGCCTAACTGTAGGAAATAAA<br>TACTTAACTGTAAATGT         |
| <b>P2-17</b>                | Non-template strand<br>ACATTTAACAGTTAAGTATTTATTTCTACAGTTAGGCAATATAATGATAAAAGATTGCTAAATCGTATAATG<br>ACAGTGAGGAGAGTGGTG<br>Template strand<br>CACCACTCTCCTCACTGTCATTATACGATTTAGACAATCTTTATCATTATATTGCCTAACTGTAGGAAATAAA<br>ACTTAACTGTAAATGT           |
| <b>P3</b>                   | Non-template strand<br>AAAATTTACAGTTAAGAATAAAAAACGACTAGTTAAGAAAAATTGAAAAATAAATGCTTTTAGCATGTTTTAA<br>TATACTAGATCACAGAGATGTG<br>Template strand<br>CACATCTCTGTGATCTAGTTATATTTAAACATGCTAAAAGCATTTATTTTCCAATTTTCTTAACTAGTCGTTTTT<br>TATTCTTAACTGTAAATTT |
| <b>P3-17</b>                | Non-template strand<br>AAAATTTACAGTTAAGAATAAAAAACGACTAGTTAAGAAAAATTGAAAAATAAATGTTTTCATGTTTTAATAT<br>AACTAGATCACAGAGATGTG<br>Template strand<br>CACATCTCTGTGATCTAGTTATATTTAAACATGAAAACATTTATTTTCCAATTTTCTTAACTAGTCGTTTTTAT<br>TCTTAACTGTAAATTT       |
| <b>P3-17AC</b>              | Non-template strand<br>AAAATTTACAGTTAAGAATAAAAAACGACTAGTTAAGAAAAATTGAAAAATAAATGTTTTCAACTTTTAATAT<br>AACTAGATCACAGAGATGTG<br>Template strand<br>CACATCTCTGTGATCTAGTTATATTTAAAGTTGAAAACATTTATTTTCCAATTTTCTTAACTAGTCGTTTTTAT<br>TCTTAACTGTAAATTT       |
| <b>P3-AC</b>                | Non-template strand<br>AAAATTTACAGTTAAGAATAAAAAACGACTAGTTAAGAAAAATTGAAAAATAAATGCTTTTAGCAACTTTTAA<br>TATACTAGATCACAGAGATGTG<br>Template strand<br>CACATCTCTGTGATCTAGTTATATTTAAAGTTGCTAAAAGCATTTATTTTCCAATTTTCTTAACTAGTCGTTTTT<br>TATTCTTAACTGTAAATTT |
| <b>P3-35</b>                | Non-template strand<br>AAAATTTACAGTTAAGAATAAAAAACGACTAGTTAAGAAAAAGACTCCAATAAATGCTTTTAGCATGTTTTAA<br>TATACTAGATCACAGAGATGTG<br>Template strand<br>CACATCTCTGTGATCTAGTTATATTTAAACATGCTAAAAGCATTTATTGGAGTCTTTTCTTAACTAGTCGTTTT<br>TTATTCTTAACTGTAAATTT |
| <b>P3-35AC</b>              | Non-template strand<br>AAAATTTACAGTTAAGAATAAAAAACGACTAGTTAAGAAAAAGACTCCAATAAATGCTTTTAGCAACTTTTAA<br>TATACTAGATCACAGAGATGTG<br>Template strand<br>CACATCTCTGTGATCTAGTTATATTTAAAGTTGCTAAAAGCATTTATTGGAGTCTTTTCTTAACTAGTCGTTTT<br>TTATTCTTAACTGTAAATTT |

Table 10: Oligonucleotides used to create *agr* P2 and P3 promoter probes.

Oligonucleotides were used in chapters 3, 4, 5, 6 and 7 and designed using Sci Ed Central Clone Manager. Oligonucleotides were synthesised by Invitrogen.

### 9.3.3. Vector constructs

| Name                           | Insert                                                                                                                      | Reference  |
|--------------------------------|-----------------------------------------------------------------------------------------------------------------------------|------------|
| <b>pJRsarA</b>                 | 0.375-Kb <i>NdeI-HindIII</i> fragment carrying <i>sarA</i> gene ligated into pET28b+                                        | This study |
| <b>pJRsarA<sub>R84A</sub></b>  | 0.375-Kb <i>NdeI-HindIII</i> fragment carrying <i>sarA<sub>R84A</sub></i> gene ligated into pET28b+                         | This study |
| <b>pJRsarR</b>                 | 0.347-Kb <i>NdeI-BamHI</i> fragment carrying <i>sarR</i> gene ligated into pET28b+                                          | This study |
| <b>pJRagrA-N</b>               | 0.716-Kb <i>NdeI-HindIII</i> fragment carrying <i>agrA</i> gene ligated into pET28b+ to encode N-terminal 6-His tag         | This study |
| <b>pJRagrA-nat</b>             | 0.716-Kb <i>NdeI-HindIII</i> fragment carrying <i>agrA</i> gene ligated into pET22b+                                        | This study |
| <b>pJRagrA-C</b>               | 0.716-Kb <i>NdeI-HindIII</i> fragment carrying <i>agrA</i> gene ligated into pET22b+ to encode C-terminal 6-His tag         | This study |
| <b>pJRagrA-MBP</b>             | 0.716-Kb <i>NdeI-EcoRI</i> fragment carrying <i>agrA</i> gene ligated into pMAL-c2X to encode N-terminal MBP fusion protein | This study |
| <b>pJRagrA<sub>H169A</sub></b> | 0.716-Kb <i>NdeI-BamHI</i> fragment carrying <i>agrA<sub>H169A</sub></i> gene ligated into pET28b+                          | This study |
| <b>pJRsigA</b>                 | 1.107-Kb <i>NdeI-HindIII</i> fragment carrying <i>rpoD</i> gene ligated into pET28b+                                        | This study |
| <b>pJRsigB</b>                 | 0.771-Kb <i>NdeI-BamHI</i> fragment carrying <i>sigma B</i> gene ligated into pET28b+                                       | This study |
| <b>pJR<sub>P2+P3</sub></b>     | 0.267-Kb <i>EcoRI-HindIII</i> fragment carrying <i>agr</i> P2 and P3 promoters and intergenic region ligated into pLSR      | This study |
| <b>pJR<sub>P2</sub></b>        | 0.213-Kb <i>NdeI-BamHI</i> fragment carrying <i>agr</i> P2 promoter and intergenic region ligated into pLSR                 | This study |
| <b>pJR<sub>P3</sub></b>        | 0.193-Kb <i>NdeI-BamHI</i> fragment carrying <i>agr</i> P3 promoter and intergenic region ligated into pLSR                 | This study |

Table 11: Vector constructs used in this study.

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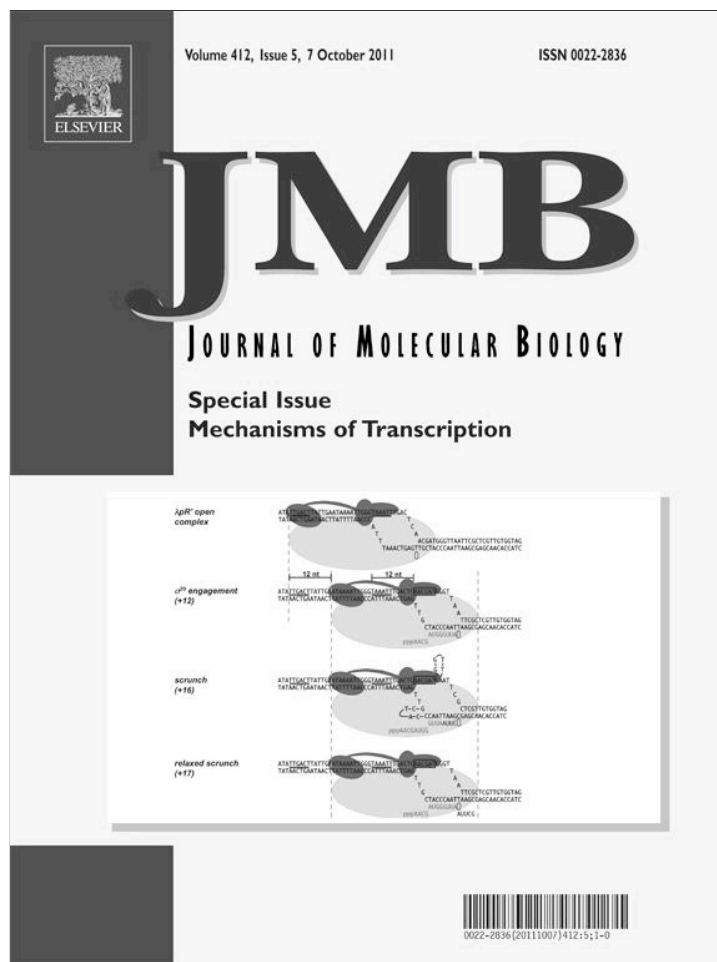
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# Molecular Insights into the Control of Transcription Initiation at the *Staphylococcus aureus* agr operon

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The accessory gene regulatory (agr) operon of the opportunistic human pathogen *Staphylococcus aureus* is a prime pathogenesis factor in this bacterium. The agr operon consists of two transcription units, RNAII and RNAPIII, which are transcribed from divergent promoters, P2 and P3, respectively. RNAII encodes a quorum-sensing system, including AgrA, the master transcription activator of the agr operon. RNAPIII is the effector RNA molecule that regulates the expression of many virulence genes. Owing to the atypical spacer lengths of P2 and P3, it is widely considered that transcription from P2 and P3 only occurs in a strictly AgrA-dependent manner. Here, using a fully native *S. aureus* *in vitro* transcription system, we provide the first molecular and mechanistic characterisation of the regulation of transcription initiation at the agr operon. Surprisingly, the results demonstrate that RNA polymerase (RNAP) can interact with P2 and P3 equally well in the absence of AgrA. However, formation of a transcription-competent open promoter complex (RPO) occurs more readily at P2 than at P3 when AgrA is absent. Reducing the atypical P3 spacer region length to the optimal length of 17 nucleotides significantly improves promoter activity by facilitating the isomerisation of the initial RNAP–P3 complexes to RPO, and the extended –10-like element of P3 is required for optimal promoter activity. AgrA increases the occupancy of both promoters by RNAP and thereby increases the amount of transcription initiated at P2 and P3. However, the AgrA-mediated effect on transcription initiation is more prominent at P3 than at P2. The effect of AgrA at P2 and P3 appears to be restricted to events leading to the formation of RPO. The relevance of AgrA-independent and AgrA-dependent transcription initiation at P2 and P3 is presented in the context of our current understanding of the role of the agr operon in the pathobiology of *S. aureus*.

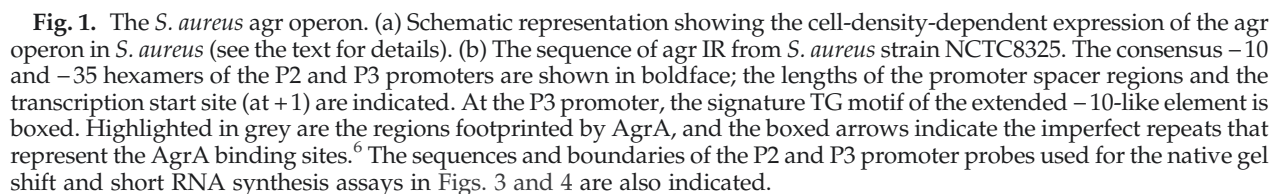
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## Introduction

*Staphylococcus aureus* is an important and opportunistic pathogen that is capable of causing a diverse range of human infections. The pathogenesis of *S. aureus* is very complex. The expression of virulence factors is controlled in a temporal and cell-density-dependent manner and is affected at multifactorial levels by a number of global regulatory systems.<sup>1–3</sup> Early in infection, the expression of cell-surface-associated virulence factors is up-regulated,

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Abbreviations used: agr, accessory gene regulator; RNAP, RNA polymerase; RPO, open promoter complex; AIP, autoinducing peptide; agr IR, agr operon intergenic region; RPO, closed promoter complex; EDTA, ethylenediaminetetraacetic acid; PEI, polyethyleneimine; BBSRC, Biotechnology and Biological Sciences Research Council.



The *agr* operon is conserved throughout the genus *Staphylococcus*, expressed from divergent promoters P2 and P3, and subject to regulation at the transcriptional level (Fig. 1a).<sup>7</sup> The P2 transcript

RNAII encodes the quorum-sensing system and consists of four proteins: AgrA, AgrB, AgrC and AgrD. AgrD is a pro-autoinducing peptide (AIP), which is processed and secreted by the transmembrane protein AgrB. AgrA and AgrC are members of a two-component system in which AgrC is the transmembrane receptor histidine kinase and AgrA is the DNA binding response regulator. The cell-density-dependent accumulation of AIP is sensed by AgrC, which undergoes autophosphorylation upon binding AIP. Phosphorylation of AgrA by AgrC allows AgrA to bind to a pair of direct repeats located in the *agr* operon intergenic region (*agr* IR), the ~115-bp region that separates the P2 and P3 promoters.<sup>6</sup> Phosphorylated AgrA is the master transcription activator of the *agr* operon, which contains an amino-terminal CheY-like domain and a carboxyl-terminal DNA binding domain that belongs to the LytTR protein family.<sup>8</sup> The P3 transcript RNAIII is the pleiotropic effector of the *agr* operon,

which modulates the expression of virulence genes generally at the translational level. RNAIII facilitates the expression of genes encoding secreted virulence factors (e.g., by countering a translation-blocking mRNA secondary structure<sup>9</sup>), whereas the expression of genes encoding cell-surface-bound virulence factors is repressed (e.g., by an antisense base-pairing mechanism<sup>10</sup>); RNAIII also encodes  $\delta$ -haemolysin at its 5'-end.<sup>11</sup> It is widely accepted that transcription initiation from the P3 promoter occurs in an RNAII-dependent manner and thus strictly relies upon AgrA. In support of this view, in *S. aureus* infection models and *in vivo* expression studies, the transcription of RNAII temporally precedes the transcription of RNAIII. A steady increase in RNAII transcription is seen during exponential growth, followed by a rapid increase in RNAIII transcription, concomitant with a cell-density-dependent expression of the agr operon.<sup>12</sup>

Notably, unlike in most bacterial promoters where the optimal spacer region that separates the conserved  $-35$  and  $-10$  hexanucleotide elements is 17 nucleotides in length,<sup>13–15</sup> the P2 and P3 promoters are atypical in that the spacer regions are 18 and 20 nucleotides in length, respectively. Shortening the P3 promoter spacer sequence by 3 nucleotides has been shown to increase the activity of the mutant P3 promoter significantly above that of the wild-type P3 promoter *in vivo*.<sup>9</sup> Therefore, it is possible that RNA polymerase (RNAP) is unable to productively interact with and utilise the P2 and P3 promoters in the absence of AgrA, which, in principle, could compensate for the suboptimal organisation of the agr operon promoters (especially P3). However, we also note the presence of an extended  $-10$ -like element at the P3 promoter (indicated by the characteristic TG nucleotides at positions  $-14$  and  $-15$ , respectively; Fig. 1b), which often contributes to additional contacts with RNAP at promoters with nonoptimally placed  $-35$  elements.<sup>16,17</sup> Intriguingly, Xiong *et al.* reported that AgrA-independent (i.e., in *S. aureus* cells in which *agrA* is inactivated) transcription from the P3 promoter can indeed occur in *S. aureus* cells in the context of a rabbit endocarditis infection model.<sup>12</sup> Conversely, since the P2 promoter better matches the consensus than does the P3 promoter, it is formally possible that AgrA-independent transcription (hereafter referred to as basal transcription) can also occur at the P2 promoter. In support of this view, Novick *et al.* proposed that basal transcription from the P2 promoter could ensure the constant presence of low levels of AgrA and AgrC, which would be sufficient to respond to the signals that up-regulate transcription from the P2 and P3 promoters.<sup>18</sup> Furthermore, basal transcription from the P2 promoter may also provide sufficient AgrA for expression of virulence genes independent of the expression of the agr operon. Consistent with the

latter view, a direct role for AgrA in the activation of leucocidal phenol-soluble modulin genes (*psmA* and *psm $\beta$* ) in an RNAIII-independent manner has been reported.<sup>19</sup>

Since the agr operon is an integral part of an exceedingly complex regulatory network, which controls the expression of the *S. aureus* virulon, the use of *in vivo* methodologies has imposed constraints on the study of the molecular mechanisms that underlie transcription regulation at the agr operon promoters P2 and P3. Compounding this problem are the strain-to-strain variations observed for the level of expression of the agr operon in many *in vivo* studies and the fact that much of our knowledge on the expression of the agr operon is derived from the laboratory strain of the RN6390 lineage, which has a mutation in the *rsbU* global regulatory gene.<sup>20–24</sup> Importantly, as yet, it is not known (i) whether RNAP can productively interact with the P2 and P3 promoters in the absence of AgrA; (ii) how the P2 and P3 promoter activities (both in the absence and in the presence of AgrA) compare; (iii) whether the extended  $-10$ -like element at the P3 promoter contributes to promoter activity; and (iv) why shortening the P3 spacer region to the optimal length of 17 nucleotides leads to increased P3 promoter activity *in vivo*. In this study, we have developed a fully native *S. aureus* *in vitro* transcription system to address the above questions. The relevance of the results we have obtained is presented in the context of the current understanding of the contribution of the agr operon to the pathobiology of *S. aureus*.

## Results

### A fully native *S. aureus* *in vitro* transcription system for studying transcription regulation at the agr operon

To study how transcription initiation at the agr operon is regulated at the molecular level, we developed a fully native *S. aureus* *in vitro* transcription system. Bacterial RNAP consists of a five-subunit catalytic core ( $\alpha_2\beta\beta'\omega$ ; abbreviated as E), which needs to associate with a dissociable sixth subunit ( $\sigma$  subunit) to form the RNAP holoenzyme ( $E\sigma$ ), which is capable of promoter-specific transcription initiation. *S. aureus* core RNAP was prepared from exponentially growing *S. aureus* strain NCTC8325 cells using a single immunoaffinity purification step. As shown in Fig. 2a, the *S. aureus* catalytic core was purified to >95% homogeneity using this procedure (lane 2). The primary housekeeping  $\sigma$  factor in *S. aureus*, [6His] $\sigma^A$ , was expressed in *Escherichia coli* and purified to >95% homogeneity by single-step

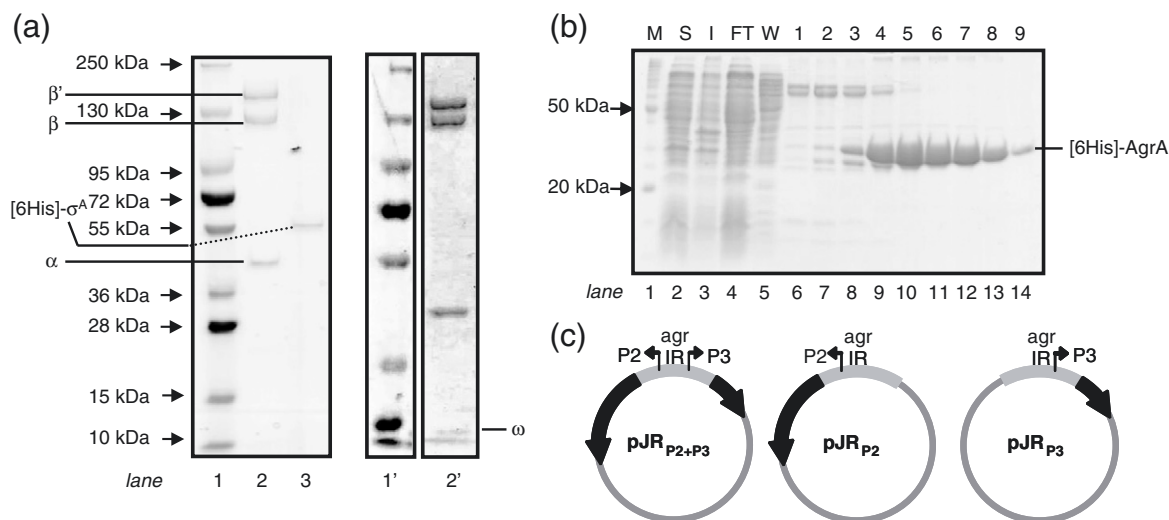


**Fig. 2.** A fully native *S. aureus* *in vitro* transcription system. (a) Image of a 10% (wt/vol) SDS denaturing polyacrylamide gel showing the purity of *S. aureus* core RNAP (lane 2) and recombinant [6His] $\sigma^A$  subunit (lane 3). The migration positions of the  $\beta$ ,  $\beta'$ ,  $\alpha$  and [6His] $\sigma^A$  subunits are indicated. The  $\omega$  subunit, which migrates at an apparent molecular mass of approximately 12 kDa, has run “off” the bottom of the gel shown on the left. To the right of the figure, a lane (lane 2') containing the *S. aureus* core RNAP sample from lane 2 of an SDS denaturing polyacrylamide gel electrophoresed for a shorter time is shown, and the migration position of the  $\omega$  subunit is indicated. (b) Image of a 12.5% (wt/vol) SDS denaturing polyacrylamide gel showing the purification of [6His]AgrA (S, soluble cell fraction; I, insoluble cell fraction; FT, flow-through from the Ni column; W, wash fraction; 1–9, peak fractions of elution from the Ni column). The migration position of [6His]AgrA is indicated. (c) Schematic representation of the plasmids used as templates for the *in vitro* transcription assay.

nickel-affinity chromatography (Fig. 2a, lane 3). In contrast to a previous study that reported difficulty in obtaining recombinant AgrA in soluble form,<sup>6</sup> we established a simple protocol to purify soluble [6His]AgrA from *E. coli* (Fig. 2b; see Materials and Methods). Promoter templates for the *in vitro* transcription assays were generated by introducing a DNA fragment containing agr IR into plasmid pLSR<sup>25</sup> between the EcoRI site and the HindIII site to generate pJR<sub>P2+P3</sub> (Fig. 2c). RNAP molecules that initiate transcription from the P2 and P3 promoters will terminate at the  $\lambda$  oop terminator, located downstream of the EcoRI and HindIII sites, respectively (Fig. 2c). In order to distinguish between the transcripts synthesised from the P2 promoter and the transcripts synthesised from the P3 promoter, we organised pJR<sub>P2+P3</sub> such that the leftward transcript from the P2 promoter terminates 143 bp beyond the HindIII site and the rightward transcript from the P3 promoter terminates 135 bp beyond the EcoRI site. Thus, pJR<sub>P2+P3</sub> can be used as a template in *in vitro* transcription assays to simultaneously monitor transcription initiation from the P2 and P3 promoters of the agr operon. Similarly, pJR<sub>P2</sub> and pJR<sub>P3</sub> were generated by introducing a DNA fragment between the EcoRI site and the HindIII site in pLSR, in which either the P2 (pJR<sub>P3</sub>) promoter or the P3 (pJR<sub>P2</sub>) promoter was missing (Fig. 2c).

### ***S. aureus* E $\sigma^A$ can productively interact with the agr operon promoters P2 and P3 equally well in the absence of AgrA**

The initiation of transcription in bacterial promoters is a multistep process that begins with the binding of RNAP to the promoter DNA to form the initial RNAP–promoter complex, the closed promoter complex (RPC). In most bacterial promoters, the competitor (heparin)-sensitive RPC is short-lived at 37 °C and is only experimentally tractable at <4 °C.<sup>26,27</sup> The transcription-competent open promoter complex (RPO), which occurs via several RNAP–promoter complex intermediates, is the predominant RNAP–promoter complex at 37 °C (in most bacterial promoters). In the RPO, promoter DNA strands are locally “melted out” (typically between position –10 and position +3 with respect to the transcription start site at +1), and the template strand is positioned in the catalytic cleft of RNAP. A hallmark feature of the RPO is that it is significantly more heparin resistant than the RPC. Inspection of the nucleotide sequences at the –10 and –35 elements of the P2 and P3 promoters indicates that 5/6 and 4/6 of the –10 and –35 nucleotides, respectively, are conserved at the P3 promoter. Similarly, 6/6 and 4/6 of the –10 and –35 nucleotides, respectively, are conserved at the P2 promoter. However, considering that the P3 promoter spacer region length deviates from the

optimal (17 nucleotides) by 3 nucleotides and that the P2 promoter spacer length deviates from the optimal by only 1 nucleotide, it would seem that the P3 promoter is a weaker promoter than the P2 promoter. Since many transcription activators improve promoter occupancy and/or usage by RNAP, and considering the atypical spacer length of the P2 and P3 promoters, we initially wanted to determine how well, if at all,  $E\sigma^A$  can interact with the P2 and P3 promoters in the absence of AgrA. We used a native gel shift assay to monitor the binding of  $E\sigma^A$  to 93 bp and 96 bp of  $^{32}$ P-labelled DNA fragments carrying the P2 (spanning the sequence from -81 to +12 with respect to the +1 site; hereafter called the P2 probe; Fig. 1b) and P3 (spanning the sequence

from -80 to +16; hereafter called the P3 probe; Fig. 1b) promoter sequences, respectively, as a function of  $E\sigma^A$  concentration. The assays were conducted at  $<4^\circ\text{C}$  to minimise the conversion of the  $E\sigma^A$ -promoter complexes (R<sub>Pc</sub>) into the R<sub>Po</sub> and therefore to enrich for the R<sub>Pc</sub>. Results show that, under these conditions,  $E\sigma^A$  is able to bind to the P2 and P3 probes equally well (Fig. 3a). Similar results were obtained when the assays were repeated at  $37^\circ\text{C}$  (Fig. 3b). Control experiments in which [6His] $\sigma^A$  was omitted from the reaction confirmed that the  $E\sigma^A$ -promoter complexes detected at  $<4^\circ\text{C}$  and  $37^\circ\text{C}$  were [6His] $\sigma^A$  dependent (data not shown; Fig. 3c). Since the P2 and P3 promoters potentially compete for  $E\sigma^A$  in the native context of the agr

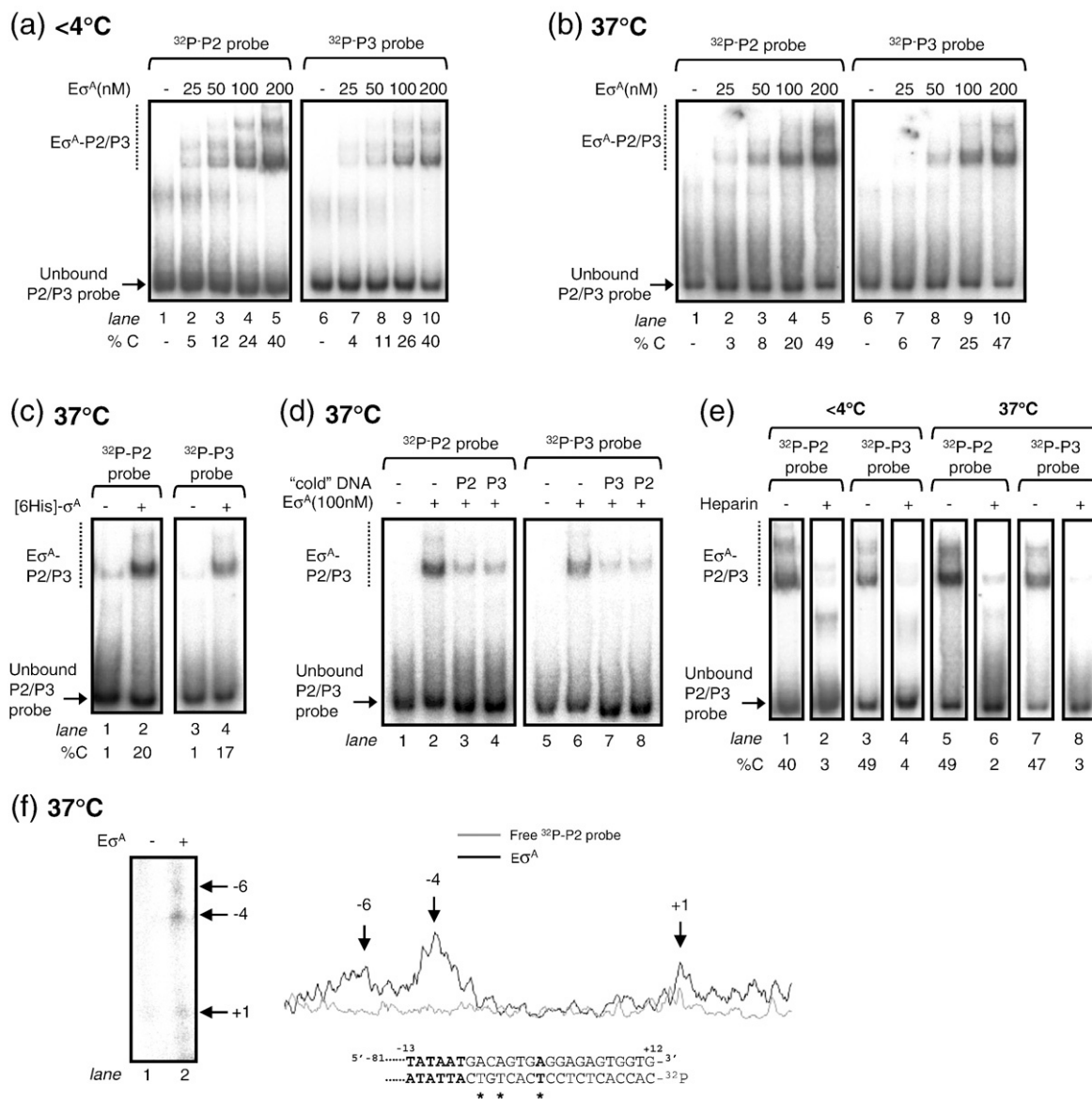


Fig. 3 (legend on next page)

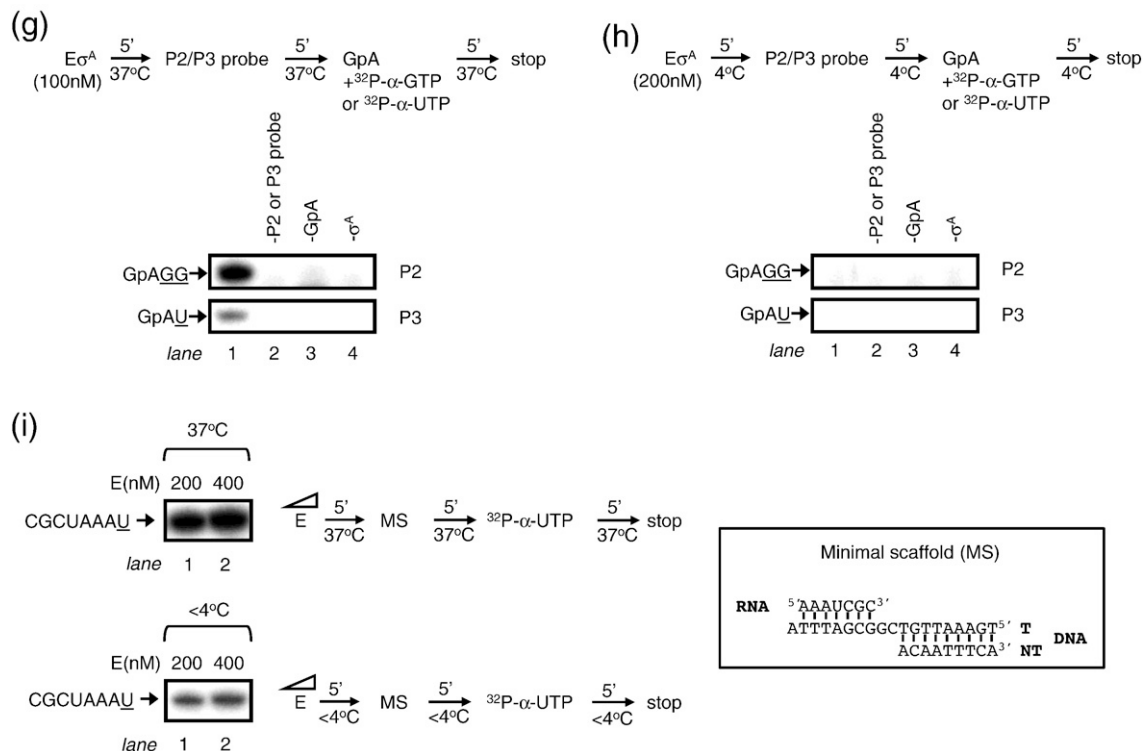


**Fig. 3.** The binding of  $E\sigma^A$  to the P2 and P3 promoter probes. (a) An autoradiograph of a 4.5% (wt/vol) native polyacrylamide gel showing the binding of  $E\sigma^A$  to the P2 (lanes 1–5) and P3 (lanes 6–10) promoter probes as a function of  $E\sigma^A$  concentration. The experiment was conducted at  $<4^\circ\text{C}$ . The migration positions of unbound P2/P3 promoter probes and  $E\sigma^A$ -P2/ $E\sigma^A$ -P3 complexes are indicated. The amount of promoter DNA bound by  $E\sigma^A$  is expressed as a percentage (% C) of the total DNA present in the reaction. In at least two independent experiments, the data obtained fall within 10% of the % C value shown. (b) As in (a), but the experiment was conducted at  $37^\circ\text{C}$ . (c) As in (a), but the reactions were conducted at  $37^\circ\text{C}$  in the absence (lanes 1 and 3) and in the presence (lanes 2 and 4) of  $[6\text{His}]\sigma^A$ , at a final concentration of 100 nM *S. aureus* core RNAP. (d) As in (a), but the reactions were conducted at  $37^\circ\text{C}$ . The  $E\sigma^A$ -P2 (lane 2) and  $E\sigma^A$ -P3 (lane 6) promoter complexes were challenged with either excess unlabelled (“cold”) P2 (lanes 3 and 8) or P3 (lanes 4 and 7) promoter probes (see the text for details). (e) The figure is organised as described above. The  $E\sigma^A$ -P2 (lanes 1, 2, 5 and 6) and  $E\sigma^A$ -P3 (lanes 3, 4, 7 and 8) promoter complexes formed at  $<4^\circ\text{C}$  (lanes 1–4) and  $37^\circ\text{C}$  (lanes 5–8) were challenged with heparin (lanes 2, 4, 6 and 8). Promoter complexes formed in the absence of heparin are shown for comparison (lanes 1, 3, 5 and 7). (f) An autoradiograph of a 20% (wt/vol) urea-denaturing polyacrylamide gel showing DNA melting with  $E\sigma^A$ -P2 promoter complexes formed at  $37^\circ\text{C}$ . Lane 1 contains a  $\text{KMnO}_4$ -treated P2 promoter probe (with  $5'$   $^{32}\text{P}$ -end-labelled template strand). In lane 2, which contains the  $E\sigma^A$ -P2 promoter complex, the  $\text{KMnO}_4$ -reactive thymine residues are marked by arrows. The promoter sequence from positions  $-13$  to  $+12$  of the P2 promoter is shown at the top of the autoradiograph, with the reactive thymine residues marked with asterisks. The transcription start position at  $+1$  is indicated. To the right of the autoradiograph, traces of lanes 1 and 2 are shown, and the positions of the  $\text{KMnO}_4$ -reactive thymines are indicated. (g) An autoradiograph of a 20% (wt/vol) urea-denaturing polyacrylamide gel showing synthesis of the short RNA products GpAGG (from P2) and GpAU (from P3) at  $37^\circ\text{C}$  (indicated by the arrow, where the underlined nucleotides are  $^{32}\text{P}$  labelled). The schematic representation of the reaction is shown at the top of the gel image (see Materials and Methods and text for details). In control lanes 2–4, the promoter template, dinucleotide and  $\sigma^A$ , respectively, are omitted from the reaction. (i) As in (g), but the reaction was conducted at  $<4^\circ\text{C}$ . (h) An autoradiograph of a 20% (wt/vol) urea-denaturing polyacrylamide gel showing the ability of *S. aureus* core RNAP (in the absence of  $\sigma^A$ ) to extend the RNA in the MS probe (schematic representation shown on the right) by 1 nucleotide using  $[\alpha\text{-}^{32}\text{P}]\text{UTP}$  at  $37^\circ\text{C}$  and  $<4^\circ\text{C}$  (left).

operon, we challenged the Eo<sup>A</sup>-promoter complexes formed on the <sup>32</sup>P-labelled P2 and P3 probes (at 37 °C) with a ~10-fold molar excess of unlabelled "cold" versions of the P2 and P3 probes to determine if Eo<sup>A</sup> prefers one promoter over the other when both are available at the same time. As shown in Fig. 3d, challenging the Eo<sup>A</sup>-<sup>32</sup>P-P2 complex with either

excess “cold” P2 or excess “cold” P3 probes results in a similar decrease (by ~90–95%) in the amount of the initial  $\text{E}\sigma^{\text{A}}\text{-}^{32}\text{P}$ -P2 complexes formed (compare lanes 3 and 4). Similar results were obtained in the converse experiment in which the  $\text{E}\sigma^{\text{A}}\text{-}^{32}\text{P}$ -P3 complex was challenged with either excess “cold” P3 or excess “cold” P2 probes (Fig. 3d, lanes 5–8).

As expected, the addition of heparin destroyed most of the  $E\sigma^A$ -promoter complexes formed at  $<4^\circ\text{C}$  (Fig. 3e, lanes 1–4). Surprisingly, the  $E\sigma^A$ -promoter complexes formed at  $37^\circ\text{C}$  were not resistant to heparin challenge (Fig. 3e, lanes 5–8). Since RPo formation is often very inefficient on short promoter templates, we repeated the heparin challenge reactions at  $37^\circ\text{C}$  using longer (–173 and +87 for the P2 promoters, and –163 and +66 for the P3 promoters) versions of the P2 and P3 promoter probes. As observed above with the short P2 and P3 probes,  $E\sigma^A$ -promoter complexes formed equally well on the long P2 and P3 probes, yet these complexes were not detectably more heparin resistant than the ones formed on the short P2 and P3 promoter probes (data not shown). Since the promoter DNA strands within the RPo are locally melted approximately between position –10 and the +1 site, we used potassium permanganate ( $\text{KMnO}_4$ ) to detect DNA melting within  $E\sigma^A$ -P2 and  $E\sigma^A$ -P3 promoter complexes formed at  $37^\circ\text{C}$  under our experimental conditions. As shown in Fig. 3f, we detected clear  $\text{KMnO}_4$  reactivity at the template strand thymines at positions –6 and –4 and weak  $\text{KMnO}_4$  reactivity at position +1 within the  $E\sigma^A$ -P2 promoter complex, thus suggesting that DNA melting (indicative of RPo formation) has indeed occurred within  $E\sigma^A$ -P2 promoter complexes in the absence of AgrA. Interestingly, no  $\text{KMnO}_4$  reactivity is detected at the thymines at either the template or the nontemplate strand within the  $E\sigma^A$ -P3 promoter complex (data not shown). Therefore, it is possible that the RPo does not form or accumulates slowly at the P3 promoter in the absence of AgrA; alternatively, the RPo formed at the P3 promoter could be unstable and therefore short-lived to detect DNA melting by  $\text{KMnO}_4$  probing. In order to directly establish whether RPo formation occurs at  $37^\circ\text{C}$  under our experiment conditions (and also to distinguish the RPo and RPo without the use of heparin or chemical probes), we used an assay that directly reports transcriptionally proficient DNA melting and RPo formation. The assay, hereafter referred to as the short RNA synthesis assay (which we routinely use to measure RPo formation by *E. coli*  $E\sigma^{70}$ ),<sup>28,29</sup> measures the ability of  $E\sigma^A$  to synthesise a dinucleotide-primed RNA product from the P2 and P3 promoters. As shown in Fig. 3g, the addition of the dinucleotide GpA (which complements positions –1 and +1 of the P2 template strand; Fig. 1b) and [ $\alpha$ - $^{32}\text{P}$ ]GTP (which complements position +2 of the P2 template strand; Fig. 1b) to the  $E\sigma^A$ -P2 complexes formed at  $37^\circ\text{C}$  results in the synthesis of the short RNA product GpAGG, thus indicating the presence of the RPo within the  $E\sigma^A$ -P2 complexes formed at  $37^\circ\text{C}$ . Similarly, the addition of the dinucleotide GpA (which complements positions +2 and +3 of the P3 template strand; Fig. 1b) and

[ $\alpha$ - $^{32}\text{P}$ ]UTP (which complements position +4 of the P3 template strand; Fig. 1b) results in the synthesis of the short RNA product GpAU, thus indicating the presence of the RPo within  $E\sigma^A$ -P3 complexes formed at  $37^\circ\text{C}$  (Fig. 3g). The synthesis of GpAGG and GpAU from the P2 and P3 promoters, respectively, is highly specific, as it strictly depends upon the presence of all the components ( $\sigma^A$ , promoter probe and dinucleotide) required for the promoter-specific short RNA synthesis (Fig. 3g, lanes 2–4). As expected, at  $<4^\circ\text{C}$ , no short RNA synthesis from the P2 and P3 promoters is detected (Fig. 3h), indicating that, as expected, the RPo is not detectably present within  $E\sigma^A$ -P2/ $E\sigma^A$ -P3 complexes formed under these conditions. Control experiments established that *S. aureus* RNAP retains catalytic activity at  $4^\circ\text{C}$ . In these assays, we used a minimal nucleic acid scaffold (Fig. 3i; hereafter referred to as the MS probe<sup>30</sup>) to measure if *S. aureus* RNAP (in the absence of  $\sigma$ , since the MS probe does not contain consensus promoter sequences) can extend the 7-nucleotides-long RNA in the MS probe by one position using [ $\alpha$ - $^{32}\text{P}$ ]UTP (Fig. 3i). The radiolabelled 8-nucleotides-long RNA product can then be analysed by urea-denaturing PAGE. As expected, *S. aureus* RNAP is able to extend the RNA in the MS probe by one position when the reaction is conducted at  $37^\circ\text{C}$  (Fig. 3i). Similarly, when the reaction is conducted at  $<4^\circ\text{C}$ , *S. aureus* RNAP retains catalytic activity and is able to extend the RNA in the MS probe (albeit with a  $\sim 7$ -fold lower efficiency than at  $37^\circ\text{C}$ ). Since  $E\sigma^A$  can form promoter complexes at  $<4^\circ\text{C}$  (Fig. 3a) and appears to retain catalytic activity at  $<4^\circ\text{C}$  (Fig. 3h), we confidently conclude that the RPo is not present within the P2 and P3 promoter complexes formed at  $<4^\circ\text{C}$ . Overall, the native gel binding assay and the short RNA synthesis assay unequivocally demonstrate that, despite the atypical spacer lengths of the P2 and P3 promoters,  $E\sigma^A$  can productively interact with both *agr* operon promoters equally well in the absence of AgrA. However, it appears that the RPo formed at the P3 promoter is less stable and/or accumulates at a slower rate than the RPo formed at the P2 promoter (see later text).

#### The extended –10-like element is required for optimal P3 promoter activity

We extended the short RNA synthesis assay to determine whether the extended –10-like element of the P3 promoter has a role in  $E\sigma^A$  binding and promoter activity. Based on previous mutagenesis analyses of *E. coli* extended –10 promoters,<sup>17,31</sup> which revealed that changing the signature TG sequence to AC leads to loss of promoter activity, we constructed a mutant version of the P3 promoter (hereafter called P3-AC) in which the TG motif at positions –14 and –15 was changed to AC. Initially,

we conducted a native gel shift assay at  $<4^\circ\text{C}$  with  $^{32}\text{P}$ -labelled P3 and P3-AC promoter probes to determine how well  $\text{E}\sigma^A$  formed the R<sub>PC</sub> on both

promoter probes. As shown in Fig. 4a,  $\text{E}\sigma^A$  is able to bind to both promoter probes equally well under our experimental conditions. Next, we measured the

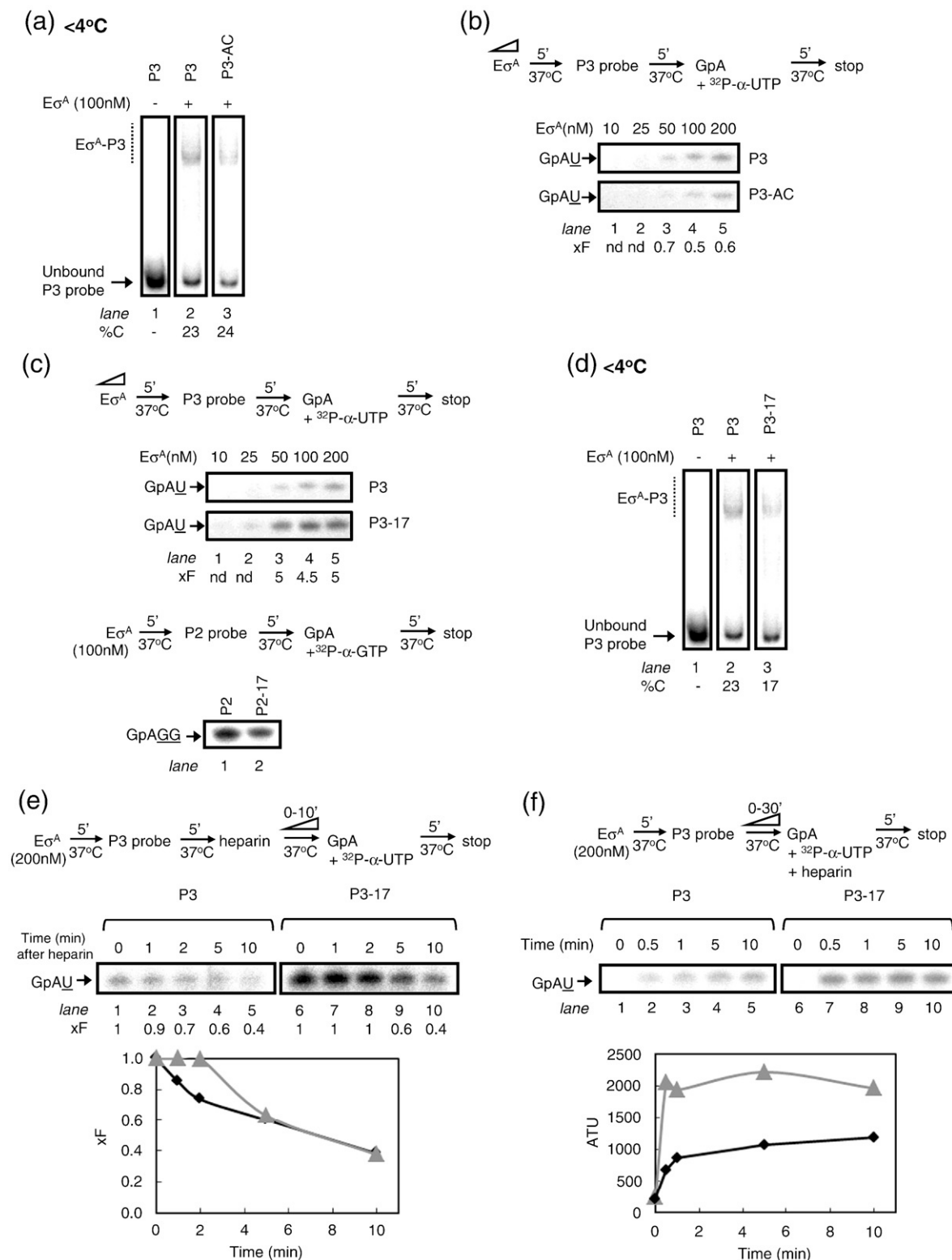


Fig. 4 (legend on next page)

amount of GpAU synthesised from the P3 and P3-AC promoter probes as a function of  $E\sigma^A$  concentration. As shown in Fig. 4b, the activity of the P3-AC promoter is reduced by  $\sim 2$ -fold when compared to the wild-type P3 promoter at all the  $E\sigma^A$  concentrations tested. We conclude that the extended  $-10$ -like element at the P3 promoter is required for optimal promoter activity.

### Shortening spacer length at the P3 promoter facilitates the isomerisation of the initial $E\sigma^A$ -P3 complexes to RPo

The optimal spacing of 17 nucleotides between the consensus  $-10$  promoter element and the consensus  $-35$  promoter element ensures that RNAP can productively and efficiently use a promoter. At the agr operon promoters, AgrA is thought to compensate for the suboptimal spacer length by distorting the DNA upon binding to specific sites located immediately upstream of the consensus  $-35$  element (Fig. 1b), thereby ensuring that the  $-35$  and  $-10$  consensus promoter elements are appropriately spaced for optimal activity.<sup>32</sup> In other words, AgrA could activate transcription from the P2 and P3 promoters by facilitating RPc and RPo formation by reducing the spacer length, thereby enabling RNAP to better "serve" the promoters. In support of this view, Morfeldt *et al.* reported that reducing the P3 spacer length by 3 nucleotides significantly increases the activity of the P3 promoter *in vivo*

and renders the mutant promoter insensitive to activation by AgrA *in vivo*.<sup>9</sup> To investigate the molecular basis by which P3 promoter activity is increased upon shortening of the spacer length, we used the short RNA synthesis assay to determine whether variants of the P2 and P3 promoters (hereafter called P2-17 and P3-17) with an optimal spacer length of 17 nucleotides are more active than the respective wild-type versions. Initially, we measured the amount of GpAU synthesised from the P3 and P3-17 promoter probes as a function of  $E\sigma^A$  concentration. Results shown in Fig. 4c unambiguously reveal that the P3-17 promoter activity is  $\sim 5$ -fold higher than P3 activity at all the  $E\sigma^A$  concentrations measured. Reducing the P2 promoter spacer length to 17 nucleotides did not lead to any detectable increase in the amount of GpAGG synthesised (Fig. 4c). We conducted native gel shift assays at  $<4^\circ\text{C}$  using  $^{32}\text{P}$ -labelled P3 and P3-17 promoter probes to test whether increased promoter activity is a consequence of increased promoter occupancy (therefore, increased RPc formation) by  $E\sigma^A$ . Results shown in Fig. 4d indicate that, despite marked differences in promoter activity,  $E\sigma^A$  binds to the P3 and P3-17 promoter probes equally well under our experimental conditions. We next extended the short RNA synthesis assay to determine if (i) the RPo formed at the P3-17 promoter is more stable than the RPo formed at the wild-type promoter, and/or (ii) RPo accumulates faster on the P3-17 promoter than on the wild-type promoter. For

**Fig. 4.** The binding of  $E\sigma^A$  to mutant variants of the P2 and P3 promoter probes. (a) An autoradiograph of a 4.5% (wt/vol) native polyacrylamide gel showing the binding of  $E\sigma^A$  to the P3 (lane 2) and P3-AC (lane 3) promoter probes. The experiment was conducted at  $<4^\circ\text{C}$ . The migration positions of unbound P3 promoter probes and  $E\sigma^A$ -P3 complexes are indicated. The amount of promoter DNA bound by  $E\sigma^A$  is expressed as a percentage (% C) of the total DNA present in the reaction. In at least two independent experiments, the data obtained fall within 10% of the % C value shown. (b) An autoradiograph of a 20% (wt/vol) urea-denaturing polyacrylamide gel showing synthesis of the short RNA product GpAU from the P3 and P3-AC promoters as a function of  $E\sigma^A$  concentration (indicated by the arrow, where the underlined nucleotides are  $^{32}\text{P}$  labelled). The experiment was conducted at  $37^\circ\text{C}$ , and the schematic representation of the reaction is shown at the top of the gel image (see Materials and Methods and text for details). The fold amount ( $x_F$ ) of the GpAU RNA product synthesised by  $E\sigma^A$  on P3-AC with respect to the GpAU RNA product synthesised from P3 is given at the bottom of the gel (nd = not determined). In at least five independent experiments, the data obtained fall within 10% of the  $x_F$  value shown. (c) As in (b), the top part of the figure, an autoradiograph of a 20% (wt/vol) urea-denaturing polyacrylamide gel, shows the synthesis of the short RNA product GpAU from the P3 and P3-17 promoters as a function of  $E\sigma^A$  concentration. At the bottom, an autoradiograph of a 20% (wt/vol) denaturing polyacrylamide gel showing the synthesis of the short RNA product GpAGG from the P2 and P2-17 promoters is shown. (d) As in (a), an autoradiograph of a 4.5% native polyacrylamide gel showing the binding of  $E\sigma^A$  to the P3 (lane 2) and P3-17 (lane 3) promoter probes at  $<4^\circ\text{C}$ . (e) As in (b), an autoradiograph of a 20% (wt/vol) urea-denaturing polyacrylamide gel showing the stability of RPo formed at P3 and P3-17 as a function of time following challenge with heparin. Heparin was added to RPo for the times indicated, and the schematic representation of the reaction is shown at the top of the gel image (see Materials and Methods and text for details). The graph at the bottom of the gel image shows the amount of GpAU synthesised from the P3 (black diamonds) and P3-17 (grey triangles) promoters at the indicated time points after heparin challenge, expressed in terms of fold decrease ( $x_F$ ) from the  $t=0$  time point (which indicates the amount of GpAU synthesised from the RPo present in the reaction at the time that heparin was added). (f) As in (b), an autoradiograph of a 20% (wt/vol) urea-denaturing polyacrylamide gel showing the time-dependent accumulation of RPo at P3 and P3-17 measured in terms of the short RNA product GpAU synthesised from the P3 and P3-17 promoters. The schematic representation of the reaction is shown at the top of the gel image (see Materials and Methods and text for details). The graph at the bottom of the gel image shows the amount of GpAU accumulation with time from the P3 (black diamonds) and P3-17 (grey triangles) promoters, expressed in terms of arbitrary transcription units (ATU).

(i), we challenged the RPo on the P3 and P3-17 probes with heparin and measured the fraction of RPo surviving the heparin challenge as a function of time. Results shown in Fig. 4e suggest that the RPo

formed at P3-17 is only slightly more stable than the RPo formed at the wild-type P3 promoter. For (ii), we measured the time-dependent accumulation of the GpAU transcript synthesised from P3 and P3-17. As

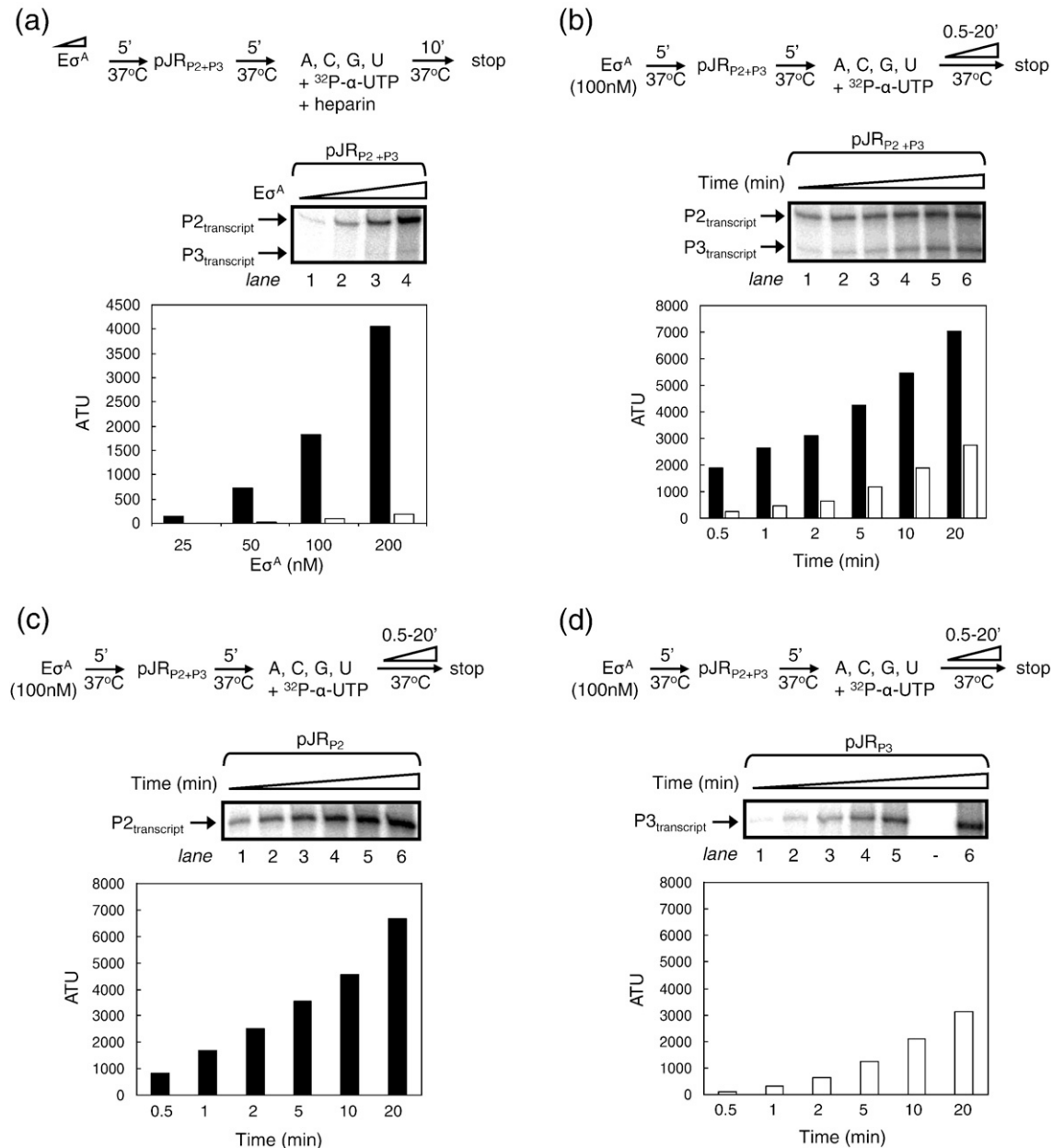


**Fig. 5.** *In vitro* transcription initiation from the P2 and P3 promoters in the absence of [6His]AgrA. (a) An autoradiograph of a 10% (wt/vol) urea-denaturing polyacrylamide gel showing transcripts synthesised from pJR<sub>P2+P3</sub> in the absence of [6His]AgrA under single-round transcription conditions (see schematic representation at the top of the gel image, as well as the text, for details) and as a function of Eσ<sup>A</sup> concentration. The amount of transcripts synthesised is expressed in terms of arbitrary transcription units (ATU). (b) As in (a), but the reactions were conducted under multiple-round transcription conditions (see schematic representation at the top of the gel image, as well as the text, for details). (c and d) As in (b), but the reactions were conducted using pJR<sub>P2</sub> and pJR<sub>P3</sub>, respectively. (e) Graph showing the stability of the AgrA-independent RPo formed at the P2 (filled circles) and P3 (empty circles) promoters in the context of pJR<sub>P2+P3</sub>, pJR<sub>P2</sub> or pJR<sub>P3</sub>. Autoradiographs of 10% (wt/vol) urea-denaturing polyacrylamide gels, from which the data in the graphs are derived, are shown at the top of each corresponding graph and labelled as in (a). For (a)–(e), an identical trend in the amount of transcripts synthesised from the P2 and P3 promoters was observed in at least three independent experiments.

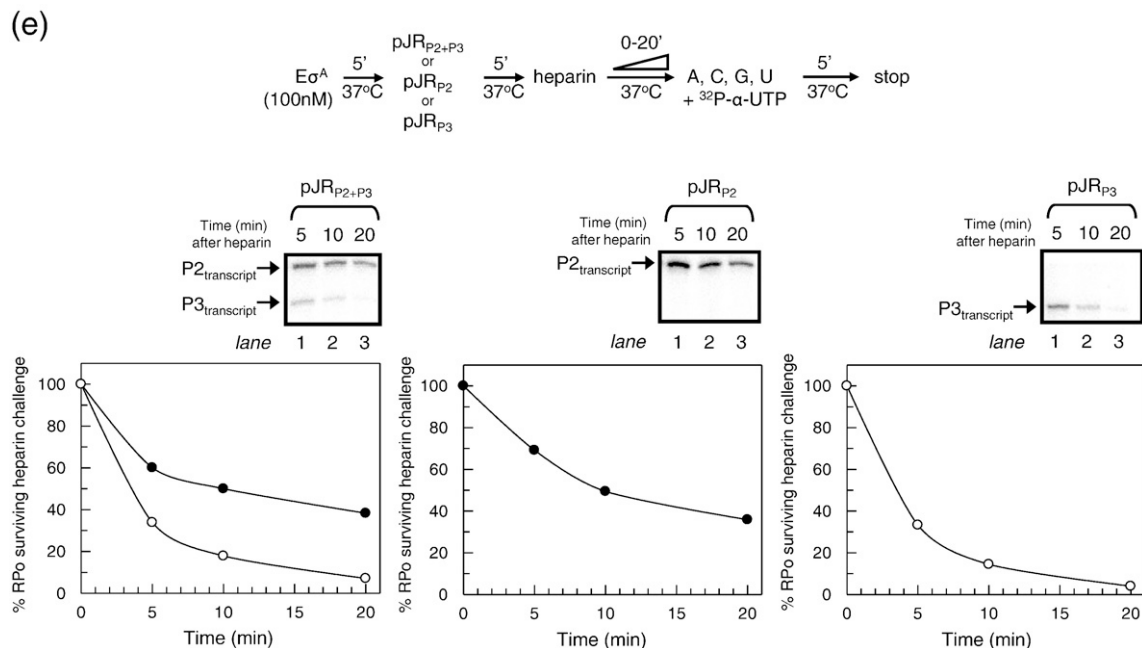


Fig. 5 (legend on previous page)

shown in Fig. 4f, the results suggest that the initial rate of RPo formation is significantly faster on the P3-17 promoter than on the wild-type P3 promoter even though the equilibrium for both promoters is reached at the same time. Overall, it seems that reducing the P3 spacer length to the optimal length of 17 nucleotides markedly improves the ability of the initial  $E\sigma^A$ -promoter complexes to isomerise to the RPo.

#### $E\sigma^A$ displays higher basal *in vitro* transcription activity from the P2 promoter than from the P3 promoter

Having established that *S. aureus* RNAP can form the RPo at both agr operon promoters in the absence of AgrA, we performed *in vitro* transcription assays using the promoter templates described in Fig. 2c to determine the basal P2 and P3 promoter activity when the promoters are available to  $E\sigma^A$  in their native back-to-back divergent context. Initially, reactions were conducted as a function of  $E\sigma^A$  concentration using pJR<sub>P2+P3</sub> as the promoter template and under conditions where only a single round of transcription can occur (see Materials and Methods). As shown in Fig. 5a, the amount of transcripts originating from the P2 promoter is consistently ~15-fold to 20-fold higher than the amount of transcripts originating from the P3 promoter under these conditions. Similar results were obtained when the assays were conducted using pJR<sub>P2</sub> or pJR<sub>P3</sub> as the promoter template (data not shown). Since  $E\sigma^A$  can interact with P2 and P3

equally well to form the RPo, the *in vitro* single-round transcription results seem to indicate that RPo formation at the P3 promoter may occur more slowly than at the P2 promoter in the absence of AgrA. Alternatively, the RPo formed on the P3 promoter is unstable and rapidly dissociates upon exposure to heparin under the single-round transcription conditions (see Materials and Methods and schematic representation in Fig. 5a). To experimentally address these possibilities and to better reflect the conditions found *in vivo*, we repeated the *in vitro* transcription assays in the absence of the competitor heparin, a condition that allows multiple rounds of transcription to occur. In these assays, the amount of transcripts originating from the P2 and P3 promoters is measured as a function of time at a fixed  $E\sigma^A$  concentration at which a good occupancy of both P2 and P3 promoters by  $E\sigma^A$  is observed (see Materials and Methods and schematic representation in Figs. 5b and 3b). As shown in Fig. 5b-d, under these conditions, the time-dependent accumulation of transcripts originating from the P3 promoter is detected at a much slower rate than from the P2 promoter. Next, we measured the stability of the RPo that evidently forms at both promoters in the absence of AgrA. To do this, we allowed the RPo to accumulate at the P2 and P3 promoters for 5 min and exposed the reaction to heparin for various lengths of time ( $t=0$  min, 5 min, 10 min and 20 min, where  $t=0$  indicates the amount of RPo present before the addition of heparin to the reaction). As shown in Fig. 5e, the RPo formed at P3 is markedly less stable than the RPo formed at the P2

promoter in the absence of AgrA in the context of pJR<sub>P2+P3</sub>, pJR<sub>P2</sub> and pJR<sub>P3</sub>. Overall, the results from the *in vitro* transcription assays are consistent with the short RNA synthesis assays and demonstrate that E $\sigma^A$  can form the RPo, albeit with different efficiencies, at both agr operon promoters in the absence of AgrA. Notably, however, the data from both assays clearly show that E $\sigma^A$  has the potential to initiate transcription from the P3 promoter independently of AgrA. Yet, the higher basal transcription activity from the P2 promoter detected in the *in vitro* transcription assays indicates that RPo formation is more efficient at P2 than at P3 in the absence of AgrA. It also seems that the RPo formed at the P2 promoter is more stable than the RPo formed at the P3 promoter in the absence of AgrA. It also appears that—judging from the results with pJR<sub>P2</sub> and pJR<sub>P3</sub>—RPo formation at the P2 promoter does not detectably influence the ability of E $\sigma^A$  to form the RPo at the P3 promoter (see Discussion).

### The effect of AgrA on transcription initiation from the P2 and P3 promoters

We next examined the effect of AgrA on transcription initiation from the P2 and P3 promoters. AgrA has been demonstrated to bind to specific sites located upstream of the P2 and P3 consensus promoter regions with low affinity in the unphosphorylated form.<sup>6</sup> *In vitro* phosphorylation of AgrA, which can be achieved using small phosphodonors such as acetyl phosphate, evidently increases the affinity of AgrA for the specific sites located upstream of the P2 and P3 consensus promoter regions (Fig. 1b).<sup>6</sup> Therefore, we used acetyl phosphate to phosphorylate [6His]AgrA *in vitro* and conducted short RNA synthesis assays using P2 and P3 probes as promoter templates to determine the effect of AgrA~P on transcription initiation. In these assays, AgrA was always pre-incubated with acetyl phosphate (for 30 min at room temperature) prior to addition of E $\sigma^A$  to the reaction. As shown in Supplementary Fig. 1, surprisingly, [6His]AgrA~P seems to antagonise efficient transcription initiation from the P2 and P3 probes. Control reactions established that the presence of acetyl phosphate alone is sufficient to inhibit E $\sigma^A$  activity under our experimental conditions (Supplementary Fig. 1), and similar results were obtained when we replaced *S. aureus* E $\sigma^A$  with *E. coli* E $\sigma^{70}$  (data not shown); this observation was not investigated further. Since there is a growing body of evidence that the unphosphorylated state of a two-component response regulator exists in dynamic equilibrium between unphosphorylated (i.e., inactive) and phosphorylated-like (i.e., active) conformations,<sup>33,34</sup> we considered whether unphosphorylated [6His]AgrA at high concentrations is sufficient to affect transcription initiation from the agr operon promoters P2 and P3. Indeed, native gel shift assays

using <sup>32</sup>P-labelled P2 and P3 probes with high concentrations of unphosphorylated [6His]AgrA revealed a pattern of migration that is identical with the previously published pattern of migration of DNA bound to phosphorylated AgrA.<sup>6,19</sup> The two bands (C1 and C2) detected for unphosphorylated [6His]AgrA binding to the P2 and P3 probes are identical with those previously observed by Koenig *et al.*<sup>6</sup> and Queck *et al.*<sup>19</sup> and thus most likely represent two conformers of unphosphorylated [6His]AgrA in the “active conformation”. Furthermore, *in vitro* phosphorylation of [6His]AgrA with acetyl phosphate only slightly increased the affinity of [6His]AgrA for the <sup>32</sup>P-labelled P2 and P3 probes under our experimental conditions (data not shown). Next, we conducted multiple-round *in vitro* transcription assays in the presence of 1  $\mu$ M and 2  $\mu$ M AgrA under conditions that support maximum basal multiple-round transcription activity from the P2 and P3 promoters (as in Fig. 5b). Judging from the amount of P2 and P3 probes shifted by unphosphorylated [6His]AgrA (Fig. 6a), we estimate the “effective” concentration of [6His]AgrA in the “active conformation” in the transcription reactions to be approximately 10–20 nM. As shown in Fig. 6b and c, we detect a ~9-fold to 14-fold increase in transcripts synthesised from the P3 promoter in the context of pJR<sub>P2+P3</sub> and pJR<sub>P3</sub>. Interestingly, under these conditions, the increase in transcription initiation from the P2 promoter is marginal (by ~1.5-fold to 2-fold) when compared to the basal transcription activity from the P2 promoter. Control reactions with a DNA binding mutant form of [6His]AgrA ([6His]AgrA<sub>H169A</sub>)<sup>32</sup> indicate that the apparent increase in P3 promoter activity is specific for the presence of [6His]AgrA (Fig. 6b, lane 4). Overall, the results unambiguously demonstrate that recombinant [6His]AgrA, albeit in the unphosphorylated form, can activate transcription from the P3 promoter but has little detectable effect on the P2 promoter under these assay conditions (but see later text). Even though the effect of unphosphorylated [6His]AgrA is unambiguous and specific, we would like to emphasise that the use of unphosphorylated [6His]AgrA may not fully reflect the conditions encountered *in vivo*, and this fact should be taken into consideration when interpreting the results. Nevertheless, the ability of unphosphorylated AgrA to activate transcription represents a valuable technical “tool” for gauging the mechanism by which AgrA could activate transcription from the P2 and P3 promoters of the agr operon.

### Occupancy of the agr operon promoters P2 and P3 by E $\sigma^A$ is increased in the presence of AgrA

We conducted experiments to determine the mechanism by which [6His]AgrA activates transcription. The proximity of the AgrA binding sites in

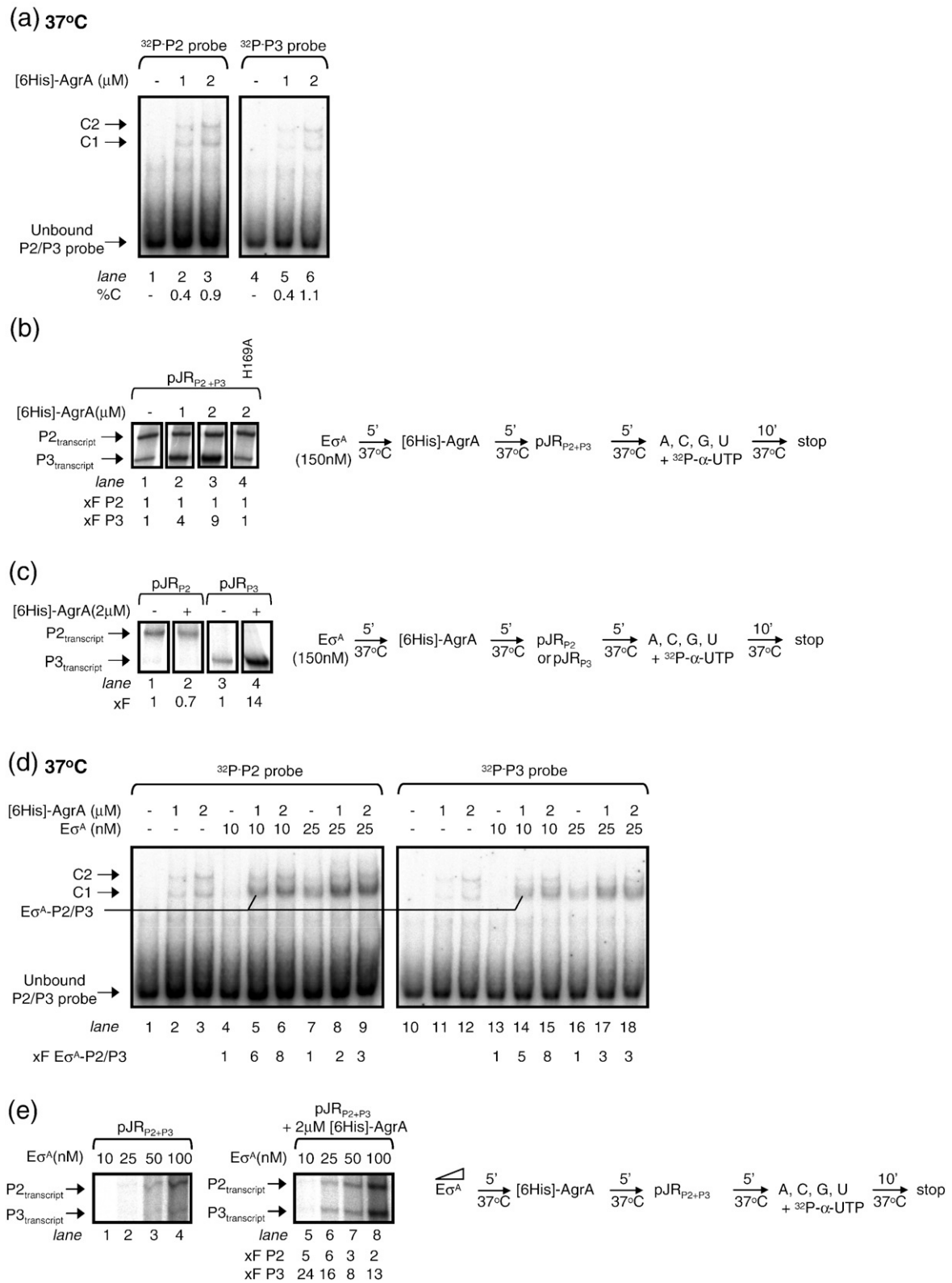


Fig. 6 (legend on next page)

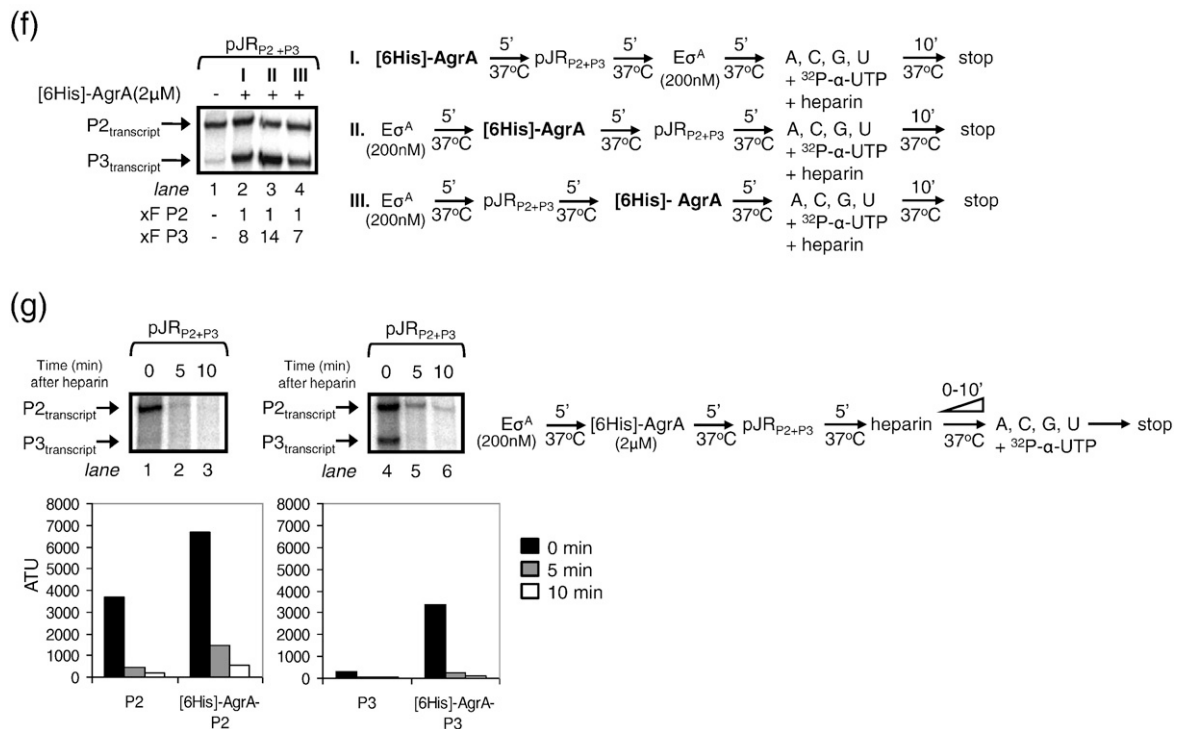


**Fig. 6.** The effect of [6His]AgrA on transcription initiation from the P2 and P3 promoters. (a) An autoradiograph of a 4.5% (wt/vol) native polyacrylamide gel showing the binding of [6His]AgrA to <sup>32</sup>P-labelled P2 and P3 promoter probes. The migration positions, C1 and C2, of the [6His]AgrA–P2/[6His]AgrA–P3 complexes are shown (see the text for details). The amount of P2 and P3 promoter probes in complex with [6His]AgrA–P2/[6His]AgrA–P3 is expressed as a percentage (% C) of the total DNA present in the reaction. In at least three independent experiments, the data obtained fall within 10% of the % C value shown. (b) An autoradiograph of a 10% (wt/vol) urea-denaturing polyacrylamide gel showing transcripts synthesised from pJR<sub>P2+P3</sub> in the presence of [6His]AgrA under multiple-round transcription conditions (see schematic representation on the right of the gel image, as well as the text, for details) in the presence of 1  $\mu$ M and 2  $\mu$ M [6His]AgrA and at a 2  $\mu$ M concentration of the [6His]AgrA H169A DNA binding mutant variant of AgrA. The fold increase in transcripts synthesised from the P2 and P3 promoters (xF P2 and xF P3, respectively) in the presence of [6His]AgrA, with respect to the reaction where no [6His]AgrA (lane 1) is added, is shown at the bottom of the gel image. (c) As in (b), but the assay was conducted using pJR<sub>P2</sub> and pJR<sub>P3</sub>. (d) An autoradiograph of a 4.5% (wt/vol) native polyacrylamide gel showing the binding of E $\sigma^A$ , in the presence and in the absence of [6His]AgrA, to the P2 and P3 promoter probes. The reaction components present in each lane are indicated, and the migration positions of the [6His]AgrA–P2/[6His]AgrA–P3 and E $\sigma^A$ –P2/E $\sigma^A$ –P3 complexes are shown (see the text for details). (e) As in (b), but the assay was conducted as a function of E $\sigma^A$  concentration. The fold increase in transcripts synthesised from the P2 (xF P2) and P3 (xF P3) promoters in the presence of [6His]AgrA, with respect to the corresponding reactions where no [6His]AgrA (lanes 1–4), is added is shown at the bottom of the gel image. (f) As in (b), but the assay was conducted under single-round transcription conditions, and [6His]AgrA was added (conditions I–III; see the text for details) to the reaction at different stages, as indicated in the schematic representation shown on the right of the gel image. (g) As in (b), but heparin was added to the transcription-competent promoter complexes for the times indicated at the top of the gel image (see schematic representation on the right of the gel image, as well as the text, for details). The transcripts synthesised from the P2 and P3 promoters at the indicated time points in the absence (lanes 1–3) and in the presence (lanes 4–6) of [6His]AgrA are shown at the bottom of the gel image and are expressed in terms of arbitrary transcription units (ATU). In at least two independent experiments, an identical trend in the amounts of transcripts synthesised from the P2 and P3 promoters was observed for this experiment. For (b)–(f), in at least three independent experiments, the data obtained for the xF P2 and/or xF P3 values fell within 10% of the values shown.

agr IR to the –35 consensus promoter regions of the P2 and P3 promoters (Fig. 1b) suggests that the effect of [6His]AgrA on transcription initiation from P2 and P3 could involve the recruitment of E $\sigma^A$  (to the P2 and P3 promoters). Therefore, we conducted native gel shift experiments with <sup>32</sup>P-labelled versions of the P2 and P3 probes to determine if

[6His]AgrA improves the ability of E $\sigma^A$  to occupy either of the P2 and P3 promoters. As shown in Fig. 6d, the E $\sigma^A$ –P2 and E $\sigma^A$ –P3 promoter complexes migrate at a slightly higher position than the faster-migrating [6His]AgrA–P2/[6His]AgrA–P3 complex, C1, on the native gel (Fig. 6d, lanes 4, 7, 13 and 16). However, when [6His]AgrA and E $\sigma^A$  are

preincubated and added to the P3 probe, as expected and consistent with the *in vitro* transcription assays (Fig. 6b and c), a significant increase (by ~5-fold to 8-fold at 10 nM  $E\sigma^A$ ) in the occupancy of the P3 promoter by  $E\sigma^A$  is detected (Fig. 6d, lanes 13–15). Surprisingly, and in contrast with the results from the *in vitro* transcription assays (Fig. 6b and c), [6His]AgrA also appears to increase (by ~6-fold to 8-fold) the occupancy of the P2 promoter by  $E\sigma^A$  (Fig. 6d, lanes 4–6). The increased binding of  $E\sigma^A$  to the P2 and P3 probes in the presence of [6His]AgrA is particularly evident at lower  $E\sigma^A$  concentrations, where  $E\sigma^A$ -P2 and  $E\sigma^A$ -P3 promoter complexes are barely detected (Fig. 6d, compare lanes 4–6 and 13–15 with lanes 7–9 and 16–18).

Based on the results obtained so far, we considered that an effect of [6His]AgrA on transcription initiation from the P2 promoter could be better detected at lower concentrations of  $E\sigma^A$  where P2 promoter occupancy (by  $E\sigma^A$ ) is limiting. Therefore, multiple-round *in vitro* transcription assays were conducted in the presence of [6His]AgrA as a function of  $E\sigma^A$  concentration. As shown in Fig. 6e, at low concentrations (25 nM) of  $E\sigma^A$ , a moderate (~6-fold increase over basal transcription activity) increase in the number of transcripts originating from the P2 promoter is detected. As expected, a relatively more significant increase (~16-fold over basal transcription activity) in the amount of transcription initiated from the P3 promoter is detected in the presence of [6His]AgrA at the lower  $E\sigma^A$  concentration. Overall, we conclude that [6His]AgrA improves the occupancy of P2 and P3 promoters by  $E\sigma^A$  and consequently increases the amount of transcripts synthesised from each promoter.

#### AgrA does not have any profound effect on the stability of the RPo formed at the P2 or P3 promoters

Since the effect of [6His]AgrA is more pronounced at the P3 promoter, we next wanted to determine which step during transcription initiation at the P3 promoter is affected by AgrA. To do so, we conducted single-round *in vitro* transcription assays in which [6His]AgrA was added to the assay at various stages of the reaction. Since transcription initiation from the P3 promoter under single-round transcription conditions is barely detectable, the assays were conducted using 200 nM  $E\sigma^A$  in order to better quantify the difference in the amount of transcripts synthesised (from the P3 promoter) in the absence and in the presence of [6His]AgrA. Under these conditions, some basal transcription activity from the P3 promoter is detected (Fig. 6f, lane 1). Results reveal that the presence of [6His]AgrA has an overall positive effect on transcription initiation at the P3 promoter, regardless of the order of addition (Fig. 6f). The effect of [6His]AgrA seems highest

under condition II, when [6His]AgrA is preincubated with  $E\sigma^A$  prior to the addition of pJR<sub>P2+P3</sub> (Fig. 6f, lane 3). However, we do not exclude the possibility that, under condition I (when [6His]AgrA is preincubated with pJR<sub>P2+P3</sub> prior to the addition of  $E\sigma^A$ ; lane 2) and condition III (when [6His]AgrA is added to the  $E\sigma^A$ -promoter complexes; lane 4), the dissociation of [6His]AgrA-promoter and  $E\sigma^A$ -promoter complexes, respectively, could revert to a scenario that eventually reflects condition II. In contrast, and as expected, there does not appear to be an order of addition effect of [6His]AgrA on transcription from the P2 promoter under the assay conditions used (Fig. 6f).

Next, we wanted to determine if the effect of [6His]AgrA is restricted to events leading to RPo formation or if [6His]AgrA influences the stability of the RPo formed at the P2 and P3 promoters. For a direct assessment of the effect of [6His]AgrA on the stability of RPo, promoter complexes, preformed on either P2 or P3 (on pJR<sub>P2+P3</sub>), were exposed to heparin for various amounts of time prior to the addition of ribonucleoside triphosphates to the reaction to allow RNA synthesis to occur (see schematic representation in Fig. 6g). Reactions were conducted in the absence and in the presence of [6His]AgrA, which, when present, was always preincubated with  $E\sigma^A$  prior to the addition of pJR<sub>P2+P3</sub> to the reaction (as under condition II of Fig. 6f). In Fig. 6g,  $t=0$  min reflects the amount of heparin-resistant transcription-competent promoter complexes formed at the P2 and P3 promoters at the time of heparin addition. In the absence of [6His]AgrA, just a 5-min exposure of the RPo at P2 to heparin results in a marked decrease in the amount of transcripts synthesised (Fig. 6g, compare lanes 1 and 2). Consistent with earlier results (Fig. 6e), under these conditions, transcripts initiated from the P3 promoter are barely detected and are only seen in the presence of [6His]AgrA (Fig. 6g, compare lanes 1 and 4). When the heparin challenge reactions were conducted in the presence of [6His]AgrA, a ~2-fold increase in transcripts originating from the P2 promoter (over basal transcription activity) is detected at  $t=0$  min. However, the presence of [6His]AgrA appears to have no profound effect on the stability of the RPo formed at the P2 and P3 promoters (Fig. 6g, compare lanes 2 and 3 with lanes 5 and 6). Overall, it seems that the effect of [6His]AgrA on transcription initiation from the P2 and P3 promoters is restricted to steps leading to RPo formation. Once the RPo has formed, [6His]AgrA appears to have no detectable effect on the longevity of these complexes under the assay conditions used here.

## Discussion

In this study, we have developed a fully native *S. aureus in vitro* transcription system to investigate

how transcription initiation is regulated at the agr operon promoters P2 and P3 at the molecular level. Despite the atypical spacer lengths of the agr operon promoters, especially the P3 promoter, the results from the *in vitro* analyses unambiguously indicate that  $\text{E}\sigma^A$  can form transcription-competent promoter complexes at both agr operon promoters in the absence of AgrA, the master transcription activator of the system. Therefore, it seems that  $\text{E}\sigma^A$  can productively interact with the P2 and P3 promoters in an AgrA-independent manner despite the atypical spacer lengths. However, the efficiency with which  $\text{E}\sigma^A$  initiates transcription from the P2 and P3 promoters varies in the absence of AgrA: The number of RPo formed at the P2 promoter is significantly higher than the number of RPo formed at the P3 promoter, which seems to accumulate at a much slower rate, and the RPo formed at P3 is significantly less stable than the RPo formed at the P2 promoter. The higher basal transcription activity at the P2 promoter is not surprising, since this promoter better matches the consensus than the P3 promoter. The agr operon promoters display a back-to-back arrangement. At divergent promoters with a back-to-back arrangement, activity in one promoter may (and often does) result in either a positive or a negative effect on the activity of the other promoter,<sup>35,36</sup> and *vice versa*. For instance, the binding of RNAP to one promoter may change the DNA topology so that it antagonises or facilitates the binding of RNAP to the other promoter. Judging from the rate and amount of P2 and P3 transcripts synthesised from pJR<sub>P2+P3</sub>, pJR<sub>P2</sub> and pJR<sub>P3</sub> (Fig. 5b–d), it appears that activity at the P2 promoter does not detectably influence activity at the P3 promoter. Therefore, it is formally possible that divergent transcription initiation can occur simultaneously from both agr operon promoters.

Clearly, the regulatory events that govern transcription initiation from the agr operon P2 and P3 promoters are very complex. Even though transcription regulators of the SarA protein family have a role in regulating P2 and P3 promoter activity,<sup>2,3</sup> the basal transcription activity of P2 and P3 promoters, as demonstrated in this study, is likely to be of regulatory significance for *S. aureus* and may help in understanding the molecular basis of results obtained from many *in vivo* studies. The basal transcription activity of P2 and P3 provides experimental support for the idea proposed by Novick *et al.* that the higher basal transcription activity at the P2 promoter may help ensure that the RNAII-encoded quorum-sensing system is always readily available to enable the *S. aureus* cells to rapidly mount a change in virulence gene expression as the cell density increases at the site of infection. Similarly, the higher basal transcription activity at the P2 promoter could also ensure that AgrA is available for the RNAIII-independent regulation of

(virulence) gene expression. Such genes, for example, include the leucocidal phenol-soluble modulins *psmA* and *psmB* that are regulated by AgrA directly binding to the promoter region of these genes.<sup>19</sup> In addition, the existence of other genes in *S. aureus* that directly depend on AgrA for activation cannot be excluded. Consistent with the latter view, genome-wide expression analyses have revealed the existence of several metabolic genes in *S. aureus* for which AgrA (but not RNAIII) appears to be indirectly required.<sup>19</sup> Furthermore, the apparent basal transcription activity at the P3 promoter observed *in vitro* may also provide a molecular explanation for the RNAII-independent (i.e., in cells in which *agrA* is inactivated) expression of RNAIII in *S. aureus* cells in the context of a rabbit endocarditis infection model.<sup>12</sup> Overall, the results presented here provide the first direct experimental evidence for the basal transcription activity of P2 and P3 promoters. We suggest that the higher basal transcription activity at the P2 promoter is of regulatory significance and potentially could represent an alternative RNAIII-independent route to couple virulence gene expression to the environmental and metabolic status of the cell at specific infection scenarios.

The presence of AgrA, despite being in the unphosphorylated form, has an overall positive and specific effect on transcription initiation from the P2 and P3 promoters. The effect of AgrA is more prominent at the P3 promoter than at the P2 promoter. However, as mentioned above, we do not exclude the possibility that AgrA in its phosphorylated form has a more pronounced effect on transcription initiation from the P2 (and P3) promoter. Nevertheless, the results obtained with the unphosphorylated form of AgrA provide useful insights into the mechanism by which AgrA might activate transcription from the P2 and P3 promoters: In the agr IR, the binding sites for AgrA at the P2 and P3 promoters overlap the –35 consensus promoter region;<sup>6</sup> therefore, the agr operon promoters resemble a class-II-like promoter.<sup>37</sup> In class II promoters, the transcription activator not only facilitates the binding of RNAP to the promoter to increase RPo formation (by increasing the binding constant of the interaction between the RNAP and the promoter) through a classical recruitment mechanism but also facilitates, in some cases, the conversion of the RPo into the RPo (by increasing the rate constant for the isomerisation of the RPo to the RPo).<sup>37</sup> Our results indicate that AgrA increases promoter occupancy by RNAP and, as a consequence, facilitates RPo formation at both promoters of the agr operon. Therefore, it seems that AgrA increases the binding constant of the interaction between RNAP and the P2 and P3 promoters. However, since RNAP can bind both promoters equally well in the absence of AgrA, despite basal transcription initiation occurring at a much slower rate from the P3 promoter (compared

to the P2 promoter), we suggest that AgrA could also increase the rate constant for the isomerisation of the P3-dependent RPc to the RPo. The AgrA-induced distortion of the DNA upon binding, as observed in the crystal structure of AgrA bound to a 16-bp DNA fragment, may indeed support a view in which AgrA contributes to the conversion of the RPc into the RPo by facilitating DNA strand separation and/or thereby by compensating for the suboptimal spacer length of the P3 promoter.<sup>32</sup> Consistent with the latter view, AgrA is unable to detectably increase the amount of transcripts synthesised from the P3 promoter with the optimal spacer length of 17 nucleotides (Supplementary Fig. 2); thus, a role for AgrA in compensating for the atypical spacer length, especially at the P3 promoter, is indeed plausible. Furthermore, the results presented in Fig. 4e and f indeed imply that the activity of the P3 promoter with the optimal spacer length (P3-17) is increased as a consequence of the increased isomerisation of the initial  $E\sigma^A$ -promoter complexes to the RPo. Thus, we do not exclude the possibility that AgrA makes different sets of contacts with RNAP when activating transcription from the P2 and P3 promoters, similarly to that observed for the *E. coli* catabolite activator protein<sup>38</sup> in class II promoters.<sup>39</sup> However, the effect(s) of AgrA on transcription initiation at the P2 and P3 promoters appears to be restricted to events leading to the formation of the RPo. However, we cannot exclude the possibility that the [6His]AgrA-dependent RPo formed at the P3 promoter is so unstable that any effect of [6His]AgrA would be obscured. Intriguingly, the order of addition experiments with AgrA indicates that the highest increase in AgrA-activated transcription initiation from P3 occurs when AgrA and  $E\sigma^A$  are preincubated. This observation, although as yet experimentally unsupported, hints at the possibility that AgrA could prerecruit RNAP, and activation of the *agr* operon promoters by AgrA could involve a pre-recruitment-like mechanism.<sup>40</sup>

In summary, the role of the *agr* operon in *S. aureus* biology appears not to be limited to the expression and regulation of the *S. aureus* virulon but also extends to controlling the expression of genes with several physiological functions.<sup>19</sup> AgrA, the master transcription activator of the *agr* operon, belongs to a family (LytTR family) of relatively poorly characterised transcription regulators that are mostly involved in the activation of virulence genes (examples include *Pseudomonas aeruginosa* AlgR, *Clostridium perfringens* VirR, *Streptococcus pneumoniae* BlpR and *Enterococcus faecalis* FsrA).<sup>8,41,42</sup> The results of this study have demonstrated, for the first time, that transcription initiation can occur from the *agr* operon promoters P2 and P3 in an AgrA-independent manner. The results also provide the first molecular insights into how AgrA might control transcription initiation at the *agr* operon in *S. aureus*. Additional

experiments in our laboratory are currently in progress to further dissect the mechanism by which (phosphorylated) AgrA activates transcription at the *agr* operon and the role of the SarA family of transcription regulators in this process. Importantly, the results of the current study further highlight the *agr* operon and AgrA as sites where intervention could be clinically attractive.

## Materials and Methods

### Plasmids, proteins and promoter probes

The genes encoding AgrA (*agrA*) and  $\sigma^A$  (*rpoD*) were PCR amplified (using primers JRagrA-F and JRagrA-R for *agrA*, and primers JRpoD-F and JRpoD-R for *rpoD*; Supplementary Table 1) from *S. aureus* strain NCTC8325 and cloned into the *E. coli* overexpression plasmid pET28b<sup>+</sup> (Merck Biosciences) to produce pJR28-[6His]agrA and pJR28-[6His]rpoD, allowing both proteins to be overexpressed as amino-terminal hexa-histidine-tagged fusion proteins in *E. coli* strain BL21(DE3). For protein overexpression, *E. coli* strain BL21(DE3) containing pJR28-[6His]rpoD was grown at 37 °C. At OD<sub>600</sub> (optical density at 600 nm) ~0.4, the cells were temperature shifted to 25 °C for 30 min, and the expression of [6His] $\sigma^A$  was induced with 1 mM IPTG. The cells were harvested after 3 h at 25 °C. *E. coli* strain BL21(DE3) containing pJR28-[6His]agrA was grown at 37 °C without induction, and the cells were harvested at OD<sub>600</sub> ~1.2. [6His]AgrA and [6His] $\sigma^A$  were purified to >95% homogeneity by a single-step nickel-affinity chromatography on an AKTA FPLC system according to the manufacturer's instructions. Briefly, for single-step nickel affinity chromatography, cells were lysed in a buffer containing 25 mM sodium phosphate (pH 8), 0.5 M NaCl and 5% (vol/vol) glycerol (buffer Ni), and centrifuged to remove cellular debris. The supernatant was then loaded onto a nickel-affinity chromatography column (5-ml Hi-Trap chelating HP column; GE Biosciences). The column was washed with 5 vol of buffer Ni+30 mM imidazole, and the bound proteins eluted with a 30-ml linear gradient of 30–300 mM imidazole in buffer Ni. *S. aureus* core RNAP was purified from exponentially growing *S. aureus* strain NCTC8325 cells (harvested at OD<sub>600</sub> ~0.5) by immunoaffinity chromatography on a homemade column packed with 2 ml of resin containing the polyol-responsive monoclonal antibody 8BR13 (Neoclone).<sup>43</sup> The procedure used for immunoaffinity chromatography with 8BR13 followed the protocol described by Thompson and Burgess.<sup>44</sup> Briefly, the cell pellet was resuspended in lysis buffer [10 mM Tris-Cl (pH 7.5), 15 mM MgCl<sub>2</sub>, 50 mM KCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 10 mM 2-mercaptoethanol and 1 tablet of protease inhibitors (Roche) per 100 ml of lysis buffer] and mechanically lysed using a French Press system. The cell lysate was centrifuged, and the nucleic acids and proteins in the supernatant were precipitated by adding polyethyleneimine (PEI) (Sigma) to a final concentration of 0.6% (vol/vol) PEI. Core RNAP was eluted from the PEI pellet with 1 M NaCl and precipitated with 35 g of ammonium sulfate per 100 ml of solution. The ammonium sulfate pellet was dissolved in

TE buffer [50 mM Tris-Cl (pH 7.9) and 0.1 mM EDTA] and loaded onto a 2-ml 8RB13 immunoaffinity column equilibrated with TE buffer. After a wash with TE buffer + 0.15 M NaCl and 0.5 M NaCl, the core RNAP was slowly eluted with elution buffer containing 40% (vol/vol) 1,2-propanediol and 0.7 M ammonium sulfate. All proteins were stored in storage buffer [10 mM Tris-Cl (pH 8), 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT and 50% (vol/vol) glycerol] in aliquots either at  $-20^{\circ}\text{C}$  (short-term storage) or at  $-80^{\circ}\text{C}$  (long-term storage). Plasmids pJR<sub>P2+P3</sub>, pJR<sub>P2</sub> and pJR<sub>P3</sub> used as templates in the *in vitro* transcription assays (see the text) were generated by PCR amplifying 267-bp (representing the whole agr IR using primers pJR<sub>P2+P3</sub>-F and pJR<sub>P2+P3</sub>-R; see Fig. 1b and Supplementary Table 1), 225-bp (representing positions  $-163$  to  $+54$  of the P2 promoter region with respect to the transcription start site at  $+1$  using primers pJR<sub>P2</sub>-F and pJR<sub>P2+P3</sub>-R; see Fig. 1b and Supplementary Table 1) and 205-bp (representing positions  $-178$  to  $+25$  of the P3 promoter region using primers pJR<sub>P2+P3</sub>-F and pJR<sub>P3</sub>-R; see Fig. 1b and Supplementary Table 1) DNA fragments from *S. aureus* strain NCTC8325 incorporating EcoRI and HindIII restriction sites and cloning into plasmid pLSR<sup>25</sup> (see the text for details). The short promoter probes (P2 and P3 probes; Fig. 1b) used in the native gel shift assays were prepared using commercially synthesised (Invitrogen) oligonucleotides, as previously described.<sup>45</sup> The long promoter probes (P2long and P3long) were generated by PCR amplifying 240-bp (representing positions  $-163$  to  $+66$  of the P3 promoter region using primers P3long-F and pJR<sub>P3</sub>-R; Supplementary Table 1) and 261-bp (representing positions  $-173$  to  $+87$  of the P2 promoter region using primers pJR<sub>P2</sub>-F and P2long-R; Supplementary Table 1) DNA fragments using pJR<sub>P2+P3</sub> as template.

### Native gel shift assays

Ten-microlitre binding reactions were set up in reaction buffer [40 mM Tris-acetate (pH 7.9), 100 mM NaCl, 20 mM MgCl<sub>2</sub> and 0.2 mM DTT] using E $\sigma^A$  and/or [6His]AgrA at the concentrations indicated in the figures and in the text (E $\sigma^A$  was reconstituted *in situ* by incubating a  $\sim 4$ -fold molar excess of  $\sigma^A$  with *S. aureus* core RNAP in reaction buffer for 5 min at  $37^{\circ}\text{C}$ ). The <sup>32</sup>P-labelled promoter DNA was present at a final concentration of 20 nM. Where indicated, heparin was present at a final concentration of 100  $\mu\text{g}/\text{ml}$ . The reactions were incubated either at  $<4^{\circ}\text{C}$  (on ice) or at  $37^{\circ}\text{C}$  (see the text for details), stopped with native gel loading dye [reaction buffer + 50% (vol/vol) glycerol and 0.05% (wt/vol) bromophenol blue] and resolved on a 4.5% (wt/vol) native polyacrylamide gel. For the reactions incubated at  $<4^{\circ}\text{C}$ , the gel was prechilled, and electrophoresis was performed in an iced water bath; for the reactions incubated at  $37^{\circ}\text{C}$ , the gel was prewarmed, and electrophoresis was performed in a water bath set to  $37^{\circ}\text{C}$ . The dried gel was visualised and quantified using a FLA-5000 PhosphorImager. The sequences of the P2 and P3 promoter probes used for the native gel shift assays are shown in Fig. 1b and Supplementary Table 1.

### KMnO<sub>4</sub> footprinting

The binding reactions for KMnO<sub>4</sub> footprinting were conducted as described above at  $37^{\circ}\text{C}$ . KMnO<sub>4</sub> (final concentration, 4 mM) was added to the reaction for

1 min before the reactions were stopped with 50 mM 2-mercaptoethanol (final concentration). The DNA was extracted through a phenol-chloroform extraction step and precipitated with 0.3 M sodium acetate (pH 5.2) and ice-cold 70% (vol/vol) ethanol for 2 h at  $-20^{\circ}\text{C}$ . The precipitated DNA was allowed to air dry, resuspended in distilled water and treated with 10% (vol/vol) piperidine for 30 min at  $90^{\circ}\text{C}$ . The reactions were rapidly cooled and precipitated with 0.3 M sodium acetate (pH 5.2) and 70% (vol/vol) ice-cold ethanol (as described above). The precipitated DNA was further washed twice with 70% (vol/vol) ice-cold ethanol and air dried. The recovery of DNA was determined by dry Cerenkov counting, and DNA was resuspended in an appropriate volume of distilled water. Equal numbers of counts were loaded onto a 10% (wt/vol) urea-denaturing polyacrylamide gel. The dried gel was visualised and quantified using a FLA-5000 PhosphorImager.

### Short RNA synthesis assays

The binding reactions for the short RNA synthesis assays were conducted as described above. The final concentrations of 0.5 mM GpA dinucleotide primer and 3  $\mu\text{Ci}$  of [ $\alpha$ -<sup>32</sup>P]UTP (for P3) or [ $\alpha$ -<sup>32</sup>P]GTP (for P2) were added to the reaction and incubated at  $37^{\circ}\text{C}$  for 5 min to allow short RNA synthesis to occur. The reaction was stopped with stop dye [3% (wt/vol) xylene cyanol, 3% (wt/vol) bromophenol blue and 20 mM EDTA in 10 ml of deionised formamide] and resolved on a 20% (wt/vol) urea-denaturing polyacrylamide gel. The dried gel was visualised and quantified using a FLA-5000 PhosphorImager. The sequences of the P2 and P3 promoter probes used in the short RNA synthesis assay are shown in Fig. 1b and Supplementary Table 1.

### RNAP catalytic activity assays

The reactions for the RNAP catalytic activity assays at  $4^{\circ}\text{C}$  and  $37^{\circ}\text{C}$  were conducted exactly as described above and as shown in the schematic representation in Fig. 3i using the *S. aureus* core RNAP (in the absence of  $\sigma^A$ ) and the minimal scaffold probe<sup>30</sup> as DNA template for the reaction (which was formed exactly as described above for the promoter probes used in the native gel binding assay using the DNA and RNA oligonucleotides shown in Fig. 3i). The reactions were stopped with stop dye and resolved on a 20% (wt/vol) urea-denaturing polyacrylamide gel. The dried gel was visualised and quantified using a FLA-5000 PhosphorImager.

### *In vitro* transcription assays

The binding reactions for the *in vitro* transcription assays were conducted as described above; however, pJR<sub>P2+P3</sub>, pJR<sub>P2</sub> or pJR<sub>P3</sub> was used as promoter template. The *in vitro* transcription assay was conducted as described by Wigneshweraraj *et al.*<sup>45</sup> Briefly, E $\sigma^A$  and the promoter template were incubated at  $37^{\circ}\text{C}$  for 5 min to allow the RPo to form. In the single-round transcription assay, RNA synthesis was initiated by adding an elongation mix (0.5 mM ATP, CTP and GTP; 0.25 mM UTP;

0.75  $\mu$ Ci of [ $\alpha$ - $^{32}$ P]UTP; and 100  $\mu$ g/ml heparin) and incubating the reaction for a further 5 min at 37 °C. Heparin was omitted from the elongation mix in the multiple-round transcription assays. The reactions were stopped with stop dye and resolved on a 10% (wt/vol) urea-denaturing polyacrylamide gel. The dried gel was visualised and quantified using a FLA-5000 PhosphorImager.

Supplementary materials related to this article can be found online at doi:10.1016/j.jmb.2011.06.018

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