Regulation of the Locus of Enterocyte Effacement in Attaching and Effacing Pathogens

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Abstract

Attaching and Effacing (AE) pathogens colonise the gut mucosa using a Type Three Secretion System (T3SS) and a suite of effector proteins. The Locus of Enterocyte Effacement (LEE) is the defining genetic feature of the AE pathogens, encoding the T3SS and the core effector proteins necessary for pathogenesis. Extensive research has revealed a complex regulatory network that senses and responds to a myriad of host and microbiota-derived signals in the infected gut to control transcription of the LEE. These signals include microbiota-liberated sugars and metabolites in the gut lumen, molecular oxygen at the gut epithelium and host hormones. Recent research has revealed that AE pathogens also perceive physical signals, such as attachment to the epithelium, and that the act of effector translocation remodels gene expression in infecting bacteria. In this review we summarise our knowledge to date and present an integrated view of how chemical, geographical and physical cues regulate the virulence program of AE pathogens during infection.

Introduction

Initially described by the German physician Theodor Escherich in 1885 as the "common colon bacillus" (1), *Escherichia coli* is a Gram-negative facultative anaerobe of the *Enterobacteriaceae* family and a major component of the normal human intestinal flora (2). Pathogenic *E. coli* have arisen through the acquisition of large groups of virulence factors on plasmids, prophages and Pathogenicity Islands (PAIs) and are capable of causing both diarrhoeal and extra-intestinal diseases, including Urinary Tract Infections (UTIs), wound infections, meningitis and sepsis (2, 3). The Attaching and Effacing (AE) pathogens Enteropathogenic *E. coli* (EPEC) and

Enterohaemorrhagic *E. coli* (EHEC) represent an important subset of pathogenic *E. coli* and are responsible for significant disease burden worldwide (4). EPEC and EHEC infections are characterised by the formation of ultrastructural lesions on the surface of the gut epithelium known as AE lesions (2, 5). The formation of these lesions, and the infectious process of the AE pathogens more broadly, depends on a Type Three Secretion System (T3SS), encoded by the Locus of Enterocyte Effacement (LEE) PAI (5), and a suite of locally and distally encoded effector proteins (6). The LEE PAI is also found in the AE pathogens *Citrobacter rodentium*, *Escherichia albertii* (previously known as *Citrobacter freundii* and *Hafnia alvei* respectively) (7, 8) and some *Edwardsiella* spp. (9, 10). Regulation of the LEE is central to the pathogenesis of AE pathogens, and has been the subject of extensive research. Our review focuses on regulation of the EPEC and EHEC LEE's, as little is currently known regarding the regulation of the LEE in these other organisms. However, it is likely that many similarities exist between the regulation of these canonical LEE's and those found in other pathogens.

The Locus of Enterocyte Effacement

The 35 kb LEE PAI consists of 41 open reading frames (ORFs) and is the defining genetic feature of the AE pathogens. The LEE carries the genes for a T3SS, a number of bacterial effector proteins, chaperones for these effectors, the adhesin intimin and regulatory elements (7). As with many of the virulence-associated elements found in the pathogenic *E. coli*, the lower G+C content of the LEE (38.3%), in comparison to the *E. coli* chromosome as a whole, (50.8%) suggests that it was originally acquired by Horizontal Gene Transfer (11, 12). The core LEEs of EPEC and EHEC are 93.9% identical at the nucleotide level, although the EHEC LEE has

an additional 13 ORFs at the 5' end due to the insertion of a cryptic prophage (13). However, the sequence of individual genes can be highly variable and ranges from complete invariance between the two organisms in the case of structural components of the T3SS, to 33.52% identity, as is seen for the effector *tir* (13).

Organisation of the LEE

The LEE is arranged as five polycistronic operons: *LEE1, LEE2, LEE3, LEE4* and *LEE5,* a bicistronic operon (*grlAB)* and multiple monocistronic transcription units (5, 14). *LEE1* contains the genes encoding the master virulence regulator Ler, the chaperones CesAB*,* structural components of the T3SS; EscR, EscS, EscT, EscU, EscK (15) and EscL (16). *LEE2* encodes for the chaperone CesD, three components of the T3SS machinery, EscC, EscJ and SepD, the effector EspZ and one ORF of unknown function. *LEE3* encodes for three components of the T3SS machinery, EscV, EscN and SepQ*,* the effector EspH and two ORFs of unknown function. *LEE4* contains the genes encoding the translocator proteins, which form the tip of the T3SS (EspB and EspD) (17), the needle filament EspA (18), the needle protein EscF (19), the chaperone CesD2*,* the gatekeeper protein SepL (20) and the effector EspF*.* Finally, *LEE5* contains the effectors Tir and intimin, and the chaperone CesT. The effector Map is located 5' to *LEE5* (21) and the effector EspG is located 5' to *LEE1*, (22). The addition of the 933L cryptic prophage 3' to the core LEE genes in EHEC makes the EHEC LEE larger than the EPEC LEE (13).

Expression of the five LEE operons is controlled through the action of the LEE-encoded transcriptional regulator Ler, the expression of which is regulated through numerous transcription factors in response to an array of intra- and extracellular stimuli, (summarised in Fig.1) which are discussed further below.

LEE-encoded regulators: Ler, GrlA and GrlR

AE pathogens sense and integrate multiple host, environmental and microbiota-derived signals to control expression of the LEE genes, with integration of these diverse signals occurring at the transcriptional, translational and post-translational level (23-28). Below 37°C the global regulator H-NS (29) represses the transcription of the master positive regulator of the LEE, *ler* (encoded in *LEE1*) as well as *LEE2, LEE3, LEE4* and *LEE5* (30-33). However, at 37°C *ler* is transcribed and, through the coordination of many cues, relieves H-NS mediated repression (30, 34) to allow expression of the LEE genes (23, 35, 36). More than 40 regulators of LEE transcription have been described to date, including both proteins and regulatory RNAs (23, 24, 31, 35, 37-49), not all of which can be discussed here. Many of these systems converge on *ler* (23, 37) (Fig. 1).

During Ler-mediated expression, the LEE-encoded regulators GrlA (positive) and GrlR (negative) (38) also influence expression of the LEE. GrlA functions in a similar way to some MerR family transcriptional activators, despite sharing no significant sequence similarity with these proteins (50) and belongs to a novel family of transcriptional activators that also contains a GrlA homologue from *Salmonella enterica* and CaiF from *E. coli* and *S. flexneri* (38, 51). Upon expression, GrlA drives the expression of virulence genes, likely through direct binding to the sub-optimal 18-base-pair spacer between the -10 and -35 elements of the distal *LEE1* promoter, P1 (Fig. 1B), via a Helix-Turn-Helix (HTH) DNA-binding motif (50, 52). This drives expression of *ler* and subsequently the entire LEE. Conversely GrlR, cellular levels of which are regulated in a growth phase dependent manner (39), antagonises the system by directly binding to GrlA, preventing its interaction with DNA (53). As demonstrated by Alsharif *et al.* GrlR is not the only antagonistic mechanism acting

upon GrlA. GlrA activity is regulated by the sRNA Arl (54) (discussed further below), whilst in some conditions GrlA also requires external queues, such as attachment to host cells and the shear forces which act upon attached bacteria in the gut, to become fully activated and drive *ler* expression (discussed further below) (26). Thus these auto-regulatory mechanisms allow the LEE to act dynamically by fine-tuning its own expression.

Regulation of the LEE by LysR and Fur-family transcriptional regulators

LysR-type transcriptional regulators are perhaps the primary family of DNA-binding regulatory proteins in bacteria and interact with DNA through a HTH domain at their amino terminus (55). Two major quorum-sensing responsive LysR-type regulators act upon the LEE, QseA and QseD (Fig. 1B). QseA is part of the LuxS quorum sensing system and responds to microbiota-derived signalling molecules, binding the *LEE1* regulatory region, inducing LEE gene expression through increased transcription of *ler* (40, 56) and direct transcription of LEE1 (57), as well as inducing virulence genes in other areas of the chromosome (58). QseD responds indirectly to Autoinducer-3 (AI-3) and is present across the AE pathogens, but exists almost exclusively in EHEC O157:H7 as a truncated short isoform (sQseD) that lacks the classical HTH domain of the LysR-type regulators (59). Whilst both Long QseD (LQseD) and sQseD repress LEE transcription in AE pathogens, each isoform achieves this in a different way. LQseD directly binds to the *LEE1* regulatory region to repress LEE transcription, whilst sQseD is thought to bind a second, as yet unidentified LysR-type transcriptional regulator in order to bind DNA and affect LEE expression (59).

In addition to the two quorum-sensing responsive regulators described above, additional cues such as the microbiota-derived Short Chain Fatty Acid (SCFA) butyrate and the essential cofactor biotin are involved in the regulation of AE pathogen virulence. Butyrate is produced through the fermentation of dietary fibre by the gut microbiota and has wide ranging effects on the health and physiology of the gut (60). Detection of intestinal butyrate, via the regulatory protein Lrp, induces expression of the LEE genes though activation of the LysR-type regulator LeuO. In turn, LeuO cooperates with the plasmid-borne virulence regulator PchA to establish a positive feedback loop and activate *LEE1* transcription (61, 62) and flagella biosynthesis (63). Thus, this accessory regulatory pathway appears to couple virulence and motility and may aid in movement towards the epithelium and the preparation of the T3SS machinery for the initiation of infection.

Biotin is an essential metabolic cofactor in all organisms, and can be both absorbed and synthesised *de novo* by many microorganisms (64). Humans cannot synthesise biotin and instead acquire it from external sources, with the majority of dietary and microbiota-derived biotin being absorbed in the small intestine (65). This creates a gradient within the gut, with high luminal biotin concentrations in the small intestine, and low concentrations in the large intestine and colon (65, 66). During infection EHEC colonises the large intestine. This is in contrast to EPEC, which preferentially infects the small intestine. During infection EHEC senses the biotin gradient in the gut through the biotin ligase BirA (66). In the presence of biotin BirA directly binds within the promoter of the global transcriptional regulator Fur, acting as a negative regulator (66, 67). The precise mechanism by which Fur then regulates the LEE is still unknown. However as Fur does not appear to affect known LEE regulators (66) is possible that an as yet undiscovered LEE regulator allows the indirect control of LEE expression by Fur. BirA therefore serves to indirectly represses LEE expression in the presence of high biotin concentrations (i.e. in the small intestine) and induce

LEE expression in low biotin environments, limiting T3SS activation and AE lesion formation to the large intestine during EHEC infection.

Regulation of the LEE by Bacterial Two-Component Systems

A number of bacterial Two-Component Systems (TCSs) control expression of the LEE. In their basic form, TCSs consist of a membrane implanted histidine kinase and a cognate response regulator (RR), but in some cases a histidine kinase can activate multiple RRs (68). The TCSs that control LEE expression can be broadly divided into two classes: those that repress transcription of the LEE and those that promote it.

Foremost amongst the TCS that repress the LEE genes is the FusKR system, which responds to the host sugar fucose (69). Fucose is found in the lumen of the intestine due to the activity of *Bacteroides thetaiotaomicron*, a major component of the intestinal microbiota, which cleaves fucose from the mucin glycoproteins that coat the gut epithelium (70). The FusR RR directly represses *ler* transcription, thus preventing LEE expression in the gut lumen (69).

Sensing of fucose by the FusKR TCS also has a secondary function, to promote the use of mannose and galactose as carbon sources by intestinal pathogenic *E. coli*, via repression of the *fuc* operon (69). As fucose is the primary carbon source for commensal *E. coli,* and these organisms are unable to metabolise mannose and galactose (71) this prevents unnecessary competition between the infecting strain and the resident commensal *E. coli* population.

Like the fucose sensing FusKR system, the CpxAR two-component system serves to repress transcription of the LEE genes (25). The Cpx envelope stress response is present across Gram-negative bacteria, responds to a variety of stimuli in the

bacterial inner membrane and periplasmic space (72), and has been linked to virulence in a number of pathogens, including *S. enterica* sv. Typhimurium (*S.* Typhimurium) (73) and *Legionella pneumophila* (74). In AE pathogens, CpxR has been shown to repress production of the T3SS (75) by interfering with the activity of Ler and GrlA (25). To achieve this, CpxR, activated by CpxA, indirectly induces the Lon protease, resulting in degradation of both Ler and GrlA (25).

In contrast, the cooperating TCS's QseCB and QseEF sense and respond to the microbiota-derived quorum sensing molecule AI-3, and the host hormones adrenaline and noradrenaline to activate the transcription of the LEE genes (68, 76, 77) and allow colonisation (78). Following autophosphorylation, QseC activates three RRs, QseB (QseC's cognate RR), QseF and KdpE (68), via a conserved 8 amino acid motif in its periplasmic sensing domain (79). QseF is also phosphorylated by its cognate membrane-bound histidine kinase QseE (77). QseB indirectly represses *LEE4* and *LEE5* via the sRNA GlmY*,* whilst activating flagellar and motility genes (24, 68, 80). QseF promotes the expression of the Shiga toxin (68, 79) and the T3SS effector TccP/EspF_U, which is necessary for AE lesion formation in EHEC (81). Importantly, QseB (directly) and QseF (indirectly) also repress the FusKR TCS, alleviating fucose-mediated repression and thus shifting the regulatory balance towards expression of the entire LEE (69). Phosphorylated KdpE promotes *ler* transcription and thus LEE expression and is discussed further below.

Sensing Metabolic Cues and Oxygen Availability

In addition to the TCSs described above, AE pathogens integrate their own metabolic status into the regulatory network controlling LEE expression. The phosphorylation-

independent transcription factor Cra directly senses metabolic intermediates in the glycolysis pathway, such as fructose-1-phosphate and fructose-1,6-bisphosphate. Both of these metabolic intermediates antagonise Cra's ability to bind to target promoters through interaction with its inducer-binding domain (82). This results in the suppression of *LEE1* expression during glucose-based metabolism and the promotion of *ler* transcription when mucin-derived sugars are the most abundant carbon source (83, 84)

In the context of the intestine, this suggests that Cra acts in opposition to fucose sensing by FusR to promote *LEE1* transcription in response to the gluconeogenic environment encountered as the pathogen moves through the mucus layer. Cra also responds to the presence of oxygen, further enhancing *LEE1* transcription as the bacteria approaches the epithelial barrier, where oxygen concentrations are higher (85). This further promotes LEE transcription, T3SS expression and AE lesion formation, as the bacterium moves from the anaerobic environment of the gut lumen to the microaerophillic environment directly adjacent to the epithelium (84).

Cra achieves efficient LEE expression in concert with the RR KdpE. As discussed above, KdpE is activated in response to AI-3, adrenaline and noradrenaline by QseC (68) and binds to the *LEE1* promoter, as Cra does. However, KdpE binds the *LEE1* promoter at a distinct site from Cra, promoting DNA bending and thus efficient initiation of *ler* transcription (83). Cra and KdpE also cooperate to control the transcription of a number of other genes, including TccP/EspF$_u$ and additional uncharacterised, putative virulence factors in EHEC (86). Interestingly, the genes regulated by Cra and KdpE don't overlap entirely, and transcription factor specific targets have been described. These include the LEE-encoded effector *espG*, which is adjacent to *ler*, and appears to be regulated by Cra but not by KdpE (86).

277 Recent work has also suggested that the regulator NagC directly influences *LEE1*
278 transcription and sugar catabolism in EHEC, in response to the mucin-derived sugars
279 N-acetylglucosamine (NAG) and N-acetylneuraminic acid (NANA). NagC directly
280 binds the *LEE1* regulatory region at a position that overlaps the -10 element of the P1
281 promoter (87) (Fig. 1B). This suggests that mucin-derived sugars in the intestine can
282 both promote and repress LEE gene expression. When taken together with the well-
283 established role of Cra in LEE expression, this suggests that metabolic status,
284 oxygen availability and virulence are intimately linked in the AE pathogens.
285

286 **Regulation of the LEE by small RNAs**

287

288 In addition to the plethora of regulatory proteins that have been shown to modulate
289 expression of the LEE genes, the influence of post-transcriptional small ribonucleic
290 acid (sRNA)-mediated regulation on the deployment of the virulence arsenal of the
291 AE pathogens is becoming increasingly apparent.
292

293 Usually between 100 and 200 nucleotides in length, sRNAs are a heterogeneous
294 population of molecules that modulate the translation of messenger RNAs (mRNAs)
295 to proteins, through base pairing with their specific target mRNA. Bacterial sRNAs
296 are grouped in to *cis*- and *trans*-encoded sRNAs, where *cis*-sRNAs are encoded on
297 the complimentary strand of the gene that they target and *trans*-sRNAs are encoded
298 distally to their target gene. Due to this, the majority of *trans*-sRNAs require an RNA
299 chaperone to facilitate base pairing with their target gene, the most common of which
300 is Hfq (88-90).
301

302 The Arl sRNA, encoded within the LEE, is the only *cis*-encoded sRNA shown to
303 influence LEE expression to date (Fig. 1A). Arl exhibits significant complementarity to

304 the *ler* mRNA, preventing translation of Ler, and thus LEE expression in response to
305 changes in cytosolic iron homeostasis (54). Hfq has also been shown to act as a
306 negative regulator of the LEE genes in both EPEC and EHEC (27) in two distinct
307 ways; through antagonism of the *grlRA* mRNA (47), which prevents any LEE-
308 mediated amplification of *ler* transcription, and through direct translational repression
309 of *ler* (27). However, in some EHEC strains large numbers of Hfq-dependent *trans*-
310 sRNAs are present, many of which have been shown to act as activators of LEE
311 gene expression (49, 91). Consistent with the observation that horizontally-acquired
312 PAIs harbour an increased number of sRNAs (92), the diversity of PAIs in AE
313 pathogens (93) and the significant strain to strain variation seen in the role of Hfq on
314 expression of the EHEC LEE (94) it is likely that differences in the role of Hfq are due
315 to the different PAIs carried in different strains. Indeed, the description of 63 novel
316 EHEC sRNAs, 55 of which are encoded in bacteriophage-derived sections of the
317 EHEC genome (49), suggests that the sRNA-mediated regulatory circuits that
318 influence LEE expression may be unique to each AE pathogen strain. The
319 exceptions to this situation are the ancestral Hfq-dependent sRNAs GlmY and GlmZ,
320 which are found in both pathogenic and non-pathogenic *E. coli* (88). These
321 molecules act to dampen expression of *LEE4* and *LEE5*, and thus assembly of new
322 T3SS injectosomes, whilst simultaneously promoting TccP/EspF_U expression (24)
323 and thus AE lesion formation (81). Interestingly, the transcription of GlmY, but not
324 GlmZ, is induced in response signals from the host that are perceived by the infecting
325 pathogen (24) (as discussed above and Fig. 1B).
326
327 Finally, the description of bacteriophage-derived anti-sRNA sRNAs, “anti-sRNAs”,
328 which silence other *trans*-sRNAs, in AE pathogens (49) exemplifies the complexity,
329 diversity and potential for variation of sRNA-mediated LEE regulation.
330
331

Regulation of the LEE by Host Attachment and Effector Translocation

It is becoming increasingly apparent that aside from responding to external cues and sensing their own internal metabolic state, pathogens respond to physical forces to regulate virulence gene expression (95). AE pathogens sense and respond to physical attachment to the epithelial surface using a number of mechanisms (26, 96) (summarised in Fig. 2). Notably, attachment to host cells, mediated by both the T3SS and non-LEE encoded adhesins, activates the *LEE1* promoter by increasing GrlA activity, which is amplified further in response to laterally acting fluid shear forces (26). However, the mechanism by which GrlA responds to host cell attachment and mechanical forces has yet to be determined.

LEE gene expression is also controlled by the translocation of effector proteins through the T3SS (97). This occurs through a novel pathway regulated by the LEE-encoded effector chaperone CesT, which modulates the global translation inhibitor CsrA, after effector translocation (97). Release of CesT following the translocation of effectors blocks CsrA activity, allowing expression of the important non-LEE encoded (Nle) effector NleA and suppression of *LEE4*. As CsrA regulates gene expression throughout the *E. coli* genome (97, 98) it is likely that effector translocation leads to widespread alteration of the translational program in actively injecting AE pathogens (97).

Perspectives and Future Directions

The regulatory mechanisms described above establish a situation in which LEE gene expression is controlled through the sensing of both chemical and physical cues, along the length of the intestine (summarised in Fig. 3). As the bacterium enters the host, the switch to 37°C alleviates negative repression by H-NS (30). Once in the gut,

359 the microbiota derived quorum-sensing molecules (56, 76) (positive regulators of *ler*)
360 provide an initial biogeographic signal as to where in the host the bacterium is
361 located. However, virulence gene expression is repressed overall whilst in the lumen
362 through the sensing of the microbiota-liberated sugars fucose (69), NAG and NANA
363 (87). This repression of the LEE is relieved as the bacterium moves laterally along
364 the intestine in response to microbiota-derived butyrate and sensing of the host
365 hormones adrenaline and noradrenaline (61, 76). LEE expression is further
366 enhanced as the bacterium moves through the mucus layer, closer to the epithelium,
367 in response to mucus-derived sugars (such as Galacturonic acid) and increasing
368 oxygen concentration (84). Perception of these signals occurs through the
369 cooperation of the QseBC and QseEF TCSs, QseA and Cra/KdpE, which together
370 remove the negative regulatory pressure provided by FusKR and stimulate the
371 transcription of the T3SS machinery, allowing infection (78). Once in contact with the
372 host epithelium, attachment to host cells and the associated shear stress produced
373 by fluid flow over the epithelial surface (26, 96), in conjunction with the act of effector
374 translocation (97) remodels gene expression in the actively infecting bacteria to allow
375 colonization of the mucosal surface. Both the synergistic and sequential effects of
376 multiple protein regulators at the *LEE1* promoter region facilitate this exquisite level
377 of control. Additionally, throughout the process described above it is likely that sRNA-
378 mediated regulation further fine-tunes the virulence programme of AE pathogens in
379 response to the changing host environment.
380
381 Unsurprisingly, given the numerous regulatory mechanisms acting upon it, correct
382 regulation of the LEE is central to virulence. Mutants lacking the sensor kinases *fusK*
383 (69), *qseC* (76, 78), *qseE* (78) or *cpxA* (25) are attenuated in *in vivo* models, whilst
384 strains lacking the transcription factor *cra* exhibit reduced pedestal formation on
385 cultured epithelial cells (83) and those lacking *cesT* exhibit reduced effector
386 translocation (97, 99, 100). These observations provide support for the notion that

targeting LEE-regulation may provide novel opportunities for therapeutic intervention. Indeed, small molecule inhibition of QseC has already been shown to be a viable anti-virulence approach for multiple Gram-negative pathogens (101)Curtis, 2014 #779}. Future work to further understand the influence of biotin-sensing, the molecular mechanisms coupling LEE expression and shear stress, the extent of the genetic reprogramming which occurs upon effector translocation and the full role of sRNAs in virulence regulation will undoubtedly prove useful in this area. Irrespective, it is clear that the complex regulatory network employed by AE pathogens provides an exquisite level of control over LEE expression, ensuring efficient, coordinated deployment of virulence factors. This undoubtedly contributes to the continued success of these important human pathogens and provides potential targets for the development of therapeutics targeting virulence rather than viability.

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Figure Legends

Figure 1. Regulation of the LEE. A) The master LEE regulator, Ler (23, 37), integrates external signals from TCS's (FusKR, QseBC, QseEF, CpxAR), LysR-type transcription factors (QseA, QseD, LeuO) and the regulators Cra, KdpE and NagC, which act in concert to

regulate transcription of *ler*. Cra also controls the expression of the LEE-encoded effector protein EspG. Ler-mediated expression of the LEE is fine-tuned by the LEE-encoded regulators GrlA and GrlR (38). The sRNA Arl directly antagonised translation of the *ler* mRNA, whist GlmY silences *LEE4* and *LEE5* upon induction by QseB/QseF. B) Binding sites within the *LEE1* regulatory region for regulators that act directly on *ler* transcription. GrlA counteracts H-NS mediated negative regulation of the LEE1 promoter and directly binds the P1 promoter of *LEE1* (50, 52). QseA binds directly upstream of the -35 element of both the P1 promoter and the EHEC-specific P2 *LEE1* promoter (57, 58). KdpE binds to AT-rich regions of DNA and as such its binding site upstream of *LEE1* is poorly defined, but is likely between 173 and 42 base pairs upstream of the *LEE1* transcription start site. Cra binds at the 5' end of the *LEE1* regulatory region acts synergistically with KdpE to promote DNA bending upstream of the P1 and P2 promoters, facilitating *LEE1* expression (83). LeuO binds AT-rich regions of the *LEE1* promoter and collaborates with PchA to removes H-NS mediated repression at these same AT-rich regions, thus enhancing transcription (62). The repressors FusR, NagC and QseD also bind directly to the *LEE1* regulatory region. FusR binds to a region between the P1 and P2 promoters (69), NagC binds up stream of FusR and the HTH-containing isoform of QseD, lQseD, binds to the same site as QseA. The binding site of sQseD remains to be determined (59). Sequence information for binding sites and regulatory elements is shown where it is known, -10 and -35 element sequences are shown in bold. Regulators are colour coded to indicate the type of signal they are responsive to: yellow = Microbiota-derived quorum sensing molecules and/or host-derived hormones, grey = butyrate, blue = mucose-derived sugars, pink = bacterial metabolic status, gold = Ler, and external mechanical cues, orange = growth phase.

Figure 2. Regulation of the LEE by physical forces and effector translocation. AE pathogens integrate physical cues to regulate expression of the LEE. Attachment to host cells, sensed by the bacterial outer membrane (OM) protein NlpE, induces the CpxAR two-component system (102). The RR CpxR indirectly promotes *ler* transcription through the LysR-type regulator LhrA (96). Attachment to host cells also induces GrlA-mediated *ler*

transcription, which is further enhanced by sensing of fluid flow at the epithelial surface (26). Finally, effector translocation by the LEE-encoded chaperone CesT, generates free CesT which causes indirect repression of *LEE4* via antagonism of the global gene regulator CsrA, as well as allowing expression of the important non-LEE encoded effector protein NleA by releaving CsrA-mediated repression (97). PM = Plasma Membrane, IM = Inner Membrane, OM = Outer Membrane

Figure 3. Cues influencing LEE expression in the gut. Upon ingestion and the accompanying shift to 37°C, H-NS mediated repression of the *ler* is relieved (30), priming the infecting bacteria for expression of the LEE. Sensing of microbiota-derived molecules (*e.g*. Autoinducer-2, AI-3 and butyrate) in the gut lumen further induces LEE-expression, but the strong negative signal provided by fucose, via the FusKR TCS, and NAG/NANA via NagC, prevents expression of the T3SS. As further positive stimuli, such as the abiotic cues SO_4^{2-} and PO_4^{3-} (77), the host hormones adrenaline and noradrenaline (sensing of which is necessary for colonisation (78)), and biotin (66) are detected *LEE* expression is gradually induced. As the infecting bacteria moves through the mucus layer and closer to the epithelium, the presence of mucin-derived sugars and the increasing oxygen concentration (left panel, blue gradient indicates increasing O_2 concentration) as the bacterium approaches the epithelial surface cooperate to activate full *LEE* expression and the deployment of the T3SS (84). Attachment to the epithelium (26, 96) and the sensing of shear forces produced by fluid flow in the gut (26) further promotes LEE expression. Finally, the act of effector translocation represses the T3SS machinery genes (*LEE4*) and causes global alterations in the expression of virulence-associated and metabolic genes (97) (right panel, feed forward regulatory mechanisms triggered by effector translocation are indicated by circular arrows).

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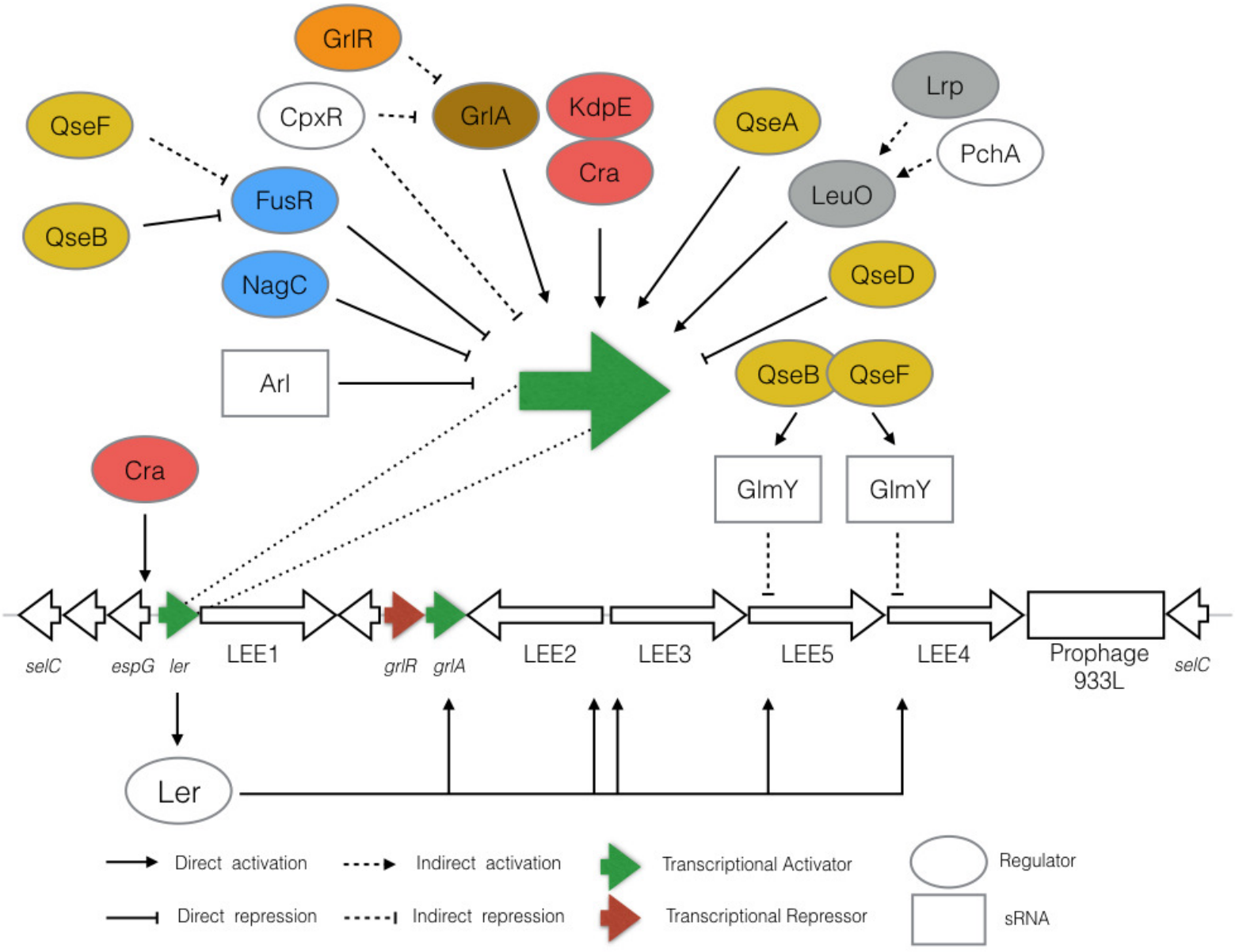
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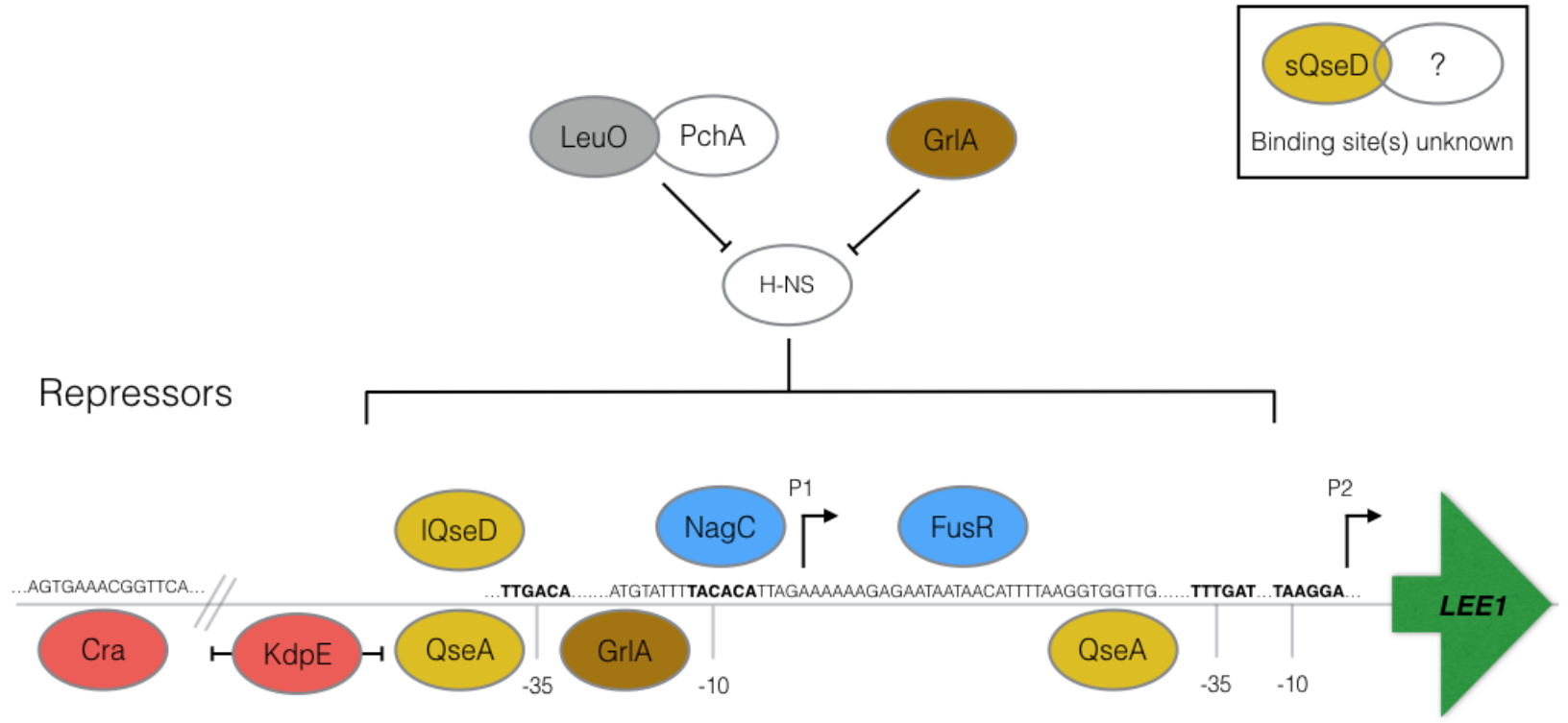
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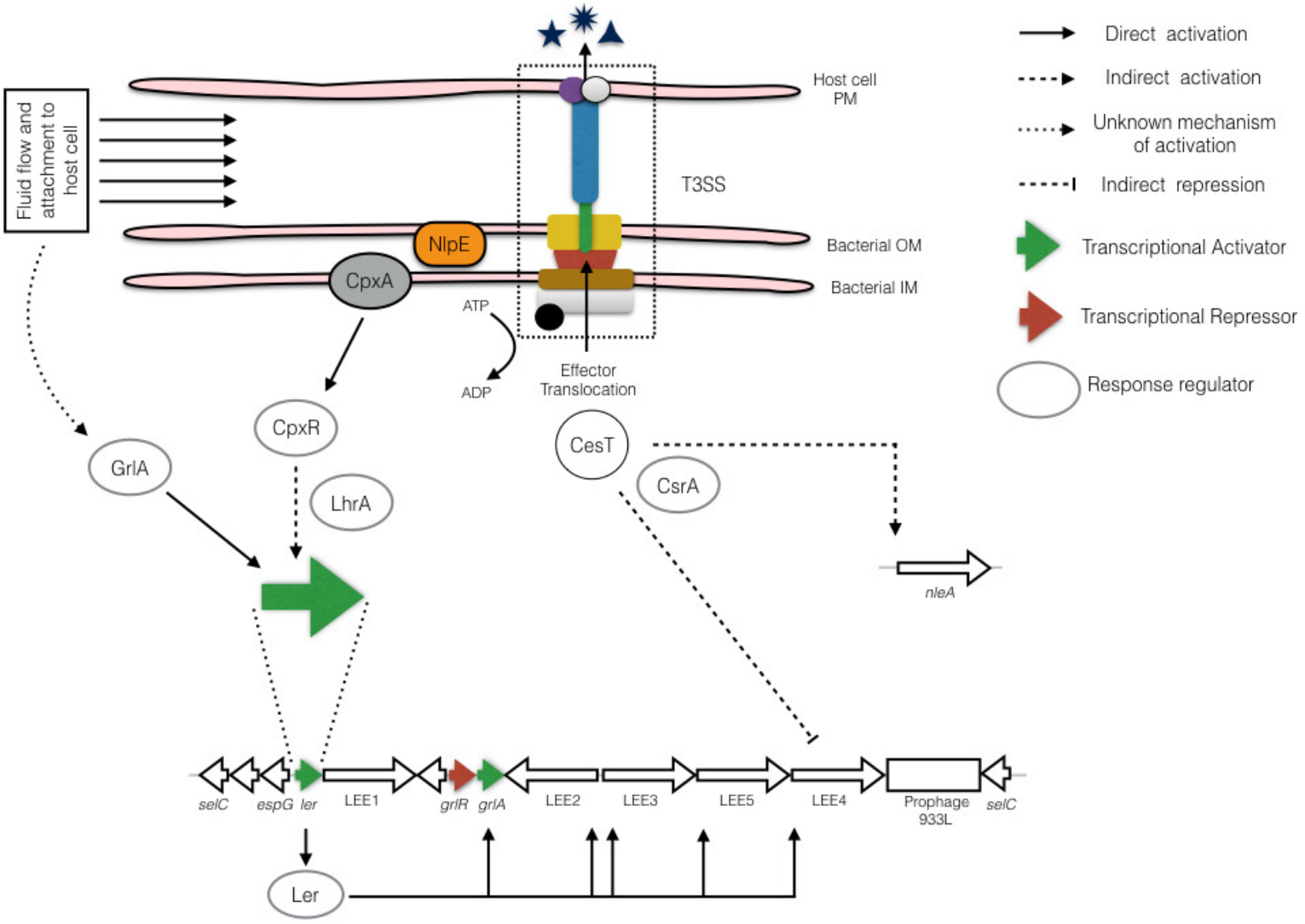
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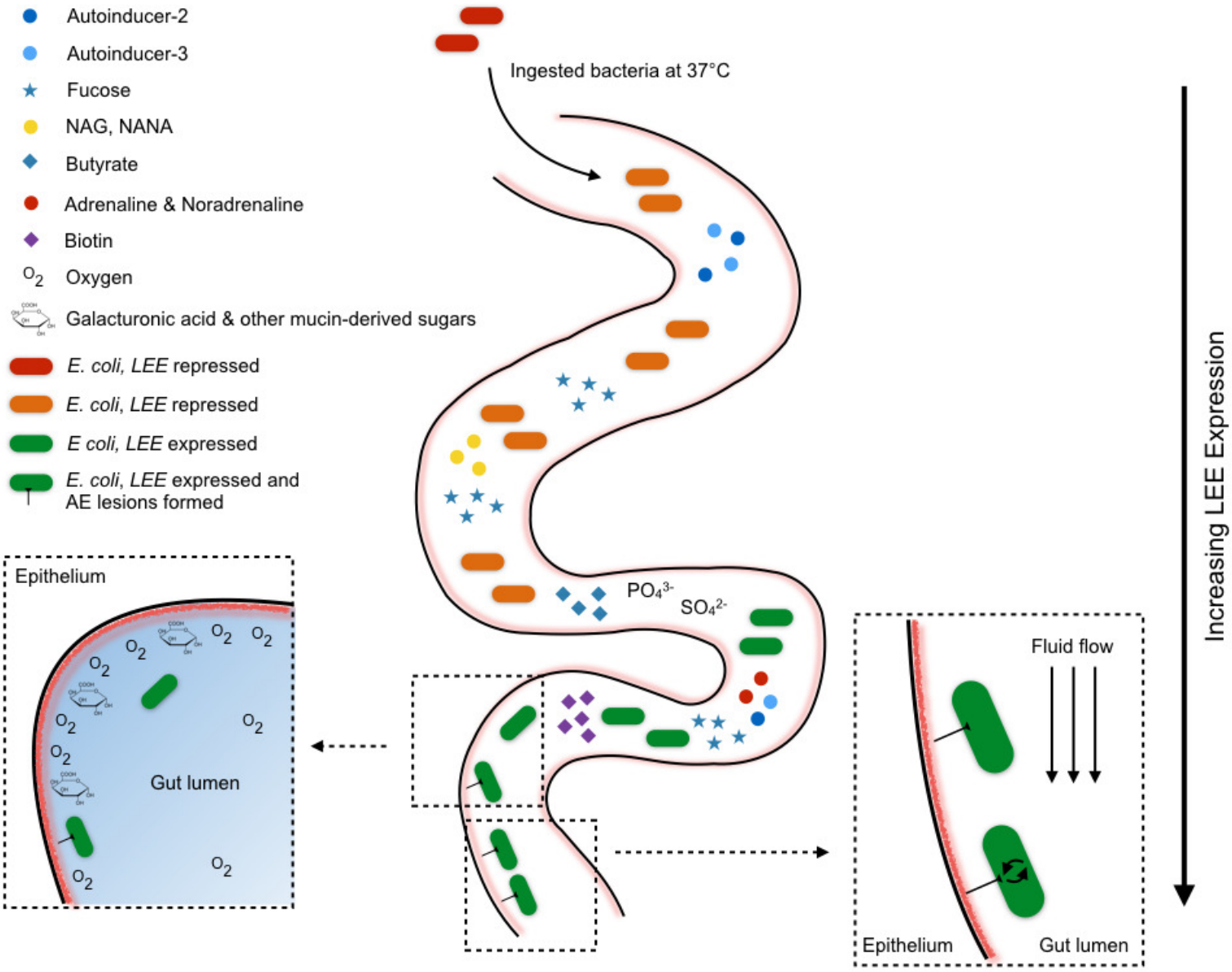
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GrlR
CpxR
GrlA
KdpE
Cra
QseF
QseB
FusR
NagC
Arl
QseA
Lrp
PchA
LeuO
QseD
QseB
QseF
GlmY
GlmY
Cra
selC
espG
ler
LEE1
grlR
grlA
LEE2
LEE3
LEE5
LEE4
Prophage 933L
selC
Ler
Direct activation
Indirect activation
Transcriptional Activator
Regulator
Direct repression
Indirect repression
Transcriptional Repressor
sRNA


sQseD
?
Binding site(s) unknown
LeuO
PchA
GrlA
H-NS
Repressors
IQseD
NagC
P1
FusR
P2
...AGTGAAACGGTTCA...
...TTGACA.....ATGTATTTTACACATTAGAAAAAAGAGAATAATAACATTTTAAGGTGGTTG.....TTTGAT...TAAGGA...
LEE1
Cra
KdpE
QseA
GrlA
-35
-10
QseA
-35
-10

Activators


Fluid flow and attachment to host cell
Host cell PM
T3SS
NlpE
CpxA
Bacterial OM
Bacterial IM
ATP
ADP
Effector Translocation
CpxR
GrlA
LhrA
CesT
CsrA
nleA
selC
espG
ler
LEE1
grlR
grlA
LEE2
LEE3
LEE5
LEE4
Prophage 933L
selC
Ler
Direct activation
Indirect activation
Unknown mechanism of activation
Indirect repression
Transcriptional Activator
Transcriptional Repressor
Response regulator


Autoinducer-2
Autoinducer-3
Fucose
NAG, NANA
Butyrate
Adrenaline & Noradrenaline
Biotin
O_2 Oxygen
Galacturonic acid & other mucin-derived sugars
E. coli, LEE repressed
E. coli, LEE repressed
E coli, LEE expressed
E. coli, LEE expressed and AE lesions formed
Ingested bacteria at 37°C
PO_4^{3-}
SO_4^{2-}
Increasing LEE Expression
Epithelium
Gut lumen
Fluid flow
Epithelium
Gut lumen