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The autoregulatory role of EsaR, a quorum-sensing regulator in *Pantoea stewartii* ssp. *stewartii*: evidence for a repressor function

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Summary

Capsular polysaccharide synthesis and virulence in the plant pathogenic bacterium *Pantoea stewartii* ssp. *stewartii* requires the quorum-sensing regulatory proteins, EsaR and EsaI, and the diffusible inducer *N*-(3-oxo-hexanoyl)-L-homoserine lactone. Prior mutational studies suggested that EsaR might function as a repressor of quorum sensing in the control of capsular polysaccharide synthesis. Further, a *lux* box-like palindromic sequence coinciding with the putative –10 element of the *esaR* promoter suggested a possible negative autoregulatory role for EsaR. This report presents genetic evidence that EsaR represses the *esaR* gene under inducer-limiting conditions, and that addition of inducer promotes rapid, dose-dependent derepression. DNA mobility-shift assays and analyses by surface plasmon resonance refractometry show that EsaR binds target DNAs in a ligand-free state, and that inducer alters the binding characteristics of EsaR. Physical measurements indicate that the EsaR protein binds *N*-(3-oxo-hexanoyl)-L-homoserine lactone, in a 1:1 protein:ligand ratio, and that inducer binding enhances the thermal stability of the EsaR protein. These combined genetic and biochemical data establish that EsaR regulates its own expression by signal-independent repression and signal-dependent derepression. Additionally, we provide evidence that EsaR does not govern the

expression of the linked *esaI* gene, thus EsaR has no role in controlling coinducer synthesis.

Introduction

Bacteria express selected gene systems in a population-dependent manner by sensing self-produced, membrane-diffusible signals in a strategy called quorum sensing (QS) (Fuqua *et al.*, 1996). The key elements of QS regulation in many Gram-negative bacteria are homologue proteins of LuxI, a *N*-acyl-homoserinelactone (AHL) signal synthase, and LuxR, an AHL-dependent response regulator. These two proteins control the expression of bioluminescence in the marine bacterium, *Vibrio fischeri* (Fuqua *et al.*, 1994; Williams *et al.*, 2000; Fuqua *et al.*, 2001; Miller and Bassler, 2001; Withers *et al.*, 2001). Alternative QS mechanisms, mediated by unrelated control factors, exist in other Gram-negative bacteria, most notably *Vibrio harveyi* (Bassler *et al.*, 1994), and in several Gram-positive organisms (Dunny and Leonard, 1997; Kleerebezem and Quadri, 2001). In general, QS governs the control of diverse phenotypes, each benefiting a bacterium in a specialized habitat (Whiteley *et al.*, 1999; Pierson, 2000; Whitehead *et al.*, 2001).

Pantoea stewartii ssp. *stewartii* (*P. stewartii*) is the causative agent of Stewart's wilt disease in sweetcorn and leaf blight in maize. Disease symptoms develop when the bacterium produces large amounts of a capsular polysaccharide (CPS), which blocks the corn xylem vessels and induces necrotic lesions (Coplin *et al.*, 1992). CPS synthesis is a QS-controlled phenotype governed by the LuxI and LuxR homologue proteins, EsaI and EsaR (von Bodman and Farrand, 1995). Disruption of the signal synthase gene, *esaI*, leads to parallel loss of AHL, CPS production, and virulence. In contrast, mutations in the *esaR* gene give a hypermucooid phenotype irrespective of AHL (von Bodman *et al.*, 1998). The simplest explanation for these observations is that EsaR functions as a repressor of CPS synthesis and that derepression requires inducing levels of AHL. The functions required for CPS synthesis are encoded by an extensive *cps* gene system (Dolph *et al.*, 1988). This gene system is closely related to the *wza* gene cluster encoding the synthesis of the group I capsules, colanic acid in *Escherichia coli* (Reeves *et al.*, 1996), and amylovoran in *Erwinia*

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amylovora (Bernhard *et al.*, 1993). It is well established that these gene systems require transcriptional activation by the environmental response regulatory complex, RcsA/B (Gottesman and Stout, 1991; Kelm *et al.*, 1997; Wehland *et al.*, 1999). The difference in *P. stewartii* is that the level of RcsA/B-mediated control of stewartan synthesis is secondary to the dominant control by EsaR.

The controlled expression of the QS regulatory elements themselves is generally an integral feature of QS regulation (Shadel and Baldwin, 1992; Fuqua *et al.*, 1994; Pesci *et al.*, 1997; Whitehead *et al.*, 2001). Thus, the strategy by which EsaR governs its own expression, and the expression of the linked *esaI* gene, may be indicative of its role as a repressor of CPS synthesis. Preliminary observations suggested that EsaR regulates its own expression by repression (von Bodman and Farrand, 1995), although the role of the AHL signal in this mode of regulation was unclear. The current study focuses on a well conserved *lux* box-like sequence, the *esaR* box associated with the *esaR* gene, to explore whether EsaR protein is genetically and biochemically programmed for a role as a repressor of QS.

Results

Autorepression of the *esaR* promoter by EsaR

LuxR, TraR and related QS transcription factors regulate their own expression by activation (Shadel and Baldwin, 1992; Hwang *et al.*, 1994; Seed *et al.*, 1995; Fuqua *et al.*, 1996; Fuqua and Winans, 1996; Fuqua *et al.*, 2001). The

linked *esaI/esaR* gene system of *P. stewartii* is convergently organized and features 3' terminal ends that overlap by 21 basepairs (bp) (von Bodman and Farrand, 1995). The promoter of the *esaR* gene, not the *esaI* gene, bears a defined *esaR* box. This palindrome coincides with the putative -10 promoter sequence, which suggests that binding of EsaR at this site may block transcription and provide a mechanism for EsaR-mediated autorepression. To test this prediction, we developed an *in vivo* assay based on the coexpression of plasmids pTDM6 and pTDM7 in the *E. coli* Top₁₀ host strain, TM67 (Table 1). In this assay, plasmid pTDM6 (Fig. 1A) contributes the *esaR* coding sequence expressed from the *E. coli* P_{BAD} promoter, which is controlled by the AraC regulator, also encoded on the plasmid, as a function of L-arabinose induction (Guzman *et al.*, 1995). Plasmid pTDM7 carries an *esaR::lacZY* reporter gene fusion designed to measure the *in vivo* activity of the *esaR* promoter (Fig. 1A). Growth of strain TM67 in glucose-supplemented medium lacking L-arabinose yielded fully induced levels of β -galactosidase, whereas growth in the presence of 0.02% L-arabinose gave nearly 10-fold lower levels of β -galactosidase (Fig. 1B). Intermediary levels of reporter activity were inversely proportional to the amount of L-arabinose provided. These data confirm that EsaR acts as a repressor, and that this repression is AHL-independent.

Derepression is AHL ligand-dependent

Proof for EsaR mediating QS through a repression

Table 1. Strains and plasmids.

| | Relevant genotype | Reference or source |
|--------------------|--|-------------------------------|
| <i>Strain</i> | | |
| TB1 | <i>E. coli</i> , JM83 <i>hsdR</i> (rk-mk+), Δ <i>ara</i> | New England Biolabs |
| Top ₁₀ | <i>E. coli</i> , <i>lacZ</i> Δ M15 Δ <i>lacX74</i> <i>araD</i> 139 Δ (<i>ara-leu</i>) 7697 | Invitrogen |
| DH5 α | <i>E. coli</i> , <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>)U169 <i>gyrA</i> 96 | Invitrogen |
| TM6 | <i>E. coli</i> Top ₁₀ (pTDM6) | This study |
| TM67 | <i>E. coli</i> Top ₁₀ (pTDM6, pTDM7) | This study |
| TDM619 | <i>E. coli</i> Top ₁₀ (pTDM6, pTDM19) | This study |
| DC283 | <i>P. stewartii</i> , SS104, Nal ^R | Dolph <i>et al.</i> (1988) |
| ESN51 | <i>P. stewartii</i> , <i>esaI</i> :Tn5 | von Bodman and Farrand (1995) |
| ES Δ IR | <i>P. stewartii</i> , Δ <i>esaI-esaR</i> | von Bodman and Farrand (1995) |
| <i>Plasmids</i> | | |
| pBluescript-II KS+ | Cloning vector, ColE1 ori, Ap ^R | Stratagene |
| pBAD22 | Arabinose inducible expression vector, Ap ^R | Guzman <i>et al.</i> (1995) |
| pBBR1MCS-3 | Broad range expression vector, Tc ^R | Kovach <i>et al.</i> (1995) |
| pSVB5-18 | <i>esaI</i> and <i>esaR</i> cloned pBluescript-II KS+, Ap ^R | von Bodman and Farrand (1995) |
| pLKC480 | Source of <i>lacZY</i> -Km ^R cassette | Tiedeman and Smith, 1988) |
| pLKC481 | Source of <i>lacZY</i> -Km ^R cassette | Tiedeman and Smith (1988) |
| pSVB60 | <i>esaR</i> re-cloned from pSVB5-18 into pBBR1MCS-3 as a <i>Pst</i> I- <i>Sal</i> I fragment | This study |
| pTDM6 | PCR-amplified <i>esaR</i> coding sequence cloned into pBAD22as a <i>Nco</i> I- <i>Hind</i> III fragment | This study |
| pTDM7 | <i>esaR::lacZY</i> -Km ^R gene fusion in pSVB60 | This study |
| pTDM18 | PCR-amplified <i>esaI</i> promoter and partial coding sequence cloned into pBBR1MCS-3 as a <i>Xba</i> I- <i>Xma</i> I fragment | This study |
| pTDM19 | <i>esaI::lacZY</i> -Km ^R gene fusion cloned into the <i>Xma</i> I site of pTDM18 | This study |

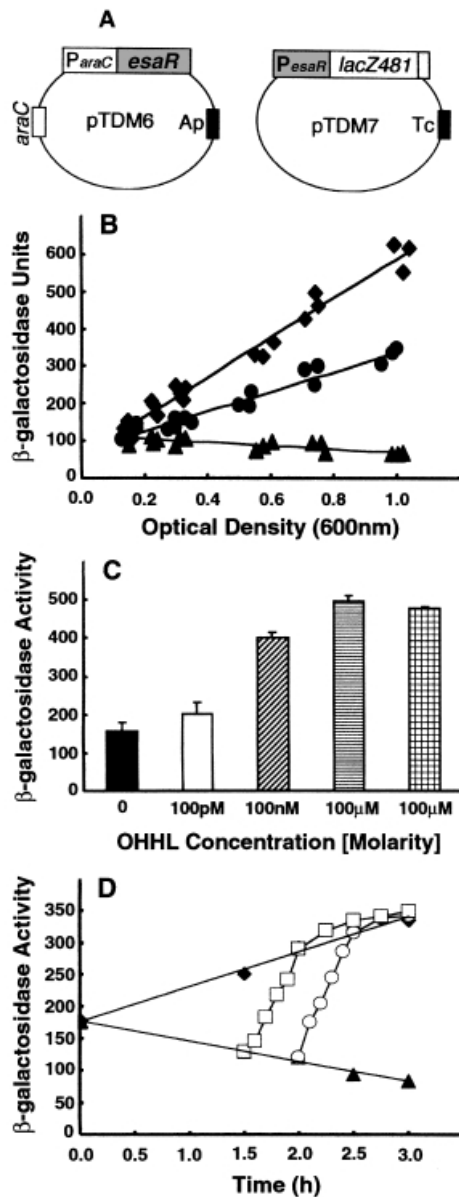


Fig. 1. Dose-dependent repression and derepression of an *esaR* translational fusion. The *E. coli* strain TM67 harbouring (A) plasmids pTDM6 and pTDM7 was grown (B) to different optical densities (600 nm) in the presence of 0% (◆), 0.0002% (●), or 0.02% (▲) L-arabinose; or (C) to an optical density (600 nm) of 0.6 in presence of 0.02% L-arabinose with 0 (■), 100 pM (□), 100 nM (▨), 100 µM (▩) *N*-(3-oxo-hexanoyl)-L-homoserine lactone (OHHL), or 0% L-arabinose and 100 µM OHHL (▤). D. Strain TM67 was grown with 0% (◆) or 0.02% L-arabinose (▲) and harvested at different times after L-arabinose induction. Parallel cultures were treated with 100 µM OHHL at 1.5 h (□) or 2 h (○). The cells were harvested and assayed for β-galactosidase expressed as units (Sambrook *et al.*, 1989) (B) or activity (Miller, 1972) (C and D).

mechanism would require evidence for inducing levels of the biologically relevant AHL signal, *N*-(3-oxo-hexanoyl)-L-homoserine lactone (OHHL), to promote derepression. Accordingly, we grew strain TM67 under stringent

repressive conditions in presence of 0.02% L-arabinose. Parallel cultures were supplemented with levels of synthetic OHHL ranging from 0 µM to 100 µM. Derepression of the reporter function correlated directly to the quantity of OHHL supplied (Fig. 1C). More importantly, derepression occurred almost immediately upon addition of the OHHL (Fig. 1D). We conclude from these data that *EsaR* repression of its own gene is abolished by AHL.

EsaR does not control the expression of the *esal* gene

We reported previously that *P. stewartii* strains mutated in the *esaR* locus synthesise wild-type levels of AHL (von Bodman and Farrand, 1995) suggesting that *EsaR* does not control the expression of the linked *esal* gene. We confirmed this observation by measuring levels of β-galactosidase expressed from an *esal::lacZY* translational gene fusion carried on plasmid pTDM19. When coexpressed with plasmid pTDM6 in *E. coli* strain TM619, this gene fusion produced identical levels of β-galactosidase regardless of whether or not the strain was induced with L-arabinose and exogenous AHL (data not shown). This therefore establishes that *EsaR* does not govern the *esal*-encoded signal synthesis; in fact, *esal* appears to be expressed constitutively.

Purification of native *EsaR* protein

DNA binding studies to establish the role of *EsaR* as a repressor *in vitro* require purified *EsaR* protein. Initial attempts to express *EsaR* as a His₆-tagged fusion protein were hampered by protein solubility problems. We therefore opted to express *EsaR* in its native form in *E. coli* strain TM6, which harbours the recombinant plasmid pTDM6. This plasmid carries the *esaR* gene under the control of the L-arabinose inducible promoter, P_{BAD} (Table 1 and Fig. 1A). *EsaR* protein was extracted from cultures grown in the presence of 0.02% L-arabinose and in the absence of the AHL ligand. This is an important point, because some LuxR-type proteins, most notably TraR, require AHL ligand to remain soluble in cellular lysates (Zhu and Winans, 1999; Qin *et al.*, 2001). Growth of strain TM6 at 28°C allowed the purification of native *EsaR* protein that, when purified by column chromatography, was soluble to 0.4 mM. Active fractions were identified by DNA mobility-shift assays against a synthetic DNA target. These fractions were pooled, aliquoted, and stored at -80°C until needed for further biochemical analysis.

Analysis of *EsaR* binding in DNA mobility-shift assays

The *in vivo* data of Fig. 1 indicated that Apo-*EsaR* represses an *esaR* promoter gene fusion, presumably by

Table 2. Oligonucleotides and primers used in this study.

| Fragment/primer | Sequence | Used for |
|----------------------|---|-------------------------------------|
| <i>Fragments</i> | | |
| <i>PesaR28</i> | 5'-TCTT <u>GCCTG</u> TACTATAGTGCAGGTTAAG 3'-AGAACGGACATGATATCACGTCCAATTC | Mobility-shift assay (Fig. 2) |
| <i>PesaR20</i> | 5'-GCCTGACTATAGTGCAGGT 3'-CGGACATGATATCACGTCCA | Mobility-shift assay (Fig. 2) |
| <i>Primers</i> | | |
| <i>F_esaRNcoI</i> | 5'-GAGCCATGGTTTCTTTTTTCC | Cloning <i>esaR</i> into pBAD22 |
| <i>R_esaRHindIII</i> | 5'-CCGCAAGCTTCAGTCACTAC | |
| <i>F_esaR70</i> | 5'-Biotin-AGAAAACATTAGGCTCCATGCTGCTTC | SPR (Fig. 3) |
| <i>R_esaR70</i> | 5'-TCTTGCCTGACTATAGTGCAGGTTAAG-Biotin | |
| <i>F_esaIXbaI</i> | 5'-CAAGTTCTAGAAAAGTGGCCAGGTCAAC | Cloning <i>esaI</i> into pBBR1MCS-3 |
| <i>R_esaIXmaI</i> | 5'-AACAGCCCAGGTCATTCCATTTC | |

The underlined sequences indicate the *esaR* box.

binding to the *esaR* box located in this region. We therefore assayed purified protein against synthetic target DNAs in DNA mobility-shift assays to define the *in vitro* DNA binding characteristics of EsaR. Apo-EsaR formed a specific complex with a 28 bp DNA fragment bearing the native *esaR* box (Table 2). Moreover, the density of the shifted band, measured in digital pixels, was proportional to the amount of EsaR protein assayed (Fig. 2A, lanes 2–4). Addition of increasing levels of unlabelled probe DNA (20 mer *esaR* box) effectively inhibited complex formation (Fig. 2A, lanes 5–7) and produced a high intensity hybrid DNA species composed of labelled 28 mer and unlabelled 20 mer oligonucleotides that migrate between the 28 mer double-stranded and single-stranded DNA forms. Higher order complexes of EsaR protein were only faintly detected at protein concentrations at or above 200 nM.

DNA mobility-shift assays using a wide range of protein concentrations against a constant amount of labelled probe DNA yielded a binding affinity of 3×10^{-8} M ($\approx K_D$) for EsaR and the *esaR* box DNA palindrome (Fig. 2B). A calculated Hill coefficient of 0.99 indicates that EsaR binds the *esaR* box with little or no cooperativity. Interestingly, the addition of the OHHL signal ligand did not induce DNA–protein complex dissociation even when used at a concentration of 500 μ M, and regardless of whether the protein was exposed to the signal ligand pre or post presentation of the DNA probe (Fig. 2A). This *in vitro* observation is puzzling considering that EsaR is highly responsive to OHHL *in vivo*.

EsaR binds to the DNA as a dimer

To further evaluate the oligomeric state of EsaR, we developed an assay based on the differential mobility of the native EsaR protein and a larger mass hybrid MBP-

EsaR protein. Specifically, we fused the *malE* gene to the 5'-end of the *esaR* coding sequence separated by a Xa factor protease recognition linker sequence (Riggs, 2000). Standard DNA binding assays, containing affinity-purified MBP-EsaR, a radiolabelled 28 bp *esaR* promoter DNA fragment, and a constant amount of Xa factor, were incubated for different times before gel electrophoresis. Figure 2C shows that at zero time incubation the larger mass MBP-EsaR homodimer was present exclusively. Prolonged periods of incubation gradually yielded the MBP-EsaR::EsaR heterodimer of intermediate mobility, followed by complete transformation to the lower migrating EsaR::EsaR homodimer. The formation of only one intermediate complex suggests that EsaR binds the DNA target as a dimer under the conditions assayed. These observations are consistent with our previous size-exclusion chromatographic results, which show that Apo-EsaR fractionates with a relative molecular mass of dimeric EsaR (data not shown).

Analysis of EsaR binding by surface plasmon resonance

Surface plasmon resonance (SPR) is a refractometry-based technique that allows measurement of biomolecular interactions in real time as changes of mass concentrations on a sensor surface (Rich and Myszka, 2001). We used this method to gain additional insight into the *in vitro* molecular recognition dynamics of EsaR for its DNA target. A 70 bp biotinylated PCR product of the native *esaR* promoter was conjugated to a dextran streptavidin-coated (SA) sensor chip at a surface density of 120 resonance units (RUs). Injection of EsaR protein analyte over a range of nM concentrations showed a steady increase in RUs with 344 nM EsaR protein yielding a sensogram that reflects saturation binding (Fig. 3A). The RU values

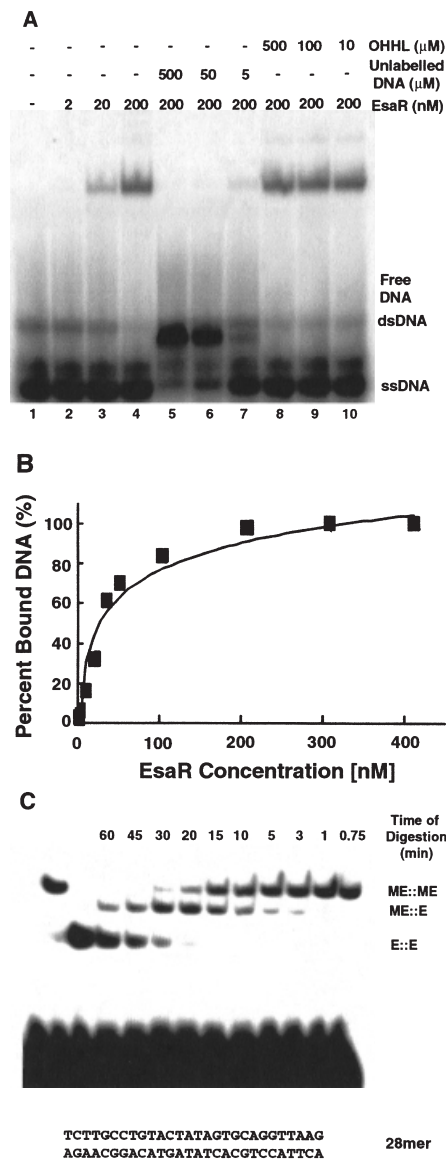


Fig. 2. Interaction of purified *EsaR* protein with target DNA. **A.** A synthetic, end-labelled 28 bp DNA fragment (1.6 μ M) containing the *EsaR* recognition sequence (lane 1) was incubated with 10-fold increasing amounts (2 to 200 nM) of purified *EsaR* protein and resolved by native gel electrophoresis (lanes 2–4). Binding specificity of *EsaR* for the probe DNA was demonstrated with 10-fold increasing amounts of excess (5 to 500 μ M) unlabelled competitor DNA bearing the 20 mer *esaR* box (lanes 5–7). Addition of OHHL at 10, 100 and 500 μ M did not result in complex dissociation (lanes 8–10). **B.** A binding constant (K_D) of 3×10^{-8} M was calculated based on the pixel intensity of free and complexed probe DNA recorded with a Bio-Rad phosphorimager and evaluated with IMAGER ONE software. **C.** DNA mobility analysis of purified MBP-*EsaR* (M) hybrid protein treated with Xa factor protease at increasing incubation times resolves probe DNA complexed with the higher mass MBP-*EsaR* (M::M) homodimer, the MalE-*EsaR*::*EsaR* (M::E) heterodimer of intermediary mobility, and eventual complete conversion to the lower mass *EsaR*::*EsaR* (E::E) homodimer. Lane 1 resolves the M::M homodimer at 0 time incubation; lane 2 shows the E::E homodimer after 2 h of Xa factor treatment.

of the sensograms displayed were corrected for non-specific interactions between *EsaR* protein and a similar length non-target DNA measured in a parallel reference cell. The data were analysed with the BIAEVALUATION 3.0 software, assuming a simple 1:1 Langmuir binding model to determine a K_D value of 9×10^{-9} M ($\chi^2 = 6.01$). This binding constant is in good agreement with the 3×10^{-8} M value derived from DNA mobility-shift assays (Fig. 2B).

Surface plasmon resonance analysis using the same sensor chip and a constant *EsaR* concentration of 344 nM, but varying amounts of AHL in the buffer system during the association phase of the experiment yielded proportionally lower RU values (Fig. 3B). The sensograms were similar to those obtained when measuring lower *EsaR* protein concentrations (Fig. 3A). These results suggest that signal ligand may prevent AHL-bound *EsaR* from interacting with the immobilized DNA target thereby reducing the effective concentration of binding competent *EsaR* protein.

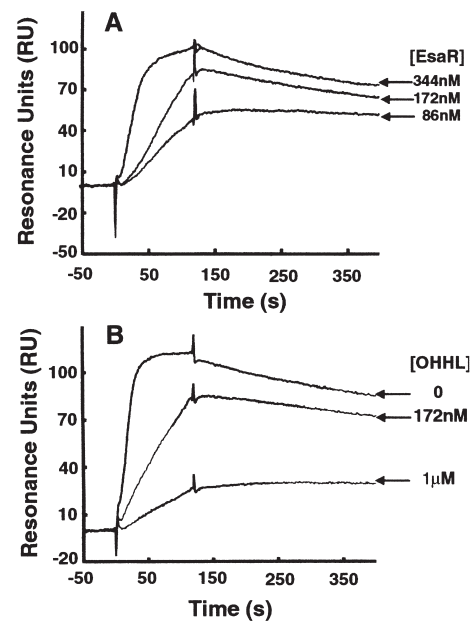


Fig. 3. Interaction of purified *EsaR* protein with a PCR-generated DNA fragment containing the *esaR* box evaluated by SPR refractometry.

A. The target DNA was immobilized on a SA streptavidin-coated gold sensory chip at 120 RU (1:1 R_{max} of 120). *EsaR* protein analyte was injected over a range of concentrations (22 nM to 1.4 μ M, displayed are 86, 172 and 344 nM) at a flow rate of $25 \mu\text{L min}^{-1}$ with an association time of 180 s, and a dissociation time of 600 s (shown only 350 s). **B.** SPR assay with 344 nM *EsaR* protein and 0, 172 nM and 1 μ M concentrations of OHHL added to the buffering system during the association phase only. The RU values shown in both sensograms are difference RU values of the sample cell and parallel reference cell measuring the non-specific protein–DNA interactions.

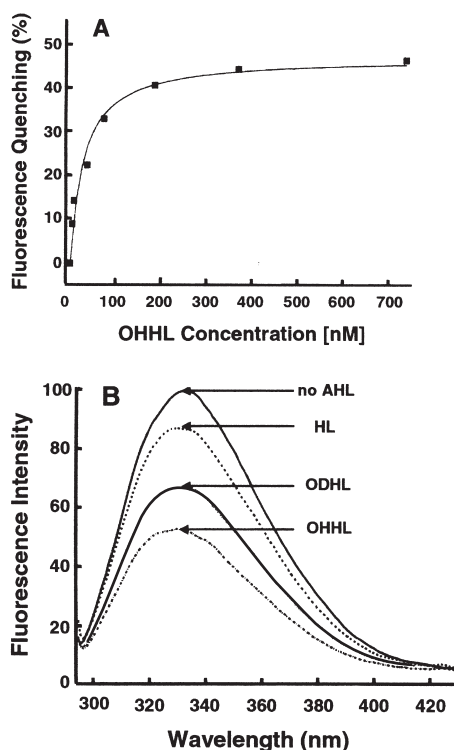


Fig. 4. Biophysical analysis of EsaR–*N*-acyl-homoserinelactone (AHL) interactions. The EsaR fluorescence was determined at a concentration of 370 nM.
 A. EsaR titration with increasing amounts of OHHL at 0, 3.7, 7.4, 37, 74, 185, 470, 740 nM and 3.7 μ M.
 B. Interaction of EsaR with 3.7 μ M OHHL, *N*-(3-oxo-dodecanoyl)-L-homoserine (ODHL), and L-homoserine lactone (HL).

Analysis of the EsaR/AHL interaction by fluorescence spectroscopy

EsaR contains three tryptophan residues; two located in the *N*-terminal region (W54 and W82) and one (W191) in the C-terminal helix–turn–helix domain. LuxR homologue proteins utilise regions in the *N*-terminus to dimerize and to bind signal ligand (Choi and Greenberg, 1992; Hanzelka and Greenberg, 1995; Qin *et al.*, 2001; Zhu and Winans, 2001). Such intermolecular interactions generally invoke structural changes, which, in turn, alter the intrinsic fluorescence intensity of the protein. We took advantage of the intrinsic fluorescence of EsaR to assess the effect of ligand binding on the structural integrity of the protein (Fig. 4A). Measurements taken at wavelengths between 300 nm and 400 nm produced a fluorescence maximum around 340 nm. This fluorescence decreased in intensity with increasing concentrations of the OHHL ligand. Maximal quenching of approximately 50% required 370 nM ligand, and higher concentrations did not further quench fluorescence. These assays employed 370 nM EsaR, therefore suggesting a 1:1 molar ratio of protein–ligand interaction. These results agree with

similar fluorescence studies reported previously for CarR (Welch *et al.*, 2000).

The specificity of EsaR for its signal ligand may vary depending on the length and substitution of the acyl side chain. Comparative fluorescence data obtained from separate experiments containing equal amounts of either L-homoserine lactone (HL), *N*-(3-oxo-dodecanoyl)-L-homoserine Lactone (ODHL) and OHHL, gave different intrinsic fluorescence spectra (Fig. 4B). The signal ligands were added in 10-fold molar excess and the recorded reductions in EsaR fluorescence were 50%, 35% and 15% respectively. These data suggest that EsaR binds the physiologically relevant OHHL species most efficiently, shows reduced affinity for ODHL, and has minimal affinity for the HL moiety alone.

EsaR is more heat-stable in the presence of the signal ligand

We wished to evaluate whether the addition of the preferred ligand, OHHL, affects the stability of the EsaR protein and/or prevents the purified EsaR protein from precipitation at extremely high concentrations. We therefore monitored the effect of OHHL on the thermal stability of purified EsaR by circular dichroism (CD) spectroscopy. As shown in Fig. 5A, the thermal stability of Apo-EsaR was relatively low with denaturation beginning at 48°C and completed at 58°C. The addition of 10-fold excess OHHL increased the thermal stability of EsaR by about 10°C, with no denaturation seen below 53°C and complete denaturation requiring 68°C (Fig. 5B).

Discussion

This study establishes several critical facts related to the role of EsaR as a repressor of QS in *P. stewartii*. First, it demonstrates that EsaR regulates its own expression by repression and AHL-mediated derepression; second, that purified EsaR dimerizes and binds DNA without AHL ligand; third, that AHL interacts specifically with EsaR protein, and induces structural changes that may neutralise its DNA binding affinity; and fourth, that EsaR has no role in the control of the linked signal synthase gene, *esal*. These data correlate well with our earlier prediction that EsaR functions as a repressor of CPS synthesis in *P. stewartii* (von Bodman and Farrand, 1995; von Bodman *et al.*, 1998).

A mechanism for QS by repression is unexpected because the majority of LuxR-type regulators are signal-responsive transcription activators (Fuqua *et al.*, 1996, 2001; Whitehead *et al.*, 2001). The LuxR class of proteins exhibits an overall sequence identity of 18–25%, with three critical regions of higher conservation (Whitehead *et al.*, 2001). These include an N-terminal region for

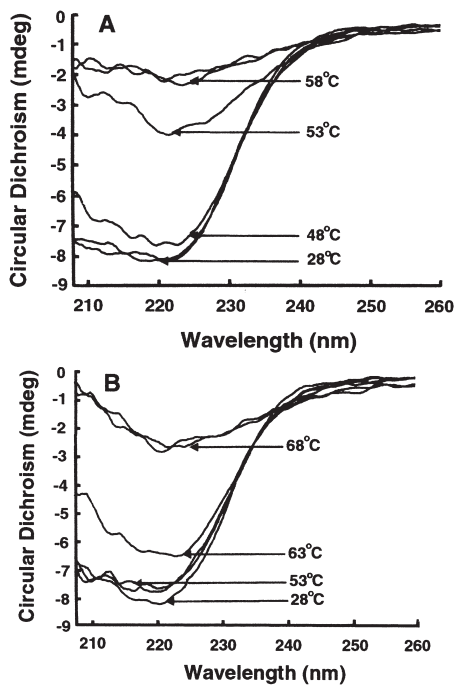


Fig. 5. Thermal stability of EsaR with and without AHL. Circular dichroism (CD) spectra of 370 nM EsaR were determined at increasing temperatures in the absence and presence of OHHL. The bottom curve in both diagrams represent the spectrum at 28°C, the top curves represent the spectrum at the highest temperature.
 A. CD spectra without OHHL measured at temperatures ranging from 28°C to 63°C.
 B. CD spectra with OHHL measured at temperatures ranging from 28°C to 73°C.

AHL signal binding, a more centrally located domain for protein oligomerization, and a helix–turn–helix C-terminal domain for DNA binding (Stevens and Greenberg, 1999; Fuqua *et al.*, 2001; Whitehead *et al.*, 2001). However, the QS regulatory proteins found in *Erwinia*-type bacterial species, including EsaR of *P. stewartii* (formerly *E. stewartii*) (von Bodman and Farrand, 1995), CarR (Welch *et al.*, 2000) and ExpR of *Erwinia carotovora* ssp. *carotovora* (*Ecc*) (Andersson *et al.*, 2000), have a higher degree of relatedness among them. This subfamily of QS regulators is also distinctive in its ability to dimerize and bind DNA in the absence of signal ligand. Based on these characteristics, it was proposed that the LuxR-type proteins fall into two basic groups (Andersson *et al.*, 2000; Qin *et al.*, 2001; Whitehead *et al.*, 2001).

We propose here that the CarR, ExpR and EsaR, subfamily of QS regulators can be divided further based on their discrete functional differences. Specifically, CarR, the QS regulator of carbapenem synthesis in *E. carotovora* (*Ecc*) dimerizes and binds DNA in the absence of AHL, but forms higher order complexes in the presence of inducing amounts of AHL to promote activation of the *car* target gene system (Welch *et al.*, 2000). In fact, muta-

tional disruption of CarR results in a null-carbapenem phenotype (McGowan *et al.*, 1995). CarR also controls its own expression by AHL-dependent activation (Whitehead *et al.*, 2001). The ExpR regulatory proteins described for two different *E. carotovora* strains (Andersson *et al.*, 2000) (Whitehead *et al.*, 2001) and *Erwinia chrysanthemi* (Nasser *et al.*, 1998) also bind target promoters in an AHL-free state, and were thought to control exoenzyme synthesis negatively. However, mutational inactivation of the *expR* gene has no, or only a minor, effect on exoenzyme expression. The implication is that in these strains QS regulation is redundant and that the repressive role of ExpR may be one of AHL sequestration rather than direct regulation (Andersson *et al.*, 2000). The ExpR protein of *E. chrysanthemi* was also shown to bind to the *expR* promoter at a well conserved *lux* box palindrome (Reverchon *et al.*, 1998; Andersson *et al.*, 2000). However, mutational inactivation of ExpR had no effect on *expR* expression (Reverchon *et al.*, 1998).

EsaR, in contrast to ExpR and CarR, has a well defined activity as a repressor of its own expression, and the hypermucoid phenotype of *esaR* null mutants suggests that it represses CPS synthesis as well. Specifically, EsaR negatively regulates an *esaR::lacZ* reporter gene expressed *in vivo* in *E. coli* in a dose-dependent manner (Fig. 1B), and EsaR responds rapidly to exogenously provided AHL for derepression (Fig. 1C). The *in vitro* binding properties of EsaR support the genetic data showing that Apo-EsaR exists primarily as a homodimer complex and exhibits specificity for the *esaR* box DNA target with an affinity characteristic of these proteins (Fig. 2A and C). In addition, higher order oligomeric complexes of EsaR, characteristic of CarR and ExpR, are detected only when assaying EsaR protein at or above 200 nM concentrations, with or without OHHL present (Fig. 2B). A significant inconsistency between the *in vivo* and *in vitro* data is the observation that addition of OHHL does not promote EsaR–DNA complex dissociation in the DNA mobility-shift assays (Fig. 2A). This is surprising for three reasons. First, the fluorescence-quenching experiments (Fig. 4) show that OHHL interacts specifically and stoichiometrically with purified EsaR protein. Second, the SPR data (Fig. 3B) show that when EsaR is exposed to increasing levels of OHHL, the concentration of binding competent EsaR decreases proportionally. Third, ExpR of *E. chrysanthemi* releases DNA in response to AHL, although the ligand concentrations used in the ExpR DNA mobility-shift assays (Reverchon *et al.*, 1998) were substantially higher than the amounts used in the current study. The SPR data indicate that AHL reduces the binding affinity of Apo-EsaR. However, additional SPR experiments are needed to establish whether AHL promotes DNA–protein complex dissociation, or whether DNA bound EsaR protein has a structural conformation that is unfit to per-

ceive the AHL signal. AHL-mediated derepression of the *esaR* reporter gene fusion *in vivo* is rapid regardless of the time lag between arabinose induction to express EsaR and the addition of OHHL (Fig. 1C). These data suggest that holoenzyme formation does not require nascent protein synthesis as is predicted for TraR protein of *Agrobacterium tumefaciens* (Zhu and Winans, 1999). In *A. tumefaciens*, AHL is thought to serve as a scaffold for proper TraR folding and protection from cellular proteolysis. Presumably, EsaR folds properly in the absence of AHL to assume a stable, DNA binding-proficient conformation. It is conceivable, that preformed Apo-EsaR undergoes an AHL-induced structural transformation that perturbs the binding domain, or causes the protein dimer to dissociate into AHL-bound monomers. In either case, AHL may render EsaR protein susceptible to proteolysis. Proteolytic inactivation of EsaR could account for the rapid derepression observed *in vivo*, while also explaining the inability of AHL to induce complex dissociation in the *in vitro* DNA mobility-shift assays. Experiments are in progress to test whether proteolysis is a factor in the mechanism of AHL-dependent derepression of EsaR.

A model by Qin and colleagues (Qin *et al.*, 2001) proposes that Apo-TraR of *A. tumefaciens* partitions to the cytoplasmic membrane to isolate monomeric TraR from the pool of intracellular AHL under non-inducing conditions. It is proposed that TraR dissociates from the membrane and dimerizes when the membrane-permeable AHL concentration reaches inducing levels. In contrast, EsaR does not appear to be membrane-associated; in fact, EsaR is soluble in its native form without AHL ligand, unlike TraR. If, as predicted, membrane association is to prevent premature TraR dimerization, then EsaR protein may not share this requirement because it is naturally dimeric and seeks DNA targets under AHL-limiting conditions.

EsaR is highly responsive to AHL-induced derepression in the control of its own gene expression. More significantly, it responds gradually and proportionally to the amount of AHL provided, not suddenly at a given inducing concentration. If this reflects the normal dynamics of EsaR and AHL co-inducer interaction, then it is unclear how EsaR governs the expression of CPS synthesis in a strictly cell density-dependent manner when AHL production is constitutive. We suggest that AHL-induced derepression of the *esaR* gene may be important in this respect. Specifically, we propose that increased cellular levels of EsaR sequester the constitutively generated cellular pools of AHL to prevent premature derepression of CPS synthesis at lower cell densities. Accordingly, a cell population would become 'quorate' when the cellular levels of AHL exceed the maximum levels of EsaR expressed. This rationale would explain also the apparent paradox of AHL-dependent derepression of the *esaR*

gene to generate more of the QS repressor when approaching AHL-inducing conditions. EsaR-mediated autorepression may also serve as an intrinsic mechanism of QS modulation.

N-Acyl-homoserinelactone (AHL) signal synthesis in *P. stewartii* is independent of EsaR and appears to be constitutive. This is in contrast to the paradigm of QS regulation that requires LuxR-AHL activation of the cognate *luxI* signal synthase gene. It is difficult to predict when and why *esaI* regulation became EsaR-independent; but it may have been in response to EsaR assuming a role as a repressor, because repression of the *esaI* gene may be counterproductive in the overall scheme of QS. Conversely, EsaR may have assumed a repressor role in response to deregulated synthesis of AHL to preserve the QS control of CPS synthesis and virulence. We know from previous studies that the deregulated synthesis of CPS is disadvantageous for *P. stewartii* to successfully colonize the plant host (von Bodman and Farrand, 1995).

Finally, we wish to emphasize that we cannot rule out the possibility that EsaR may function under certain conditions also as a gene activator, nor can we predict at this stage whether the expression of the *cps* gene system is under the direct control of EsaR. We have to consider the formal possibility that the main role of EsaR is to sequester cellular AHL and that a second QS regulatory system may actually govern CPS synthesis. However, such a mechanism is difficult to reconcile with the fact that the *esaI-esaR* double mutant *P. stewartii* strain ES Δ IR exhibits a hypermuroid phenotype in absence of cellular EsaR or AHL. Experiments to define the role of EsaR in the control of CPS and to search for additional QS systems in *P. stewartii* are in progress.

Experimental procedures

Bacterial strains, plasmids, oligonucleotides and DNA techniques

The *Escherichia coli* strains DH5 α (Life Technologies) and Top₁₀ (Invitrogen) were used as cloning hosts and were grown at 37°C on nutrient agar plates, Luria-Bertani (LB) broth, M9 minimal, or RM medium (1 × M9 salts, 1 mM MgCl₂, 2% casamino acids, 0.4% glucose) in presence of 100 µg ml⁻¹ of ampicillin, 10 µg ml⁻¹ of tetracycline, and 10 µg ml⁻¹ of kanamycin, where applicable. All relevant plasmids and strains are listed in Table 1. The *stewartii* strains were grown at 28°C in LB in presence of 30 µg ml⁻¹ of nalidixic acid. DNA techniques were performed by standard methods as described (von Bodman and Farrand, 1995; von Bodman *et al.*, 1998). DNA fragments were amplified using *Ex Taq* Polymerase (Takara/Panvera) and synthetic oligonucleotides (Table 2) ordered to specification from Sigma Genosys.

Plasmid constructions

We amplified the *esaR* gene using PCR primers, F_esaRN-

col and R_esaRHindIII (Table 2) to create a 5'-NcoI cloning site to overlap and include the ATG translation initiation codon, and a 3' HindIII site downstream of the stop codon. This fragment was inserted into the similarly digested expression vector, pBAD22 (Guzman *et al.*, 1995) resulting in plasmid pTDM6 (Fig. 1A). The *esaR* gene was re-cloned as a PstI-SalI fragment from plasmid pSVB5-18 (von Bodman and Farrand, 1995) into the broad-host-range vector, pBBR1MCS-3 (Kovach *et al.*, 1995) to generate plasmid pSVB60 (Table 1). This plasmid was used to create a translational reporter gene fusion by inserting the *lacZYKm^r* cassette from plasmid pLKC481 (Tiedeman and Smith, 1988) cloned as an XmaI fragment into the internal MroI site to generate pTDM7 (Fig. 1A and Table 1). Polymerase chain reaction (PCR) amplification of the *esaI* promoter and partial coding sequence used primers F_esaIXbaI and R_esaIXmaI that provided 5'-XbaI and 3'-XmaI restriction sites (Table 2) for cloning into plasmid pBBR1MCS-3 to create pTDM18 (Table 1). Plasmid pTDM18 was used to create an in-frame reporter gene fusion by linking the *lacZYKm^r* cassette released as a XmaI fragment from plasmid pLKC480 (Tiedeman and Smith, 1988) to the *esaI* coding sequence at the XmaI site to create pTDM19 (Table 1).

β-Galactosidase activity assay

Production of β -galactosidase was quantified as described (von Bodman and Farrand 1995). Cells were grown in RM minimal medium, diluted to an optical density (OD)₆₀₀ of 0.05 and allowed to grow to an OD₆₀₀ of 0.1 before inducing with L-arabinose. *N*-(3-oxo-hexanoyl)-L-homoserine lactone (OHHL) was supplied at the indicated concentrations either at the point of L-arabinose induction, or at defined times post L-arabinose induction. β -Galactosidase levels were expressed as β -galactosidase units (Sambrook *et al.*, 1989) or β -galactosidase activity (Miller, 1972). Each experiment was performed in triplicate and was repeated at least two times.

Expression and purification of EsaR protein

Native EsaR protein was purified from *E. coli* strain TOP₁₀ (pTDM6). This strain was grown at 28°C in 1 l of LB containing 100 μ g ml⁻¹ of ampicillin. The cells were harvested when the culture reached an optical density OD₆₀₀ of 0.6, resuspended in 10% glycerol, and stored as stock inocula at -80°C. Cells from 18 l volume fermentations, induced with 0.02% L-arabinose at an OD₆₀₀ of 0.6 were harvested 4 h post induction. The cells were resuspended in 180 ml of buffer (50 mM Tris, pH 7.5, 10% glycerol) and broken by three passages through a French press (20 000 psi). Cell debris was removed by centrifugation (30 000 *g* for 30 min) and the soluble fraction was passed through a 0.2 μ m cellulose filter (Millipore) before fractionation by heparin affinity perfusion chromatography (BioCAD, Poros 20HE). The column was equilibrated with 450 ml of TBP buffer (20 mM Tris bis-propane buffer, pH 7.5). The soluble lysate was applied to the column in four 5 ml-volume injections and the column was washed with 20 ml of TBP buffer after each injection. Bound protein was eluted with a two-step gradient of TBP buffer con-

taining 400 mM and 800 mM NaCl respectively. Fractions containing EsaR were identified by standard 15% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using protein molecular weight markers (prestained, broad range, New England Biolabs) for size reference, and differential expression of EsaR protein in un-induced and arabinose-induced lysates. Fractions containing EsaR were pooled and concentrated 16-fold with a Centriprep YM-10 filter (Millipore). The filtrate was applied to a size exclusion column (S-100 Sephacryl, 100KD exclusion) and eluted with a 1 bed-volume of TNG buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 10% glycerol). Fractions containing EsaR were identified by SDS-PAGE. EsaR was stored in TNG at -80°C.

Gel retardation assays

Synthetic DNA fragments used in these assays are listed in Table 2. Double-stranded DNA was obtained by mixing complementary oligodeoxyribonucleotides heated to 95°C and slowly cooled to room temperature. These DNAs were labelled at their 3'-ends with [α -³²P]-dATP, specific activity 3000 Ci mmol⁻¹ (Perkin Elmer) in presence of Klenow DNA polymerase (Amersham). DNA binding reactions used varying concentrations of purified EsaR incubated at 28°C for 30 min with 1.6 μ M concentration of ³²P-labelled DNA fragments in 20 μ l reactions using a buffer consisting of 20 mM Hepes, pH 7.6, 1 mM EDTA, 10 mM (NH₄)₂SO₄, 1 mM DTT, 0.2% Tween-20, 30 mM KCl, 50 mg ml⁻¹ of uncut λ -DNA and 150 μ g ml⁻¹ of BSA. After adding 5 μ l of loading buffer, each reaction mixture was fractionated by electrophoresis at 4°C on a native 6% polyacrylamide gel in 1 \times TBE buffer (Tris-Borate-EDTA, electrophoresis grade, Fisher Biotech). Gels were dried using a vacuum gel drier. An imaging screen-K (Bio-Rad) was exposed overnight and phosphorescence signal was detected with the Molecular Imager FX system (Bio-Rad). The bands were analysed with QUANTITY ONE quantification software (Bio-Rad).

Surface plasmon resonance technique

A BIAcore X instrument (BIAcore) was used to perform SPR measurements (Rich and Myszka, 2001). Activation of the SA streptavidin-coated sensor chip was performed following the manufacturer's recommended procedure. Non-specific and specific biotinylated DNA were coupled to a streptavidin matrix of two separate chips to yield approximately 120 resonance units (RU). The resulting sensor chips were analysed in parallel flowcells. All experiments were carried out at a flow rate of 25 μ l min⁻¹. EsaR analyte was presented in a running buffer (50 mM Tris, pH 7.5, 100 mM NaCl, 10 mM DTT, 0.1 mM EDTA). Bovine serum albumin (BSA) (200 ng μ l⁻¹) and λ -DNA (8 ng μ l⁻¹) were added to each protein sample before injection. Protein mixtures containing various concentrations (ranging from 22 nM to 1.4 μ M) of purified EsaR protein were injected allowing an association time of 180 s and a dissociation time of 350 s. The reference flow cell featuring a random DNA target of the same size as the probe DNA was used to subtract unspecific DNA-protein interactions. Regeneration of the chip surface was achieved by removing all bound proteins with a pulse of 5 ml of 0.05%

SDS in running buffer. Data analysis was performed using the BIAEVALUATION 3.0 program to determine the binding properties of the protein assuming 1:1 Langmuir kinetics.

MBP-EsaR hybrid protein

The EsaR protein was expressed from plasmid pMBP-EsaR that features the *esaR* coding sequence cloned into the pMAL-c2x plasmid (New England Biolabs) to generate a C-terminal fusion to the maltose-binding protein for expression in *E. coli* host strain TB1. The hybrid protein was purified by amylose resin (New England Biolabs) affinity chromatography. Bound proteins were eluted using four volumes of buffer (20 mM Tris, 1 mM EDTA, 200 mM NaCl) with a linear gradient of maltose from 0 mM to 0.6 mM. Proteolytic cleavage of the hybrid protein was achieved with Xa factor (New England Biolabs), using supplier-recommended conditions and varying times of incubation. DNA mobility-shift assays were performed as described above.

Fluorescence spectroscopy

Fluorescence measurements were performed with a Shimadzu RF-5001 PC spectrofluorimeter equipped with a Hellma Cuv-O-Stir magnetic cuvette stirrer and a Lauda RM6 thermostat. All experiments were carried out in 50 mM Tris pH 7.5, 100 mM NaCl, 0.1 mM EDTA. Purified EsaR protein was used for all experiments. Before measuring, the cuvettes containing 1.5 ml of the samples were equilibrated to 28°C and carefully stirred to ensure homogeneity. Spectra between 290 nm and 480 nm were recorded with an excitation wavelength of 284 nm and a scan speed of 0.8 nm per second. To avoid errors caused by sample dilution, the autoinducer titrations were performed by adding 1 µl of suitable stock solutions.

Circular dichroism measurements

Measurements were obtained with a Jasco J-600 spectropolarimeter equipped with a Lauda RC6 thermostat. All experiments were carried out in 20 mM Hepes pH 7.0, 500 mM NaCl, 5% glycerol. Spectra from 200 nm to 260 nm were recorded using a cuvette of 2 mm path length at a scanning speed of 20 nm per minute. For each temperature, the cuvette was allowed to equilibrate for 10 min before recording the spectrum.

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