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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.

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-acylhomoserine lactone (AHL) synthase. LuxR is an AHL-dependent transcriptional activator with affinity for a 20 base pair (bp) palindromic sequence, termed the lux box (Englebrecht and Silverman, 1987; Stevens and Greenberg, 1997). In contrast, interspecies-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 aetiologic 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 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

**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 (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 esal 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 σ70 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 auto-regulated 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 esal, esaR double mutant, hypermucoid ESΔIR strain with the Tn5gfp-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 (esal::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.
ally higher in response to 3-oxo-C6-HSL. The transcript
HSL, respectively. The transcript level of
genes, particularly those located
between 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).

**Table 1.** Oligonucleotides and primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Introduced restriction site</th>
<th>Used for</th>
</tr>
</thead>
<tbody>
<tr>
<td>PrcsA5</td>
<td>5’-cgacgcaagtctggtgcatgct</td>
<td>EcoRI</td>
<td>EMSA (Fig. 4A), deletion mutation (Fig. 3A)</td>
</tr>
<tr>
<td>PrcsA3</td>
<td>5’-tctctgtgatcccagctcttgcc</td>
<td>EcoRI</td>
<td>EMSA (Fig. 4A)</td>
</tr>
<tr>
<td>PrcsA3-1</td>
<td>5’-tcctgagcgttttcttcttcct</td>
<td>EcoRI</td>
<td>EMSA (Fig. 4A)</td>
</tr>
<tr>
<td>WceG</td>
<td>5’-cgacgcaagtctggtgcatgct</td>
<td>EcoRI</td>
<td>EMSA (Fig. 4A)</td>
</tr>
<tr>
<td>WceG2</td>
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<td>EcoRI</td>
<td>EMSA (Fig. 4A)</td>
</tr>
<tr>
<td>Wza1</td>
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<td>EcoRI</td>
<td>EMSA (Fig. 4A)</td>
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<tr>
<td>Wza2</td>
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<td>EcoRI</td>
<td>EMSA (Fig. 4A)</td>
</tr>
<tr>
<td>WceL1</td>
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<td>EcoRI</td>
<td>EMSA (Fig. 4A)</td>
</tr>
<tr>
<td>WceL2</td>
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<td>EcoRI</td>
<td>EMSA (Fig. 4A)</td>
</tr>
<tr>
<td>16s1</td>
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<td>EcoRI</td>
<td>EMSA (Fig. 4A)</td>
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<tr>
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<td>EcoRI</td>
<td>EMSA (Fig. 4A)</td>
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<tr>
<td>RseC1</td>
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<td>EcoRI</td>
<td>EMSA (Fig. 4A)</td>
</tr>
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<td>RseC2</td>
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<td>EcoRI</td>
<td>EMSA (Fig. 4A)</td>
</tr>
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<td>EMSA (Fig. 4A)</td>
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<tr>
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<td>EcoRI</td>
<td>EMSA (Fig. 4A)</td>
</tr>
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<td>GalE1</td>
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<td>EcoRI</td>
<td>EMSA (Fig. 4A)</td>
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<td>GalE2</td>
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<td>EcoRI</td>
<td>EMSA (Fig. 4A)</td>
</tr>
<tr>
<td>RcsB1</td>
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<td>EcoRI</td>
<td>EMSA (Fig. 4A)</td>
</tr>
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<td>RcsB2</td>
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<td>EcoRI</td>
<td>EMSA (Fig. 4A)</td>
</tr>
<tr>
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<td>EcoRI</td>
<td>EMSA (Fig. 4A)</td>
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<tr>
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<td>5’-ctctgagcgttttcttcttcct</td>
<td>EcoRI</td>
<td>EMSA (Fig. 4A)</td>
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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 (esal::cat) and strain PSS11 (esal::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
Quorum sensing control of stewartan EPS

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 PrcsA::lacZ transcriptional fusion and not the Pcps::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. 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.](image)

![Fig. 3. Epistasis experiments to establish the regulatory dominance of EsAR over RcsA.](image)
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 σ70 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 PrcsA::gfp promoter gene fusion carried on plasmid pAUC30 for parallel expression in strains ESN10 (esaR−, esaR+) and PSS11 (rcsA−, esaR−, esaA+). 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

**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.
so, the \textit{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 \textit{esaR} box in the \textit{rcsA} promoter compared to the corresponding palindrome in the \textit{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 \textit{lon} mutant strains of \textit{P. stewartii} analogous to \textit{lon} mutants of \textit{E. coli} and \textit{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 \textit{rcsA} gene. In this context, it is important to note that EsaR does not govern the expression of the \textit{rcsB} gene, and \textit{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 \textit{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 \textit{esaR} or \textit{esaI/esaR} double mutant strain of \textit{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 \textit{cps} of \textit{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 \textit{cps} gene system and stewartan EPS synthesis in \textit{P. stewartii}. However, it is interesting to note that the promoter proximal genes (\textit{wceG}, \textit{wza}) of the \textit{cps} gene cluster are induced to a significantly higher degree by 3-oxo-C6-HSL than genes located toward the middle (\textit{wceL}) and 3' end (\textit{galE}) of the \textsim 18 kb operon. The primary promoter upstream of \textit{wceG} features a conserved JUMPstart sequence (just upstream of many polysaccharide starts).

Fig. 6. Schematic depiction of the \textit{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) $\sigma^70$ recognition sequence. An arrow indicates a possible transcriptional start. Comparison of the published \textit{rcsA} DNA sequence (GenBank Accession X58707) with the sequences obtained from the flanking regions of the \textit{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 \textit{P. stewartii} strains confirmed the discrepancies. We deposited a corrected DNA sequence for the \textit{rcsA} promoter in GenBank as Accession AY819768.

Fig. 7. A model depicting the hierarchical EsaR QS regulatory pathway. At low cell density, ligand-free EsaR represses the transcription of \textit{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 \textit{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 \textit{rcsA} and activation of the \textit{cps} gene cluster.
(Hobbs and Reeves, 1994; Wehland et al., 1999). This sequence contains an eight bp element termed \( \text{ops} \) (operons polarity suppressor), which recruits the RsaH 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; Artsimovich and Landick, 2002). The \( \text{cps} \) operon of \( P. \text{stewartii} \) also features a putative stem-loop structure at the 3’ end of the \( \text{wzc} \) gene, analogous to the Rho-independent termination stem-loop structure found in the K30 \( \text{cps} \) cluster of \textit{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 \( \text{cps} \) genes in \( P. \text{stewartii} \). It is conceivable that under optimal RcsA/B-mediated activation of the \( \text{cps} \) operon, a subpopulation of transcripts escape antitermination leading to the accumulation of truncated transcripts. This scenario would explain the differential levels detected of genes located upstream and downstream of the termination loop in response to 3-oxo-C6-HSL induction (Fig. 2). The dual \( \text{galF/galE} \) genes positioned at the 3’ end of the \( \text{cps} \) 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 \( \text{galE} \) gene. The GaF and GaE 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 \( K_{\text{ps}} \). 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 \( \text{wceG} \) and the putative stem-loop structure upstream of \( \text{wceL} \) in \( P. \text{stewartii} \). We therefore envision a model in which basal level expression of the \( \text{cps} \) gene system directs oligosaccharide repeat units into O-antigen and or \( K_{\text{ps}} \) synthesis, while RcsA/B-mediated activation of the \( \text{cps} \) 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 \( P. \text{stewartii} \). It should be noted that the \( \text{cps} \) gene cluster of \( P. \text{stewartii} \) lacks a \( \text{wzi} \) 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 \textit{Erwinia} strains and \( P. \text{stewartii} \) (Cui et al., 1995). Additionally, this group and others showed that RsmA functions by destabilizing transcripts of LuxI homologue QS signal synthases in different \textit{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 \( \text{cis} \) binding site (von Bodman et al., 2003). Preliminary data indicate that EsaR may positively control one or more genes in \( P. \text{stewartii} \) 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 \textit{E. coli} strains used as cloning hosts include DH5\( \alpha \) (Life Technologies), Top10 (Invitrogen), DH10B (Invitrogen), and
Table 2. Strains and plasmids.

<table>
<thead>
<tr>
<th>Strain</th>
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<th>Reference or source</th>
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<tr>
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<td>Invitrogen</td>
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<td>DC283</td>
<td>P. stewarti wild type, Nafl</td>
<td>Dolph et al. (1988)</td>
</tr>
<tr>
<td>ES(\Delta IR)</td>
<td>P. stewartii (\Delta esal-esaR)</td>
<td>von Bodman et al. (1998)</td>
</tr>
<tr>
<td>ESN51</td>
<td>P. stewartii esal::TnSeq5N51</td>
<td>(von Bodman et al. 1998)</td>
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<td>S17-1</td>
<td>RP4 Mobs</td>
<td>Simon et al. (1982)</td>
</tr>
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<td>This study</td>
</tr>
<tr>
<td>PSS11</td>
<td>P. stewartii esal::cat rcsA::kan</td>
<td>This study</td>
</tr>
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<td>Cloning vector Ap(\beta)</td>
<td>Invitrogen</td>
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<tr>
<td>pBluescriptII KS+</td>
<td>Cloning vector, ColE1 ori, Ap(\beta)</td>
<td>Stratagene</td>
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<td>pTGN</td>
<td>Tn5gfp-km, Ap(\beta), Km(\beta), R6K ori</td>
<td>Tang et al. (1999)</td>
</tr>
<tr>
<td>pKNG101</td>
<td>Suicide vector, R6K ori, Sp(\beta), sacB</td>
<td>Kaniga et al. (1991)</td>
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<tr>
<td>pKD4</td>
<td>Km(\beta), source of kan cassette</td>
<td>Datsenko and Wanner (2000)</td>
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<td>pBBR1MCS</td>
<td>Broad host range vector, Cm(\beta)</td>
<td>Kovach et al. (1995)</td>
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<td>pKOK5</td>
<td>Ap(\beta), Km(\beta), lacZ-Km cassette</td>
<td>Kokotek and Lotz (1989)</td>
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<tr>
<td>pBAD22::esaR</td>
<td>esal coding region driven by ParA</td>
<td>von Bodman et al. (2003)</td>
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<td>genomic clone (wceG-galE), Tc(\beta)</td>
<td>Dolph et al. (1988)</td>
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<td>pES4S07</td>
<td>genomic clone (rcsA), Tc(\beta)</td>
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<td>pCM1</td>
<td>Chloramphenicol cassette</td>
<td>Close and Rodriguez (1982)</td>
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<td>pSBV806</td>
<td>esaR driven by native esaR promoter</td>
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<td>PrcsA::gfpmut cloned in pFPV25</td>
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a. Ap\(\beta\), ampicillin; Cm\(\beta\), chloramphenicol; Km\(\beta\), kanamycin; Nafl, nalidixic acid; Sp\(\beta\), 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 (Takara/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: PrcsA5/PrcsA3, PwceG5/PwceG3, PwceL5/PwceL3, PwceB5/PwceB3, and Pwzx5/Pwzx3 (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 SalI 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 PrcsA5 and PrcsA3 (Table 1). The PCR fragment was cloned into Topo pCR2.1 plasmid by TA cloning (Invitrogen). The resulting construct was digested with BamHI 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 esal/esaR double mutant strain, ES\(\Delta IR\) (Table 2) was mutagenized with the transposon Tn5gfp-km carried on the pTGN plasmid (Table 2). Escherichia coli strain S17-1 (pTGN) served as a conjugal donor to mobilize pTGN into strain ES\(\Delta IR\). Each strain was grown separately to an OD\text{\textsubscript{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.

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Screening Tn5gfp-km mutants

Colonies exhibiting stable expression of Tn5gfp-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 Tn5gfp-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 ES5IR Tn5gfp-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 Kpnl (Invitrogen), HinDIII (Invitrogen) or Xmal (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, KmR-resistant, ApR-negative 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 Tn5gfp-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 ES5IR. PCR amplicons were cloned using the pCR2.1-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

Pantoaea 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 Skaltsky, 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 rscA 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^{ΔΔC_{T} target gene – ΔC_{T} internal reference gene} or (2^{ΔC_{T}}) 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 esal/esaR locus into pUC18 as a Smal/PstI fragment resulting in plasmid pMDK2. The chloramphenicol acetyl-transferase cassette (caf) was released from pCM1 (Close and Rodriguez, 1982) as a SalI fragment. This fragment was inserted into the SalI restriction site located in the esal gene. The resulting construct was digested with Smal/Hpal and the released 2.8 kb fragment was cloned into the Smal 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 (esal′, rcsA′) double mutant strain was created by the amplification of partial 5′- and 3′ fragments of the rcsA gene using the primer pairs PrcsA5′/RcsAαup and RcsAα′/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 EcoRI 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 KmR cassette, was excised from the pBluescript and sub-cloned 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-32P]-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 (pH7.6), 1 mM EDTA, 10 mM (NH4)2SO4, 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 ¥ TBE buffer (pH 8.3) (Fisher Scientific). Gels were dried using a vacuum gel drier. Radioactivity was detected using a Molecular Imager FX phosphorimagery 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 PrcsA5 and PrcsA3-1 (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)2SO4, 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. The 0.9 mM 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 10² units μl−1 in dilution buffer (10 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 5 mM CaCl2 and 0.1 mg ml−1 (BSA) and incubating at 26°C for 4 min. The DNase I digestion was stopped by the addition of 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).

Acknowledgements

The authors gratefully acknowledge Dr David Coplin (The Ohio State University) for providing cps clones used for genetic complementation in this study and Tonia Vassilovitch for assisting in the transposon mutagenesis phase of this study. This work was supported by Grant MCB-0211687 from the National Science Foundation.

References


