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Cell wall stress activates expression of a novel stress response facilitator (SrfA) under σ^{22} (AlgT/U) control in *Pseudomonas aeruginosa*

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The ECF (extracytoplasmic function) alternative sigma factor, σ^{22} (AlgT/U), is required for expression of the *algD* promoter of the operon for alginate biosynthesis in *Pseudomonas aeruginosa*. Alginate production promotes chronic pulmonary infections by this opportunistic pathogen in patients with cystic fibrosis and chronic obstructive pulmonary disease. σ^{22} is normally sequestered, but its deregulation and activation occur either by mutation in *mucA* (encoding an anti-sigma factor) or in response to envelope stress, such as inhibition of peptidoglycan synthesis. The σ^{22} stress response system includes many genes in addition to those for alginate. In the present study, we characterized an intergenic region between ORFs PA2559 and PA2560 in PAO1 for a σ^{22}-dependent, stress-responsive transcript, described here as PA2559.1. Northern analysis and transcript end-mapping indicated the PA2559.1 transcript was ~310 nt in length. Examination of the DNA sequence upstream of +1 revealed a σ^{22} core promoter motif, GAATTT-N_{16}-TCTGT, and site-directed mutagenesis confirmed this to be a σ^{22}-dependent promoter that was highly activated during cell wall stress. PA2559.1 also contained an ORF that demonstrated increased translational activity upon cell wall stress. As determined by mutant analysis, the protein encoded by PA2559.1 was shown to play a positive role in the σ^{22}-dependent activation of the *algD* promoter under stress in both sessile (i.e. biofilm) and planktonic conditions. Thus, it appeared to act as a stress response facilitator and so was named SrfA. The sequence of SrfA was found to be novel in nature and extremely well conserved only in *P. aeruginosa*, suggesting that it is of high evolutionary importance in this species.

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INTRODUCTION

The cell envelope of Gram-negative bacteria provides the first line of defence against harsh environmental conditions for these organisms. In addition to the intrinsic physical properties of the envelope that provides a barrier to destructive agents, stress response systems monitor the integrity of this compartment and respond to inducing cues. The stress response adjusts gene expression to maintain the critical functions performed in the envelope, including nutrient uptake, motility, peptidoglycan and lipopolysaccharide biosynthesis, as well as the elimination of toxic metabolic byproducts.

The Gram-negative bacterium *Pseudomonas aeruginosa* is a ubiquitous, nutritionally versatile opportunistic human pathogen that is capable of adapting to and thriving in a wide range of environmental niches, from the human lung airways to lettuce leaves. One weapon in its stress response arsenal is a repertoire of alternative sigma factors that control expression of sophisticated regulatory networks, which are in part responsible for *P. aeruginosa*'s adaptability, and thus importance in medical, agricultural and industrial settings. At least 19 of these alternative sigma factors belong to the ECF (extracytoplasmic function) subfamily and regulate activities within the envelope in response to environmental changes or stress (Lonetto *et al.*, 1994). One well-characterized member of the ECF sigma family in *P. aeruginosa* is σ^{22} (also known as AlgT and AlgU), which is essential for the production of alginate – a capsule-like exopolysaccharide (Jain & Ohman, 2004; Leid *et al.*, 2005; Ramsey & Wozniak, 2005). Alginate forms a protective barrier around *P. aeruginosa* cells that increases resistance to phagocytic killing, antibody-dependent bactericidal mechanisms and antibiotics (Govan & Deretic, 1996; Leid *et al.*, 2005; Pier *et al.*, 2001; Schwarzmann & Boring, 1971). Overexpression of alginate by *P. aeruginosa*

Abbreviations: ECF, extracytoplasmic function; IgR, intergenic region; MU, Miller units; RIP, regulated intramembrane proteolysis; sRNA, small RNA.

The GenBank/EMBL/DDBJ accession number for the stress response facilitator *srfA* transcript is BK008866.

results in a mucoid colony morphology, which is a common phenotype of strains infecting patients with cystic fibrosis and chronic obstructive pulmonary disease (Govan & Deretic, 1996; Murphy *et al.*, 2008). This is usually due to adaptive mutations in *mucA*, which encodes the anti-sigma factor of σ^{22} (Martin *et al.*, 1993). Transcriptional activation of the *algD* operon for alginate biosynthesis via σ^{22} activation can also occur by envelope stress such as inhibition of peptidoglycan biosynthesis, outer membrane perturbation or the overexpression of certain outer-membrane proteins. In the presence of such σ^{22}-inducing signals, an inner membrane protease called AlgW initiates a regulated intramembrane proteolysis (RIP) cascade that leads to the rapid degradation of MucA, which normally sequesters σ^{22} at the cytoplasmic membrane (Cezairliyan & Sauer, 2009; Qiu *et al.*, 2007, 2008; Wood & Ohman, 2009; Wood *et al.*, 2006). The destruction of MucA results in the release of σ^{22} and coordinate transcription of its regulon gene members, which include the *algD* operon.

In a transcriptome analysis of *P. aeruginosa* using Affymetrix DNA gene chips, we previously identified 293 ORFs in PAO1 that were upregulated twofold or more in response to cell wall stress induced by D-cycloserine and are under σ^{22} control (Wood *et al.*, 2006). In addition to the biosynthetic and regulatory genes required for the biosynthesis of alginate, members of the σ^{22} stimulon included genes encoding drug efflux pumps and mechano-sensitive channels, numerous lipoproteins predicted to have roles in outer membrane repair and/or maintenance, as well as genes involved in peptidoglycan and lipopoly-saccharide biosynthesis. However, most genes of the σ^{22} stimulon encoded hypothetical proteins for which there was limited biological information. In this study, we revisited this original transcription analysis to investigate an intergenic region (IgR) located between PA2559 and PA2560 that was dramatically activated for transcription by cell wall stress and appeared to be under σ^{22} control.

METHODS

Bacterial strains and culture conditions. Strains of *P. aeruginosa* used in this study are shown in Table 1. PAO1 (also known as PDO1) used in this study was a spontaneous chloramphenicol-sensitive revertant of the commonly studied PAO1 strain originally obtained from B. W. Holloway (Holloway, 1969). *Escherichia coli* DH5α was used for routine plasmid manipulations. Bacteria were routinely cultured in L broth (g l^{-1}: tryptone, 10; yeast extract 5; NaCl, 5) or on L agar (LB Miller agar; Fisher Scientific) at 37 °C. LPIA plates were a 1 : 1 mix of L agar and *Pseudomonas* isolation agar (Difco), and were used to counter-select against *E. coli* following conjugal transfers of plasmids to *P. aeruginosa*. Plasmids and their derivatives were maintained in *E. coli* and *P. aeruginosa* using antibiotics (Sigma) at the following concentrations: gentamicin, 20 and 100 μg ml^{-1} for *E. coli* and *P. aeruginosa*, respectively; tetracycline, 20 and 60 μg ml^{-1} for *E. coli* and *P. aeruginosa*, respectively; kanamycin, 30 μg ml^{-1}; ampicillin, 100 μg ml^{-1}; carbenicillin, 100 μg ml^{-1}; and chloramphenicol, 50 μg ml^{-1}.

Construction of *lacZ* transcriptional and translational reporter constructs. High-fidelity *Pfu* Turbo (Stratagene) was used to amplify DNA by PCR with primers listed in Table 2 synthesized by Eurofins MWG Operon using sequences available in the Pseudomonas Genome Database (Winsor *et al.*, 2009). PCR products were cloned into the broad-host range transcriptional *lacZ* reporter pSS269 or translational *lacZ* reporter pSS361. After sequence confirmation of the correct in-frame juncture between PA2559.1 and *lacZ* in pSS361, all reporter plasmids were transferred from *E. coli* to *P. aeruginosa* strains by triparental mating as described previously (Suh *et al.*, 2004) using helper plasmid pRK2013 to mobilize the *oriT*-containing plasmids to *P. aeruginosa* with selection on LPIA plates containing the appropriate antibiotic.

***β*-Galactosidase assays.** Transcriptional and translational activity of reporter constructs were determined by measuring β-galactosidase activity, reported in Miller units (MU). To determine activity in response to cell wall stress induced by D-cycloserine, strains were grown in 25 ml L broth at 37 °C to OD_{600} 0.3; cultures were split in half, D-cycloserine being added to one half (400 μg ml^{-1}) and then both halves were incubated for an additional 60 min, at which time 100 μl samples of untreated and treated cultures were assayed for β-galactosidase activity.

Generation of PA2559.1 promoter mutations. Site-directed mutants of the predicted −35 promoter site of PA2559.1 were generated using a QuikChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies). A small plasmid (pLW253, conducive in size for the mutagenesis protocol) was constructed by moving the *Eco*RI/*Bam*HI fragment from pLW210, containing the WT IgR fragment between PA2559 and PA2560, into pUC19 (*Eco*RI/*Bam*HI). Following sequence confirmation, this plasmid was used as a template for all mutagenesis reactions. Primers were designed to contain the appropriate nucleotide changes (Table 2) using PAO1 genomic sequences from the *Pseudomonas* Genome Database (Winsor *et al.*, 2009) and each construct was verified by DNA sequencing (ACGT DNA Technologies). Confirmed mutant fragments were excised by *Eco*RI/*Bam*HI digestion and ligated into the *lacZ* reporter plasmid pLW210 at *Eco*RI/*Bam*HI. Following sequence confirmation, the reporter constructs (pLW265–269) were moved by triparental mating into *P. aeruginosa* strains and tested for promoter activity in β-galactosidase assays as described above.

Isolation of RNA and Northern blot analysis. Total RNA was prepared with a miRNeasy Mini kit (Qiagen). Residual contaminating DNA was removed from RNA preparations using Ambion TURBO DNA-*free* (Life Technologies). RNA was fractionated on an 8 % urea/polyacrylamide gel (Ultrapure Sequagel System; National Diagnostics), and then transferred and cross-linked to a MagnaCharge nylon membrane (GE Water & Process Technologies). 5′-DIG-gene-specific oligonucleotides (Table 2) were used for detection of PA2559.1 and 5S RNAs following protocols using a DIG Luminescent Detection kit (Roche). Blots were exposed to X-ray film for 8 h before development. ImageJ software (http://imagej.nih.gov/ij/) was used to estimate the relative densities of the *srfA* bands.

PA2559.1 transcript end-mapping. The procedure for 5′ and 3′ RNA end-mapping was as described previously (Urban & Vogel, 2008), and was used here with some modifications. PAO1 and its *mucA* mutant (PDO351) were grown in L broth to OD_{600} 0.3 and then exposed to D-cycloserine (400 μg ml^{-1}) for 60 min. Total RNA was isolated from the cultures using a miRNeasy Mini kit (Qiagen) and treated with Ambion TURBO DNA-*free* (Life Technologies) to remove contaminating DNA. A 6.5 μg sample of RNA was treated with 5 U tobacco acid pyrophosphatase (Epicentre) for 1 h at 37 °C to convert triphosphates to monophosphates on the 5′ ends. Following phenol/chloroform extraction and ethanol precipitation, RNA was treated for 16 h with 10 U T4 RNA Ligase I (New England Biolabs) at 16 °C to allow circularization. After phenol/chloroform extraction and ethanol precipitation, 1.5–5 μg self-ligated RNA was reverse transcribed using a PA2559.1-RT primer (Table 2) and a

Table 1. *P. aeruginosa* strains and plasmids used in this study

All *P. aeruginosa* strains were derived from strain PAO1 (originally obtained from B. W. Holloway), but using a spontaneous Cm^S laboratory isolate called PDO1.

Strain or plasmid	Characteristic(s)	Source or reference
Strains		
PAO1/PDO1	WT, Cm^S	This laboratory
PDO-LS586	*algT*::*aacCI* (Gm^R)	Silo-Suh *et al.* (2002)
PDO351	*mucA*::*aacCI* (Gm^R) Alg^+	Wood *et al.* (2006)
PDO374	PAO1 ΔPA2559.1, Gm^R	This study
PDO375	PDO351 ΔPA2559.1, Tc^R	This study
Plasmids		
pCR2.1	Cloning vector, f1 *ori*, pBR322 *ori*, Ap^R Km^R	Invitrogen
pKK61	pCP19 ($oriV_{RK2}$, Tc^R) P*algD-cat*	Wood *et al.* (2006)
pLW149a	pSS269 P*algD-lacZ*	Wood & Ohman (2009)
pLW197	pSS269, IG2893827–2894451-*lacZ*	This study
pLW208	pSS269, IG2893827–2894007-*lacZ*	This study
pLW209	pSS269, IG2893827–2894127-*lacZ*	This study
pLW210	pSS269, IG2893827–2894247-*lacZ*	This study
pLW250	pUC19, ΔPA2559.1, *oriT*, Gm^R	This study
pLW252	pUC19, ΔPA2559.1, *oriT*, Tc^R	This study
pLW253	pUC19, IG2893827–2894247 WT	This study
pLW254	pLW253, $P_{PA2559.1}$ CAATTT	This study
pLW255	pLW253, $P_{PA2559.1}$ AAATTT	This study
pLW256	pLW253, $P_{PA2559.1}$ GCCTTT	This study
pLW257	pLW253, $P_{PA2559.1}$ GAACTT	This study
pLW258	pLW253, $P_{PA2559.1}$ AGGTTT	This study
pLW265	pLW210, $P_{PA2559.1}$ CAATTT	This study
pLW266	pLW210, $P_{PA2559.1}$ AAATTT	This study
pLW267	pLW210, $P_{PA2559.1}$ GCCTTT	This study
pLW268	pLW210, $P_{PA2559.1}$ GAACTT	This study
pLW269	pLW210, $P_{PA2559.1}$ AGGTTT	This study
pLW279	pSS361, PA2559.1 ORF-*lacZ* translational	This study
pRK2013	ColE1-Tra(RK2)$^+$, Km^R	Figurski & Helinski (1979)
pSS192	pUC1819, Tc^R	This laboratory
pSS223	*oriV* (ColE1), *trp-lacZ1* (transcriptional) Ap^R	Suh *et al.* (2004)
pSS231	*oriV* (ColE1), *lacZ2* (translational) Ap^R	Suh *et al.* (2004)
pSS269	pSS223, SF (*Pst*I site)	This laboratory
pSS361	pSS231, SF (*Pst*I site)	This laboratory
pUC19	*oriV* (ColE1), Ap^R	This laboratory
pUCGm	*aacCI*, non-polar Gm^R cassette	Schweizer (1993)

Alg^+, mucoid due to alginate overproduction; *lacZ*, β-galactosidase reporter in transcriptional and translational fusions; SF, stabilization fragment for replication in *P. aeruginosa*. Numbers preceding *lacZ* indicate genomic coordinates from the Pseudomonas Genome Database (www.pseudomonas.com).

SuperScript III First Strand Synthesis System kit (Invitrogen). A sample (10%) of the reverse transcription reaction served as template in a subsequent standard 50 μl PCR using 0.5 U *Taq* polymerase (Applied Biosystems) and primer pairs designed to amplify the ligated end junctions (Table 2). PCR products were gel purified using a QIAEX II Gel Extraction kit (Qiagen) and cloned into the pCR2.1 vector (Invitrogen). Plasmids from positive clones were sequenced with M13 reverse and M13 F(−20) primers (ACGT DNA Technologies).

Bioassay for P*algD* induction on solid media. Bioassays of PAO1 and mutant derivatives carrying reporter plasmid pKK61 (Wozniak & Ohman, 1991) containing the *algD* operon promoter fused transcriptionally to a gene for chloramphenicol acetyltransferase (P*algD-cat*) were performed as previously described (Wood *et al.*, 2006). Briefly, expression of P*algD-cat* was tested by incubating growing cultures to OD_{600} 1.2 and then a 1.5 ml sample was centrifuged; the bacterial cell pellet was resuspended in 10 mM $MgSO_4$ to OD_{600} 0.2. A 25 μl sample of this cell suspension was spread onto L agar plates containing chloramphenicol at 50 μg ml^{-1}. A 5 mm filter disc impregnated with 1 mg D-cycloserine (i.e. 10 μl of a 100 mg ml^{-1} stock solution) was placed in the centre of the plate, incubated at 25 °C for 3 days and examined for a ring of Cm^R growth around the disc, indicating induced expression of *algD-cat*.

Construction of PA2559.1 deletion strains. To construct a PAO1 ΔPA2559.1 mutant (PDO374) and PDO351 *mucA* ΔPA2559.1

Table 2. Oligonucleotides used in this study

Category/name	Sequence 5′→3′	Description
PA2559.1 deletion		
PA2559.1-KOF3-HIII	CATCTAAGCTTGCGCAGGTAGCTCTGCCGG	
PA2559.1-KOR3-Pst	CATAGCTGCAGCCATACCTGTACGCCAGCCG	
PA2559.1-KOF4	ATCAGCGCCACGCCGGGAG	
PA2559.1-KOR-RI	CATCTGAATTCAATTGCAGGTTGGCCTCGTCG	
Northern analysis		
PA2559.1-DIG	DIG-GGGTGTACTTGTCCTGGGGTTCAGCCAT	Northern probe
5S-DIG	DIG-TCAGGTGGTTCCAACGCTCTATGATCG	
Promoter mutagenesis		
$P_{2559\text{-}2560}$-Mut1F	CCGTCAGTTTTTTTTT**C**AATTTTTGCCATTGG	−35, CAATTT
$P_{2559\text{-}2560}$-Mut1R	CCAATGGCAAAAATT**G**AAAAAAAACTGACGG	−35, CAATTT
$P_{2559\text{-}2560}$-Mut2F	CCGTCAGTTTTTTTT**A**AATTTTTGCCATTGG	−35, AAATTT
$P_{2559\text{-}2560}$-Mut2R	CCAATGGCAAAAATT**T**AAAAAAAACTGACGG	−35, AAATTT
$P_{2559\text{-}2560}$-Mut3F	CCGTCAGTTTTTTTTG**CC**TTTTTGCCATTGG	−35, GCCTTT
$P_{2559\text{-}2560}$-Mut3R	CCAATGGCAAAAA**GG**CAAAAAAAACTGACGG	−35, GCCTTT
$P_{2559\text{-}2560}$-Mut4F	CCGTCAGTTTTTTTTGAA**C**TTTTGCCATTGG	−35, GAACTT
$P_{2559\text{-}2560}$-Mut4R	CCAATGGCAAAA**G**TTCAAAAAAAACTGACGG	−35, GAACTT
$P_{2559\text{-}2560}$-Mut5F	CCGTCAGTTTTTTTT**AGG**TTTTTGCCATTGG	−35, AGGTTT
$P_{2559\text{-}2560}$-Mut5R	CCAATGGCAAAAA**CCT**AAAAAAAACTGACGG	–35, AGGTTT
Transcript end-mapping		
PA2559.1-RT	GGGTGTACTTGTCCTGGGGTTCAGCCAT	
PA2559.1-F-end	TTCTCCTTGCGGGCCGGATG	
PA2559.1-R-end	GGCGCACAGGCAGGACCTG	
***lacZ* fusions**		
$IgR_{2559\text{-}2560}$-F	CATGCGAATTCATAGCATTTCCTTACTCGCAAAAC	
$IgR_{2559\text{-}2560}$-R1	CTGACGGATCCCCATACCTGTACGCCAGCC	
$IgR_{2559\text{-}2560}$-R2	CTGACGGATCCGGAAAAACGCTGCGCTGAACAG	
$IgR_{2559\text{-}2560}$-R3	CTGACGGATCCCTTCTGGCGAGCGGCGTCG	
$IgR_{2559\text{-}2560}$-R4	CTGACGGATCCGCCGACGCCAGGAGCACGC	
PA2559.1-ORF-F2trans	GACATCGGTACCAATAGCGAAG	
PA2559.1-ORF-R2trans	ACGGTTGTAGGTCGCCTGGTC	*lacZ* fusion at aa 25

(PDO375) mutant, PCR fragments containing ~800 bp of the DNA upstream and downstream of PA2559.1 were inserted into the multi-cloning site of pUC19, at *Hin*dIII/*Pst*I and *Eco*RI/*Sma*I, respectively. Between these two regions, either the non-polar *aacCI* (Gm^R) cassette from pUCGm (Schweizer, 1993) or the non-polar Tc^R cassette from pSS192 (Suh *et al.*, 2004) was inserted at *Xba*I. After confirmation of the correct forward orientation of transcription, the *P. aeruginosa oriT* sequence was inserted at *Ssp*I to allow for mobilization of the plasmids from *E. coli* to *P. aeruginosa.* The resulting allelic exchange constructs, pLW250 (Gm^R) and pLW252 (Tc^R), were moved by triparental mating into PAO1 or PDO351 (*mucA*::Gm^R), respectively, with selection for Gm^R or Tc^R, and screened for Cb^S. Northern analysis of total RNA was performed on resulting candidates to verify the absence of PA2559.1 mRNA.

Alginate production. To assay the amount of alginate secreted, cultures were grown for 24 h at 37 °C to confluence on three plates of L agar. The mucoid growth was resuspended in saline and then bacteria were removed by centrifugation. Supernatants were tested for alginate using a carbazole spectrophometric method for the assay of uronic acids (Knutson & Jeanes, 1968), with alginic acid from *Macrocystic pyrifera* (Sigma) used as the standard.

Disc diffusion assay. The relative level of resistance to antibiotics was determined by a standard disc diffusion assay. A sample (0.1 ml) of culture in the early exponential phase was spread onto an L agar plate and then a 5 mm paper disc impregnated with an antibiotic was placed in the centre to permit radial diffusion. After 24 h of incubation at 37 °C, the diameter of the ring of growth inhibition was measured.

Motility assays. Swimming and twitching motility were determined as described previously (Rashid & Kornberg, 2000) with some modifications. To assess twitching motility, L agar plates containing 1 % agar at 3 mm depth were stab-inoculated to the bottom of the Petri dish from 24 h L agar plates. After incubation at 37 °C for 24 h, the zone of motility at the agar/Petri dish interface was measured. To assess swimming motility, L agar plates containing 0.3 % agar were inoculated by deposition of 1 µl 24 h culture on the surface in the centre of the plate. Plates were covered with plastic wrap to prevent dehydration and incubated at 30 °C for 18–20 h, after which time the zones of motility were measured.

Biofilm assay. Static L broth cultures of strains were compared for their ability to adhere to the walls of polystyrene tubes after 24 h of incubation at 37 °C as described previously (Friedman & Kolter, 2004).

Prediction of protein structure. NovaFold (DNASTAR), a 3D protein structure prediction program, was used to build a structure model from a sequence file using iterative assembly simulations. The NovaFold client provides an interface for submitting structure

prediction tasks to a high-performance computing cluster on the Amazon EC2 web service. NovaFold uses the I-TASSER algorithm in predicting protein structure.

RESULTS AND DISCUSSION

Characterization of a novel σ^{22}-dependent transcript in *P. aeruginosa*

We previously employed microarray chips (Affymetrix) to define the set of 293 ORF genes in strain PAO1 that were both rapidly induced by cell wall stress and under σ^{22} control; these included the genes for the production of an important virulence factor – alginate (Wood *et al.*, 2006). Affymetrix arrays also contain a limited number of oligonucleotide probe sets complementary to ~100 *P. aeruginosa* IgRs, which are useful in identifying non-coding regulatory small RNAs (sRNA). We observed high RNA expression in response to cell wall stress within an IgR located between the two annotated ORFs PA2559 (19.3 kDa) and PA2560 (10.4 kDa), which encode hypothetical proteins (unpublished data). Transcription within this 623 bp DNA sequence was directionally opposite to that of the adjacent genes and increased 14-fold following 15 min exposure to D-cycloserine (400 μg ml^{-1}) to induce cell wall stress when compared with transcript levels in untreated PAO1. As a cyclic analogue of D-alanine, D-cycloserine is an antibiotic that inhibits cell wall biosynthesis by acting on alanine racemase and D-alanine : D-alanine ligase. Elevated IgR transcription was not observed in an *algT* (i.e. σ^{22}-deficient) mutant. Studies were undertaken here to verify this observation using microarray chips, and to characterize the newly identified σ^{22}- and stress-dependent transcript in this IgR, called PA2559.1.

In order to confirm that this transcriptional activity was indeed σ^{22}-dependent and stress-responsive, the 623 bp IgR sequence was cloned into pSS269 – a broad-host range *lacZ* transcriptional reporter vector (Table 1). The resultant PA2559.1-*lacZ* construct (pLW197) was tested for β-galactosidase activity (MU) in PAO1 with and without exposure to cell wall stress. A moderate basal activity level (398 MU) was observed from PA2559.1-*lacZ* in PAO1(pLW197) under non-stress growth conditions, but was elevated 33.4-fold (to 13 293 MU) following 60 min of exposure to D-cycloserine (400 μg ml^{-1}) (Fig. 1). Consistent with the expected expression pattern for a gene under σ^{22} control, the β-galactosidase activity level in a *mucA* mutant (PDO351) lacking the σ^{22} anti-sigma factor, PA2559.1-*lacZ* expression was eightfold higher (3198 MU) than that detected in PAO1 under routine laboratory conditions. However, it was interesting that PA2559.1-*lacZ* expression was fourfold higher following D-cycloserine treatment (13 293 MU) compared with constitutive σ^{22} activation due to *mucA* mutation (3198 MU); this suggested that other factors may activate PA2559.1 transcription in addition to deregulated σ^{22}. In addition, PA2559.1-*lacZ* expression in an *algT* mutant lacking σ^{22} was at the lower limit of detection with stress (53 MU) or without stress induction (22 MU). These results confirmed the identification of a stress-inducible, σ^{22}-dependent novel transcript within the IgR between PA2559 and PA2560 called PA2559.1. We suspected that this could be a regulatory sRNA under σ^{22} control, similar to those described under ECF σ^{E} in *E. coli* (Johansen *et al.*, 2006).

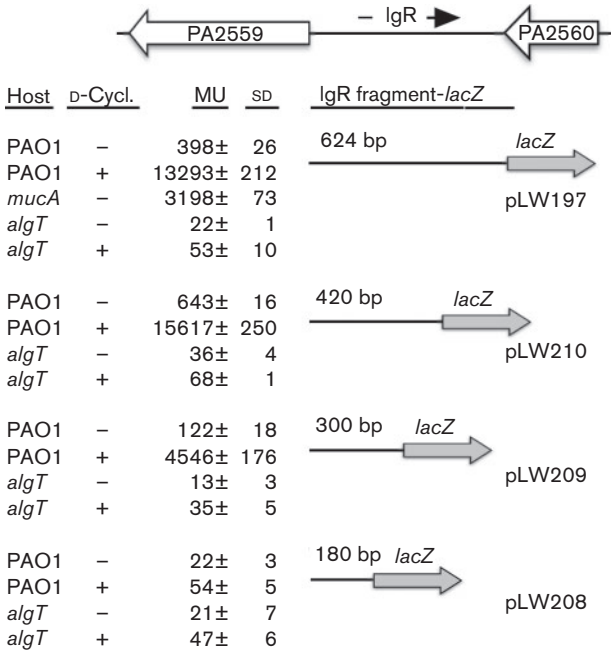


Fig. 1. Transcriptional analysis of the IgR between PA2559 and PA2560 (PA2559.1). A map of the IgR region of interest is shown at the top, which contains a novel transcript in the σ^{22} stimulon. Aligned below are the relative locations of DNA fragments from this region that were cloned into the *lacZ* transcriptional reporter vector, pSS269. The size of the insert (bp) and name of each plasmid are indicated. Hosts were PAO1 (WT), PDO351 (*mucA* mutant) or PDO-LS586 (*algT* mutant). 'D-Cycl.' indicates whether or not (+ or –) cultures were treated with D-cycloserine (400 μg ml^{-1}) for 15 min before β-galactosidase activities were assayed (MU). Experiments were performed in triplicate and values represent mean ± SD.

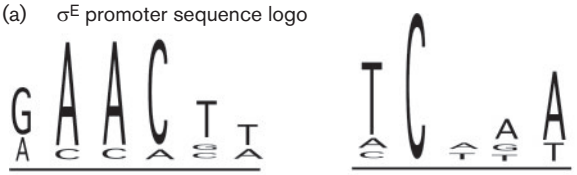
To begin to determine the location of the promoter for PA2559.1, a deletion analysis was performed on the IgR by constructing incrementally shorter clones in the same *lacZ* transcriptional reporter vector, pSS269 (Fig. 1). When activity levels were measured from the 5′ 420 bp sequence (pLW210), no change was observed in the expression pattern, although there was a slight increase overall. Analysis of the expression from 300 bp of the 5′ IgR sequence (pLW209) also showed the predicted expression pattern for a σ^{22}-dependent promoter, although overall expression with this construct was reduced. Following treatment with D-cycloserine, a 37-fold increase in expression from PAO1(pLW209) was observed (from 122 to 4546 MU).

Activity from pLW209 in an *algT* mutant (i.e. lacking σ^{22}) was very low under non-stress and stress conditions (13 and 35 MU, respectively), suggesting that this 300 bp fragment still contained a σ^{22}-dependent promoter. Consistent with this hypothesis, expression from pLW209 was eightfold higher in a *mucA* mutant (PDO351 lacking the anti-sigma factor) when compared with levels measured in PAO1(pLW209) not exposed to envelope stress (data not shown). However, β-galactosidase activity from a shorter 180 bp construct (pLW208) was essentially negligible under all conditions (Fig. 1). These results suggested that a σ^{22}-dependent, stress-responsive promoter resided in the region between 180 and 300 bp of this IgR, which is defined by the PAO1 genome coordinates as nucleotides 2 893 926–2 894 034.

Mutational analysis of the predicted PA2559.1 promoter

Upon close examination of the stress-responsive promoter region of PA2559.1 identified above, a putative σ^{22} core promoter recognition sequence (−35 and −10 motif) was observed: GAATTT-N_{16}-TCTGT. This is a close match to the known consensus promoter sequence described for σ^{22}: GAACTT-$N_{16/17}$-TCTnn (Firoved *et al.*, 2002). *P. aeruginosa* σ^{22} is a homologue (66 % identity) of the well-characterized *E. coli* ECF alternative sigma factor, σ^{E} (DeVries & Ohman, 1994), and both sigma factors respond to envelope stress via RIP. *P. aeruginosa* σ^{22} is sufficiently functional in *E. coli* such that it can activate σ^{E} promoters (Yu *et al.*, 1995), and both recognize similar core promoter motifs. A library of 60 σ^{E}-controlled promoters has been analysed to develop a sequence logo for σ^{E} promoter binding (Rhodius *et al.*, 2006). Such logos display promoter DNA sequences as stacks of possible nucleotides found at each position in the binding site, where the relative height of individual letters indicates their frequency in the DNA sequences compared (Fig. 2a). In light of the high degree of similarity between the σ^{E} and σ^{22} consensus binding patterns, the σ^{E} logo was used as a guide to identify important determinants for mutational analysis of σ^{22} recognition and activation of the proposed PA2559.1 promoter. Based on the high sequence conservation of the first 4 nt in the σ^{E} −35 sequence logo, variants at these positions were constructed in the 420 bp fragment (pLW210), cloned into the *lacZ* transcriptional reporter and moved by conjugation into PAO1 as well as the *algT* or *mucA* mutants. When tested under routine growth conditions, *lacZ* transcriptional fusion activity from the PA2559.1 native promoter increased sixfold (from 643 to 3958 MU) in the *mucA* mutant (PDO351, where σ^{22} is deregulated) compared with WT PAO1 and activity was very low (36 MU) in the *algT* mutant (PDO-LS586, devoid of σ^{22}) (Fig. 2b). Cell wall stress increased β-galactosidase activity in PAO1(pLW210) 24-fold (from 643 to 15 617 MU). Changing the first nucleotide from G to C (pLW265) resulted in a low level of promoter expression in all strains compared with the WT native promoter. Expression from pLW265 was nearly identical in WT (40 MU) and the *algT* mutant (32 MU), suggesting loss of σ^{22} control as a result of this single base pair change. Upon cell wall stress, expression from pLW265 was severely dampened compared with WT pLW210 levels under stress, but expression still increased 14-fold when stressed (548 MU) compared with unstressed (40 MU). Interestingly, a substitution of A for G at position 1 (pLW266), which is less favoured in the σ^{E} logo, resulted in lower expression overall, but caused little difference between

(a) σ^{E} promoter sequence logo



(b) σ^{22} promoter sequence mutations-*lacZ*

Host	D-Cycl.	MU	SD	−35	−10
PAO1	−	643 ±	16	GAATTT	TCTGT
PAO1	+	15617 ±	250		pLW210
algT	−	36 ±	4		
mucA	−	3958 ±	1		
PAO1	−	40 ±	4	GAATTT	TCTGT
PAO1	+	548 ±	20		pLW265
algT	−	32 ±	10		
mucA	−	105 ±	4		
PAO1	−	541 ±	63	AAATTT	TCTGT
PAO1	+	2123 ±	117		pLW266
algT	−	423 ±	43		
mucA	−	737 ±	90		
PAO1	−	39 ±	2	GCCTTT	TCTGT
PAO1	+	97 ±	10		pLW267
algT	−	38 ±	0.5		
mucA	−	56 ±	3		
PAO1	−	73 ±	6	AGGTTT	TCTGT
PAO1	+	134 ±	4		pLW269
algT	−	83 ±	17		
mucA	−	80 ±	1		
PAO1	−	6278 ±	609	GAACTT	TCTGT
PAO1	+	35501 ±	2170		pLW268
algT	−	26 ±	3		
mucA	−	11150 ±	148		

Fig. 2. Mutational analysis of the putative σ^{22} promoter found in PA2559.1. (a) The *E. coli* σ^{E} promoter sequence logo, which was used to guide mutagenesis of the putative σ^{22} promoter. (b) σ^{22} promoter sequence mutations. Base pair substitutions made in the −35 region are underlined and all are in the pLW210 (420 bp) fragment for comparison. The name of each plasmid is indicated. Hosts were PAO1 (WT), PDO-LS586 (*algT* mutant) or PDO351 (*mucA* mutant). 'D-Cycl.' indicates whether or not (+ or −) cultures were treated with D-cycloserine (400 μg ml^{-1}) for 60 min before β-galactosidase activities were assayed (MU). Experiments were performed in triplicate and values represent mean ± SD.

WT (541 MU) and the *algT* mutant (423 MU). Again, stress-induced activation was strong (2123 MU), suggesting that this new synthetic promoter may still be recognized by σ^{22}. More drastic sequence changes in positions 1, 2 and 3 (pLW267, pLW269) that completely alter the σ^{E} consensus resulted in loss of any significant activity from these promoters under all conditions tested. This suggested that there is no second promoter site recognized by another sigma factor. A substitution was also made at position 4 (pLW268) that changed T to C at the −35 site, which improved consensus to the motif of σ^{E}; this resulted in a greater than twofold enhancement of activity in all strains, except in the *algT* mutant lacking σ^{22}, which showed that this promoter was under strict σ^{22} control. Overall, this promoter analysis of PA2559.1 suggested that it is highly dependent on σ^{22} for expression, with and without stress. However, other unknown factors may also play a role during the stress response, in that expression was higher under stress than in a *mucA* mutant where σ^{22} is presumed to be totally deregulated. The discovery of such stress-induced positive factors will be part of future studies.

PA2559.1 RNA end-mapping

A Northern blot analysis was used to determine the approximate size of the PA2559.1 transcript. Total cellular RNA was harvested from PAO1 with and without exposure to D-cycloserine, and from the *mucA* mutant (PDO351) grown under routine laboratory conditions (i.e. L broth, 37 °C, aeration to OD_{600} 1.0). Equal concentrations of fractionated RNA and standards were transferred to a nylon membrane and probed with a 5′-DIG-PA2559.1-specific oligonucleotide and a 5′-DIG oligonucleotide designed to detect 5S RNA as an internal control (Table 2). A unique PA2559.1 band migrating just above the 300 nt standard was detected in all strains (Fig. 3), although barely detectable in unstressed PAO1 under these conditions (lane 1). ImageJ software was used to measure the intensity of the PA2559.1 bands, which showed around ninefold greater expression when PAO1 was treated with D-cycloserine (lane 2) and around fourfold greater expression in the *mucA* mutant (lane 3) as compared with unstressed PAO1 (lane 1).

In order to identify the +1 and last nucleotide(s) of the PA2559.1 transcript, 5′ and 3′ RNA end-mapping was performed as described previously (Urban & Vogel, 2008) with some minor modifications. Briefly, RNA used in the Northern blot above was self-ligated to form circularized RNA molecules with their 5′- and 3′-end sequences juxtaposed. Following reverse transcription of this RNA population, the resulting cDNA was cloned and sequenced with PA2559.1-specific primers (Table 2) across the end junction to identify the 5′- and 3′-end positions. Of the 15 candidates sequenced, all but one identified the same G nucleotide located just downstream of the −10 motif as the +1 start of transcription (Fig. 4a). The majority of the transcripts (13 out of 15) terminated within a 13 nt region,

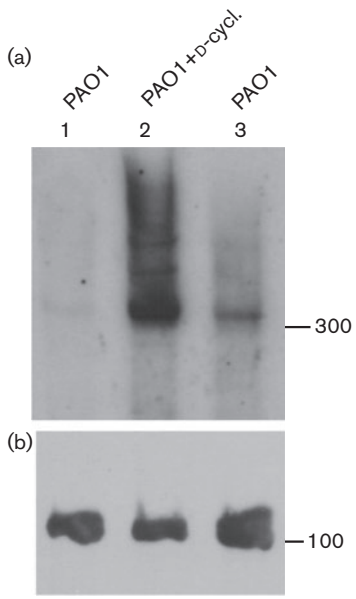


Fig. 3. Northern blot analysis of the PA2559.1 transcript in the IgR. (a) A DIG-labelled oligonucleotide probe specific for PA2559.1 was used to detect relative amounts of transcripts from this region. The migration location of the 300 nt marker is indicated. (b) A DIG-labelled probe for 5S RNA was used as a positive control for equal loading of total RNA. Lane 1, PAO1; lane 2, PAO1 treated with D-cycloserine (D-cycl.) for 60 min; lane 3, PDO351 (PAO1 *mucA* mutant).

from +303 to +316 relative to +1. Two transcripts were found to be slightly longer with the addition of 31 and 59 nt, suggesting some occasional RNA polymerase read-through could occur or that a longer transcript could be processed. Close examination of the 3′ sequence of this transcript did not uncover any sequences necessary for the formation of a stem–loop typical of intrinsic termination (i.e. GC-rich inverted repeat followed by a stretch of consecutive thymidines), suggesting that PA2559.1 transcript termination may be factor dependent.

PA2559.1 contains a protein-encoding ORF

Non-coding sRNAs have been found in unannotated IgRs of multiple bacterial species that vary in size between 50 and 400 nt (Argaman *et al.*, 2001; Livny & Waldor, 2007). sRNAs are also known to play regulatory roles in many stress responses in bacteria and so we sought to determine whether this was the case for PA2559.1. However, when we examined its ~310 nt transcript for potential translational signals, we found a putative ATG start codon at +43 that was preceded by an appropriately positioned ribosome-binding site (AGGA) and followed by a TGA stop codon at +229 (Fig. 4a). In order to determine whether PA2559.1 RNA was translated, DNA from −78 to +113 bp that included the promoter and the first 24 putative codons was cloned in-frame with the *lacZ* gene into the broad-host range

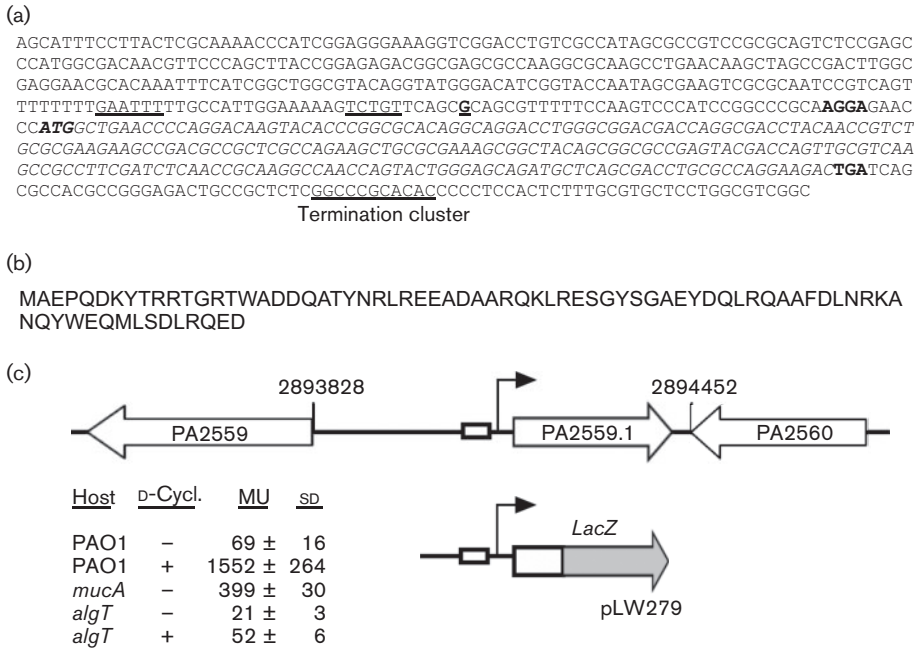


Fig. 4. Transcriptional and translational analysis of PA2559.1. (a) The entire sequence of the IgR between PA2559 and PA2560 is shown. Underlined nucleotides indicate the −35, −10, start of transcription (bold G) and termination cluster. The ribosome-binding site (AGGA), start of translation (ATG) and termination (TGA) are in bold. The ORF of PA2559.1 is in italics. (b) Amino acid sequence of the 9 kDa protein encoded by PA2559.1. (c) The DNA fragment (−78 bp to +113 bp) that was translationally fused to *lacZ* is shown relative to the start of transcription (thin arrow) in the PA2559.1 region. Hosts were PAO1 (WT), PDO351 (*mucA* mutant) or PDO-LS586 (*algT* mutant). 'D-Cycl.' indicates whether or not (+ or −) cultures were treated with D-cycloserine (400 μg ml^{-1}) for 60 min before β-galactosidase activities were assayed (MU). Experiments were performed in triplicate and values represent mean ± SD.

translational reporter vector, pSS361. After sequence confirmation, the construct (pLW279) was moved into *P. aeruginosa* test strains. β-Galactosidase activity was at a low level (69 MU) in PAO1 cultures, barely detectable (21 MU) in the *algT* (σ^{22} knockout) strain, but increased sixfold (399 MU) in the *mucA* mutant (Fig. 4c). Also, translational activity increased markedly (22.5-fold) in PAO1(pLW279) when treated with D-cycloserine to activate σ^{22} activity. These results provided evidence that the PA2559.1 transcript encodes a previously unannotated protein of 9078 Da (Fig. 4b) in PAO1 that is synthesized in response to cell wall stress.

PA2559.1 acts as a stress response facilitator

The PA2559.1 protein sequence was novel and showed no homology to any protein of known function. In order to determine the biological function for this newly identified member of the σ^{22} stimulon, a PA2559.1-null mutant was constructed in PAO1 (PDO374) for analysis of alterations in stress-related phenotypes. This mutation showed no effect on bacterial growth under normal conditions (data not shown). In a recent study, we found that some σ^{22}-dependent genes affect the stress response itself, sometimes by playing a role in envelope homeostasis that is dependent upon planktonic or sessile conditions (Wood & Ohman, 2012). To determine whether PA2559.1 might have such a role in envelope homeostasis, we examined the PA2559.1 mutant for effects on σ^{22} activation, which responds with high sensitivity to disturbances in the cell wall. Expression of P*algD*, a well-characterized σ^{22}-dependent promoter for an important virulence factor, alginate, was examined under sessile conditions. We have previously reported a plate bioassay for P*algD* induction using a reporter plasmid (pKK61) that carries the promoter of the *algD* operon fused to a promoterless chloramphenicol resistance gene (P*algD-cat*) (Wood *et al.*, 2006). P*algD-cat* undergoes strong induction when PAO1 is subjected to cell wall-damaging agents such as D-cycloserine. When a culture of PAO1(P*algD-cat*) is spread onto an L agar plate containing chloramphenicol and then D-cycloserine is spotted in the centre of the plate, a zone of growth forms around the antibiotic due to activation of the P*algD-cat* fusion, which confers Cm^R (Fig. 5a). When the P*algD-cat* fusion is in a *mucA* mutant (PDO351), a lawn of growth forms on the chloramphenicol plate because σ^{22} is constitutively active and does not require stress induction (Wood & Ohman, 2012). However, the P*algD-cat* phenotype in the PA2559.1 mutant produced a weak ring of growth, indicating a reduced responsiveness to cell wall stress. This indicated

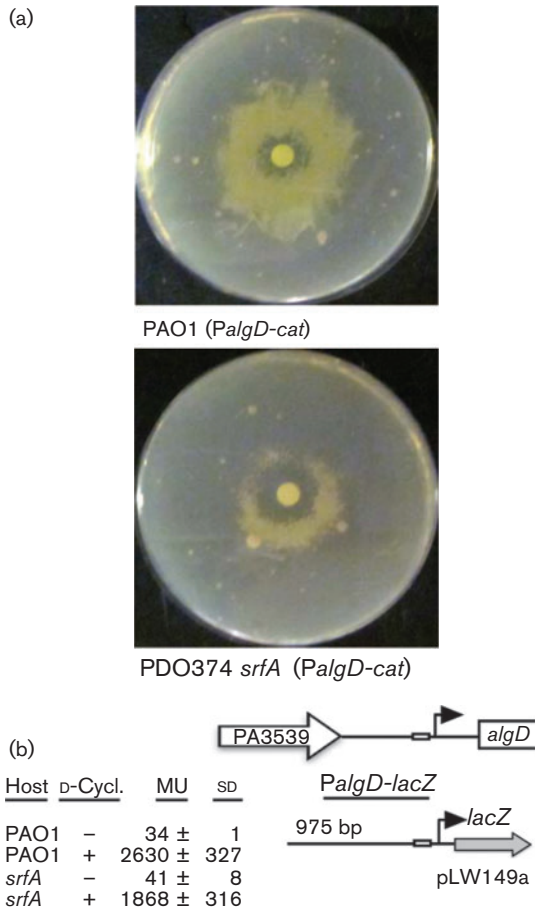


Fig. 5. Effect of a PA2559.1 (*srfA*Δ) mutation on the *algD* promoter for alginate biosynthesis, P*algD*. (a) A sessile growth assay was used to observe the effects of *srfA* mutation on the σ^{22}-dependent promoter, P*algD*. Shown are plates of L agar plus chloramphenicol coated with PAO1 (top) or PDO374 (*srfA*Δ mutant, bottom), both containing P*algD-cat* (pKK61), which confers Cm^R and thus growth when activated. A 5 mm filter disc impregnated with D-cycloserine (1 mg in 10 μl) was placed in the centre of the plate. Following incubation, PAO1 showed a large ring of Cm^R growth, whereas the *srfA*Δ mutant showed a weak reaction indicating reduced activation of the σ^{22}-dependent P*algD*. (b) A planktonic assay was used to observe the effects of *srfA* mutation on the σ^{22}-dependent promoter, P*algD*. Shown is a map of the σ^{22}-dependent promoter region upstream of the *algD* gene and the relative location of a 975 bp fragment (−925 to +50 relative to the start of transcription) from this region that was transcriptionally fused to *lacZ*. Hosts were PAO1 (WT) and PDO374 (*srfA* mutant). 'D-Cycl.' indicates whether or not (+ or −) cultures were treated with D-cycloserine (400 μg ml^{-1}) for 60 min before β-galactosidase activities were assayed (MU). Experiments were performed in triplicate and values represent mean ± SD. P*algD-lacZ* was consistently higher in the WT than in the PA2559.1/*srfA* mutant following stress activation with D-cycloserine.

that the PA2559.1 protein apparently acts a stress response facilitator and so its gene was given the name *srfA*. However, the effects of mutations in the hypothetical genes surrounding *srfA* (oppositely transcribed) have not yet been examined for responsiveness to cell wall stress.

To examine the effect of *srfA* mutation on P*algD* expression under planktonic conditions, a P*algD-lacZ* transcriptional fusion (pLW149a) was tested. In L broth without stress, PAO1 and the *srfA* mutant (PDO374) showed very low β-galactosidase activity (34 and 41 MU, respectively). When stressed with D-cycloserine, PAO1 P*algD-lacZ* showed a 77-fold increase in activity (from 34 to 2630 MU) (Fig. 5b). However, the *srfA* mutant PDO374 with P*algD-lacZ* consistently showed a lower (45-fold) increase in activity (from 41 to 1868 MU) upon stress. To rule out the possibility that *srfA* has a role in regulating the alginate operon apart from facilitating the stress response, an *srfA* mutation was made in a mucoid *mucA* mutant (PDO351) and it was still highly mucoid. Alginate production levels accumulating from cultures were quantitatively compared between the *mucA* and *mucA srfA* mutants, and they were indistinguishable (data not shown). Thus, the activity of this stress-responsive σ^{22} promoter, P*algD*, was reduced under stress conditions as a result of a *srfA* mutation under both planktonic and sessile plate (i.e. biofilm-like) conditions. This phenotype is similar to mutants with defects in two other σ^{22} stress-inducible genes: PA3459, encoding an osmoprotectant, and PA5107, encoding a putative membrane protein (Wood & Ohman, 2012).

As the σ^{22} regulon is highly activated following stress from peptidoglycan damage, the PAO1 *srfA* mutant (PDO374) was tested for changes in sensitivity to cell wall-inhibiting antibiotics (e.g. D-cycloserine, fosfomycin, carbenicillin and pipericillin), but no obvious differences were observed in a standard disc diffusion assay (data not shown). Mutants defective in their ability to recover from stress can be sensitive to elevated temperatures, yet when growth patterns were compared for PAO1 and PDO374 at 37 or 43 °C in L broth with aeration, PDO374 exhibited no alternation in temperature sensitivity (data not shown). Additionally, PA2559.1 mutant strains displayed no changes in microbial adherence to polystyrene tubes or in twitching and swimming motilities (data not shown).

SrfA encoded by PA2559.1 is highly conserved only within *P. aeruginosa*

PA2559.1 is a monocistronic locus encoding a 76 aa protein (9 kDa), here called SrfA for its role as a stress response facilitator. BLAST searches using the protein sequence showed that it is found in all sequenced *P. aeruginosa* strains, but not in any other species. Also, the SrfA amino acid sequence is 100 % conserved in nearly all of the strains in the *Pseudomonas* Genome Database (Winsor *et al.*, 2009). This high level of conservation suggests that SrfA has evolutionary importance in *P. aeruginosa*. Its synteny in the chromosome is also highly conserved; unfortunately, its adjacent genes (PA2559 and PA2560) have no known function that could shed light on SrfA's specific role as a stress response facilitator. A signal

peptide was not apparent nor was evidence for membrane translocation/insertion, so it is likely to be a cytoplasmic protein. SrfA contained no apparent DNA-binding motif. A prediction of its 3D structure was performed (using NovaFold), which suggested that SrfA is entirely α-helical in nature (molecular model not shown). For such a small protein with limited 3D structure, it is unlikely to have an enzymic function. Thus, SrfA may perform a role as an inhibitor or facilitator of protein–protein interactions, perhaps by providing chaperone functions. Future studies will continue to investigate the direct role of SrfA as a stress response facilitator.

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