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The cell density-dependent expression of stewartan exopolysaccharide in *Pantoea stewartii* ssp. *stewartii* is a function of EsaR-mediated repression of the *rcsA* gene

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Summary

The LuxR-type quorum-sensing transcription factor EsaR functions as a repressor of exopolysaccharide (EPS) synthesis in the phytopathogenic bacterium *Pantoea stewartii* ssp. *stewartii*. The cell density-dependent expression of EPS is critical for Stewart's wilt disease development. Strains deficient in the synthesis of a diffusible acyl-homoserine lactone inducer remain repressed for EPS synthesis and are consequently avirulent. In contrast, disruption of the *esaR* gene leads to hypermucoidy and attenuated disease development. Ligand-free EsaR functions as a negative autoregulator of the *esaR* gene and responds to exogenous acyl-homoserine lactone for derepression. The focus of this study was to define the mechanism by which EsaR governs the expression of the *cps* locus, which encodes functions required for stewartan EPS synthesis and membrane translocation. Genetic and biochemical studies show that EsaR directly represses the transcription of the *rcsA* gene. RcsA encodes an essential coactivator for RcsA/RcsB-mediated transcriptional activation of *cps* genes. *In vitro* assays identify an EsaR DNA binding site within the *rcsA* promoter that is reasonably well conserved with the previously described *esaR* box. We also describe that RcsA positively controls its own expression. Interestingly, promoter proximal genes within the *cps* cluster are significantly more acyl-homoserine lactone responsive than genes located towards the middle or 3′ end of the gene cluster. We will discuss a possible role of EsaR-mediated quorum sensing in the differential expression of the *cps* operon.

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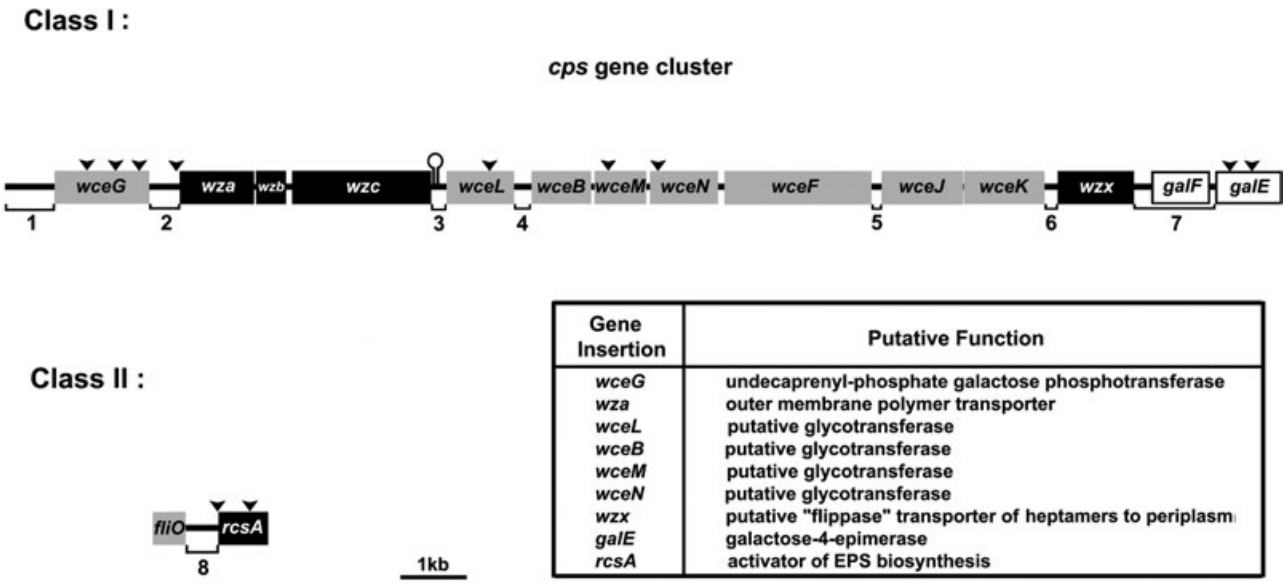
Introduction

Bacterial populations communicate and gauge their own population densities through production and perception of self-produced membrane diffusible or secreted autoinducer (AI) molecules in a process known as quorum sensing (QS) (Kaplan and Greenberg, 1985; Fuqua *et al*., 1996; Pearson *et al*., 1999). As a result, bacterial communities can co-ordinate and adjust the expression of specialized target genes in response to external AI concentrations. The paradigm for intraspecies-specific QS in Gram-negative bacteria is the LuxI/LuxR regulatory system that controls bioluminescence in the marine bacterium *Vibrio fischeri* (reviewed in Fuqua *et al*., 1994; 2001; Miller and Bassler, 2001). LuxI is a *N*-acyl-homoserine lactone (AHL) synthase. LuxR is an AHL-dependent transcriptional activator with affinity for a 20 base pair (bp) palindromic sequence, termed the *lux* box (Engebrecht and Silverman, 1987; Stevens and Greenberg, 1997). In contrast, intraspecies-specific QS in Gram-positive bacteria typically utilizes secreted oligopeptide AIs and cognate two-component transduction systems (Dunny and Leonard, 1997; Lazazzera *et al*., 1997; Kleerebezem and Quadri, 2001; Sturme *et al*., 2002). Both bacterial groups commonly also express a second type of QS system characterized by a LuxS signal synthase for production of furanone-based AI-2 signals and a LuxP/LuxQ two-component signal transduction system (Miller and Bassler, 2001; Chen *et al*., 2002; Henke and Bassler, 2004). AI-2 QS systems are thought to play a role in interspecies communication among mixed, natural bacterial communities (Federle and Bassler, 2003). The broad spectrum of physiological processes governed by various QS regulatory systems underscores their biological significance in supporting bacterial colonization of diverse niches including animal and plant hosts (Davies *et al*., 1998; Williams *et al*., 2000; Withers *et al*., 2001).

Pantoea stewartii ssp. *stewartii* (*P. stewartii*) is the aetiological agent of Stewart's wilt disease in maize. The bacterium colonizes the xylem of the plant host and produces large amounts of stewartan exopolysaccharide (EPS), a major factor in the cause of Stewart's vascular wilt (Leigh and Coplin, 1992). Mutants deficient in EPS synthesis are avirulent. Stewartan EPS is an acidic, high molecular weight polymer of heptameric oligosaccharide

repeat units that are composed of glucose, galactose, and glucuronic acid in a 3:3:1 proportion (Nimtz *et al*., 1996). The synthesis and translocation of EPS is encoded by a ~18 kilobase (kb), 14 gene *cps/galF/galE* DNA region, which is linked to the *rfb/his* chromosomal genetic locus analogous to other group I *cps* gene systems including the colanic acid biosynthetic operon in *Escherichia coli* (*E. coli*) (Coplin *et al*., 1992; Leigh and Coplin, 1992). The nomenclature of individual *cps* genes has been changed to conform to the proposed *wce* designation according to Reeves *et al*. (1996) (Fig. 1). The genetic conservation of these systems has allowed the putative assignment of *cps*-encoded functions in *P. stewartii* (Bernhard *et al*., 1993; Whitfield and Roberts, 1999; Nesper *et al*., 2003). Stewartan EPS is classified as a group 1 polysaccharide, in part, because polymerization initiation is undecaprenol-lipid carrier dependent, and the *cps* gene system is regulated by an RcsC/YojN/RcsB/A multicomponent phosphorelay signal transduction system (Gottesman and Stout, 1991; Kelm *et al*., 1997; Whitfield and Roberts, 1999; Takeda *et al*., 2001). RcsC is a transmembrane sensor kinase that responds to an unknown signal, possibly desiccation, changes in osmolarity and/or other membrane perturbations (Parker *et al*., 1992; Ophir and Gutnick, 1994; Sledjeski and Gottesman, 1996). YojN is an inner membrane protein that is thought to shuttle phosphoryl groups from the RcsC sensor kinase to the RcsB regulator (Takeda *et al*., 2001; Rogov *et al*., 2004). RcsB forms an activation complex with RcsA for the cooperative activation of promoters containing an RcsAB-specific binding sequence (Wehland *et al*., 1999). The expression of the *E. coli* RcsA coactivator is negatively regulated by H-NS, a transcriptional silencer, and positively by *DsrA*, a small RNA molecule that acts as an antisilencer (Sledjeski and Gottesman, 1995). Also, the RcsA protein is highly unstable in presence of a functional Lon protease (Stout *et al*., 1991).

Several previous studies confirmed that EPS synthesis in *P. stewartii* is Rcs-dependent (Torres-Cabassa *et al*., 1987; Bernhard *et al*., 1990; Wehland *et al*., 1999). However, in *P. stewartii,* QS regulation involving the EsaI signal synthase and AHL-responsive EsaR transcription factor is dominant to RcsAB-mediated activation of *cps* (Beck von Bodman and Farrand, 1995). Disruption of the *esaI* gene blocks the synthesis of AHL and EPS even in presence of a functional Rcs system. In contrast, a mutation in the



Gene Insertion	Putative Function
wceG	undecaprenyl-phosphate galactose phosphotransferase
wza	outer membrane polymer transporter
wceL	putative glycotransferase
wceB	putative glycotransferase
wceM	putative glycotransferase
wceN	putative glycotransferase
wzx	putative "flippase" transporter of heptamers to periplasm
galE	galactose-4-epimerase
rcsA	activator of EPS biosynthesis

Fig. 1. Gene organization of the EsaR controlled gene systems, *cps* and *rcsA*. Class I mutants are distributed throughout the EPS biosynthetic locus, *cps*; Class II mutants localized to the promoter or coding region of the *rcsA* gene (insertions are indicated by black arrows). The gene organization and putative functions of the *cps* gene cluster (Bernhard *et al*., 1993; Dolph *et al*., 1998) are shown with genes highlighted in grey encoding putative functions for precursor biosynthesis; genes indicated in black required for translocation and higher order polymerization. A putative stem-loop is shown immediately following the *wzc* gene. Heptameric repeat unit (3:3:1, glucose : galactose : glucuronic acid) synthesis initiates with the transfer of galactose-1-phosphate from UDP-galactose to an undecaprenyl phosphate lipid carrier catalysed by the membrane-localized WceG protein (grey). Additional *wce*-encoded glycosyltransferases (grey) complete heptamer polymerization through sequential addition of appropriate hexose constituents. A *wzx*-encoded function (black) 'flips' the lipid-linked heptamers across the plasma membrane into the periplasm. Further translocation and higher order polymerization requires the membrane-associated functions encoded by *wzc* and *wzb* (black). Export across the outer membrane and EPS surface assembly requires the *wza*-encoded *trans*-membrane protein complex (for detailed discussion of this process see Bernhard *et al*., 1993; Nesper *et al*., 2003). Class II mutants localize to the promoter or coding region of the *rcsA* gene, which encodes the RcsA transcription factor. The *rcsA* gene is located next to a gene with homology to *fliO*. Underlying brackets represent the primary *cps* promoter (indicated by the number 1) and potential internal *cps* promoters (numbers 2–7). The promoter region of the *rcsA* gene is indicated by the number 8.

esaR gene, or a double mutation in *esaI* and *esaR*, leads to maximal synthesis of EPS (von Bodman *et al*., 1998). These findings suggested that EsaR-mediated QS regulation functions by gene repression in a mechanism fundamentally different from the paradigm QS model of AHL-dependent gene activation (Beck von Bodman and Farrand, 1995).

Studies related to the autoregulatory role of EsaR provided experimental proof for a QS repressor mechanism. The promoter of the *esaR* gene features a well-conserved *lux* box-like palindrome, the *esaR* box, which spans the predicted –10 region of a σ^{70} promoter consensus sequence (Beck von Bodman and Farrand, 1995). Genetic and biochemical evaluation of EsaR function at the *esaR* promoter differentiates EsaR from the LuxR paradigm in three fundamental aspects. First, EsaR dimerizes and becomes DNA binding competent in absence of the cognate AHL signal (Qin *et al*., 2000). Second, EsaR exhibits reduced affinity for the *esaR* box DNA target in presence of AHL ligand. Third, EsaR represses an *esaR* reporter gene fusion, and exogenous addition of AHL promotes dose-dependent derepression (von Bodman *et al*., 1998; Qin *et al*., 2000; Minogue *et al*., 2002).

This study focused on defining the mechanism by which EsaR governs EPS synthesis by gene repression. We utilized an unbiased random transposon mutagenesis approach to consider all potential EsaR regulatory scenarios. This approach yielded two classes of transposon insertion mutations, one that localized to genes within the *cps* biosynthetic locus, and the other to the *rcsA* regulatory gene. Genetic experiments and DNA binding studies detailed here allow us to conclude that EsaR functions as a transcriptional repressor of the *rcsA* gene by binding to an imperfect palindromic DNA sequence located in the *rcsA* promoter. We also show that RcsA is positively autoregulated and that maximal expression of the *rcsA* gene requires AHL inducing conditions.

Results

Insertional mutagenesis of ESΔIR

We reported previously that EsaR, the QS regulator of *P. stewartii,* governs the autoregulation of its own gene, *esaR*, and the cell density-dependent synthesis of EPS by transcriptional repression and AHL-dependent derepression (von Bodman *et al*., 1998; Minogue *et al*., 2002). These studies did not resolve whether repression of EPS synthesis was by direct EsaR control of the *cps* gene cluster, by indirect control through the Rcs phosphorelay system or by other potential intermediary or alternate regulatory pathways. None of the *cps* and *rcs* promoters revealed obvious conserved *esaR* box-like DNA sequences, even though EsaR genetically controlled different *rcsA* and *cps* reporter gene fusions. We therefore mutagenized the *esaI*$^-$*, esaR*$^-$ double mutant, hypermucoid ESΔIR strain with the Tn*5gfp-km* transposon (Tang *et al*., 1999) to locate EsaR controlled genes with a role in EPS synthesis. A screen of approximately 40 000 kanamycin resistant transconjugants yielded nearly 300 EPS deficient mutants that actively expressed the transposon-encoded promoterless green fluorescent protein (GFP) gene. Of these, 11 mutants showed a significant reduction in GFP fluorescence after coexpression of a functional *esaR* gene from plasmid pSVB60. These 11 EPS deficient, GFP positive, EsaR responsive mutants were selected for further study.

EPS deficient strains carry insertions primarily in the cps locus and the rcsA *gene*

Genomic DNA, separately isolated from the 11 mutant strains, was subcloned into pBluescript II SK$^+$ and expressed in *E. coli* DH10B. DNA isolated from kanamycin resistant, GFP positive transformants was sequenced with a set of transposon-specific primers (Table 1) to determine the flanking sequences of each insertion. NCBI BLAST searches revealed two classes of mutants designated class I and II. Class I mutants localized to the *cps* gene cluster, while class II mutants carried allelic insertions in the *rcsA* gene (Fig. 1). All of the class I mutants were readily complemented with the pES2144 plasmid that carries the entire *cps* gene system plus *galF* and *galE*. All of our class II mutants were complemented with plasmid pES4507 that carries a wild type copy of the *rcsA* gene. These data are consistent with our original model that EsaR governs the negative control of *cps* genes directly and/or indirectly through control of *rcsA*.

Relative expression of rcsA *and* cps *genes under AHL-limiting and AHL-inducing conditions*

The location of the transposon insertions (Fig. 1) suggested that QS represses EPS synthesis through direct interaction with the *rcsA* promoter, and potentially, with select promoters of the *cps* gene system. If the expression of the *cps* genes depends strictly on RcsA, then the transcript levels of genes within the *cps* locus should increase in parallel with *rcsA* transcription in response to *N*-(3-oxohexanoyl)homoserine lactone (3-oxo-C6-HSL) induction. Real time reverse transcription polymerase chain reaction (RT-PCR) of cDNAs generated from total mRNA isolated separately from 3-oxo-C6-HSL induced and uninduced strain ESN51 (*esaI::kan, esaR*$^+$) allowed us to measure the relative transcript induction levels of the *rcsA, rcsB* regulatory genes, and the *cps*-encoded structural genes *wceG, wza, wceL* and *galE* against a 16S rRNA

Table 1. Oligonucleotides and primers.

Primer	Sequence	Introduced restriction site	Used for
P*rcsA*5	5′-ccataggatccaaattcacaactatcc	*Bam*HI	EMSA (Fig. 4A), deletion mutation (Fig. 3A)
P*rcsA*3	5′-aagctaagcttgatgatagtggacagac	*Hin*DIII	EMSA (Fig. 4A)
P*rcsA*3-1	5′-acaccaagcttgggagcaatgtcactat	*Hin*DIII	EMSA, DNase I protection Assays (Fig. 5A)
P*wceG*5	5′-aagctaagcttgatgatagtggacagac	*Hin*DIII	EMSA (Fig. 4A)/promoter *lacZ* fusion
P*wceG*3	5′-gcataaagctttctttattttatttcct	*Hin*DIII	EMSA (Fig. 4A)/promoter *lacZ* fusion
P*wceL*5	5′cacgaggatccaaggcgctaagtgagaa	*Bam*HI	EMSA (Fig. 4A)/promoter *lacZ* fusion
P*wceL*3	5′-atggaaagcttgtgtgattccttaaatc	*Hin*DIII	EMSA (Fig. 4A)/promoter *lacZ* fusion
P*wceB*5	5′-tgactggatcctcaacccggcgatcgtc	*Bam*HI	EMSA (Fig. 4A)/promoter *lacZ* fusion
P*wceB*3	5′-gaatgaagcttattgccagcacctcatt	*Hin*DIII	EMSA (Fig. 4A)/promoter *lacZ* fusion
P*wza*5	5′-atggtggatcctaacccgcagaaaaagg	*Bam*HI	EMSA (Fig. 4A)
Pwza3	5′-catttaagctttaatcatttcgctcttc	*Hin*DIII	EMSA (Fig. 4A)
P*rcsA*(60)5	5′-catcgggatccttgttttggtcataaaa	*Bam*HI	EMSA (Fig. 4B)
P*rcsA*(60)3	5′-acaagaagcttcacacaatatttttct	*Hin*DIII	EMSA (Fig. 4A)
P*wzx*5	5′-tgactggatcctcaacccggcgatcgtc	*Bam*HI	Promoter lacZ fusion
P*wzx*3	5′-gaatgaagcttattgccagcacctcatt	*Hin*DIII	Promoter lacZ fusion
RcsAΔup	5′-gcgaccctcacgaattcgttatc	*Eco*RI	Deletion mutation (Fig. 3A)
RcsAΔlow	5′-acgctgaattctctccttagca	*Eco*RI	Deletion mutation (Fig. 3A)
RcsAlow	5′-gttctaagcttcggcaaactatcttacg	*Hin*DIII	Deletion mutation (Fig. 3A)
Tn5Seq	5′-cagtttgtttcagttaaaac		Sequencing ESΔIR Tn5 mutants (Fig. 1)
*RcsA*1	5′-acatgtctccgcgtatttcc		Real time RT-PCR (Fig. 2)
*RcsA*2	5′-atgacccgacatccacattt		Real time RT-PCR (Fig. 2)
*WceG*1	5′-ctgaaattccgttcgatggt		Real time RT-PCR (Figs 2 and 3B)
*WceG*2	5′-gcataaagctttctttattttatttcct		Real time RT-PCR (Figs 2 and 3B)
*Wza*1	5′-tatattggtcgggtccgtgt		Real time RT-PCR (Figs 2 and 3B)
*Wza*2	5′-cgcaccggttacgtaagttt		Real time RT-PCR (Figs 2 and 3B)
*WceL*1	5′-gctctgtattgctgccatga		Real time RT-PCR (Figs 2 and 3B)
*WceL*2	5′-tcctcgaagaaactccggta		Real time RT-PCR (Figs 2 and 3B)
16*s*1	5′-gttagccggtgcttcttctg		Real time RT-PCR (Fig. 2)
16*s*2	5′-aggccttcgggttgtaaagt		Real time RT-PCR (Fig. 2)
*RseC*1	5′-cataccgaagccaaaacctc		Real time RT-PCR (Figs 2 and 3B)
*RseC*2	5′-attctttggccccagcttat		Real time RT-PCR (Figs 2 and 3B)
*EsaR*1	5′-tgaccgatccggttattctc		Real time RT-PCR (Fig. 2)
*EsaR*2	5′-aggtcggacatcagcgtaat		Real time RT-PCR (Fig. 2)
*GalE*1	5′-ggcattgcacagattatcca		Real time RT-PCR (Fig. 2)
*GalE*2	5′-ttacatcggctctcataccg		Real time RT-PCR (Fig. 2)
*RcsB*1	5′-ggtaacggaaattgcgaaga		Real time RT-PCR (Fig. 2)
*RcsB*2	5′-gttgagcagggcaatatcgt		Real time RT-PCR (Fig. 2)
FPprimer	5′-lightsabre green-caggaaacagctatgaccaatgatt		DNase I protection assays (Fig. 5A)
T7	5′-taatacgactcactataggg		DNase I protection assays (Fig. 5A)

internal calibrator. Transcripts were also measured for the *esaR* and *rseC* genes as representative internal standards for well-characterized 3-oxo-C6-HSL responsive and unresponsive genes, respectively. The *rseC* gene encodes an enhancer of the RpoE stress response sigma factor (Missiakas *et al*., 1997). Real time RT-PCR was performed using the specific sets of primers listed in Table 1. The data presented in Fig. 2 show that the relative *rcsA* transcript levels increased approximately fivefold under 3-oxo-C6-HSL inducing conditions, while those of *rcsB* remained largely unchanged. The *wceG* and *wza* genes, which represent the first and second genes of the *cps* gene cluster (see Fig. 1) yielded about five and eightfold enhanced transcript levels in response to 3-oxo-C6-HSL, respectively. The transcript level of *wceL*, a gene located in the middle of the *cps* locus, was nearly threefold higher, while the *galE* gene located at the 3′ end of the 18 kb *cps* gene cluster appeared to be expressed marginally higher in response to 3-oxo-C6-HSL. The transcript levels measured for *esaR* increased threefold, which is in good agreement with previous genetic induction studies (Minogue *et al*., 2002). As expected, 3-oxo-C6-HSL had no effect on the transcript levels of *rseC*. These data support the hypothesis that EsaR negatively controls the transcription of the *rcsA* gene, not *rcsB*, and affects the transcription of the *cps* genes, particularly those located closest to the *wceG* promoter (see Fig. 1).

RcsA is a central factor in the EsaR-mediated QS control of EPS synthesis

To determine whether EsaR controls EPS synthesis exclusively through repression of *rcsA* or dual control of *rcsA* and *cps,* we designed an epistasis experiment to compare the phenotypes of strain ESN10 (*esaI::cat*) and strain PSS11 (*esaI::cat, rcsA::kan*). As shown in Fig. 3A, both strains exhibit a non-mucoid phenotype when grown in absence of exogenous 3-oxo-C6-HSL. However, growth

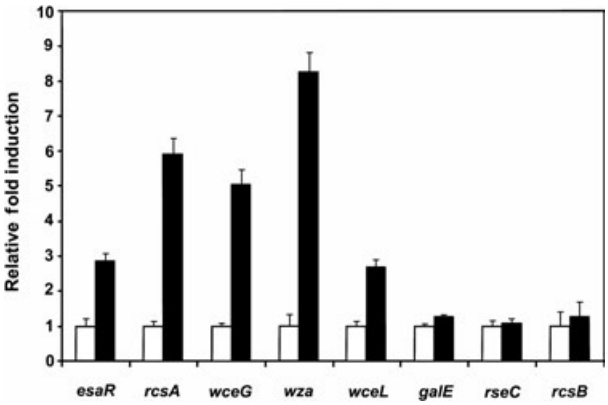


Fig. 2. Induction of specific gene expression in response to 3-oxo-C6-HSL. The relative transcript induction of specific *cps*-encoded EPS biosynthetic genes, the *esaR, rcsA* and *rcsB* regulatory genes, and *rseC*, as an example of a 3-oxo-C6-HSL neutral gene, was measured by real time RT-PCR. Template cDNAs were generated from total RNA extracts of strain ESN51 grown to exponential phase separately in the absence (□) or presence (■) of 10 μM 3-oxo-C6-HSL. Target transcript levels were normalized using 16S rRNA as an internal reference. Relative fold induction (RFI) was calculated using the mathematical equation: $RFI = 2^{-\Delta\Delta CT}$ (see *Experimental Procedures*). Each experiment was repeated three times and error bars represent the standard deviation.

in the presence of 3-oxo-C6-HSL induces mucoidy in strain ESN10, but not in strain PSS11. Expression of the *rcsA* gene from several different plasmid vectors overrides the EsaR-mediated repression of EPS production in strain ESN10 and complements the defect in strain PSS11 (data not shown). These genetic data establish that QS signal-mediated inducibility of mucoidy in *P. stewartii* depends on a functional *rcsA* gene. Direct *in vitro* transcriptional analysis supports this genetic conclusion. Specifically, real time RT-PCR showed that strain ESN10 (*esaI::cat, esaR*$^+$) has induced levels of *wceG, wceL* and *wza* transcripts in response to 3-oxo-C6-HSL (data not shown) similar to those measured for ESN51 (*esaI::kan, esaR*$^+$) in Fig. 2. In contrast, the transcript levels of these genes in PSS11 (*esaI::cat; rcsA::kan*) remained roughly the same, even under 3-oxo-C6-HSL inducing conditions (Fig. 3B). Correspondingly, genetic assays to measure the activity of several *cps* and *rcsA* promoter *lacZ* fusions in *E. coli* demonstrated that EsaR only repressed the P*rcsA::lacZ* transcriptional fusion and not the P*cps::lacZ* fusions (data not shown). Together these data confirm that *rcsA* is the primary target for EsaR-mediated repression and 3-oxo-C6-HSL specific derepression, and, that the effect of EsaR regulation on EPS synthesis is indirect and dependent on RcsA.

EsaR directly and specifically binds to the RcsA promoter

The above experimental data establish a role for EsaR as a direct negative regulator of the *rcsA* gene under 3-oxo-C6-HSL restrictive conditions and related derepression of the *rcsA* gene under inducing conditions. Therefore, ligand-free EsaR (Apo-EsaR) should physically interact with a target sequence of the *rcsA* promoter. Correspondingly, if the hierarchical regulatory model is correct, then EsaR should not bind to promoters associated with the *cps* gene system. We employed electromobility shift assays (EMSAs) to measure relative binding of purified native Apo-EsaR to specific sequences of the *rcsA* promoter and sequences corresponding to the primary *wceG* promoter, and potential intergenic promoters upstream of

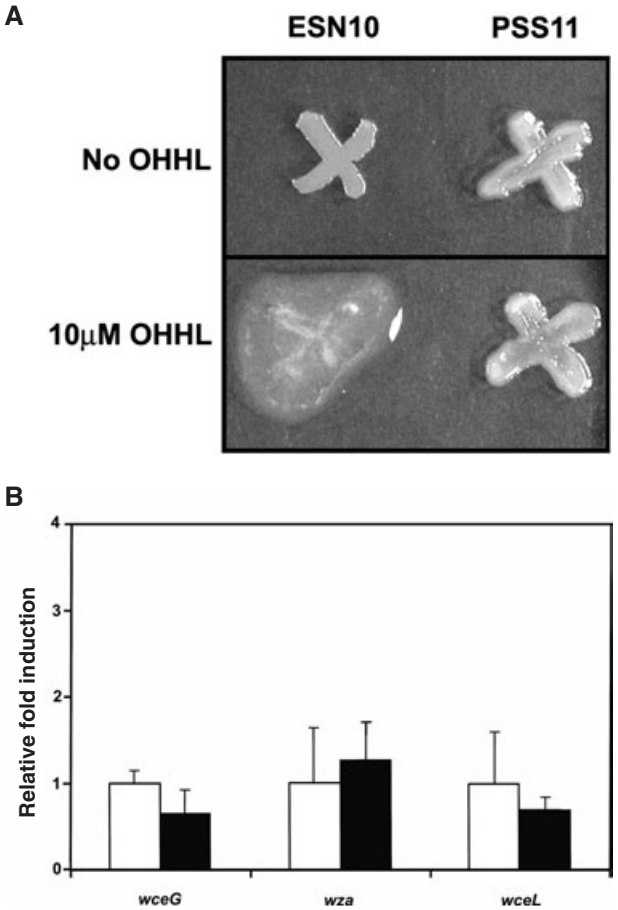


Fig. 3. Epistasis experiments to establish the regulatory dominance of EsaR over RcsA.
A. Strains ESN10 (*esaI::cat*) and PSS11 (*esaI::cat, rcsA::kan*) were grown on glucose-rich agar supplemented with (lower panel) or without (upper panel) 10 μM 3-oxo-C6-HSL and incubated at 28°C. Both strains lack a typical mucoid phenotype when grown in absence of 3-oxo-C6-HSL. Growth in presence of 3-oxo-C6-HSL induced mucoidy in strain ESN10, but not in strain PSS11.
B. Transcript levels of *wceG, wza* and *wceL* were determined by real time RT-PCR. The cDNAs generated from total RNA of PSS11 cultures grown separately to exponential phase in the absence (□) or presence (■) of 10 μM 3-oxo-C6-HSL. Relative transcript levels were calculated based on *rseC* levels as an internal 3-oxo-C6-HSL neutral standard. Experiments were repeated three times and error bars represent the standard deviation.

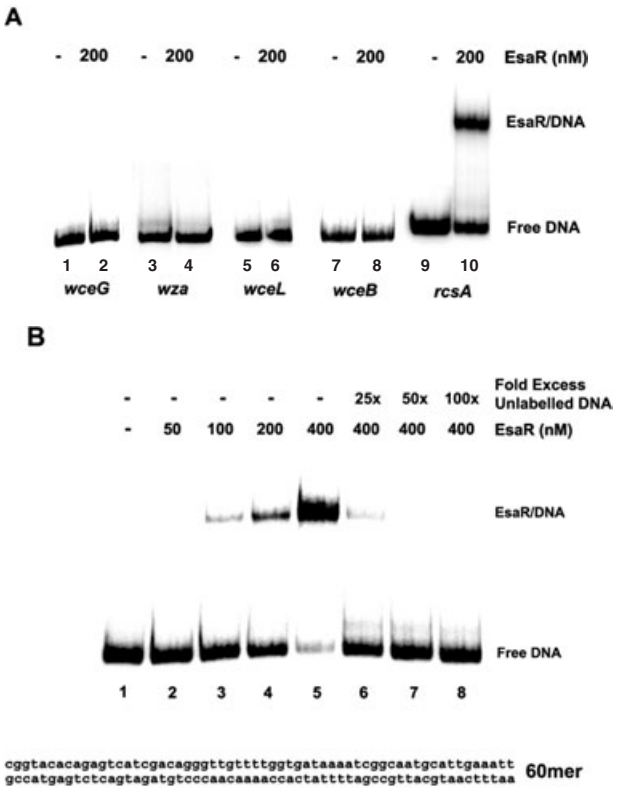


Fig. 4. *In vitro* EsaR binds to the *rcsA* promoter.
A. Radiolabelled PCR products (20 nM) from the upstream regions of *wceG*, *wza*, *wceL*, *wceB*, and *rcsA* were incubated without (lanes 1, 3, 5, 7, 9) and with 200 nM purified Apo-EsaR (lanes 2, 4, 6, 8, 10) and resolved by native polyacrylamide gel electrophoresis.
B. A 60 bp radiolabelled PCR product (40 μM), amplified from the *rcsA* promoter (lane 1) was incubated with increasing concentrations of purified Apo-EsaR (lanes 2–5) and resolved by native polyacrylamide gel electrophoresis. Addition of excess (25×, 50×, 100×) 60 bp unlabelled competitor effectively excluded the labelled target DNA indicative of a specific EsaR/target DNA interaction (lanes 6–8).

the *wza, wceL, wceB,* and *wzx* genes (see Fig. 1). The autoradiograph shown in Fig. 4A shows that Apo-EsaR formed a specific protein/DNA complex only with the *rcsA* promoter, and failed to complex with DNA fragments of the primary promoter and intergenic sequences of the *cps* gene cluster. The *rcsA* promoter sequence did not reveal an obvious EsaR binding site. Therefore, we assayed smaller PCR fragments of the *rcsA* promoter region using primers listed in Table 1, which allowed us to localize an *rcsA*-specific EsaR binding site to a 60 bp DNA fragment (Fig. 4B). Binding specificity of EsaR for this fragment was demonstrated by addition of excess unlabelled 60 mer competitor DNA, which effectively excluded the radiolabelled probe from the EsaR/DNA complex in a concentration-dependent manner. These data confirm that EsaR physically and specifically interacts with an operator site located within the *rcsA* promoter.

Footprint analysis defines a 20 bp DNA fragment with characteristics of an esaR box

We used DNase I protection assays to define the specific EsaR DNA binding site within the *rcsA* promoter. This study employed a plasmid-borne 260 bp DNA fragment of the *rcsA* promoter that includes the 60 bp binding region (see Fig. 4B). This DNA served as a template to generate separate sense and antisense, fluorescently labelled PCR fragments, which were incubated with DNase I in the presence or absence of EsaR before resolution by capillary electrophoresis. The results, which are displayed as superimposed electropherograms in Fig. 5A, draw attention to a single 20 bp region in both the sense (black) and antisense strand (grey) that was specifically protected by EsaR. Careful analysis of this protected region reveals a limited, but significant DNA sequence conservation with other *lux* box-like palindromes (Fig. 5B) including the previously characterized *esaR* box (Minogue *et al.*, 2002). The protected site overlaps a putative –10 σ^{70} consensus sequence located in the *rcsA* promoter (Fig. 6), analogous to the position of the *esaR* box in the *esaR* promoter (Beck von Bodman and Farrand, 1995; Minogue *et al.*, 2002). From these data, we conclude that EsaR binds at a semi-conserved *esaR* box to repress *rcsA* expression presumably through steric interference with RNA polymerase transcription initiation.

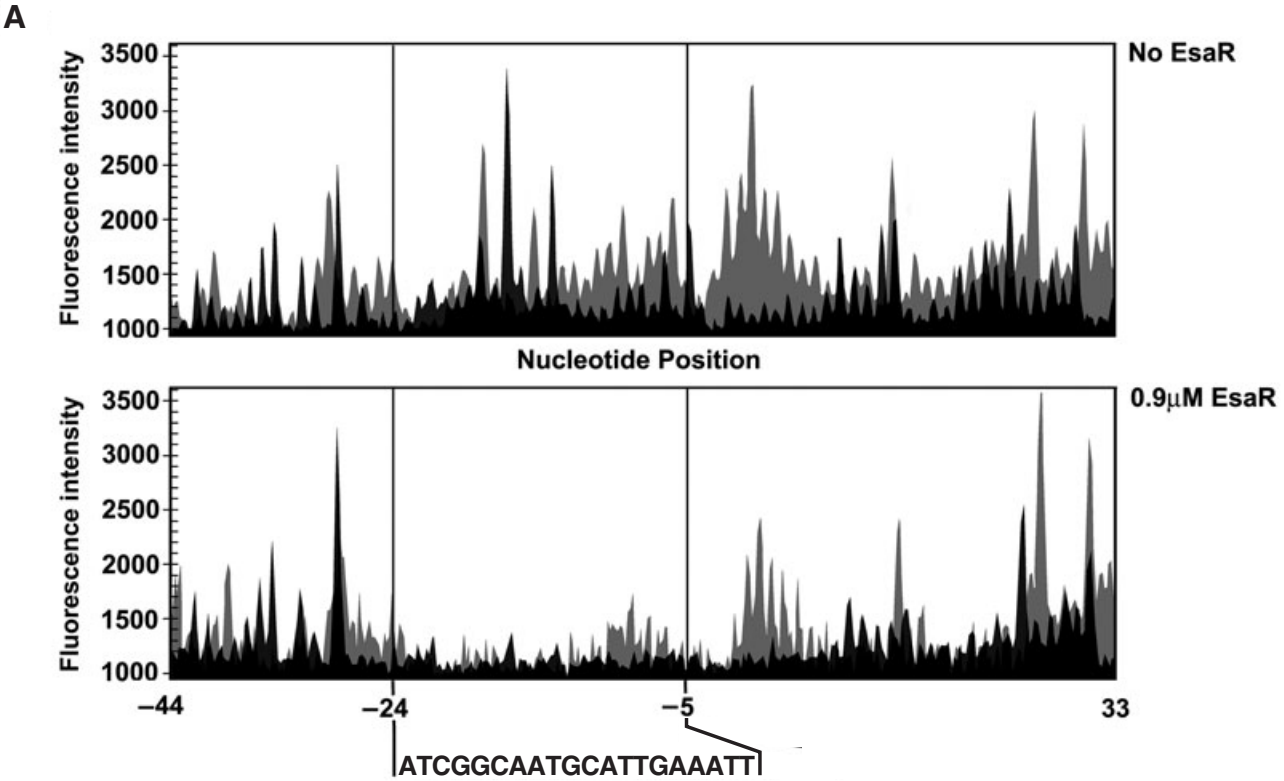
RcsA autoregulates its own expression in a 3-oxo-C6-HSL dependent fashion

RcsA of *E. coli* has positive autoregulatory characteristics (Ebel and Trempy, 1999). An RcsAB binding site is located in the *rcsA* promoter of *P. stewartii* with 71% identity to other confirmed RcsAB box sequences (Wehland and Bernhard, 2000). To experimentally test the autoregulatory role of RcsA in *P. stewartii,* we constructed a P*rcsA::gfp* promoter gene fusion carried on plasmid pAUC30 for parallel expression in strains ESN10 (*esaI*$^-$*, esaR*$^+$) and PSS11 (*rcsA*$^-$, *esaI*$^-$*, esaR*$^+$). Strain ESN10 exhibited a fivefold increase in GFP fluorescence, while the fluorescence of strain PSS11 increased only slightly (1.2-fold) in response to 3-oxo-C6-HSL induction (data not shown). We conclude from these data that RcsA activates its own expression from the *rcsA* promoter. More significantly, maximal RcsA autoregulation requires 3-oxo-C6-HSL to remove the EsaR-specific transcriptional block.

Discussion

This study establishes that the fundamental mechanism for QS regulation of EPS production in *P. stewartii* involves the direct repression of *rcsA* transcription by EsaR. The critical evidence for this conclusion is threefold. First, the

esaI mutant strain ESN51 (*esaR*$^+$) can be induced for EPS synthesis by exogenous addition of 3-oxo-C6-HSL, while the corresponding *esaI/rcsA* double mutant strain, PSS11, is non-inducible and remains blocked for EPS synthesis even in presence of the signal. Second, Apo-EsaR binds specifically to the *rcsA* promoter, but does not interact with sequences of the primary *wceG* promoter or intergenic regions within the *cps* operon. Third, DNase protection assays identify a region within the *rcsA* promoter that corresponds to a semiconserved *esaR* box element. This element spans the predicted −10 promoter consensus sequence. We also show that the activation of *rcsA* is subject to positive feedback regulation by RcsA, similar to *rcsA* in *E. coli* and *Erwinia amylovora* (Ebel and Trempy, 1999; Wehland *et al*., 1999). These data permit us to formulate a hierarchical model for QS regulation of EPS synthesis in *P. stewartii*, as summarized in Fig. 7. At low cell density, in absence of threshold concentrations of 3-oxo-C6-HSL ligand, Apo-EsaR is DNA binding competent and acts as a direct repressor of *rcsA* transcription. Even



B

Species	Gene	*lux*-box sequence
P. stewartii	*rcsA*	ATCGGCAATGCATTGAAATT
	esaR	GCCTGTACTATAGTGCAGGT
S. marcescens	*spnR*	ACCTGACCGAAAGGTCAGGT
V. fischeri	*luxI*	ACCTGTAGGATCGTACAGGT
P. aeruginosa	*lasB* OP1	ACCTGCCAGTTCTGGCAGGT
Concensus matrix		RYCKGHMVKDHMKKDMARKT

Fig. 5. DNase I footprinting analysis to detect the EsaR binding motif in the *rcsA* promoter.
A. DNase I digestion reactions of a 260 bp *rcsA* promoter fragment spanning the full-length *rcsA* promoter (35 nM) were resolved by capillary electrophoresis on a Beckman Coulter CEQ 2000XL. The electropherograms shown are of the sense (■) and antisense (▒) strands in the absence (upper panel) or presence of purified EsaR (0.9 μM) (lower panel). Fluorescence Intensity, *y*-axis, is proportional to the relative fragment abundance and elution time, *x*-axis, correlates to fragment length. Numeric values (bottom scale) refer to nucleotide position relative to the putative transcription start of the *rcsA* promoter. The protected sequence is as indicated and falls between nucleotides −24 and −5.
B. The EsaR binding site from the *rcsA* promoter was compared to other *lux* box-like DNA sequences found in *P. stewartii* (*esaR*) (Minogue *et al*., 2002), *Vibrio fischeri* (*luxI*) (Egland and Greenberg, 1999), *Serratia marcescens* (*spnR*) (Horng *et al*., 2002) and *Pseudomonas aeruginosa* (*lasB*) (Rust *et al*., 1996). Highly conserved nucleotides are highlighted in black, while other conserved nucleotides are displayed in grey; R = A or G; Y = T or C; M = A or C; K = G or T; H = A, C or T; V = A, C or G; D = A, G or T.

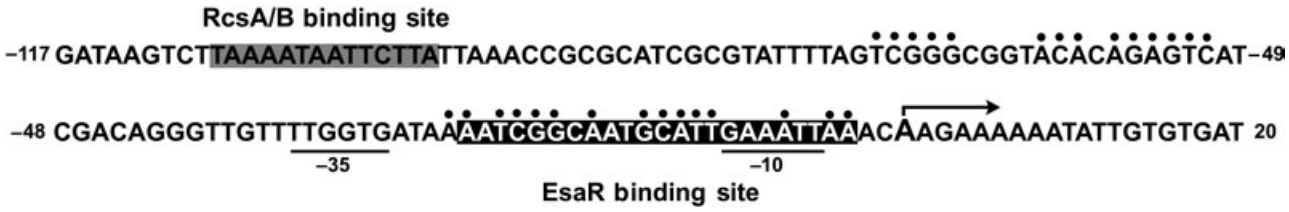


Fig. 6. Schematic depiction of the *rcsA* promoter. The promoter features a well-conserved RcsA/B binding site (grey), an EsaR binding motif (black) positioned between a putative −35 (underlined) and overlapping a putative −10 (underlined) σ^{70} recognition sequence. An arrow indicates a possible transcriptional start. Comparison of the published *rcsA* DNA sequence (GenBank Accession X58707) with the sequences obtained from the flanking regions of the *rcsA* transposon insertions revealed minor, but significant discrepancies (black circles above individual nucleotides). Sequence analyses of PCR fragments amplified from the genome of several different *P. stewartii* strains confirmed the discrepancies. We deposited a corrected DNA sequence for the *rcsA* promoter in GenBank as Accession AY819768.

so, the *rcsA* gene expresses at measurable basal levels under EsaR repressive conditions (data not shown). This is likely a function of the less stringent conservation of the *esaR* box in the *rcsA* promoter compared to the corresponding palindrome in the *esaR* promoter (Minogue *et al*., 2002). RcsA is also subject to rapid proteolysis by Lon protease presumably to keep cellular RcsA protein below a functionally relevant concentration. Consistent with this assessment is the observed enhanced mucoidy of *lon* mutant strains of *P. stewartii* analogous to *lon* mutants of *E. coli* and *E. amylovora* (Stout *et al*., 1991; Eastgate *et al*., 1995; M. D. Koutsoudis, unpubl.). At high cell density, or after exogenous addition of 3-oxo-C6-HSL, EsaR repressor activity relaxes, thus permitting the rapid expression of the *rcsA* gene. In this context, it is important to note that EsaR does not govern the expression of the *rcsB* gene, and *rcsB* transcript levels remain constant under inducing conditions. This model also indicates that EsaR-mediated QS is functionally dominant to the RcsC-YojN-RcsB environmental sensing phosphorelay system. The genetic evidence for this assertion is twofold. First, the *esaI* mutant strains ESN10 and ESN51 grown on high glucose medium remain repressed for EPS synthesis unless exposed to inducing levels of 3-oxo-C6-HSL. Second, an *esaR* or *esaI/esaR* double mutant strain of *P. stewartii* exhibits a mucoid phenotype even when grown on low glucose medium (von Bodman *et al*., 1998).

The role of RcsA/B activation of several group 1 capsule gene clusters including *cps* of *P. stewartii* is well documented (Poetter and Coplin, 1991; Stout *et al*., 1991; Kelm *et al*., 1997; Wehland *et al*., 1999). Our data confirm that the RcsA protein is essential for the activation of the *cps* gene system and stewartan EPS synthesis in *P. stewartii.* However, it is interesting to note that the promoter proximal genes (*wceG, wza*) of the *cps* gene cluster are induced to a significantly higher degree by 3-oxo-C6-HSL than genes located toward the middle (*wceL*) and 3′ end (*galE*) of the ~18 kb operon. The primary promoter upstream of *wceG* features a conserved JUMPstart sequence (just upstream of many polysaccharide starts)

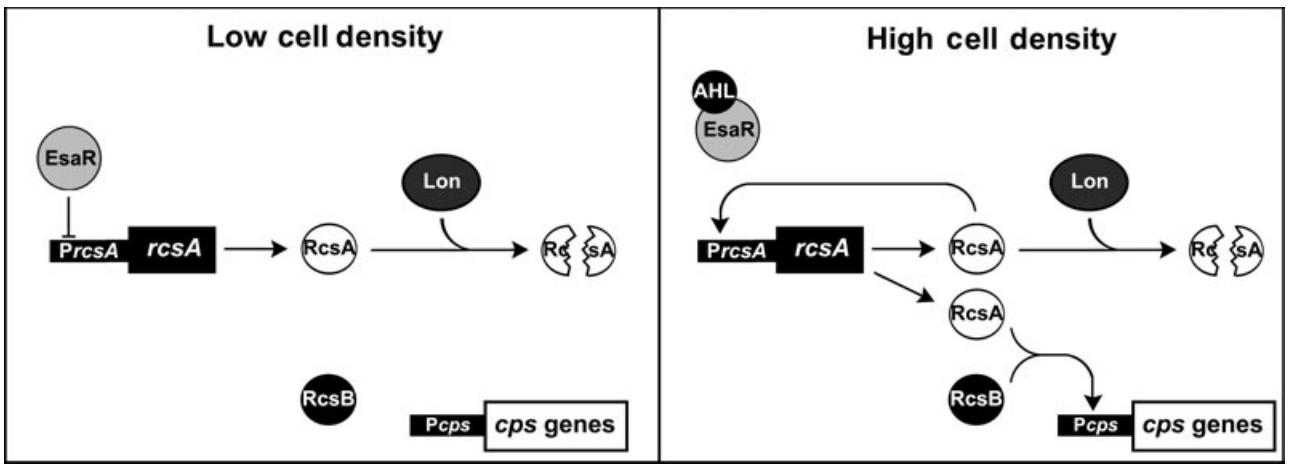


Fig. 7. A model depicting the hierarchical EsaR QS regulatory pathway. At low cell density, ligand-free EsaR represses the transcription of *rcsA*, yielding basal levels of RcsA protein that is subject to degradation by Lon protease (Gottesman and Stout, 1991) preventing significant RcsA/RcsB activation complex formation. At high cell density or 3-oxo-C6-HSL inducing conditions, EsaR repression of *rcsA* is relieved resulting in RcsA levels exceeding the degradation capacity of Lon. RcsA recruits RcsB to form an activation complex for the positive feedback regulation of *rcsA* and activation of the *cps* gene cluster.

(Hobbs and Reeves, 1994; Wehland *et al*., 1999). This sequence contains an eight bp element termed *ops* (operons polarity suppressor), which recruits the RfaH antitermination protein into the transcription complex to promote the synthesis of full-length operonic transcripts (Bailey *et al*., 1997; Stevens *et al*., 1997; Marolda and Valvano, 1998; Rahn *et al*., 1999; Artsimovitch and Landick, 2002). The *cps* operon of *P. stewartii* also features a putative stem-loop structure at the 3′ end of the *wzc* gene, analogous to the Rho-independent termination stem-loop structure found in the K30 *cps* cluster of *E. coli* (Rahn *et al*., 1999; Rahn and Whitfield, 2003). By analogy, this terminator region separates genes involved in higher-order polymerization and surface expression of EPS from the genes that encode specific glycosyltransferase enzymes for the biosynthesis of oligosaccharide repeat units. We assume that this putative stem-loop structure plays an important role in the differential expression of these two blocks of *cps* genes in *P. stewartii*. It is conceivable that under optimal RcsA/B-mediated activation of the *cps* operon, a subpopulation of transcripts escape antitermination leading to the accumulation of truncated transcripts. This scenario would explain the differential transcript levels detected of genes located upstream and downstream of the termination loop in response to 3-oxo-C6-HSL induction (Fig. 2). The dual *galF/galE* genes positioned at the 3′ end of the *cps* gene system could be expressed from an independent promoter (Torres-Cabassa *et al*., 1987; Dolph *et al*., 1988), although additional experiments are needed for unequivocal proof. In any case, we show that EsaR and 3-oxo-C6-HSL do not significantly control the expression of the *galE* gene. The GalF and GalE enzymes serve important functions in UDP-glucose and UDP-galactose synthesis, which are important precursors of stewartan EPS (Dolph *et al*., 1988; Nimtz *et al*., 1996).

Group 1 polysaccharides can be produced in several distinct forms. A short, or low molecular weight form assembles on a lipid A-core and consists of one or a few oligosaccharide repeat units (MacLachlan *et al*., 1993; Drummelsmith and Whitfield, 1999; Rahn and Whitfield, 2003). This form is referred to as K_{LPS}. Multiple oligosaccharide repeat units attached to the lipid A-core leads to the synthesis of O-antigen, sometimes referred to as smooth LPS (S-LPS). A high molecular weight capsular or EPS form is assembled on the cell surface in a translocation pathway that requires functions including Wza, an outer membrane lipoprotein, Wzb, an acid phosphatase, and Wzc, an inner membrane tyrosine kinase (Stevenson *et al*., 1996; Drummelsmith and Whitfield, 1999; Geider, 2000; Beis *et al*., 2004). These proteins are encoded by genes located between *wceG* and the putative stem-loop structure upstream of *wceL* in *P. stewartii*. We therefore envision a model in which basal level expression of the *cps* gene system directs oligosaccharide repeat units into O-antigen and or K_{LPS} synthesis, while RcsA/B-mediated activation of the *cps* gene system may be a mechanism to shunt these same oligosaccharide repeat units into high molecular weight stewartan EPS biosynthesis. Thus, EsaR-mediated QS regulation may serve as a key switch between LPS and EPS synthesis in *P. stewartii*. It should be noted that the *cps* gene cluster of *P. stewartii* lacks a *wzi* gene, which encodes an outer membrane protein thought to anchor the high molecular weight polymer to the cell surface typical of capsular polysaccharides (CPS) (Rahn *et al*., 2003). It is therefore likely that most of stewartan is in the cell-free EPS form.

We recognize that EPS synthesis is controlled by other global regulatory mechanisms in addition to QS. For example, the role of Lon protease in EPS synthesis is well established (Gottesman *et al*., 1985). Chatterjee and colleagues reported a role of the CsrA/*csrB* (Romeo *et al*., 1993) homologue pair, RsmA*/rsmB,* in the control of EPS synthesis in several *Erwinia* strains and *P. stewartii* (Cui *et al*., 1995). Additionally, this group and others showed that RsmA functions by destabilizing transcripts of LuxI homologue QS signal synthases in different *Erwinia* species (Cui *et al*., 1995; Whitehead *et al*., 2002). It is therefore possible that the effect of RsmA on EPS synthesis is a consequence of controlled intrinsic levels of 3-oxo-C6-HSL.

Finally, one must ask why EsaR, a reasonably conserved LuxR orthologue, should have evolved to function as a repressor with affinity for its DNA binding target in a ligand-free state while LuxR requires the signal cofactor for DNA binding and transcriptional activation? We recently reported that EsaR retains the ability to function also as a transcriptional activator in the ligand free state if provided a properly positioned *cis* binding site (von Bodman *et al*., 2003). Preliminary data indicate that EsaR may positively control one or more genes in *P. stewartii* under signal-limiting conditions. In the overall context of QS regulation, such dual functionality would be possible only if EsaR is DNA binding proficient in absence of the signal ligand to correspondingly activate genes required at low cell density, while repressing genes needed for cellular function at a higher cell density. This regulatory scenario would be an attractive mechanism for bacteria to transition between different stages of growth or development particularly when colonizing a specific niche or host.

Experimental procedures

Bacterial strains, growth conditions and DNA techniques

The *E. coli* strains used as cloning hosts include DH5α (Life Technologies), Top10 (Invitrogen), DH10B (Invitrogen), and

Table 2. Strains and plasmids.

	Relevant genotype[a]	Reference or source
Strain		
DH10B	*E. coli* Δ*lac*X74 *ara*Δ139 Δ(*ara-leu*)	Invitrogen
DC283	*P. stewartii* wild type, *Nal*^{R}	Dolph *et al.* (1988)
ESΔIR	*P. stewartii* Δ(*esaI-esaR*)	von Bodman *et al.* (1998)
ESN51	*P. stewartii esaI*::Tn5seqN51	(von Bodman *et al.* (1998)
S17-1	RP4 *Mob*$^+$	Simon *et al.* (1982)
ESN10	*P. stewartii esaI*::*cat*	This study
PSS11	*P. stewartii esaI*::*cat rcsA*::*kan*	This study
Plasmid		
Topo pCR2.1	Cloning vector *Ap*^{R}	Invitrogen
pBluescriptII KS+	Cloning vector, *ColE1 ori, Ap*^{R}	Stratagene
pTGN	Tn5*gfp-km, Ap*^{R}*, Km*^{R}*, R6K ori*	Tang *et al.* (1999)
pFPV25	Ap^R, promoterless *gfpmut gene*	Valdivia and Falkow (1996)
pKNG101	Suicide vector, *R6K ori, Sp*^{R}*, sacB*	Kaniga et al. (1991)
pKD4	*Km*^{R}, source of kan cassette	Datsenko and Wanner (2000)
pBBR1MCS	Broad host range vector, *Cm*^{R}	Kovach *et al.* (1995)
pKOK5	*Ap*^{R}*, Km*^{R}*, lacZ-Km cassette*	Kokotek and Lotz (1989)
pBAD22::*esaR*	*esaR* coding region driven by P*araC*	von Bodman *et al.* (2003)
pES2144	genomic clone (*wceG-galE*), *Tc*^{R}	Dolph *et al.* (1988)
pES4507	genomic clone (*rcsA*), *Tc*^{R}	Poetter and Coplin (1991)
pCM1	Chloramphenicol cassette	Close and Rodriguez (1982)
pSVB60	*esaR* driven by native *esaR* promoter	Minogue *et al.* (2002)
pAUC1	P*rcsA*::*lacZ* cloned in pBBR1MCS	This study
pAUC2	P*wceG*::*lacZ* cloned in pBBR1MCS	This study
pAUC3	P*wceB*::*lacZ* cloned in pBBR1MCS	This study
pAUC4	P*wceL*::*lacZ* cloned in pBBR1MCS	This study
pAUC5	P*wzx*::*lacZ* cloned in pBBR1MCS	This study
pAUC10	P*rcsA* cloned in pCR2.1 (sense orientation)	This study
pAUC11	P*rcsA* cloned in pCR2.1 (antisense orientation)	This study
pAUC20	*rcsA*::*kan* cloned in pKNG101	This study
pAUC30	P*rcsA*::*gfpmut* cloned in pFPV25	This study

a. *Ap*^{R}, ampicillin; *Cm*^{R}, chloramphenicol; *Km*^{R}, kanamycin; *Nal*^{R}, nalidixic acid; *Sp*^{R}, streptomycin; resistance.

S17-1 (Simon *et al.*, 1982) for conjugal transfer of RK2-based plasmid constructs into *P. stewartii* strains. *Escherichia coli* strains were grown at 37°C on nutrient agar (NA) plates or Luria–Bertani broth (LB) in presence of appropriate antibiotics, where applicable. The *P. stewartii* strains were grown at 28°C in LB in presence of 30 μg ml^{-1} of nalidixic acid on NA plates, glucose-rich CPG (0.1% casamino acids, 1% peptone and 1% glucose) (Bradshaw-Rouse *et al.*, 1981), AB minimal medium (Clark and Maaløe, 1967) or LB. All relevant strains and plasmids are listed in Table 2. DNA techniques were performed by standard methods as previously described (Beck von Bodman and Farrand, 1995; von Bodman *et al.*, 1998). DNA fragments were amplified using *Ex Taq* Polymerase (Takera/Panvera), *Taq* polymerase (Applied Biosystems), or Deep Vent (NEB) and synthetic oligonucleotides ordered to specification from Qiagen Operon.

Plasmid cloning strategies

The DNA fragments containing the putative *rcsA, wceG, wceL, wceB* and *wzx* promoters were amplified by PCR using purified genomic DNA from wild type *P. stewartii* strain DC283 as template in presence of the following primer pairs: P*rcsA*5/P*rcsA*3, P*wceG*5/P*wceG*3, P*wceL*5/P*wceL*3, P*wceB*5/P*wceB*3, and P*wzx*5/P*wzx*3 (Table 1). Amplicons were digested with the appropriate restriction enzymes (Table 1) and ligated into plasmid pBBR1MCS (Kovach *et al.*, 1995). The constructs were digested with *Sal*I for insertion of a similarly digested *lacZ-kan* cassette from plasmid pKOK5 (Kokotek and Lotz, 1989), to generate plasmids pAUC1 through pAUC5 (Table 2). The putative *rcsA* promoter was PCR amplified using wild type genomic DNA and primers P*rcsA*5 and P*rcsA*3 (Table 1). The PCR fragment was cloned into Topo pCR2.1 plasmid by TA cloning (Invitrogen). The resulting construct was digested with *Bam*HI and the released ~800 bp fragment was ligated into pFPV25 plasmid (Valdivia and Falkow, 1996), resulting in pAUC30 (Table 2).

Tn5gfp-km mutagenesis

The *P. stewartii esaI/esaR* double mutant strain, ESΔIR (Table 2) was mutagenized with the transposon Tn5*gfp-km* carried on the pTGN plasmid (Table 2). *Escherichia coli* strain S17-1 (pTGN) served as a conjugal donor to mobilize pTGN into strain ESΔIR. Each strain was grown separately to an OD_{600} of 0.6 in AB minimal medium. Cells were collected by centrifugation at 7000 *g* and washed with sterile PBS. Donor and recipient strains were combined and transferred to 0.2 μM nitrocellulose filters (Millipore). Filters were placed on NA plates and incubated at 28°C for 6 h. Stable transposition events were selected on NA supplemented with 30 μg ml^{-1} kanamycin and 30 μg ml^{-1} nalidixic acid.

Screening Tn5gfp-km mutants

Colonies exhibiting stable expression of Tn5*gfp-km* were viewed under a stereomicroscope for identification of EPS deficient mutants. Such mutants were patched onto AB minimal medium to ensure prototrophy. Secondary screening was based on the comparative expression of the Tn5*gfp-km* encoded GFP in the presence or absence of EsaR. Plasmid pSVB60 (Table 2) was introduced into independent mutants using *E. coli* S17-1 (Table 2) as a conjugal donor. The ESΔIR Tn5*gfp-km* mutants and corresponding strains carrying pSVB60 were cultured in AB minimal medium and grown to an OD_{600} of 0.6. Aliquots of 5 μl cell suspensions, standardized to OD_{600} of 1.0, were spotted onto fresh AB minimal plates in replicates of six, and evaluated over the course of 3 days using a Molecular Imager FX (FITC) (Bio-Rad). GFP levels of each sample were quantified using QUANTITY ONE software (Bio-Rad). Insertional mutants showing less than 50% GFP-specific fluorescence in the presence of EsaR were selected for further characterization.

Cloning and sequencing of the genomic DNA flanking Tn5gfp-km insertions

Genomic DNA was extracted using the MasterPure™ DNA Purification Kit (Epicentre) and digested to completion with *Kpn*I (Invitrogen), *Hin*DIII (Invitrogen) or *Xma*I (NEB). Digested DNA was cloned into pBluescriptII KS^+ (Stratagene) using T4 ligase (Invitrogen). Ligation reactions were transformed into *E. coli* strain DH10B and transformants were analysed for GFP production. Plasmid DNA from GFP expressing, Km^R/Ap^R transformants was isolated using QIAPrep Spin Mini-prep Kit (Qiagen). The purified DNA was sequenced at the W.M. Keck Foundation Biotechnology Resource Center (Yale University) using a primer specific to the 5′ region of Tn5*gfp-km* (Tn5 seq, Table 1).

Sequence analysis of the rcsA *promoter*

The *rcsA* promoter region was PCR amplified from the following sources: plasmid pES4507, DC283, ESN51 and ESΔIR. PCR amplicons were cloned using the pCR®2.1-TOPO® TA cloning kit (Invitrogen) as per manufacturer's recommendations. Resulting plasmids were isolated using QIAPrep Spin Mini-prep Kit (Qiagen) and sequenced at the W.M. Keck Foundation Biotechnology Resource Center (Yale University). The *rcsA* promoter sequence was deposited to GenBank under Accession (AY819768).

Real time RT-PCR analysis

Pantoea stewartii strains were grown in AB minimal medium to an OD_{600} of 0.6. Total RNA was extracted using the Ribopure™-Bacteria (Ambion) RNA extraction kit following the manufacturer's instructions. RNA concentrations were quantified by absorbance at 260 nm. Total cDNAs were synthesized using 500 ng of total RNA and the iScript™ cDNA Synthesis Kit (Bio-Rad). Reactions were incubated for 5 min at 25°C, 30 min at 42°C, 5 min at 85°C. Real time RT-PCR was performed using iQ™SYBR®Green Supermix and an iCycler (Bio-Rad) using the appropriate primers (Table 1). Primers were designed using the Primer3 algorithm (Rozen and Skaletsky, 1998). The 25 μl standard reaction volume consisted of 12.5 μl of iQ™SYBR® Green Supermix, 1 μl of cDNA, 1.25 μl of each 5′- and 3′ primer (10 μM), and 9 μl of water. Amplifications were performed using the following conditions: an initial 4 min incubation at 95°C followed by 40 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. Fluorescence was monitored at the end of each cycle using a *SYBR-490* filter setting. Melt curve analysis, 80 increments of 0.5°C every 30 s starting at 55°C, was performed after experiment completion to check for primer-dimer formation. All experiments were performed in triplicate. The 16S rRNA or *rseC* mRNAs were used as internal references. Data analysis to determine the cycle threshold (C_T) values was performed using the MyiQ software (Bio-Rad) and ΔC_T values were calculated as the average C_T of target DNA – average C_T of reference DNA. The calculation of comparative expression levels, or relative fold induction (RFI) (Applied Biosystems), used the formula $2^{(\Delta CT\ \text{target gene} - \Delta CT\ \text{internal reference gene})}$ or ($2^{-\Delta\Delta CT}$) to reflect the difference between each samples ΔC_T and the baseline or reference ΔC_T. Statistical analysis was performed using Microsoft Excel (Microsoft).

Deletion mutagenesis and allelic replacement

The ESN10 mutant (Table 2) was created by cloning the *esaI/esaR* locus into pUC18 as a *Sma*I/*Pst*I fragment resulting in plasmid pMDK2. The chloramphenicol acetyl-tranferase cassette (*cat*) was released from pCM1 (Close and Rodriguez, 1982) as a *Sal*I fragment. This fragment was inserted into the *Sal*I restriction site located in the *esaI* gene. The resulting construct was digested with *Sma*I/*Hpa*I and the released 2.8 kb fragment was cloned into the *Sma*I digested pKNG101 (Kaniga *et al.*, 1991) to create pMDK10. This plasmid was mobilized into *P. stewartii,* wild type strain, DC283 by conjugal transfer using *E. coli* S17-1 (pMDK10) as donor strain. Allelic replacement events were select on the basis of chloramphenicol resistance and sucrose sensitivity.

The PSS11 ($esaI^-$, $rcsA^-$) double mutant strain was created by the amplification of partial 5′- and 3′ fragments of the *rcsA* gene using the primer pairs PrcsA5′/RcsAΔup and RcsAΔ–low/RcsA3′ (Table 1). The PCR products were digested with the appropriate endonucleases and ligated into pBluescript SK+ cloning vector. The resulting construct lacking a 355 bp internal fragment was digested with *Eco*RI to allow the insertion of a kanamycin resistance cassette (*kan*) released from plasmid pKD4 (Datsenko and Wanner, 2000). The construct, which contained the 5′- and 3′ regions of *rcsA* and an internal Km^R cassette, was excised from the pBluescript and subcloned into the suicide vector pKNG101 to yield pAUC20. This plasmid was introduced into *E. coli* strain S17-1 and transferred by conjugation into the *P. stewartii*, ESN10. Allelic replacement events were select on the basis of chloramphenicol resistance and sucrose sensitivity. Southern Blot hybridization (DIG Detection Kit, Roche) and PCR analysis were used to verify all allelic replacement events.

Purification of EsaR

Native EsaR was purified from *E. coli* strain DH10B carrying

the pBAD22::*esaR* essentially as previously described (Minogue *et al*., 2002).

Gel retardation assays

DNA/protein complexes were resolved essentially as previously described (Minogue *et al*., 2002). DNA fragments were amplified from genomic DNA using the primers listed in Table 1 to obtain the desired *rcsA* and *cps* promoter fragments. PCR products were digested with the appropriate enzymes (Table 1), and labelled by a fill-in reaction using Deep Vent polymerase in presence of [α-^{32}P]-dATP, specific activity 3000 Ci mmol^{-1} (Perkin Elmer). DNA binding reactions, using varying concentrations of EsaR and labelled DNA product, were incubated at 28°C for 30 min The reaction buffer consisted of 20 mM Hepes (pH 7.6), 1 mM EDTA, 10 mM $(NH4)_2SO_4$, 1 mM DTT, 0.2% Tween-20, 30 mM KCl, 50 μg ml^{-1} λ-DNA, and 150 μg ml^{-1} BSA. Each reaction was resolved by electrophoresis on a native 6% polyacrylamide gel in 0.25 $\times$ TBE buffer (pH 8.3) (Fisher Scientific). Gels were dried using a vacuum gel drier. Radioactivity was detected using a Molecular Imager FX phosphorimager system and analysed using QUANTITY ONE software (Bio-Rad).

DNase I nucleotide protection assay

A 260 bp DNA fragment was PCR amplified from the *rcsA* promoter using the primers P*rcsA*5 and P*rcsA*3-1 (Table 1). Products were cloned into the vector pCR2.1® Topo® (Invitrogen) (Table 2). Inserts in both orientations yielded, respectively, plasmids pAUC10 (sense) and pAUC11 (antisense). Insert DNAs were confirmed by automated DNA sequencing. The LightSabre Green Primer, FPprimer (Synthegen) (Table 1), was used to generate fluorescently labelled double stranded DNA by PCR using pAUC10 and pAUC11 as templates. The PCR product was purified using the Qiagen PCR Purification kit. Binding reactions of 20 μl consisted of binding buffer (20 mM Hepes (pH 7.6), 1 mM EDTA, 10 mM $(NH4)_2SO_4$, 1 mM DTT, 0.2% Tween-20, 30 mM KCl), 100 ng of labelled DNA (0.4 pmol), 500 ng of λ DNA (NEB), and 9 μg of total protein (0.9 μM purified EsaR + BSA or BSA alone). Binding reactions were incubated for 30 min at 25°C. Footprint assays were performed using a protocol adapted from Yindeeyoungyeon and Schell (2000). DNase I digestion was performed by adding 10 μl of DNase I (Amersham) diluted to 10^{-2} units μl^{-1} in dilution buffer (10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 5 mM $CaCl_2$ and 0.1 mg ml^{-1} (BSA) and incubating at 26°C for 4 min The DNase I digestions were stopped by the addition 30 μl of 0.5 M EDTA (pH 8.0). Digested DNA was extracted using the Qiagen Nucleotide Removal Kit and resuspended in 40 μl of Sample Loading Solution (Beckman Coulter). Before loading, 0.5 μl of size standard 400 (Beckman Coulter) was added to each sample. Samples were resolved using a Beckman Coulter CEQ 2000XL capillary electrophoresis unit under the following conditions: denaturation for 2 min at 90°C; injection at 2.0 kV for 30 s; separation at 7.5 kV for 45 min The resulting electropherograms were analysed using Beckman Coulter CEQ 2000 software (Beckman Coulter).

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