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Structure–activity relationships of *Erwinia carotovora* quorum sensing signaling molecules

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Abstract—Production of virulence factors and secondary metabolites is regulated in the phytopathogen *Erwinia carotovora* by quorum sensing involving *N*-acylated homoserine lactone (AHL) signaling molecules. Non-hydrolyzable AHL analogues were synthesized and screened in vivo. The biological activity of each compound was correlated with its ability to bind *Erwinia* AHL receptor proteins (LuxR homologues) in vitro. There is an excellent correlation between carbapenem production in vivo and in vitro binding to CarR. However, no such correlation could be found between exoprotease production and analogue binding to EccR. Our data are consistent with the involvement of a third, as yet uncharacterized LuxR homologue.

Many microorganisms communicate intercellularly by synthesising and detecting the presence of specific small molecules in a cell density-dependent fashion; this phenomenon is known as quorum sensing (QS).[1–3] This mode of cell–cell communication has been studied intensely in Gram-negative bacteria, where *N*-acyl homoserine lactones (AHL) are signaling molecules and the AHL receptor proteins are known as LuxR homologues.[4] Clinically important and commercially relevant pathogens often use QS to control virulence factor production, secondary metabolite biosynthesis and biofilm formation.[5] For example, some strains of the phytopathogen *Erwinia carotovora* ssp. *carotovora* (Ecc) use 3-oxo-hexanoyl-L-homoserine lactone (OHHL, Fig. 1) to regulate the production of exoenzymes (proteases, cellulases and pectinases) and to control carbapenem antibiotic synthesis.[6,7] At least three *luxR* homologues have been identified in different *Erwinia* species on the basis of sequence similarity.[8–11] The LuxR homologue, EccR, may play a role in the regulation of exoenzyme production in some Ecc strains, since mutation of the equivalent gene (*expR*) in Ecc strain SCC3193 results in small increase in pectate lyase production and virulence.[10] However, in Ecc strain ATTn10, an *eccR* mutation has no significant impact on exoenzyme production,[11] while carbapenem antibiotic production is controlled by the LuxR homologue, CarR.[9] The mechanisms for QS regulation mediated by these various LuxR homologues remain to be elucidated fully. In order to investigate this, we used OHHL, HHL and non-hydrolyzable analogues **1** and **2** (Fig. 1)

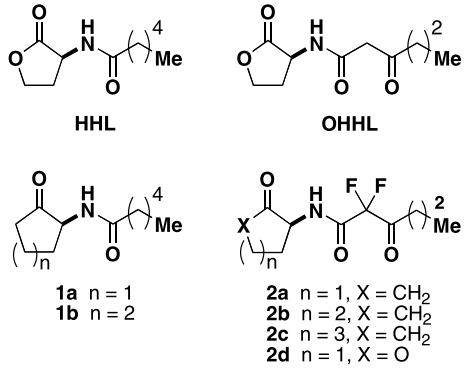

Figure 1. Structures of the compounds used in this study.

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to correlate biological activity in vivo with the ability to bind *Erwinia* LuxR homologues in vitro.

The major endogenous AHL produced by Ecc strain ATTn10 is OHHL, with smaller amounts of HHL also being made; both ligands are synthesized by the CarI protein.[9] We generated a defined *carI* knockout by allelic exchange and verified that this strain did not produce OHHL (data not shown). A variety of synthetic C_6 chain compounds were tested to see how they affected carbapenem (Table 1) and protease (Table 2) production in the mutant. Most of the compounds did not facilitate carbapenem synthesis, even when applied at concentrations of up to 50 μM (Table 1). Only OHHL and HHL were able to induce substantial carbapenem biosynthesis, although the difluorinated OHHL derivative (**2d**) was able to elicit some production of the antibiotic at the highest concentration applied. It seems that even minor alterations of the AHL head group are detrimental to the induction of carbapenem synthesis by Ecc.

The QS-dependent regulation of protease production by Ecc was less sensitive to head group modifications (Table 2), consistent with the involvement of a different LuxR homologue in the regulation of exoenzyme production.[12] OHHL was able to promote protease production by the *carI* mutant at concentrations as low as 50 nM. Introduction of the difluoro-group into the acyl chain of OHHL (**2d**) reduced the activity of the ligand about 100-fold, as did removal of the 3-oxo moiety from the acyl chain (HHL). Replacement of the homoserine lactone group in the difluorinated OHHL derivative with either a cyclohexanone (**2b**) or a cycloheptanone (**2c**) moiety[13] abolished biological activity of the ligand. However, the cyclopentanone derivative (**2a**) showed some protease-inducing activity, albeit weak. This suggests that the head group preference in Ecc is for a 5-membered ring, unlike *Pseudomonas aeruginosa*.[13] In line with this, cyclopentanone **1a** showed weak protease-inducing activity, while the corresponding cyclohexanone **1b** was completely inactive. It seems that the AHL receptor proteins in Ecc are exquisitely sensitive to even minor alterations in the ligand, since any modification to either the acyl chain or the head group of the ligands reduces their biological activity.

Table 1. Effect of analogues on carbapenem production by the Ecc ATTn10 *carI* mutant

Compound	Concentration (μM)			
	50	5	0.5	0.05
(±)-**1a**	–	–	–	–
(±)-**1b**	–	–	–	–
OHHL	+++	++	+	–
(*S*)-**2a**	–	–	–	–
(*S*)-**2b**	–	–	–	–
(*S*)-**2c**	–	–	–	–
HHL	+++	–	–	–
(*S*)-**2d**	+	–	–	–

Halo sizes were scored by measuring the diameter of the clear zone around each colony spot. +++, wild type halo size; ++, reduced halo size (20–99% of wild type halo diameter); +, very small halo (<20% of wild type halo diameter); –, no halo.

Table 2. Effect of analogues on protease production by the Ecc ATTn10 *carI* mutant

Compound	Concentration (μM)			
	50	5	0.5	0.05
(±)-**1a**	+	–	–	–
(±)-**1b**	–	–	–	–
OHHL	+++	+++	+++	++
(*S*)-**2a**	++	–	–	–
(*S*)-**2b**	–	–	–	–
(*S*)-**2c**	–	–	–	–
HHL	+++	+++	+	–
(*S*)-**2d**	+++	+++	–	–

Halo sizes were scored by measuring the diameter of the clear zone around each colony spot after developing the plates with saturated ammonium sulfate. +++, wild type halo size; ++, reduced halo size (20–99% of wild type halo diameter); +, very small halo (<20% of wild type halo diameter); –, no halo.

We have shown previously that equilibrium binding of AHL to a purified soluble portion of CarR (from Ecc strain ATTn10) can be monitored by measuring changes in the intrinsic tryptophan fluorescence of the protein.[14] Given that a highly conserved Trp residue is likely to be directly involved in ligand binding by all LuxR homologues,[15,16] fluorescence is probably a generally useful way of investigating ligand binding to these proteins. Therefore, we attempted to purify all of the known LuxR homologues in Ecc and to investigate their ligand-binding properties.

Overexpression of full-length CarR and EccR gave mostly inclusion bodies, with only very low yields of soluble protein (data not shown). However, when we expressed defined N-terminal hexa-histidine-tagged truncates composed of approximately the first two-thirds of each protein, we obtained high yields (ca. 10 mg/l culture) of soluble protein. (In the TraR crystal structure[15,16], this region spans from the N-terminus to the junction between α6 and α7.) These constructs ($CarR_{1–167}$ and $EccR_{1–182}$) are predicted to include the ligand-binding and putative dimerization domain of each protein. In both cases, the fluorescence of the protein was quenched (albeit to differing extents) upon addition of the AHL analogues. The apparent dissociation constants were derived from the raw data by applying a binding model.[14] Ligands that caused fluorescence changes of <20% (relative to F_0, the fluorescence in the absence of added ligand) were considered to be weak binders (e.g., **2c**) and were excluded from this analysis. Examples of the data fits obtained for $CarR_{1–167}$ and $EccR_{1–182}$ binding to OHHL and **2d** are shown in Figure 2.

The obtained dissociation constants are given in Table 3. The K_d obtained for OHHL binding to $CarR_{1–167}$ was 1.5 μM, which compares favourably with previous measurements (1.8 μM.[14]). The only other synthetic analogue that bound reasonably well to $CarR_{1–167}$ was **2d** (K_d = 37 μM). This correlates well with the phenotypic observations (Table 1), showing that of the analogues tested, only **2d** was able to elicit carbapenem production. Analogues **1a** and **b** also bound to $CarR_{1–167}$, but the dissociation constants were well above the maximal

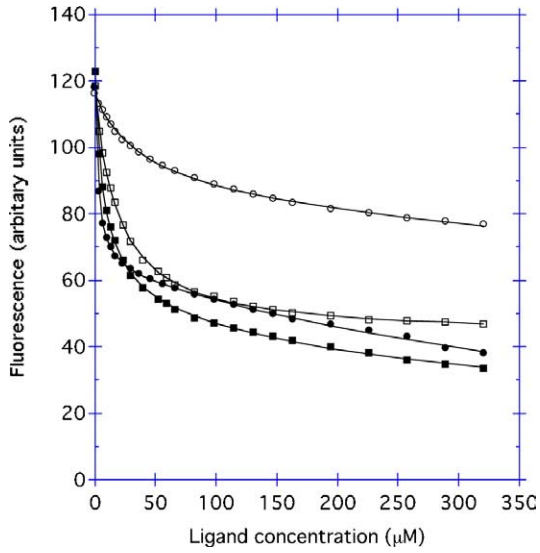

Figure 2. Effect of OHHL and **2d** on the fluorescence of CarR and EccR. Solid lines: best curve fits to the data calculated by the sum of least squares method; (○): CarR/**2d**; (●): CarR/OHHL; (□): EccR/**2d**; (■): EccR/OHHL. The calculated dissociation constants are given in Table 3.

Table 3. Apparent dissociation constants for selected AHL and analogues binding to soluble fragments of the CarR and EccR proteins

Compound	K_d CarR (μM)	K_d EccR (μM)
OHHL	1.5	7.5
(*S*)-**2d**	37	5.2
(*S*)-**2a**	—	6.7
(*S*)-**2b**	—	45
(±)-**1a**	349	109
(±)-**1b**	286	316

concentration that was feasible to test in the carbapenem assay (Table 1).

The binding data for $EccR_{1–182}$ are less consistent with the phenotypic data (induction of exoprotease activity). The presumed natural ligand of EccR (OHHL) bound to the protein with K_d = 7.5 μM. It should be noted that very little is known about the precise role of EccR in the control of virulence factor production and this is the first direct evidence that this homologue physically binds any AHL. However, although OHHL was by far the most potent inducer of exoprotease activity (Table 2), **2d** (K_d = 5 μM) and **2a** (K_d = 6.7 μM) displayed tighter binding to $EccR_{1–182}$. Cyclohexanone **2b**, which was essentially inactive at eliciting protease production in vivo, exhibited a low affinity for $EccR_{1–182}$ (K_d = 45 μM), suggesting that EccR has a preference for 5-membered rings. Consistent with this, the cyclopentanone **1a**, which activated slightly protease production in the *carI* mutant at the highest concentration assayed (50 μM), bound to $EccR_{1–182}$ with higher affinity than the less active corresponding 2-aminocyclohexanone **1b**.

Taken together, our findings suggest that although there is good agreement between the binding of ligands to CarR and induction of carbapenem biosynthesis, the correlation is much weaker between the binding of these ligands to EccR and their ability to induce exoprotease activity. One explanation for this might be that Ecc contains one (or possibly more) additional LuxR homologues that are involved in regulating protease production. Indeed, Bell et al. recently reported that a novel LuxR homologue (denoted ECA1561) is present in the genome of a closely related *Erwinia* species, *E. carotovora* ssp. *atroseptica*, and speculated that the protein might be involved in the control of virulence.[8] We used PCR to amplify the corresponding gene from Ecc and expressed the predicted N-terminal domain region (residues 1–169) of the encoded protein in *Escherichia coli*. Although the resulting protein was soluble at high concentration (ca. 5 mg/ml) in storage buffer, it immediately precipitated upon dilution into the fluorescence assay buffer. We tried diluting the protein into storage buffer and performing the assays in this medium, but none of the ligands induced any fluorescence change in these conditions. Addition of various detergents (sarkosyl, zwittergents 3–16 and 3–14, β-octyl glucoside) did not improve solubility, neither did altering the ionic strength or the pH. We conclude that, the presence of this LuxR homologue (or heterodimers formed between this gene product and EccR, as is the case in other systems[17]) in vivo may account for the disparity between the phenotypic observations and the direct binding data.

In conclusion, we have purified the ligand-binding domains of all known Ecc LuxR homologues and assayed the ability of these constructs to bind a range of non-hydrolyzable AHL analogues in vitro. Non-hydrolyzable AHL analogues should be generally useful reagents for investigating the mechanism and consequences of QS in a variety of organisms. CarR is known to bind ligands as a preformed dimer, and preliminary cross-linking studies suggest that EccR is likely to behave in a similar way (M.W., unpublished data). The in vitro binding data with CarR are in good overall agreement with the observed carbapenem production in vivo data; however, no such correlation could be found between exoprotease production and analogue binding to EccR. Our data are consistent with the involvement of a third, as yet uncharacterized LuxR homologue.

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Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bmcl.2005.06.066.

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