

SUPPLEMENTAL MATERIALS AND METHODS

Structure solution and refinement

Multiple datasets were collected from a single crystal at 100K at the SOLEIL synchrotron at the PROXIMA2 beamline (Saint-Aubin, France). The protein belonged to space group $P2_1$ with the unit cell parameters $a = 51.86 \text{ \AA}$, $b = 88.64 \text{ \AA}$, $c = 61.42 \text{ \AA}$; $\alpha = 90.0^\circ$, $\beta = 114.88^\circ$, $\gamma = 90.0^\circ$. The datasets were indexed and reduced together to $2.50 \text{ \AA}$ resolution using XIA2 (63) with the XDS and XSCALE procession options (64). The R_{free} set was generated randomly in Unique (65). The structure was solved by molecular replacement using Rosetta MR, as implemented in the PHENIX packages (66, 67) and using the MJ0100 protein structure from *Methanocaldococcus jannaschii* (PDB code 3KPB) as a model. Briefly, global and local alignment files and 9 or 3 amino acid fragments were generated using the HHRpred server (<http://toolkit.tuebingen.mpg.de/hhrpred>) and used as input files in Rosetta MR for phasing (68). The MR solution was only partial as it included only 2 identical monomers, accounting for approximately 50% of the predicted monomers in the asymmetric unit (a.u.). The initial model was also fragmented around the inner core of the protein. Next, one of the two OpuCA CBS domain monomers was trimmed to the inner core of 101 continuous residues from residues 250 to 351 and used as search model in PHASER (69). Reiterated PHASER runs allowed the placement of all 4 predicted monomers in the a.u.. The MR solution was further extended in several runs with PHENIX Autobuild (66) leading to initial $R_{\text{factor}}=0.37$ and $R_{\text{free}}=0.46$. Restrained refinement was performed in REFMAC 5 (resolution range $88.26\text{-}2.50 \text{ \AA}$) (70) and Phenix (66), and re-iterated model building was performed manually in COOT (71). Jelly body refinement and map sharpening were included in the final stages of the refinement in REFMAC yielding $R_{\text{factor}}=0.24$ and $R_{\text{free}}=0.28$. Clear electron density was missing for the side chains of

several residues (probably due to disorder) and were set to occupancy zero in the model. Structure validation was performed using MOLPROBITY (72). Crystallographic data and refinement statistics are reported in Table S1.

Structural analysis

The PDBePISA and PDBeFold servers (32, 33) were used for protein assembly and structural homology analysis, respectively. The PDB2PQR (73) was used prior to APBS (36) for the analysis of electrostatic surface properties. The STRAP programme (74) was used to perform sequence-based structural alignment with the OpuCA CBS domain and a set of 10 bacterial AMP-binding CBS domains with known structures.

SUPPLEMENTARY FIGURES

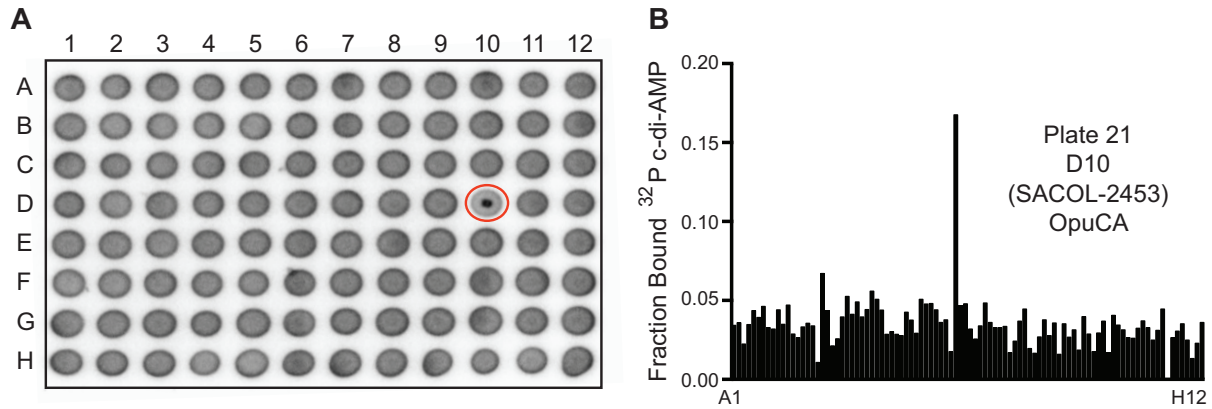


Fig. S1. Identification of SACOL2453 (OpuCA) as a potential c-di-AMP target protein using a genome wide DRaCALA screen. Cell lysates were prepared from an *E. coli* strain library allowing the production of 2,337 His-MBP *S. aureus* COL strain fusion proteins. The *E. coli* lysates were arrayed in 96-well plates and mixed with radiolabeled c-di-AMP. Using a 96-well pin tool, 2.5 μ l of the reactions were spotted onto nitrocellulose membranes, air dried and (A) spots visualized using a phosphorimager and (B) fraction bound values determined as previously described (30) and plotted. A positive interaction was detected on plate 21 well D10 with a lysate prepared from an *E. coli* strain expressing the His-MBP-SACOL2453 (annotated as OpuCA) fusion protein. The genome wide screen was conducted only once (n=1), and the candidate c-di-AMP receptor protein thoroughly verified afterwards.

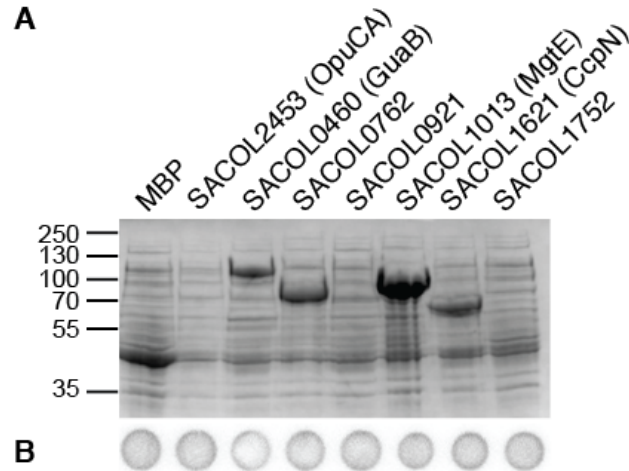


Fig. S2. DRaCALAs with cleared cell lysates derived from *E. coli* strains producing different CBS domain-containing *S. aureus* proteins. (A) Cell lysates from Fig. 3B were centrifuged for 1 h at 21,000 x g to remove protein aggregates, aliquots subsequently separated on a 10% SDS polyacrylamide gel and proteins visualized by Coomassie staining. Proteins SACOL2543 (OpuCA), SACOL0921 and SACOL1752 were depleted compared to uncleared lysates. (B) Samples from (A) were then used in DRaCALA assays with radiolabeled c-di-AMP to determine binding ability. One representative experiment is shown (n=4).

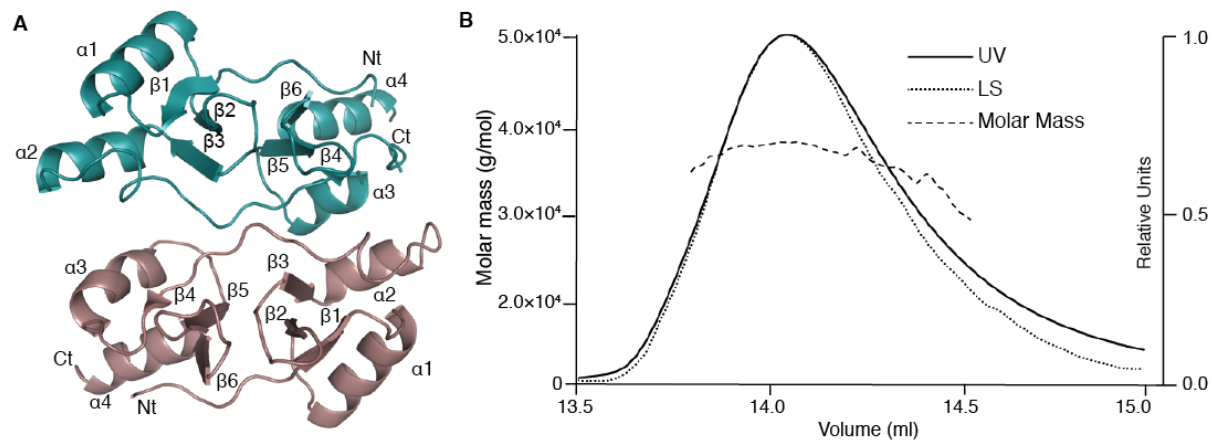


Fig. S3. Oligomeric state of the *S. aureus* OpuCA CBS domain in the crystal structure and in solution. (A) Oligomeric state of OpuCA in the crystal structure. The OpuCA CBS domain is found in a dimeric arrangement in the crystal structure. (B) SEC-MALS profile of the OpuCA CBS domain protein. The purified His-CBS protein was analyzed by SEC-MALS and a graph of the elution profile (solid line) is shown with the molar mass value (dashed line) plotted against the elution volume (X-axis). The His-CBS monomer has a calculated molecular mass of 21.8 kDa, while independent experiments yielded a mass between $34.5 \text{ kDa} \pm 3.2\%$ and $36.6 \text{ kDa} \pm 1.9\%$, with a median of $35.9 \text{ kDa} \pm 4.3\%$ ($n=3$). This suggests, that OpuCA forms a compact dimer in solution.

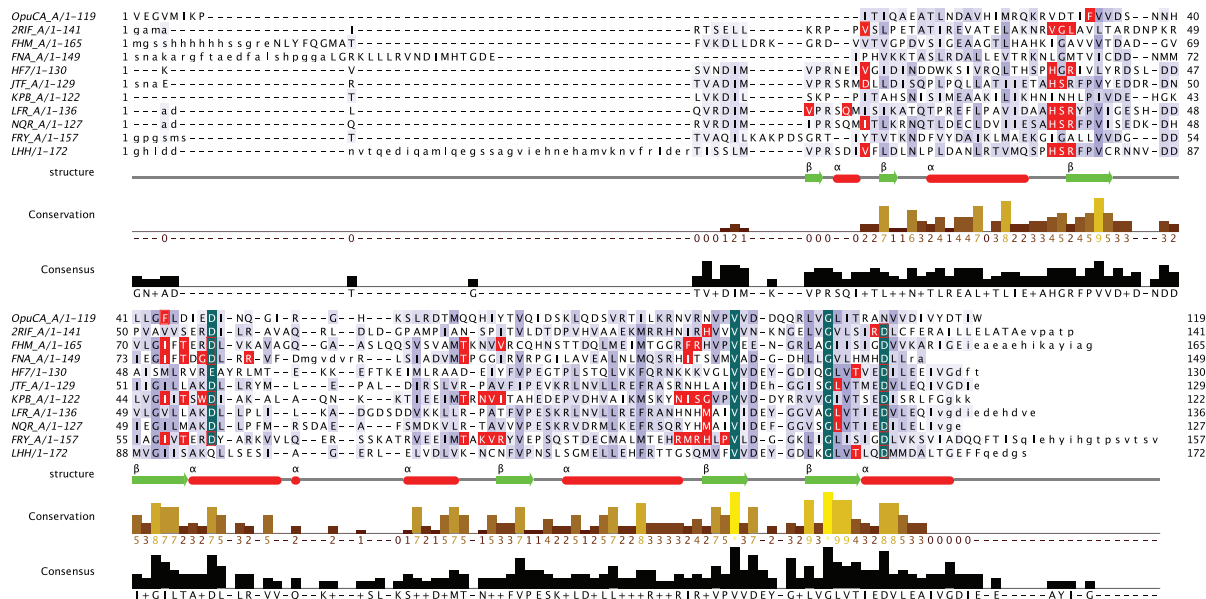


Fig. S4. Structure-based sequence alignment of the *S. aureus* OpuCA CBS domain with other ligand-bound CBS domains. The proteins used for the alignment are: PAE2072 from *Pyrobaculum aerophilum* (PDB 2RIF); ATU1752 from *Agrobacterium tumefaciens* (PDB 3FHM); YrbH from *Escherichia coli* (PDB 3FNA); YfjD from *Klebsiella pneumonia* (PDB 3HF7); CorC from *Bordetella parapertussis* (PDB 3JTF) and *Salmonella typhimurium* (PDB 3NQR); MJ0100 from *M. jannaschii* (PDB 3KPB); PSPTO_4807 from *Pseudomonas syringae* (PDB 3LFR); BamMC406_4587 from *Burkholderia ambifaria* (PDB 4FRY); SO_2815 from *Sheiwanella oneidensis* (PDB 3LHH). All protein structures used for the alignment were in the AMP-bound form except MJ0100, which was in a SAM-bound form. Residues involved in ligand binding are colored in red, and conserved residues are shaded in teal. Residues with both features are shaded in teal with a red border. Secondary structure elements are shown on top of the alignment.

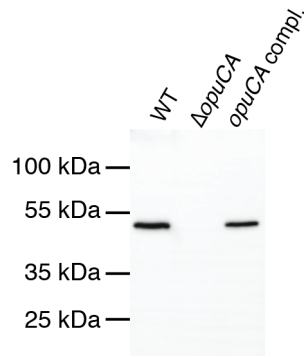


Fig. S5. OpuCA protein amounts in wild-type *S. aureus*, mutant, and complementation strains. Detection of OpuCA by western-blot. The *S. aureus* strains LAC* pCL55 (WT), the *opuCA* mutant LAC*Δ*opuCA* pCL55 (Δ*opuCA*) and the complementation strain LAC*Δ*opuCA* pCL55-*opuCA* (*opuCA* compl.) were grown in 0.5 M NaCl CDM to an OD₆₀₀ of 0.5 and samples prepared for western-blot analysis as described in the materials and method section. Samples were separated on a 12% PAA gel and the OpuCA protein detected by western-blot using a polyclonal *S. aureus* OpuCA-CBS domain rabbit antibody. Shown is one representative experiment (n=3).

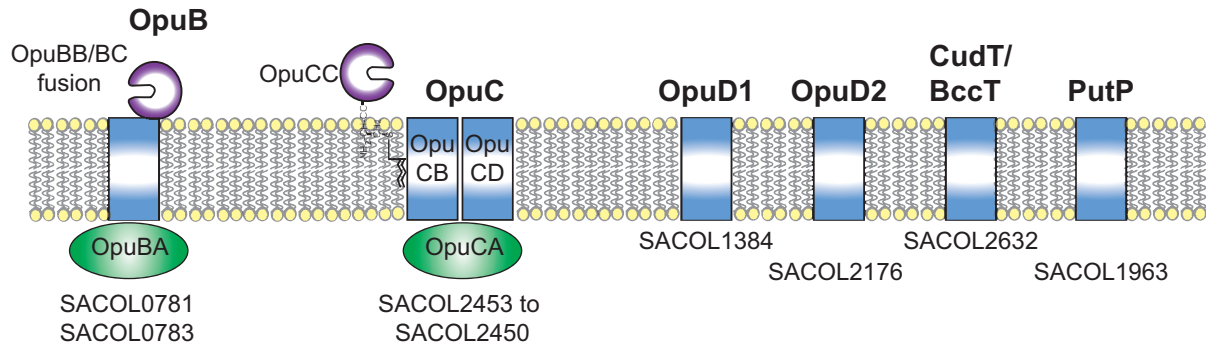


Fig. S6. Confirmed and putative osmolyte uptake systems in *S. aureus* strains. Schematic representation of the ATP-binding cassette transport systems OpuB and OpuC, the BCCT-type transport systems OpuD1, OpuD2 and BccT and the SSF transporter PutP. *S. aureus* strain COL locus tag number coding for the indicated proteins are denoted below each system.

Table S1. Data collection and refinement statistics (molecular replacement)

Data collection	
Space group	P1 21 1
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	52.15 88.26 61.79
α , β , γ (°)	90 114.79 90
Resolution (Å)	88.27 (2.5) *
Unique reflections	17664
<i>R</i> _{merge}	0.057 (1.16)
<i>R</i> _{<i>pin</i>}	0.016 (0.315)
<i>CC</i> (1/2)	0.999 (0.820)
<i>I</i> / <i>sdI</i>	34.2 (3.21)
Completeness (%)	96.8 (99.7)
Redundancy	13.4 (14.3)
Refinement	
<i>R</i> _{work} / <i>R</i> _{free}	0.244/0.280
Mean <i>B</i> -factor	42.55
R.m.s. deviations	
Bond lengths (Å)	0.004
Bond angles (°)	0.717

*Values in parentheses refer to highest-resolution shell.

Table S2. Bacterial strains used in this study

Strain name	Strain description and features	Reference
	<i>Escherichia coli</i> strains	
XL1-Blue	Cloning strain, Tet ^R ; ANG127	Stratagene
T7IQ	Protein production strain: Cam ^R ; ANG2712	NEB
BL21(DE3)	Protein production strain: ANG191	Novagen
IM08B	<i>E. coli</i> strain with <i>S. aureus</i> restriction modification system: ANG3724	(58)
ANG243	XL1-Blue pCL55; <i>S. aureus</i> single-site integration vector: Amp ^R	(75)
ANG1824	XL1-Blue pET28b; Kan ^R	Novagen
ANG2154	DH10B pIMAY; allelic exchange vector: Cm ^R	(59)
ANG2815	XL1-Blue pVL847; Amp ^R	(76)
ANG3156	T7IQ pVL847; Amp ^R , Cam ^R	This study
ANG2890	BL21(DE3) pVL847; Amp ^R	This study
ANG3048	BL21(DE3) pET28b- <i>disA</i> _{BT} ; Kan ^R	(18)
ANG3128	T7IQ pVL847 Gn- <i>SACOL2453</i> (<i>opuCA</i>); Gent ^R , Cam ^R	(29)
ANG3172	XL1-Blue pVL847-ATPase (<i>OpuCA</i> amino acids 1-236); Amp ^R	This study
ANG3173	BL21(DE3) pVL847-ATPase; Amp ^R	This study
ANG3199	XL1-Blue pET28b-CBS (<i>OpuCA</i> amino acids 237-408); Kan ^R	This study
ANG3218	BL21(DE3) pET28b-CBS; Kan ^R	This study
ANG3560	XL1-Blue pET28b-CBS _{F283A} ; Kan ^R	This study
ANG3561	XL1-Blue pET28b-CBS _{F294A} ; Kan ^R	This study
ANG3562	XL1-Blue pET28b-CBS _{Y319A} ; Kan ^R	This study
ANG3563	XL1-Blue pET28b-CBS _{Y365A} ; Kan ^R	This study
ANG3564	XL1-Blue pET28b-CBS _{W369A} ; Kan ^R	This study
ANG3565	BL21(DE3) pET28b-CBS _{F283A} ; Kan ^R	This study
ANG3566	BL21(DE3) pET28b-CBS _{F294A} ; Kan ^R	This study
ANG3567	BL21(DE3) pET28b-CBS _{Y319A} ; Kan ^R	This study
ANG3568	BL21(DE3) pET28b-CBS _{Y365A} ; Kan ^R	This study
ANG3569	BL21(DE3) pET28b-CBS _{W369A} ; Kan ^R	This study
ANG3570	XL1-Blue pET28b-CBS _{A365} ; Kan ^R	This study
ANG3571	XL1-Blue pET28b-CBS _{A374} ; Kan ^R	This study
ANG3572	BL21(DE3) pET28b-CBS _{A365} ; Kan ^R	This study
ANG3573	BL21(DE3) pET28b-CBS _{A374} ; Kan ^R	This study
ANG3580	XL1-Blue pIMAY- <i>ΔopuCA</i> ; Cam ^R	This study
ANG3581	XL1-Blue pCL55- <i>opuCA</i> ; Amp ^R	This study
ANG3589	T7IQ pVL847 Gn- <i>SACOL0460</i> ; Gent ^R , Cam ^R	(29)
ANG3592	T7IQ pVL847 Gn- <i>SACOL1621</i> ; Gent ^R , Cam ^R	(29)
ANG3593	XL1-Blue pVL847- <i>SACOL1752</i> ; Amp ^R	This study
ANG3597	T7IQ pVL847- <i>SACOL1752</i> ; Amp ^R , Cam ^R	This study
ANG3728	IM08B pIMAY- <i>ΔopuCA</i> ; Cam ^R	This study
ANG3732	IM08B pCL55; Amp ^R	This study
ANG3733	IM08B pCL55- <i>opuCA</i> ; Amp ^R	This study
ANG4046	XL1-Blue pVL847 SACOL0762 (amino acids 162-449); Amp ^R	This study
ANG4047	XL1-Blue pVL847 SACOL0921 (amino acids 140-346); Amp ^R	This study
ANG4048	XL1-Blue pVL847 SACOL1013 (amino acids 2-294); Amp ^R	This study
ANG4049	T7IQ pVL847 SACOL0762 (amino acids 162-449); Amp ^R , Cam ^R	This study
ANG4050	T7IQ pVL847 SACOL0921 (amino acids 140-346); Amp ^R , Cam ^R	This study
ANG4051	T7IQ pVL847 SACOL1013 (amino acids 2-294); Amp ^R , Cam ^R	This study
	<i>Staphylococcus aureus</i> strains	
LAC*	Erm ^S CA-MRSA LAC strain (AH1263); ANG1575	(77)
ANG1961	LAC* <i>ΔgdpP::kan</i> ; Kan ^R	(6)
ANG3744	LAC* <i>ΔopuCA</i>	This study
ANG3795	LAC* pCL55; Cam ^R	This study
ANG3829	LAC* <i>ΔopuCA</i> pCL55; Cam ^R	This study
ANG3830	LAC* <i>ΔopuCA</i> pCL55- <i>opuCA</i> ; Cam ^R	This study

Antibiotics were used at the following concentrations: for *E. coli* cultures: Kanamycin (Kan^R) 30 µg/ml, Ampicillin (Amp^R) 100 µg/ml, Gentamicin (Gent^R) 20 µg/ml, Chloramphenicol (Cam^R) 10 µg/ml; for *S. aureus* cultures: Chloramphenicol (Cam^R) 10 µg/ml.

Table S3. Primers used in this study

Number	Name	Sequence
ANG1785	3-EcoRI-OpuCA	CGGAATTCTCATGATTTATCATCTCCTATGTCACGTAC
ANG1821	5' XhoI OpuCA ATPase	TCGACTCGAGTTAAGTATTAAGCATTTAACGAAAAATTTATTCTGG
ANG1822	3' HindIII OpuCA ATPase	AGCTAAGCTTTCATTGTATCGTGATTGGTTAATCATTACACC
ANG1842	5-NheI-OpuCA-CBS (237-408)	CTAGGCTAGCGGACAAAAATAGACTGATTCAAGACCGTCCC
ANG1923	3-BamHI-SACOL0762	GATCGGATCCCTATTCTGATTTTTCATCTTCATCAGACTGTCC
ANG1924	5-XhoI-SACOL1752	TCGACTCGAGACAAAACATGAACAAATTTAGATTATATTGAATCG
ANG1925	3-BamHI-SACOL1752	GATCGGATCCCTATACTTCGTCAAACATTTGGCATATCAC
ANG2025	5' OpuCA comp (-643) XmaI	CCGGCCCGGGCAACAACATACAAGATGACACGAAACAACCAATGGC
ANG2027	3' OpuCA comp EcoRI	AATTGAATTCTCATGATTTATCATCTCCTATGTCACGTAC
ANG2028	5' OpuCA (-1000) EcoRV	GATATCGGTGCAAGCAGCTCGTCAGTTACG
ANG2029	3' OpuCA (10aa)	TATGTCACGTACAATTTTCGTTAAATGCTTAATACTTAACAT
ANG2030	5' OpuCA (10aa)	TTAACGAAAATTGTACGTGACATAGGAGATGATAAATCATGA
ANG2031	3' OpuCA (+1000) NotI	GGCCGCGGCCGCCCTCCCGTTAAATCTGTGCCATTATATCTAACACCTG
ANG2092	OpuCA-F283A-fw	CAAAAACGTGTTGATGCTATT gc TGTAGTAGATAGTAATAACCATTTAC
ANG2093	OpuCA-F283A-rev	GTAAATGGTTATTACTATCTACTAC gc AATAGCATCAACACGTTTTTG
ANG2094	OpuCA-F294A-fw	GATAGTAATAACCATTTACTAGGT gca TTAGACATTGAAGATATAAAATCAGGG
ANG2095	OpuCA-F294A-rev	CCCTGATTTATATCTTCAATGTCTAA atgc ACCTAGTAAATGGTTATTACTATC
ANG2096	OpuCA-Y319A-fw	GAGACACCATGCAACAACATATT gc TACCGTTCAAATTGATTCTAAATTAC
ANG2097	OpuCA-Y319A-rev	GTAATTTAGAATCAATTTGAACGGTAG gc AATATGTTGTTGCATGGTGTCTC
ANG2098	OpuCA-Y365A-fw	GTGCCAATGTTGTTGATATTGT agc TGACACGATTTGGGGCGATAGTGAG
ANG2099	OpuCA-Y365A-rev	CTCACTATCGCCCCAAATCGTGTCA gc TACAATATCAACAACATTGGCAC
ANG2100	OpuCA-W369A-fw	GTTGTTGATATTGTATaTGACACGATT gca GGCGATAGTGAGGATACAGTGCAAA C
ANG2101	OpuCA-W369A-rev	GTTTGCACTGTATCCTCACTATCGCC tgca AATCGTGTCAtaTACAATATCAACAAC
ANG2102	OpuCA 364 EcoRI r	GATCGAATTC TTA TACAATATCAACAACATTGGCACGC
ANG2103	OpuCA 373 EcoRI r	GATCGAATTC TTA CTCACATCGCCCCAAATCGTGTC
ANG2363	5-XhoI-SACOL0762-trunc_162	TCGACTCGAGTCTGCACGTGTTATTATTAGAATGTTTGGTGTAATCC
ANG2364	5-XhoI-SACOL0921-trunc_140	TCGACTCGAGACGGACAGTATTAATCGAAGTTTATCTAAGGGCCAAC
ANG2365	3-BamHI-SACOL0921	GATCGGATCCTTATATAGATACGTTGCGATTTTCCGTTGTTGAAATTG
ANG2366	5-XhoI-SACOL1013	TCGACTCGAGTCAATGAACACAGATGAAAAAGAGCGTGTCAAGAG
ANG2367	3-BamHI-SACOL1013-trunc_294	GATCGGATCCCTAACGTTTTTAATGCTGTTTAAATGATTGAATCATTAG

Restriction sites in primer sequences are underlined and mutated bases are shown in bold and lower case letters.