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The *Serratia* LuxR family regulator $CarR_{39006}$ activates transcription independently of cognate quorum sensing signals

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Summary

In Gram-negative bacteria, quorum sensing control of gene expression is mediated by transcription factors of the LuxR family, whose DNA-binding affinity is modulated by diffusible *N*-acyl homoserine lactone (AHL) signalling molecules. In *Serratia* sp. ATCC 39006 and the plant pathogen *Erwinia carotovora* ssp. *carotovora* (*Ecc*), the biosynthesis of the β-lactam antibiotic 1-carbapen-2-em-3-carboxylic acid (Car) is under quorum sensing control. This study has revealed that, uniquely, the LuxR family transcriptional activator $CarR_{39006}$ from *Serratia* 39006 has no detectable affinity for cognate AHL molecules. Furthermore, $CarR_{39006}$ was shown to be naturally competent to bind to its target promoter with high affinity, activate transcription and resist cellular proteolysis, and was unaffected by AHL signals. Experiments with chimeric proteins suggest that the C-terminal DNA-binding domain of $CarR_{39006}$ may be responsible for conferring AHL independence. In contrast, we show that the homologous $CarR_{Ecc}$ protein binds to its 3O-C6-HSL ligand with high affinity, and that the highly conserved Trp-44 residue is critical for this interaction. Unlike TraR from *Agrobacterium tumefaciens*, $CarR_{Ecc}$ is not directly protected from cellular proteolysis by AHL binding, but via AHL-induced DNA binding. At physiological protein concentrations, AHL binding induces $CarR_{Ecc}$ to bind to its target promoter with higher affinity and activate transcription.

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Introduction

A diverse array of bacterial species regulate gene expression in response to population cell density, in a process termed quorum sensing (QS). Gram-negative proteobacteria sense population density using diffusible *N*-acyl homoserine lactone (AHL) molecules. AHLs act as ligands to modulate the DNA-binding ability of cytoplasmic transcription factors of the LuxR family, thereby controlling expression of key gene sets (Whitehead *et al.*, 2001). LuxR family proteins are highly divergent in primary sequence, but all share conserved secondary structure and a common two-domain architecture (Fig. S1). The N-terminal domains of LuxR proteins contain an amphipathic AHL-binding pocket (Vannini *et al.*, 2002; Zhang *et al.*, 2002). In TraR from *Agrobacterium tumefaciens*, the cognate 3O-C8-HSL inducer is completely buried within this pocket, and forms four key hydrogen bonds with the highly conserved TraR residues Tyr-53, Trp-57, Asp-70 and Thr-129 (Vannini *et al.*, 2002; Zhang *et al.*, 2002) (Fig. S1). In the LuxR family transcriptional activators TraR, LuxR from *Vibrio fischeri* and LasR from *Pseudomonas aeruginosa*, binding of the AHL inducer drives the formation of homodimers (Choi and Greenberg, 1992; Qin *et al.*, 2000; Zhu and Winans, 2001; Kiratisin *et al.*, 2002; Schuster *et al.*, 2004; Bottomley *et al.*, 2007). Dimerization facilitates binding of a helix–turn–helix motif in the C-terminal domain of the LuxR protomers to palindromic DNA sequences upstream of target promoters, thereby recruiting RNA polymerase for transcription (Vannini *et al.*, 2002; Zhang *et al.*, 2002; Schuster *et al.*, 2004; Urbanowski *et al.*, 2004). AHL binding was also shown to protect TraR from degradation by the cellular proteases ClpP and Lon (Zhu and Winans, 2001).

The LuxR family transcriptional activator CarR controls production of the β-lactam antibiotic carbapenem (1-carbapen-2-em-3-carboxylic acid; Car) in the plant pathogen *Erwinia carotovora* ssp. *carotovora* (*Ecc*) and in *Serratia* ATCC 39006 (S39006) (Parker *et al.*, 1982; Bycroft *et al.*, 1988; McGowan *et al.*, 1995; Cox *et al.*, 1998). In *Ecc*, the 3O-C6-HSL inducer synthesized by the AHL synthase CarI interacts with the $CarR_{Ecc}$ protein,

allowing it to bind to the promoter of the Car biosynthetic operon (*carA-H*) and activate transcription (McGowan *et al*., 1995; 1996; 1997; 2005; Welch *et al*., 2000). Unlike other LuxR proteins, data from *in vitro* experiments suggested that $CarR_{Ecc}$ may exist as a pre-formed dimer, which forms a higher-order multimer upon the addition of 3O-C6-HSL (Welch *et al*., 2000). In addition to its role as an AHL-dependent regulator, $CarR_{Ecc}$ was also reported to activate Car biosynthesis in the absence of AHL molecules, when overexpressed *in trans* (McGowan *et al*., 1995). This phenomenon, which is indicative of a mass-action effect caused by high concentrations of apo-$CarR_{Ecc}$, has not been reported for any other LuxR protein. Even when strongly overexpressed, TraR, LuxR from *V. fischeri* and RhlR and LasR from *P. aeruginosa* all required their cognate AHL molecule to function (Zhu and Winans, 1999; Lamb *et al*., 2003; Schuster *et al*., 2004; Urbanowski *et al*., 2004).

Serratia ATCC 39006 possesses a homologous *carA-H* biosynthetic cluster under the transcriptional control of the $CarR_{39006}$ protein (Cox *et al*., 1998; Thomson *et al*., 2000). $CarR_{39006}$ and $CarR_{Ecc}$ share strong overall amino acid identity (59.3%) and similarity (75.0%) (Fig. S1). Carbapenem production in *Serratia* 39006 is completely dependent upon production of the short-chain C4-HSL signal by the SmaI synthase (Thomson *et al*., 2000). However, in an AHL-deficient *smaI* mutant, a subsequent mutation in the gene encoding a second LuxR homologue (the SmaR repressor) restored wild-type carbapenem production (Slater *et al*., 2003), suggesting that $CarR_{39006}$ can activate *carA-H* transcription in the absence of AHLs. According to this model, QS control of Car production is imposed by the AHL-responsive SmaR protein, via transcriptional repression of the $carR_{39006}$ gene (and the *carA* promoter itself) in the absence of AHLs (Slater *et al*., 2003; Fineran *et al*., 2005a). In support of this model, expression of $carR_{39006}$ *in trans* complemented the carbapenem production defect in an *Ecc* $carR_{Ecc}$ mutant, in the absence of AHLs (Cox *et al*., 1998). However, the mass-action effect observed with overexpressed $CarR_{Ecc}$ made the significance of this result unclear. It was also recently reasoned that $CarR_{39006}$ might be inherently transcriptionally active, but could become deactivated by binding to cognate AHLs (Tsai and Winans, 2010), as has been proposed for EsaR from *Pantoea stewartii* (Schu *et al*., 2009).

The aim of this study was therefore to confirm and investigate the phenomenon of AHL independence in $CarR_{39006}$, in comparison with its more extensively studied homologue $CarR_{Ecc.}$ We show that, uniquely, the *Serratia* $CarR_{39006}$ protein has no detectable affinity for AHLs, but can still bind to its target promoter with high affinity and activate transcription in the absence of AHLs. We further show that, unlike TraR, protection of $CarR_{Ecc}$ and $CarR_{39006}$ from cellular proteolysis relies on DNA binding. Finally, our data suggest that the C-terminal domain of $CarR_{39006}$ may be responsible for conferring AHL independence.

Results

$CarR_{39006}$ has no detectable affinity for Erwinia *or* Serratia *AHLs*

First, we analysed the binding of $CarR_{Ecc}$ and $CarR_{39006}$ to cognate AHL signals, using isothermal titration calorimetry (ITC). The TraR protein from *A. tumefaciens* forms a hydrogen bond between its Trp-57 residue and the carbonyl group of the AHL lactone ring (Vannini *et al*., 2002; Zhang *et al*., 2002). This H-bond is also formed between the equivalent Trp-60 residue of LasR from *P. aeruginosa* and its 3O-C12-HSL ligand (Bottomley *et al*., 2007; Zou and Nair, 2009). This functional tryptophan residue is very highly conserved among LuxR homologues, including $CarR_{Ecc}$ (Cox *et al*., 1998) (Fig. S1). In contrast, $CarR_{39006}$ contains a cysteine at this position (Cys-44), which forms much weaker H-bonds than tryptophan (Fig. S1). It was previously hypothesized that this W44C substitution might confer AHL independence to $CarR_{39006}$ (Slater *et al*., 2003). The remaining three H-bonding residues are conserved in $CarR_{39006}$ (Fig. S1).

To test the importance of the strongly conserved tryptophan residue for AHL binding, the highly soluble His_6-tagged AHL-binding domain of $CarR_{Ecc}$ (residues 1 to 167) was purified by Ni-NTA affinity chromatography, and ITC analysis demonstrated its strong affinity for the *Ecc* inducer 3O-C6-HSL (Fig. 1A). Based on the observed stoichiometry of the interaction, binding of 3O-C6-HSL to $CarR_{Ecc}^{1\text{-}167}$ was fitted to a two binding site model, probably reflecting one molecule of 3O-C6-HSL binding to each protomer in a $CarR_{Ecc}$ dimer (Welch *et al*., 2000) (Fig. 1A). The values for the calculated dissociation constants (K_d = 0.72 μM and 3.53 μM) are consistent with previous fluorescence quenching data (Welch *et al*., 2000). Site-directed mutagenesis of the Trp-44 residue to cysteine completely abolished any detectable interaction between $CarR_{Ecc}$ and 3O-C6-HSL (Fig. 1A). This strongly suggests that H-bonding by the Trp-44 residue is essential for AHL binding.

The AHL-binding ability of the N-terminal domain of $CarR_{39006}$ was then compared with that of His_6-$CarR_{Ecc}^{1\text{-}167}$. No detectable interaction was observed between the His_6-$CarR_{39006}^{1\text{-}167}$ protein and either 3O-C6-HSL or the *Serratia* C4-HSL ligand (Fig. 1B), suggesting that $CarR_{39006}$ is unresponsive to the cognate *Erwinia* and *Serratia* AHL signals.

At physiological concentrations, $CarR_{Ecc}$ requires AHLs to activate transcription

To directly isolate the activity of $CarR_{Ecc}$ and $CarR_{39006}$ in the absence of any endogenous host regulation, these

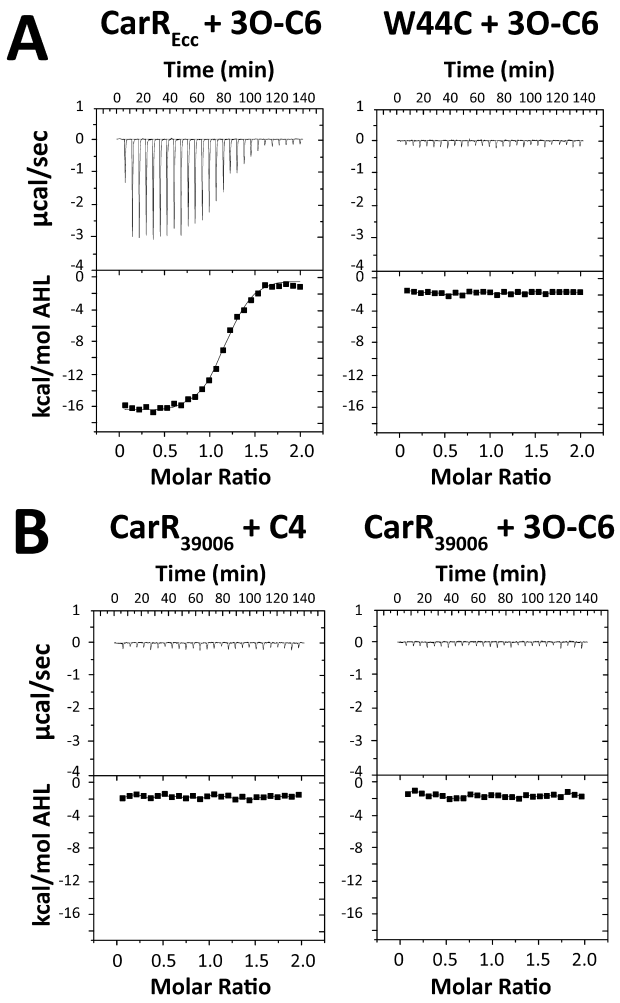

Fig. 1. CarR$_{39006}$ does not detectably bind to *Serratia* or *Erwinia* AHLs.
A. ITC of synthetic 3O-C6-HSL titrated into wild-type (left) and W44C (right) variants of His$_6$-CarR$_{Ecc}^{1-167}$. For His$_6$-CarR$_{Ecc}^{1-167}$, K_d values were calculated as 0.72 ± 0.15 µM (binding site 1) and 3.53 ± 0.41 µM (binding site 2).
B. Titration of synthetic C4-HSL (left) and 3O-C6-HSL (right) into a His$_6$-CarR$_{39006}^{1-167}$ solution.
Upper panel, raw heat uptake data for sequential 10 µl injections of 600 µM of the indicated AHL into a solution containing 60 µM of the relevant His$_6$-CarR$^{1-167}$ variant at 30°C.
Lower panel, integrated heat data corrected for heats of dilution with theoretical fit to a two binding site model using the Origin VP-ITC software.

proteins were expressed in the heterologous host *Escherichia coli* DH5α and assessed for their ability to activate transcription of their target carbapenem operon promoters in the presence of AHL inducers.

When expressed in *E. coli* DH5α from the pQE80L-derived plasmid pSP78 induced with 1 µM IPTG, hexahistidine-tagged CarR$_{Ecc}$ required 1 µM 3O-C6-HSL to activate transcription from the *Ecc* P*carA*::*lacZ* transcriptional reporter plasmid pSP14 (Fig. 2A). No β-galactosidase activity was detected with either the empty pQE80L vector (Fig. 2A), or 3O-C6-HSL in the absence of His$_6$-CarR$_{Ecc}$ (data not shown). His$_6$-tagged CarR$_{Ecc}$ retained ~ 85 % of the transcriptional activity of the untagged protein (data not shown). Non-cognate AHLs of varying acyl chain length and oxidation state were unable to induce His$_6$-CarR$_{Ecc}$ to activate *Ecc carA* transcription (Fig. 2A), consistent with the observation that CarR$_{Ecc}$ has a low affinity for these signals (Welch *et al.*, 2000). As previously reported, the non-cognate 3O-C8-HSL signal also induced His$_6$-CarR$_{Ecc}$ to activate *carA* transcription, to a higher level than the endogenous 3O-C6-HSL (Welch *et al.*, 2000) (Fig. 2A).

Binding to AHLs protects CarR$_{Ecc}$ from cellular degradation

Previous studies demonstrated that binding to AHLs protected the *A. tumefaciens* TraR protein from cellular

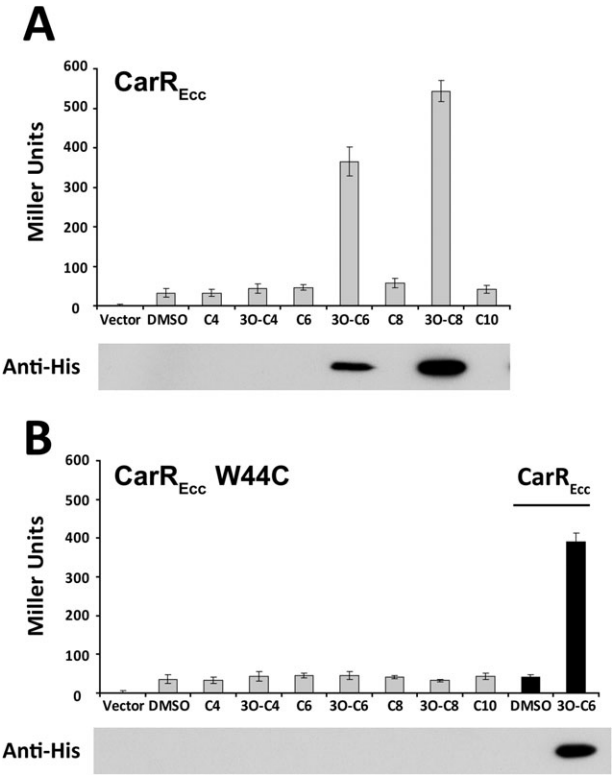

Fig. 2. Binding to cognate acyl homoserine lactones protects CarR$_{Ecc}$ from cellular proteolysis and activates *carA* transcription. Full-length N-terminal His$_6$-CarR$_{Ecc}$ (A) and site-directed variant His$_6$-CarR$_{Ecc}$ W44C (B) were expressed from pSP78 and pSP90, respectively, in *E. coli* DH5α carrying the *Ecc* P*carA*::*lacZ* transcriptional reporter plasmid pSP14. LB broths were supplemented with 1 µM IPTG, and either DMSO or a 1 µM final concentration of the indicated AHLs. Expression of the *carA* promoter was measured as β-galactosidase activity (expressed as Miller Units). A second sample was blotted with anti-His antibodies to detect His$_6$-CarR$_{Ecc}$. DH5α carrying pSP14 and the empty pQE80L vector was induced with IPTG as a negative control. Data shown are the means ± SD of three independent experiments.

proteolysis (Zhu and Winans, 2001). In contrast, MrtR from *Mesorhizobium tianshanense* and RhlR from *P. aeruginosa* were stably expressed, but unable to activate transcription, in the absence of AHLs (Lamb *et al*., 2003; Yang *et al*., 2009). To assess whether binding to cognate AHLs was having any stabilizing effect on the $CarR_{Ecc}$ protein *in vivo*, duplicate samples from the P*carA* transcription experiment were separated by SDS-PAGE and probed with anti-His antibodies to detect His_6-$CarR_{Ecc}$. Consistent with the model for TraR, His_6-$CarR_{Ecc}$ was only detected in *E. coli* DH5α cultures grown in the presence of 3O-C6-HSL and 3O-C8-HSL (Fig. 2A). Based on the experiments with TraR, it was hypothesized that binding to cognate AHLs protected His_6-$CarR_{Ecc}$ from degradation by cellular proteases.

To test this hypothesis, a full-length version of the 'signal-blind' His_6-$CarR_{Ecc}$ W44C mutant protein was expressed from pSP90 in *E. coli* DH5α carrying pSP14 (Fig. 2B). The signal-blind W44C variant was unable to resist cellular proteolysis or activate *carA* transcription in the presence of 3O-C6-HSL (Fig. 2B), suggesting that the ability to bind to AHLs is essential for $CarR_{Ecc}$ to function and resist degradation at physiologically relevant concentrations. When the wild type and W44C variants were expressed with higher IPTG concentrations, $CarR_{Ecc}$ protein levels were comparable via Western blotting, suggesting that the W44C mutation was not intrinsically attenuating $CarR_{Ecc}$ production or stability (Fig. S2). These data also do not support the previous hypothesis that the W44C substitution in $CarR_{39006}$ might be responsible for conferring AHL independence (Slater *et al*., 2003).

When overexpressed, $CarR_{Ecc}$ accumulates and activates carA *transcription in the absence of AHLs*

Based on the AHL-dependent nature of *carA* transcription (Fig. 2A) it was predicted that induction of pSP78 with 1 μM IPTG resulted in approximately wild-type His_6-$CarR_{Ecc}$ protein levels. We next investigated the previously reported phenomenon of AHL-independent *carA* transcriptional activation, by overexpressing His_6-$CarR_{Ecc}$. Consistent with previous studies in *Ecc* and with the data in Fig. 2A, in *E. coli* DH5α cultures induced with lower IPTG concentrations (0 μM and 1 μM) P*carA* transcription was dependent upon 3O-C6-HSL (Fig. 3A). However, as IPTG induction increased to 2 μM, *carA* transcription was activated in the absence of AHL, to ~ 60% of the level induced by 3O-C6-HSL. With 5 μM IPTG, *Ecc carA* transcription was identical in the presence or absence of 3O-C6-HSL. In each case, His_6-$CarR_{Ecc}$ protein levels (assessed by Western blot) correlated with the increase in AHL-independent *carA* transcription. At higher IPTG induction levels, His_6-$CarR_{Ecc}$ appeared to somehow overcome degradation by cellular proteases, and activate *carA* transcription.

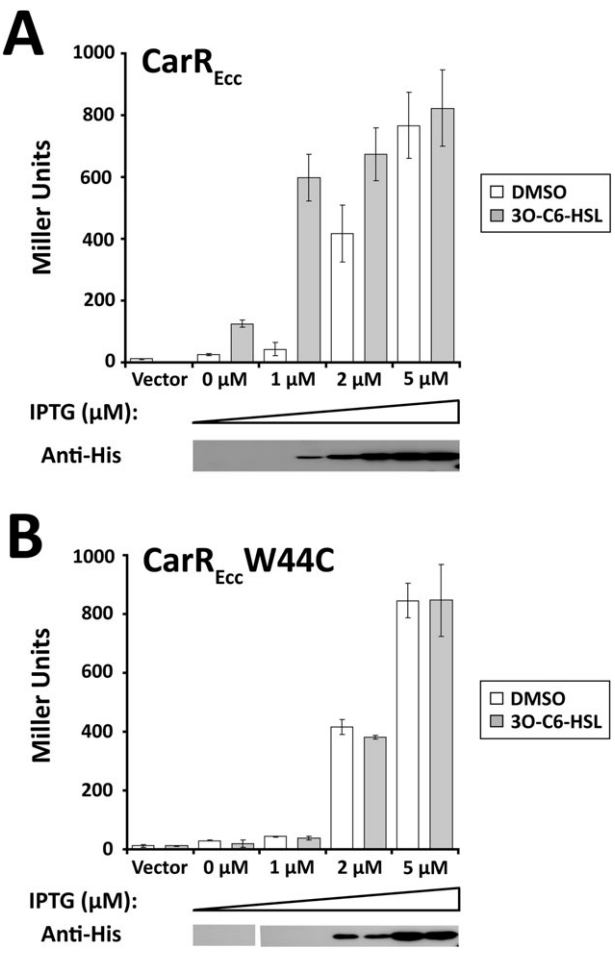

Fig. 3. AHL-independent $CarR_{Ecc}$ accumulation directly correlates with *Erwinia carA* transcription. His_6-$CarR_{Ecc}$ (A) and site-directed variant His_6-$CarR_{Ecc}$ W44C (B) were expressed from pSP78 and pSP90, respectively, in *E. coli* DH5α carrying the *Ecc* P*carA*::*lacZ* reporter plasmid pSP14. LB broths were supplemented with the indicated concentration of IPTG, and either DMSO (open bars) or 1 μM 3O-C6-HSL (grey bars). Expression of the *carA* promoter was measured as β-galactosidase activity (expressed as Miller Units). A second sample was blotted with anti-His antibodies to detect His_6-$CarR_{Ecc}$. DH5α carrying pSP14 and the empty pQE80L vector was induced with 5 μM IPTG as a negative control. Data shown are the means ± SD of three independent experiments.

AHL-independent *Ecc carA* transcription and protein accumulation at higher IPTG induction levels was also observed with the signal-blind His_6-$CarR_{Ecc}$ W44C variant (Fig. 3B). This supports the hypothesis that AHL-independent $CarR_{Ecc}$ function is caused by a mass-action effect that is unrelated to the ability to bind to AHLs.

$CarR_{39006}$ activates transcription and resists proteolysis in the absence of AHLs

We next tested the effect of AHLs on the transcriptional activity of the *Serratia* $CarR_{39006}$ protein. In previous

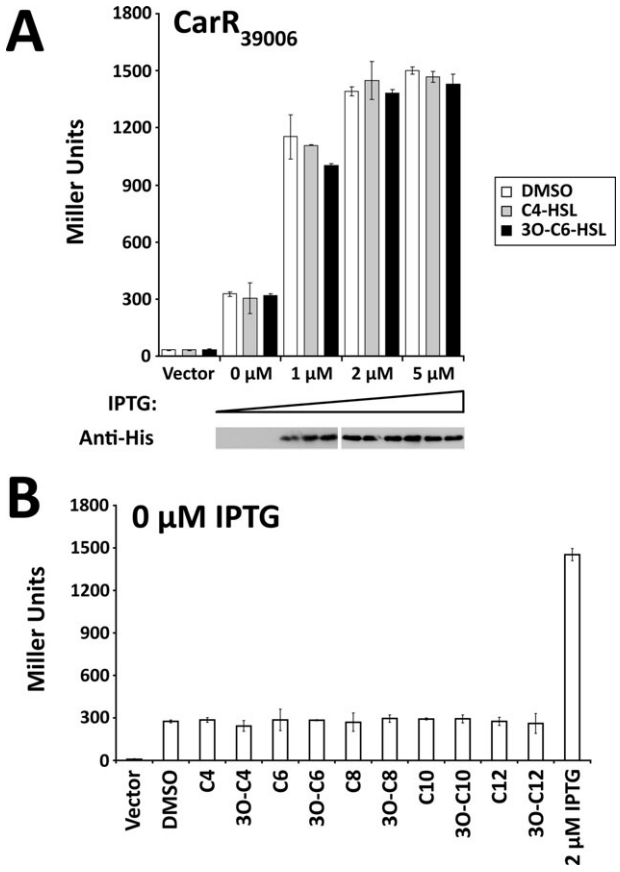

Fig. 4. $CarR_{39006}$ accumulation and *Serratia carA* transcription is unaffected by synthetic AHLs.
A. N-terminal His_6-$CarR_{39006}$ was expressed from pSP79 in *E. coli* DH5α carrying the *Serratia* P*carA*::*lacZ* transcriptional reporter plasmid pTA16. LB broths were supplemented with the indicated concentration of IPTG, and either DMSO (open bars), 1 μM C4-HSL (grey bars) or 1 μM 3O-C6-HSL (black bars). Expression of the *carA* promoter was measured as β-galactosidase activity (expressed as Miller Units). A second sample was blotted with anti-His antibodies to detect His_6-$CarR_{39006}$. DH5α carrying pTA16 and the empty pQE80L vector was induced with 5 μM IPTG as a negative control.
B. His_6-$CarR_{39006}$ was expressed in *E. coli* DH5α (pTA16), in the absence of IPTG, with 1 μM of the indicated AHLs, and assayed for β-galactosidase activity. A culture was also induced with 2 μM IPTG, as a positive control. Data shown are the means ± SD of three independent experiments.

studies, it was unclear whether the apparent AHL-independent *carA* transcription in *Serratia* 39006 was the result of some inherent property of the $CarR_{39006}$ protein, or due to other *Serratia*-specific regulatory factors or features of the *Serratia carA* promoter. To investigate AHL independence in the absence of potential complications from *Serratia*-specific regulation, $CarR_{39006}$ was expressed in a heterologous host, *E. coli* DH5α. In this host background, transcription from the *Serratia* P*carA*::*lacZ* transcriptional reporter plasmid pTA16 was activated by expression of His_6-$CarR_{39006}$ from pSP79 (Fig. 4A). However, unlike $CarR_{Ecc}$, His_6-$CarR_{39006}$ activated transcription in the absence of AHLs at all IPTG concentrations tested, consistent with previous genetic studies in *Ecc* and *Serratia* (Cox *et al.*, 1998; Fineran *et al.*, 2005a). Moreover, the addition of 1 μM C4-HSL or 3O-C6-HSL to cultures did not significantly alter *carA* transcription (Fig. 4A). Western blotting also showed that the accumulation of the His_6-$CarR_{39006}$ protein was not altered by the addition of either C4-HSL or 3O-C6-HSL (Fig. 4A). His_6-$CarR_{39006}$ was also unresponsive to a broader range of non-cognate AHLs (Fig. 4B). Taken together, these data are consistent with a transcriptional activator that functions independently of AHLs.

Site-directed mutagenesis to convert the Cys-44 residue back to the highly conserved tryptophan slightly reduced the stability of His_6-$CarR_{39006}$, but did not restore AHL dependence (Fig. S3), strongly suggesting that the W44C substitution is not the sole cause of AHL independence in $CarR_{39006}$.

$CarR_{39006}$ has a naturally strong affinity for its target promoter

It has been demonstrated for other LuxR family proteins that binding to their cognate AHL increases their affinity for target DNA (Qin *et al.*, 2000; Schuster *et al.*, 2004; Urbanowski *et al.*, 2004). Unlike other LuxR family proteins, the full-length His_6-$CarR_{Ecc}$ and His_6-$CarR_{39006}$ proteins were estimated to be 60–70% soluble in the absence of AHLs, when expressed under low-temperature (16°C) conditions (data not shown). We were therefore able to assess the DNA-binding affinity of Ni-NTA purified His_6-CarR proteins by *in vitro* electrophoretic mobility shift assays (EMSA), using digoxygenin (DIG)-labelled *carA* promoter fragments (Fig. 5).

In the absence of 3O-C6-HSL, His_6-$CarR_{Ecc}$ displayed an intrinsic specific affinity for the *Ecc carA* promoter, with 100 nM His_6-$CarR_{Ecc}$ forming a DNA–protein complex (Complex 1, Fig. 5A). Binding was assessed to be relatively weak, as only part of the DIG-labelled DNA was shifted. The weak affinity of apo-$CarR_{Ecc}$ for its promoter DNA is consistent with AHL-independent *carA* transcription when the protein was overexpressed (Fig. 3A). When 3O-C6-HSL was added, His_6-$CarR_{Ecc}$ formed DNA–protein complexes at lower protein concentrations (20 nM), indicating enhanced affinity of $CarR_{Ecc}$ for the *carA* promoter (Fig. 5B). Addition of 3O-C6-HSL to the reaction mixture containing 40 nM and 100 nM His_6-$CarR_{Ecc}$ also caused the formation of a more highly retarded DNA–protein complex (Complex 2, Fig. 5B). This complex may be compatible with *carA* DNA binding to an AHL-induced higher-order CarR multimer, as previously suggested (Welch *et al.*, 2000). However, this EMSA method is not appropriate for accurate assessment of multimeric state. Alternatively, this DNA–protein complex

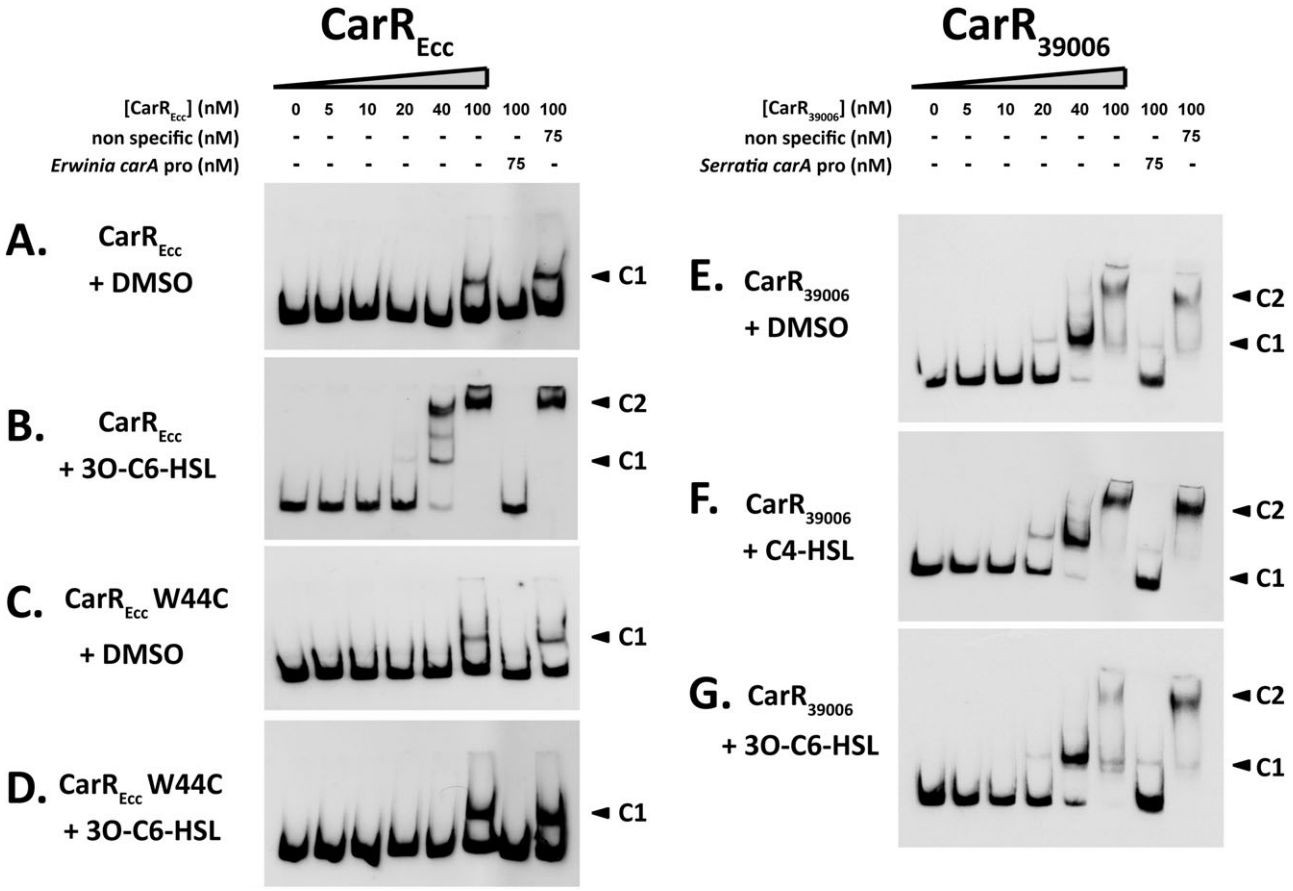

Fig. 5. Binding of His_6-CarR proteins to *carA* promoter DNA. EMSA assays were performed with Ni-NTA purified His_6-$CarR_{Ecc}$ (A and B), His_6-$CarR_{Ecc}$ W44C (C and D) or His_6-$CarR_{39006}$ (E, F and G), as described in *Experimental procedures*. *Ecc* and *Serratia carA* promoter DNA fragments were generated by PCR. Each lane contained 1.5 nM of appropriate DIG-labelled *carA* promoter DNA, the indicated concentration of the relevant His_6-CarR protein, and either DMSO, 5 μM C4-HSL or 5 μM 3O-C6-HSL. Competition experiment lanes contained 1.5 nM DIG-labelled *carA* DNA, 100 nM of the relevant His_6-CarR protein, and a 50-fold excess (75 nM) of either unlabelled *carA* promoter DNA or a non-specific fragment. High and low mobility DNA–protein complexes are indicated as Complex 1 (C1) and Complex 2 (C2) respectively.

could represent CarR dimers bound to two distinct sites in the same *carA* promoter fragment, perhaps facilitating transcription via DNA looping (De Carlo *et al*., 2006). Regardless of the $CarR_{Ecc}$ multimeric state, the more highly retarded DNA–protein complex completely shifted the *carA* DNA, indicating that AHL-bound $CarR_{Ecc}$ protein had a higher DNA affinity than apo-His_6-$CarR_{Ecc}$. Enhanced binding of $CarR_{Ecc}$ to *carA* promoter DNA correlates with activation of *carA* transcription in the presence of 3O-C6-HSL (Fig. 3A).

Like the wild-type protein, His_6-$CarR_{Ecc}$ W44C had an intrinsic weak affinity for the *carA* promoter (Fig. 5C), but this was not altered by 3O-C6-HSL (Fig. 5D). This is consistent with a signal-blind $CarR_{Ecc}$ variant that is unresponsive to AHLs, and therefore locked in a state with low DNA-binding affinity.

In contrast, His_6-$CarR_{39006}$ displayed a strong intrinsic affinity for the *Serratia carA* promoter in the absence of AHLs, with 20 nM His_6-$CarR_{39006}$ forming a DNA–protein complex (Fig. 5E). In fact, the DNA-binding ability of His_6-$CarR_{39006}$ in the absence of AHLs closely matched that of His_6-$CarR_{Ecc}$ in the presence of 3O-C6-HSL (Fig. 5B) consistent with the hypothesis that $CarR_{39006}$ is a 'locked-on' version of $CarR_{Ecc}$. Reactions containing 100 nM His_6-$CarR_{39006}$ formed the more highly retarded DNA–protein complex (Complex 2), formation of which required 3O-C6-HSL with His_6-$CarR_{Ecc}$. The affinity of His_6-$CarR_{39006}$ for the *Serratia carA* promoter was not altered by either C4-HSL or 3O-C6-HSL (Fig. 5F and G).

CarR protease resistance requires target promoter DNA

The ability of His_6-$CarR_{Ecc}$ to bind to 3O-C6-HSL correlated with enhanced DNA affinity (Fig. 5B) but it was unclear how this was linked with protection from cellular proteolysis (Fig. 2). In TraR, AHL-mediated protease protection occurred in the absence of target promoter DNA, suggesting that AHL binding was protecting the protein directly

(Zhu and Winans, 2001). Like the TraR model, 3O-C6-HSL could be directly protecting His_6-$CarR_{Ecc}$, thereby allowing it to accumulate and activate *carA* transcription. Alternatively, $CarR_{Ecc}$-3O-C6-HSL complexes could be resisting proteolysis via enhanced binding to target DNA. In this second model, protection from proteolysis would only occur in the presence of promoter DNA.

To test if either of the two models was correct, His_6-$CarR_{Ecc}$ accumulation was assessed in the absence of the *carA* promoter, by expressing pSP78 (encoding His_6-$CarR_{Ecc}$) in *E. coli* DH5α carrying the promoterless pRW50 progenitor plasmid instead of the P*carA*::*lacZ* reporter pSP14. In the presence of 3O-C6-HSL, His_6-$CarR_{Ecc}$ was only detectable by Western blot in *E. coli* cells containing the *carA* promoter (pSP14) but not in its absence (pRW50) (Fig. 6A). Similarly, His_6-$CarR_{39006}$ expressed from pSP79 was only detectable in the presence of the *Serratia carA* promoter (pTA16) (Fig. 6B). In fact, His_6-$CarR_{Ecc}$ and His_6-$CarR_{39006}$ expressed in the presence of pRW50 required approximately 50- to 200-fold higher IPTG induction to accumulate to a similar level as when expressed with pSP14 or pTA16 (Fig 6C and D). This suggests that *carA* promoter DNA is involved in stabilizing the His_6-CarR proteins.

More importantly, addition of 3O-C6-HSL did not significantly protect His_6-$CarR_{Ecc}$ from proteolysis in the absence of target promoter DNA (Fig. 6C). This result disputes the model that $CarR_{Ecc}$ is directly protected from cellular proteolysis by AHL binding, and instead suggests that the protective action of 3O-C6-HSL may be mediated indirectly, via binding of His_6-CarR proteins to the *carA* promoter. This model of CarR protease protection is strikingly different from that currently proposed for TraR (Zhu and Winans, 1999; 2001).

The C-terminal domain of $CarR_{39006}$ may confer AHL independence

The C-terminal DNA-binding domains of $CarR_{Ecc}$ and $CarR_{39006}$ share an amino acid identity of 67.4% (86.0% similarity). The two proteins are especially well-conserved in the predicted recognition helix (α9) of the helix–turn–helix DNA-binding motif (Fig. S4). $CarR_{39006}$ expressed from a multicopy plasmid can also complement a *carR*$_{Ecc}$ mutation in *Ecc* 39048 (Cox *et al.*, 1998). By expressing pSP78 in the presence of pTA16, and pSP79 with pSP14, we next showed that $CarR_{Ecc}$ and $CarR_{39006}$ were functionally interchangeable, activating maximal transcription from the heterologous *carA* promoter (Fig. S5). Moreover, the response of each CarR protein to AHLs was retained while activating either *carA* promoter, suggesting that the ability of $CarR_{39006}$ to activate *carA* transcription in the absence of AHLs was due to an inherent property of the protein itself (Fig. S5).

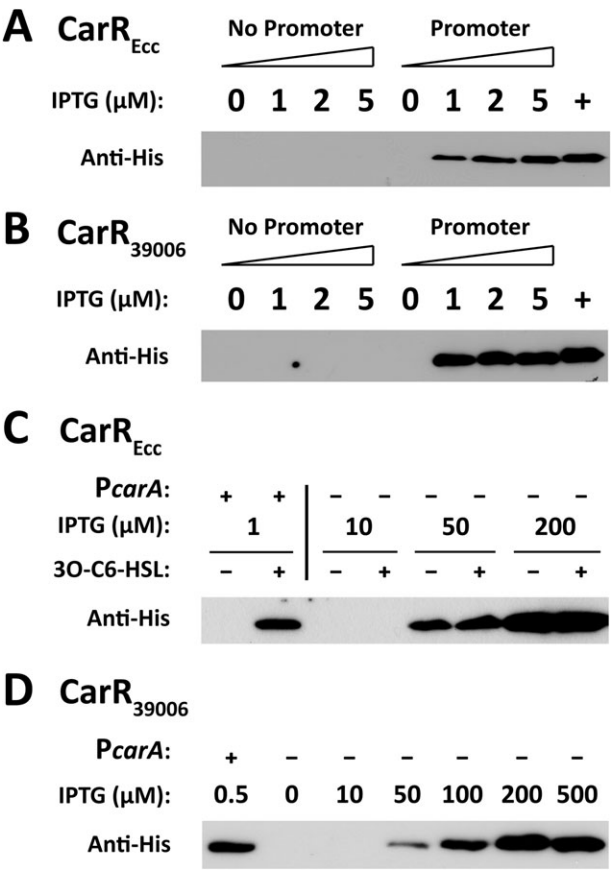

Fig. 6. Accumulation of His_6-CarR proteins requires the presence of target *carA* promoter DNA. His_6-$CarR_{Ecc}$ (A) and His_6-$CarR_{39006}$ (B) were expressed from pSP78 and pSP79, respectively, in *E. coli* DH5α carrying the empty pRW50 vector (No Promoter) or either pSP14 or pTA16 (Promoter). LB broths were supplemented with the indicated concentration of IPTG. For $CarR_{Ecc}$, cultures were also supplemented with 1 μM 3O-C6-HSL. Proteins were separated by SDS-PAGE and blotted with anti-His antibodies. Purified His_6-$CarR_{Ecc}$ (+) was used as a positive control. His_6-$CarR_{Ecc}$ (C) and His_6-$CarR_{39006}$ (D) were also expressed in the presence or absence of the *carA* promoter, in the absence (DMSO control) or presence of 1 μM 3O-C6-HSL, with increasing concentrations of IPTG.

However, these data gave no clues as to the functional differences between the $CarR_{Ecc}$ and $CarR_{39006}$ proteins that caused them to behave so differently. Given the strong sequence identity between the C-terminal DNA-binding domains of the two CarR proteins, it was previously hypothesized that AHL independence might be conferred by the N-terminal domain of $CarR_{39006}$ (Slater *et al.*, 2003). To test this hypothesis, the $CarR_{39006}$ N-terminal domain (residues 1 to 158) was fused to the C-terminal domain of the $CarR_{Ecc}$ protein (residues 159 to 244) to form a $CarR_{39006\text{-}Ecc}$ domain chimera, and vice versa to create a $CarR_{Ecc\text{-}39006}$ chimera.

To identify which domain of the $CarR_{39006}$ protein was responsible for AHL independence, these CarR domain chimeras were expressed in *E. coli* DH5α carrying pSP14.

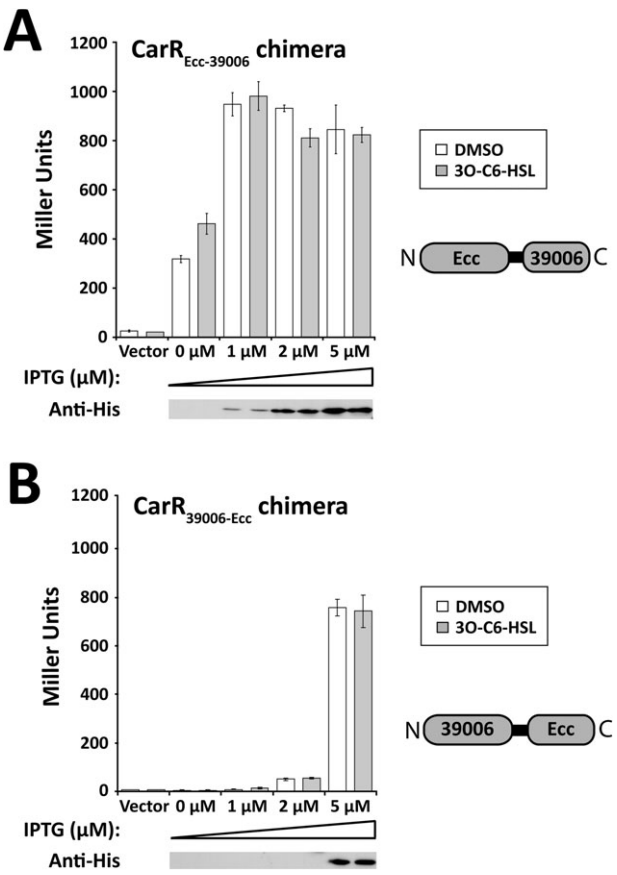

Fig. 7. The C-terminal domain of $CarR_{39006}$ may confer AHL independence. His_6-tagged $CarR_{Ecc\text{-}39006}$ (A) and $CarR_{39006\text{-}Ecc}$ (B) chimera proteins were expressed from pSP89 and pSP88, respectively, in *E. coli* DH5α carrying the transcriptional reporter plasmid pSP14. LB broths were supplemented with the indicated concentration of IPTG, and either DMSO (open bars) or 1 µM 3O-C6-HSL (grey bars). Expression of the *carA* promoter was measured as β-galactosidase activity (expressed as Miller Units). A second sample was blotted with anti-His antibodies. Data shown are the means ± SD of three independent experiments. Schematic diagrams show the domain structure of the chimera proteins, which were fused at amino acid 158 of the relevant CarR protein.

The His_6-$CarR_{Ecc\text{-}39006}$ chimera expressed from pSP89 accumulated and activated *carA* transcription at low IPTG induction levels, and was not significantly affected by the addition of 3O-C6-HSL (Fig. 7A). This result was very similar to that induced by wild-type His_6-$CarR_{39006}$, suggesting that the C-terminal domain of $CarR_{39006}$ may be conferring ligand independence, contrary to our previous hypothesis. Control experiments showed that the $CarR_{39006}$ C-terminal domain alone could not activate *carA* transcription (data not shown). In contrast, the His_6-$CarR_{39006\text{-}Ecc}$ chimera expressed from pSP88 was far less able to accumulate, or activate transcription (Fig. 7B). In fact, the His_6-$CarR_{39006\text{-}Ecc}$ protein acted in a similar fashion to that of wild-type His_6-$CarR_{Ecc}$ grown in the absence of 3O-C6-HSL, or His_6-$CarR_{Ecc}$ W44C grown in the presence of 3O-C6-HSL (Fig. 3).

Discussion

This study has furthered our understanding of the mode of action of the $CarR_{Ecc}$ and $CarR_{39006}$ proteins. Our data confirm that the $CarR_{Ecc}$ protein binds to its 3O-C6-HSL ligand *in vitro* with high affinity, and that the hydrogen bond formed between 3O-C6-HSL and the highly conserved Trp-44 residue is critical for this interaction. The complete abolition of detectable AHL binding in the W44C mutant protein suggests that this key hydrogen bond may be required for the formation or stabilization of additional H-bonds between $CarR_{Ecc}$ residues and the AHL ligand. *In vitro*, AHL binding induced the $CarR_{Ecc}$ protein to bind to the *carA* promoter with higher affinity. In the heterologous host *E. coli* DH5α, enhanced DNA binding had the twin effect of preventing proteolytic turnover and activating *carA* transcription. The W44C variant of His_6-$CarR_{Ecc}$ cannot bind to 3O-C6-HSL, and so remains in a low-affinity state, which cannot resist proteolysis or activate *carA* transcription at physiologically relevant concentrations. The mechanism by which DNA-bound $CarR_{Ecc}$ resists cellular proteolysis is unclear, but must differ significantly from the direct protection afforded to the TraR protein from *A. tumefaciens* by 3O-C8-HSL (Zhu and Winans, 1999; 2001).

This study has also confirmed the ability of $CarR_{Ecc}$ to bind to target DNA and activate transcription in the absence of AHLs, when overexpressed. This is in stark contrast to other activators of the LuxR family, in which the apo-form had no detectable affinity for target DNA (Qin *et al.*, 2000; Schuster *et al.*, 2004). The window between AHL-dependent and AHL-independent *carA* transcription was narrow, with mild overexpression of $CarR_{Ecc}$ sufficient to activate *carA* transcription. The AHL-independent behaviour of $CarR_{Ecc}$ when overexpressed also demonstrates that care must be taken when attempting to interpret the physiological mechanism of proteins based purely on *in vitro* data and the expression of physiologically artefactual protein concentrations from multicopy plasmid systems.

This study has also revealed that, uniquely, the previously uncharacterized $CarR_{39006}$ protein from *Serratia* 39006 has no detectable affinity *in vitro* for AHL molecules, and that its ability to bind to its target *carA* promoter with high affinity, activate transcription and successfully resist cellular proteolysis is unaffected by AHLs. The results of *in vivo* experiments in the heterologous *E. coli* host closely match those obtained from several genetic studies in *Serratia*, implying that AHL independence is conferred by the $CarR_{39006}$ protein itself, and not by other *Serratia*-specific regulatory factors. High-affinity binding of purified His_6-$CarR_{39006}$ to purified target DNA, in the absence of any other molecule, suggests that $CarR_{39006}$ does not require a ligand to function. Before this study, it had been demonstrated that some QS systems can respond to non-AHL

molecules, such as *Rhodopseudomonas palustris*, which responds to a *p*-coumaroyl-homoserine lactone derived from lignin (Schaefer *et al*., 2008). Similarly, OryR from *Xanthomonas oryzae* pv. *oryzae* is activated by undefined plant signals from rice (Ferluga and Venturi, 2009). Other LuxR family proteins have been shown to be inherently transcriptionally active, but are inactivated by cognate AHLs (Schu *et al*., 2009). In contrast, binding of $CarR_{39006}$ to DNA is neither improved nor attenuated by AHLs, thereby representing the first truly ligand-independent LuxR family protein.

The binding of apo-$CarR_{Ecc}$ and apo-$CarR_{39006}$ to DNA is reminiscent of the 'repressor' class of LuxR proteins, such as EsaR from *P. stewartii* (Von Bodman *et al*., 1998). Members of this class are more accurately described as 'apo-functional', as in some cases their competence to bind DNA in the absence of AHLs can result in transcriptional activation (Von Bodman *et al*., 2003; Schu *et al*., 2009). Members of this class are inactivated by AHL signals, via a poorly defined mechanism, resulting in derepression (or deactivation) of transcription. A very recent review of LuxR family proteins proposed that these apo-functional LuxR proteins fall into a single phylogenetic clade (Tsai and Winans, 2010). Interestingly, the $CarR_{Ecc}$ and $CarR_{39006}$ activators were identified as the closest relatives of this apo-functional class (Fig. S6). Thus, $CarR_{Ecc}$ and $CarR_{39006}$ may represent evolutionary intermediates between AHL-activated and AHL-inactivated LuxR proteins. Our data show that $CarR_{39006}$ represents a third class of LuxR protein, which is neither activated nor inactivated by AHLs.

Experiments with domain chimeras suggest that the C-terminal DNA-binding domain of $CarR_{39006}$ may be responsible for conferring AHL independence. Attaching this C-terminal domain onto the $CarR_{Ecc}$ N-terminus resulted in high-affinity DNA binding and transcriptional activation, abolishing the need for an AHL signal. The N-terminal domain residues involved in AHL binding are probably no longer selectively advantageous in the AHL-independent $CarR_{39006}$, so the loss of the critical Trp-44 residue may have occurred by neutral drift. In contrast, attachment of the signal-blind $CarR_{39006}$ N-terminal domain onto the $CarR_{Ecc}$ C-terminal domain (which is normally activated by 3O-C6-HSL binding to the N-terminus) resulted in low affinity binding to the *carA* promoter, protease sensitivity and poor transcriptional activation.

The C-terminal DNA-binding domains of $CarR_{Ecc}$ and $CarR_{39006}$ are extremely similar, containing only three amino acid differences within alpha helices α7, α8, α9 and α10 (Fig. S4). This strong amino acid identity is consistent with the ability of these two proteins to activate transcription from the two *carA* promoters interchangeably (Fig. S5). Interestingly, secondary structure prediction suggests that the extreme C-terminus of $CarR_{39006}$ may contain an additional short alpha helix, which is not predicted for $CarR_{Ecc}$ or any other LuxR family protein analysed, and which may be functionally significant (Fig. S4). Further studies are required to identify the C-terminal regions that are responsible for the unique mechanism by which the signal-blind $CarR_{39006}$ protein has evolved to overcome the requirement for an AHL ligand.

Experimental procedures

Bacterial strains and culture conditions

Escherichia coli DH5α was grown at 37°C in Luria–broth (LB) at 300 r.p.m. or on LB agar containing 1.5% (w/v) agar. Media were supplemented with ampicillin (Ap, 100 μg ml^{-1}) and tetracycline (Tc, 35 μg ml^{-1}), and growth (OD_{600}) was measured as described previously (Poulter *et al*., 2010). When required, an appropriate amount of isopropyl-β-D-thiogalactopyranoside (IPTG) was added (see *Results*). Synthetic *N*-acyl homoserine lactones were synthesized as previously described (Glansdorp *et al*., 2004). All experiments were performed in triplicate and plotted as mean ± SD.

DNA manipulation and sequence analysis

Molecular biology techniques and sequencing were performed as described previously (Poulter *et al*., 2010). Oligonucleotide primers are shown in Table S1. All plasmids were verified by DNA sequencing. Sequence data were analysed using GCG (Genetics Computer Group, University of Wisconsin) and compared with GenBank DNA or non-redundant protein sequence databases using BLAST (Altschul *et al*., 1997). Multiple sequence alignments were performed using MultAlin (Corpet, 1988), and images were generated with ESPript (Gouet *et al*., 1999). Secondary structure prediction analysis was performed using PSIpred (McGuffin *et al*., 2000).

Construction of CarR expression vectors

All proteins expressed in this study contained N-terminal hexahistidine (His_6) tags, and all plasmids are shown in Table 1. CarR expression vectors pSP78 (His_6-$CarR_{Ecc}$) and pSP79 (His_6-$CarR_{39006}$) were created by PCR cloning of the $carR_{Ecc}$ and $carR_{39006}$ genes into pQE80L (Qiagen) BamHI and PstI sites, using primers SP277 and SP257, and SP278 and PF67 respectively. For expression of the CarR AHL-binding domains (amino acids 1 to 167), the primers SP277 and SP279 ($carR_{Ecc}$) and SP278 and SP280 ($carR_{39006}$) were used to PCR clone the first 501 bp of the *carR* genes into pQE80L BamHI and PstI sites, creating plasmids pSP76 (His_6-$CarR_{Ecc}^{1\text{-}167}$) and pSP77 ($His_6$-$CarR_{39006}^{1\text{-}167}$).

Site-directed mutagenesis of CarR proteins

Substitution of residue 44 of $CarR_{Ecc}$ and $CarR_{39006}$ was performed by overlap extension PCR (Warrens *et al*., 1997). A $CarR_{Ecc}$ W44C variant was created by PCR amplification of the 5′ 148 bp of $carR_{Ecc}$ using SP277 and the mutagenic primer SP273, and the 3′ 618 bp of $CarR_{Ecc}$ using mutagenic

Table 1. Bacterial strains and plasmids used in this study.

Strain/plasmid	Genotype/phenotype	Reference
Escherichia coli		
BL21/DE3	B strain; F^- *ompT* $hsdS_B$ (r_B^-·m_B^-) *dcm gal* λ(DE3[*lacI lacUV5* -T7 *gene 1 ind1 sam7 nin5*])	Novagen
DH5α	F^-, ϕ80Δ*dlacZM15*, Δ(*lacZYA-argF*)U169, *endA1, recA1, hsdR17 (rk-mk+), deoR, thi-1, supE44*, λ^-, *gyrA96, relA1*	Gibco/BRL
Serratia		
ATCC 39006	Wild type	Bycroft *et al.* (1988)
Erwinia carotovora		
Ecc ATCC 39048	Wild type	Bainton *et al.* (1992)
Plasmids		
pQE80L	Cloning vector for N-terminal hexahistidine proteins, $lacI^q$, Ap^R	Qiagen
pRW50	Promoterless *lacZ* fusion reporter plasmid, RK2 replicon, Tc^R	Lodge *et al.* (1992)
pSP14	*Erwinia carA* promoter *lacZ* fusion (–210 to +127), Tc^R	This study
pSP76	His_6-$CarR_{Ecc}^{1-167}$ expression vector, derivative of pQE80L, Ap^R	This study
pSP77	His_6-$CarR_{39006}^{1-167}$ expression vector, derivative of pQE80L, Ap^R	This study
pSP78	Full size His_6-$CarR_{Ecc}$ vector, derivative of pQE80L, Ap^R	This study
pSP79	Full size His_6-$CarR_{39006}$ vector, derivative of pQE80L, Ap^R	This study
pSP88	His_6-$CarR_{39006\text{-}Ecc}$ chimera vector, derivative of pQE80L, Ap^R	This study
pSP89	His_6-$CarR_{Ecc\text{-}39006}$ chimera vector, derivative of pQE80L, Ap^R	This study
pSP90	Full-length His_6-$CarR_{Ecc}$ W44C vector, derivative of pQE80L, Ap^R	This study
pSP91	Full-length His_6-$CarR_{39006}$ C44W vector, derivative of pQE80L, Ap^R	This study
pSP111	His_6-$CarR_{Ecc}^{1-167}$ W44C vector, derivative of pQE80L, Ap^R	This study
pTA16	*Serratia carA* promoter *lacZ* fusion (–199 to +50), Tc^R	Gristwood *et al.* (2008)

primer SP274 and SP257. Using the resulting products as template, PCR amplification with SP277 and SP257 resulted in a spliced 735 bp product, which was cloned into pQE80L BamHI and PstI sites to generate plasmid pSP90. An identical strategy was used for the $CarR_{39006}$ C44W variant plasmid pSP91, using primers SP278 and SP95 (5′ end) and SP96 and PF67 (3′ end). The $CarR_{Ecc}$ W44C ligand-binding domain (His_6-$CarR_{Ecc}^{1-167}$ W44C) was expressed from plasmid pSP111, created by PCR cloning the 5′ 501 bp of $carR_{Ecc}$ W44C from pSP90 into pQE80L BamHI and PstI sites, using primers SP277 and SP279.

Construction of CarR domain chimeras

Chimera proteins were constructed, in which the $CarR_{39006}$ N-terminal AHL-binding domain (residues 1 to 158) was fused to the $CarR_{Ecc}$ C-terminal DNA-binding domain (residues 159 to 244), and vice versa. The 5′ 474 bp of $carR_{39006}$ was PCR amplified using primers SP278 and SP299, and the 3′ 261 bp of $carR_{Ecc}$ amplified using SP300 and SP257. Using the resulting products as template, PCR amplification with SP278 and SP257 resulted in a final 735 bp spliced product in which the gene fragment encoding the $CarR_{39006}$ N-terminal domain was fused to the fragment encoding the $CarR_{Ecc}$ C-terminal domain. An identical strategy was used using primers SP277 and SP307, and primers SP308 and PF67, resulting in a spliced $carR_{Ecc\text{-}39006}$ PCR product. These products were cloned into pQE80L BamHI and PstI sites to create plasmid pSP88 and pSP89 respectively.

Purification of His_6-tagged proteins

CarR proteins were purified from *E. coli* BL21/DE3 carrying plasmids pSP76, pSP77, pSP78, pSP79, pSP90 or pSP111. 5 ml overnight cultures were grown in LB with Ap at 37°C, and used to inoculate 1 l of pre-warmed LB and Ap. Expression cultures were grown at 37°C with shaking at 200 r.p.m. to an OD_{600} of 0.5, then rapidly cooled to 20°C, induced with 1 mM IPTG and incubated overnight at 16°C. His_6-tagged proteins were purified from harvested cells using Ni-NTA resin (Qiagen) as described previously (Gristwood *et al.*, 2008). Elution fractions containing protein were pooled and dialysed overnight against 1 l Dilution Buffer (50 mM NaH_2PO_4/300 mM NaCl/pH 8.0) at 4°C.

carA *promoter* lacZ *fusion experiments*

The *Ecc carA* promoter (nucleotides –210 to +127, relative to the transcriptional start site) was PCR cloned with primers SP152 and SP153 into the BamHI and HindIII sites of pRW50 (Lodge *et al.*, 1992) to give plasmid pSP14. The *Serratia carA* promoter (-199 to +50) was previously cloned into pRW50, to create plasmid pTA16 (Gristwood *et al.*, 2008). *E. coli* DH5α cells carrying pRW50, pSP14 or pTA16, plus pSP78, pSP79, pSP88, pSP89, pSP90 or pSP91, were grown in 5 ml of LB, plus Ap, Tc, IPTG and synthetic AHLs to an OD_{600} of 0.5. Promoter expression (measured as Miller Units) was determined as described previously (Fineran *et al.*, 2005b).

Western blot analysis for CarR proteins

Western blot analysis was performed against bacterial cell samples taken from promoter *lacZ* fusion experiments (normalized to OD_{600} and separated by SDS-PAGE), using a primary mouse monoclonal anti-His antibody (Novagen) and a goat anti-mouse polyclonal HRP conjugated secondary antibody (Sigma).

EMSA

Electrophoretic mobility shift assays experiments were performed with 3′ DIG-labelled *carA* promoter fragments from pSP14 and pTA16, using a DIG Gel Shift Kit (Roche). EMSA reaction mixtures (20 μl) contained the indicated amount of His_6-CarR protein and 1.5 nM of DIG-labelled DNA, in binding buffer [20 mM HEPES, pH 7.6, 1 mM EDTA, 10 mM $(NH_4)_2SO_4$, 1 mM DTT, 0.02% Tween and 30 mM KCl], 5 μg ml^{-1} poly-L-lysine and 50 μg ml^{-1} poly[d(A-T)]. Specific competition reactions contained 50-fold excess (75 nM) unlabelled *carA* promoter DNA. An *rsmA* promoter fragment from *Erwinia carotovora* ssp. *atroseptica* SCRI1043 (Bell *et al*., 2004) was used as a non-specific competitor.

ITC

Isothermal titration calorimetry was performed on a VP-ITC micro-calorimeter (MicroCal) at 30°C. Proteins were dialysed against ITC Buffer (50 mM NaH_2PO_4/300 mM NaCl/1% DMSO/pH 8.0). Synthetic AHLs (50 mM in DMSO) were diluted into Dilution Buffer (50 mM NaH_2PO_4/300 mM NaCl/pH 8.0) to 600 μM. Standard settings were: 27 injections (2 μl, 5 μl, then 25 × 10 μl), 120 s initial delay, 30°C, reference power 17, stirring speed 310 r.p.m. Based on the observed stoichiometry of binding, raw data were processed and plotted using a two binding site model (Origin software). Dissociation constants (K_d) were derived from K_a values ($K_d = 1/K_a$) calculated from the slope of the isotherm curve.

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