Purification and characterisation of a quorum quenching AHL-lactonase from the endophytic bacterium Enterobacter sp. CS66

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One sentence summary: A quorum quenching enzyme originally associated with Gram-positive bacterial species is also found in Gram-negative bacteria too.

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ABSTRACT

The quorum quenching (QQ) activity of endophytic bacteria associated with medicinal plants was explored. Extracts of the Gram-negative Enterobacter sp. CS66 possessed potent N-acylhomoserine lactone (AHL) hydrolytic activity in vitro. Using degenerate primers, we PCR-amplified an open reading frame (denoted aiiE) from CS66 that was 96% identical to the well-characterised AHL-lactonase AiiA from Bacillus thuringiensis, but only 30% was identical to AHL-lactonases from other Gram-negative species. This confirms that close AiiA homologs can be found in both Gram-positive and Gram-negative bacteria. Purified AiiE exhibited potent AHL-lactonase activity against a broad range of AHLs. Furthermore, aiiE was able to reduce the production of secreted plant cell wall-degrading hydrolytic enzymes when expressed in trans in the economically important plant pathogen, Pectobacterium atrosepticum. Our results indicate the presence of a novel AHL-lactonase in Enterobacter sp. CS66 with significant potential as a biocontrol agent.

Keywords: quorum sensing; Pectobacterium atrosepticum; quorum quenching

INTRODUCTION

Many pathogens cause tissue damage by secreting a welter of exceptionally active proteases and phospholipases, and in many species of bacteria, the secretion of these exoproducts is now known to be coordinated by a cell–cell communication mechanism called ‘quorum sensing’ (QS). In essence, each cell in the
Table 1. Bacterial strains and plasmids used in this study.

| Strain or plasmid | Description | Source or references |
|-------------------|-------------|---------------------|
| **Strains**       |             |                     |
| Rosetta DE3       | E. coli BL21 derivative, pRare2, Cm' | Tegel et al. 2010 |
| Serratia sp. SP19 | Serratia sp. ATCC 39006 derivative | Pouiller et al. 2010 |
| Escherichia coli JM109 | E. coli strain for cloning and expression | New England Biolabs |
| Enterobacter sp. CS66 | Endophytic AHL-degrading strain | This study |
| Pectobacterium atrosepticum (Eca1043) | Wild type | Bowden et al. 2013a |
| P. atrosepticum (Δexipl, SB1031) | Quorum sensing mutant | This study |
| **Plasmids**      |             |                     |
| pMAL-c2X          | MBP fusion cloning vector; Ap' | New England Biolabs |
| pET19m            | Expression vector to make His6-tagged fusion proteins; Ap' (modified pET-19b, Novagen) | Dolan et al. 2017 |
| pMAL-c2X-aiiE      | pMAL containing aiil from Enterobacter sp. CS66; Ap' | This study |
| pET19m-aiiE       | pET19m containing aiil from Enterobacter sp. CS66; Ap' | This study |

Because of the key role it plays in controlling virulence and biofilm formation by pathogenic bacteria, QS has become a popular target for the development of anti-virulence strategies. Some of these approaches employ small molecules to block QS molecule synthesis or reception (Hodgkinson et al. 2012). For example, inhibition of QS lowers the production of virulence factors and depresses biofilm formation in both Gram-negative and Gram-positive bacterial pathogens (Hentzer and Givskov 2003). An alternative approach exploits the fact that some bacteria produce enzymes capable of degrading AHL signalling molecules. Such quorum quenching (QQ) enzymes exhibit AHL-lactonase, AHL-acylase, or AHL-oxidoreductase activity (Fetzner 2015). Of these, the AHL-lactonases have been most extensively studied, with examples including AiiA from a Bacillus sp. (Dong et al. 2000), AiiD from Arthrobacter sp. IBN110 (Park et al. 2003), AidC from Chryseobacterium sp. StRB126 (Wang et al. 2012), AttM from Agrobacterium tumefaciens (Carlier et al. 2003), AiiL from Microbacterium testaceum (Wang et al. 2010) and AiiLS from Solibacillus silvestris (Morohoshi et al. 2012).

The microflora associated with many plants of medicinal interest have been under-investigated, and this environment remains a rich and unexploited reservoir of microbes with biotechnological potential. Recently, we reported on an Enterobacter sp. (denoted VT66) isolated from Ventilago madraspatana, which encodes a ca. 30 kDa enzyme with AHL-degrading activity (Rajesh and Rai 2015). However, the gene encoding the AHL degrading enzyme was not cloned, and the protein was only partially characterized. In the present study, we identified a gene encoding an AHL-lactonase from a different endophyte, Enterobacter sp. CS66. This gene (denoted aiil) was cloned for recombinant expression and further characterisation, and its potential application in controlling the expression of virulence factors by the QS phytopathogen, P. atrosepticum, was explored.

MATERIALS AND METHODS

Bacterial strains and plasmids

The strains used in the study are listed in Table 1. Rosetta DE3 (derived from Escherichia coli BL21) was grown in the presence of chloramphenicol (Cm, 34 μg/mL) at 37°C. Escherichia coli JM109 was grown at 37°C. Pectobacterium atrosepticum wild type (strain Eca1043) and the isogenic P. atrosepticum mutant Δexipl (SB1031) were grown at 30°C. Where required, ampicillin (Ap) was used.
at a final concentration of 50 μg/mL. *Serratia* sp. SP19 and *Chromobacterium violaeum* CV026 were used as biosensors to detect C4-HSL. These strains were grown at 30°C.

**Isolation and identification of Enterobacter sp. CS66**

A sample of *Coscinium fenestratum* Gaertn. was collected from forest of Western Ghats in Karnataka, India (13.08° N, 75.45° E). The plant was identified by consulting taxonomists and the herbarium of the plant was preserved in the Department of Studies in Microbiology (MGMB/001/2013-14), University of Mysore, Mysore, India. Endophytic bacteria were isolated as previously described and screened for their ability to degrade AHLs (Rajesh and Rai 2015). Isolates capable of degrading AHLs were classified following 16S rRNA sequence analysis (Araújo et al. 2002).

**Cloning and expression of aiiE from Enterobacter sp. CS66**

The ORF encoding the AHL-degrading gene *aiiE* from *Enterobacter* sp. CS66 was cloned using a previously described method (Rajesh and Rai 2015). Briefly, the *aiiE* ORF was amplified from extracted genomic DNA using the polymerase chain reaction (PCR) with the forward primer 5'-AAAGATCTCATGACGATATAA GAAGCTTTATTTCAT-3' and the reverse primer 5'-AAAGTTCAGCTATATACCTAGGGAACACTTTAC-3'. These primers contained BamHI and SalI restriction sites (underlined) as indicated. Following digestion with XhoI and BamHI, the gel-purified amplicon was ligated with XhoI and BamHI, the gel-purified amplicon was ligated to appropriately digested pMAL-c2X to yield pMAL-c2X-aiiE. Cultures of *E. coli* JM109 containing pMAL-c2X-aiiE were grown in LB medium at 37°C with good aeration (shaking at 200 rpm) until OD600 0.5. The temperature was then lowered to 20°C, and isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 1 mM final concentration to induce expression of the cloned gene. The induced culture was grown for a further 16 h before assaying the cells for AHL degradation activity.

**Purification of AiiE**

The *aiiE*-coding region was PCR-amplified using the forward primer 5'-AAAGATCTCATGACGATATAA GAAGCTTTATTTCAT-3' and the reverse primer 5'-AAAGATCTCATATATACCTAGGGAACACTTTAC-3'. These primers contained XhoI and BamHI restriction sites (underlined) as indicated. Following digestion with XhoI and BamHI, the gel-purified ampiclon was ligated to pET-19m that had been previously digested with the same enzymes and gel-purified. This yielded construct pET-19m-aiiE. For purification of the His6-tagged AiiE, the cells were grown in 1 L LB medium at 37°C with good aeration (shaking at 200 rpm) until OD600 0.5. The temperature was then lowered to 20°C and IPTG was added to 0.5 mM final concentration to induce expression of the cloned gene. The induced culture was grown for a further 16 h and then harvested by centrifugation (6000× g, 4°C, 15 min). The cell pellet was resuspended in 20 mL of lysis buffer (50 mM sodium phosphate, 200 mM NaCl, 10% (v/v) glycerol, pH 8.0), and the cells were ruptured by sonication (3 × 10 s, Soniprep 150, maximum power output). The cell lysate was clarified by centrifugation (11 000× g, 4°C, 30 min), and the supernatant was filtered through a 0.45-μm filter. The filtered lysate was then loaded onto an Ni-NTA column (2 mL packed resin bed volume) and the column was washed overnight at 4°C with lysis buffer containing 10 mM imidazole. The His6-AiiE was eluted with lysis buffer containing 250 mM imidazole. The purified protein was dialyzed overnight against 2 L dialysis buffer (20 mM Tris-HCl, 50 mM NaCl, 5% (v/v) glycerol, 1 mM EDTA, 1 mM DTT, pH 7.5) in the presence of His6-tagged TEV-protease. The AiiE thus released was cleaned up by batch extraction in a slurry of Ni-NTA resin equilibrated in dialysis buffer. The purity of the AiiE was confirmed by SDS-PAGE, and loss of the His6-tag was confirmed by western blot analysis using commercially available anti-His6 antibodies.

**AHL lactonase activity**

Two cultures were used to monitor the AHL degradation activity of AiiE. In the first, AHL levels were monitored using an agar overlay assay, as previously described (McClean et al. 1997). Briefly, a 200 μL reaction containing 100 μM C4-HSL and 100 μg/mL purified AiiE or MBP-tagged AiiE was prepared in 10 mM potassium phosphate buffer (pH 7.2). The samples were incubated at 30°C for 6 h. Following this, the reaction mixture was heat inactivated (95°C for 10 min) and then filtered through a 0.45-μm filter. LB plates were overlaid with soft agar seeded with an overnight culture of either CV026 or *Serratia* SP19 (as indicated) and 5 μL of the heat-inactivated sterile reaction mixture was spotted onto the plates. The plates were then incubated at 30°C for 24 h to allow development of the pigment (violacein or prodigiosin, depending on the indicator strain used) halo.

For kinetic analyses, the catalytic activity of AiiE was measured spectrophotometrically as previously described (Liu et al. 2013) with some modifications. Proton release from the hydrolysis of the AHL substrate was measured in weakly buffered solutions using the pH sensitive dye, phenol red. The 1 mL reaction mixture contained 1 mM HEPES, 20 mM NaCl, 5 μM phenol red (pH 7.5) and 0 to 10 mM C4-HSL substrate. The reaction was initiated by adding 10 μg AiiE. AHL hydrolysis was measured by monitoring the decrease in A557 over time. A standard curve was generated by titrating hydrochloric acid.

**Exoenzyme production by P. atrosepticum**

The well-characterised wild-type strain, Eca1043, was used to examine whether AiiE affects secreted virulence factor production in *P. atrosepticum*. An isogenic AHL-deficient exp1 mutant, SB1031, served as a control. Plasmid pMAL-c2X-aiiE harbouring the *aiiE* gene from *Enterobacter* sp. CS66 was introduced into each genetic background. Plasmid pMAL-c2X without the *aiiE* gene served as a control. Production of secreted pectate lyase (Pel) was monitored as previously described (Bowden et al. 2013a). Overnight cultures of the *P. atrosepticum* strains were grown in LB supplemented with ampicillin (to maintain the plasmid) and 5 μL aliquots were spotted onto each Pel plates. The plates were then incubated at 30°C for 48 h. After incubation, plates were developed by flooding with 7.5% copper acetate to reveal the Pel halos. The production of secreted proteases (Prt) was followed using gelatin-agar plates, as previously described (Bowden et al. 2013a). The Prt plates were inoculated as described for the Pel plates and incubated at 30°C for 48 h. The plates were developed by flooding with 4 M ammonium sulphate solution to reveal the halos.

**Statistical analyses**

Virulence assays were analysed by one-way ANOVA using Graphpad Prism 5.03 software.
RESULTS

Identification of aiiE in Enterobacter sp. CS66

An isolate of Enterobacter sp. CS66 was obtained from samples of the critically endangered tree, Coscinium fenestratum (also known as yellow vine or tree turmeric), a producer of the benzylisoquinoline alkaloid, berberine. One of the endophytic bacteria (denoted Enterobacter sp. CS66) associated with the C. fenestratum samples was able to degrade AHLs (data not shown). Primers designed to anneal to the previously characterised aiiA gene from Bacillus sp. 240B1 (Dong et al. 2000) were used to PCR-amplify a ca. 750 bp product from the genomic DNA of Enterobacter sp. CS66. BLAST analysis of the amplicon sequence revealed that the encoded ORF exhibited 92% identity at the amino acid level with the AiiA protein from Bacillus sp. 240B1, and 96% identity with the AiiA protein from Bacillus thuringiensis serovar kurstaki, which has been structurally characterised (Kim et al. 2005). This was unexpected because close relatives of AiiA have not been reported in Gram-negative bacteria such as Enterobacter sp., and of the AHL-lactonases that have been identified in Gram-negative organisms; these share only distant similarity with AiiA. We therefore named the new gene aiiE (autoinducer inactivation gene from Enterobacter). A sequence alignment of AiiE against the best-characterised AHL-lactonases from a variety of organisms is shown in Fig. 1A, and a relationship tree is shown in Fig. 1B. The aiiE ORF was cloned into pMAL-c2X to generate an MBP-fusion protein, which was expressed and purified using an amylose column. The purified MBP-AiiE protein was able to completely degrade 100 μM C4-HSL within 6 h (Fig. 2, inset). This confirmed that AiiE is an AHL-lactonase with C4-HSL degradation activity.
Expression and purification of AiiE

His6-tagged AiiE was overexpressed from pET-19m in Rosetta DE3 cells and purified to homogeneity using an Ni-NTA affinity column. The His6-tag was removed using His6-tagged TEV protease. The purified protein was approximately 29 kDa in mass (Fig. 3), which was in agreement with the molecular mass of AiiE based on its predicted amino acid sequence. Western analysis (data not shown) confirmed that the purified protein no longer contained a His6-tag. To further characterise the purified protein, it was mixed with 100 μM C6-HSL incubated at 30°C. At different times, aliquots of the reaction mixture were withdrawn and assayed for their ability to restore production of red prodigiosin pigment by Serratia SP19. This strain is unable to produce prodigiosin in the absence of exogenous AHL because it contains a mutation in the AHL synthase gene, smal. However, in the presence of exogenous AHL (especially short chain AHLs such as C4-HSL), the amount of prodigiosin pigment produced is proportional to the concentration of AHL present. The sensitivity and dynamic range of SP19 is enhanced by the presence of additional mutations in the pigX and pigZ genes of the strain (Poulter et al. 2010). AiiE was able to completely degrade a 10-fold molar excess of C4-HSL within 30 min (Fig. 2).

Enzyme kinetics

To more accurately determine the steady state kinetic constants of AiiE, we measured the initial rates in the presence of AHL substrates with increasing acyl chain lengths (C4-HSL, C6-HSL, C8-HSL, C10-HSL and C12-HSL). Lineweaver-Burk plots (1/v0 versus 1/[S]) were used to determine the kinetic constants (kcat, Km) and the results are shown in Table 2. The kcat values for all substrates were in the range of ca. 61 to 101 s⁻¹, whereas the Km values varied between ca. 6 and 15 mM. These values are very comparable with those reported previously for AiiA from Bacillus sp. B240 (Wang et al. 2004). From the pseudo-second order rate constant (kcat/Km), it is clear that AiiE exhibits a slight preference for shorter chain AHLS. This contrasts with the findings of Wang et al. who reported that AiiA from Bacillus sp. B240 exhibits a slight preference for longer chain AHLS, although in both studies, the acyl chain length preference is only marginal (Wang et al. 2004).

### Table 2. Steady-state kinetic constants of AHL hydrolysis.

| AHL-lactonase | Substrate | kcat (s⁻¹) | Km (mM) | kcat/Km (mM⁻¹ s⁻¹) |
|---------------|-----------|------------|---------|---------------------|
| AiiE          | C4-HSL    | 61.14      | 6.01    | 10.17               |
|               | C6-HSL    | 69.43      | 7.88    | 8.81                |
|               | C8-HSL    | 80.07      | 10.35   | 7.74                |
|               | C10-HSL   | 98.31      | 14.88   | 6.61                |
|               | C12-HSL   | 101.5      | 15.21   | 6.67                |

When AiiE was expressed from a plasmid in trans in wild-type P. aeruginosa, it was found that the production of secreted pectate lyase (Pel) and secreted protease (Prt) diminished to levels equivalent to that of an OHHL-deficient expI mutant (Fig. 4A and B). In contrast, Pel and Prt production by the wild-type strain containing an empty vector (pMAL-c2X) was unaffected. As a further control, we also examined whether the presence of the plasmid (pMAL-c2X) or AiiA had any effect on growth; it did not (Fig. 4C). We conclude that expression of AiiE in trans in P. aeruginosa abolishes production of secreted protease (Prt) and reduces production of secreted pectate lyase (Pel) to levels equivalent to that of an expI mutant.

**DISCUSSION**

An isolate of the Gram-negative endophytic Enterobacter sp. CS66 was identified. This isolate was capable of rapidly degrading exogenously supplied AHLS, and this activity was associated with an AiiA-like protein, denoted here as AiiE. The first AHL-lactonase to be described, AiiA, was originally isolated from a Gram-positive soil-dwelling bacterium, Bacillus sp. 240B1. Remarkably, sequence comparisons revealed that AiiA and AiiE are 90% identical at the amino acid level (rising to 96% when comparing AiiE with the more recently characterised AiiE enzyme from B. thuringiensis serovar kurstaki), suggesting that the gene encoding these enzymes may have been acquired by relatively recent horizontal gene transfer, either between species or from a common source. Although this is not the first discovery of an AiiA homolog in Gram-negative bacteria—the AttM protein from A. tumefaciens is also an AHL lactonase (Zhang, Wang and Zhang 2002)—it is worth noting that AttM and AiiA are only distantly related (sharing just 31% amino acid identity). Furthermore, other AiiE-lactonases from Gram-negative bacteria, such as the AhkL protein from Klebsiella pneumoniae, are much more similar to AttM than they are to AiiA/AiiE (Fig. 1B). It is therefore surprising to find two almost identical AHL-lactonases conserved between Gram-positive and Gram-negative species.

AiiE showed broad specificity, although it exhibited a preference for hydrolysing shorter chain AHLS. Consistent with a previous study reporting on the kinetics of AiiA from Bacillus sp. B240 (Wang et al. 2004), the very best kcat/Km value measured here (6012 M⁻¹ s⁻¹, for C4-HSL) is much lower than the diffusion limit (10⁹-10¹⁰ M⁻¹ s⁻¹), suggesting that the enzyme is inefficient and that AHLS are unlikely to be its ‘true’ substrate. Nevertheless, AiiE contained all the conserved amino acids known to be required for AHL-lactonase activity (especially the dinuclear

Figure 3. Purification of AiiE. The figure shows a Coomassie Brilliant Blue G250-stained 10% polyacrylamide gel run in SDS buffer showing the purification of AiiE. Lane 1; protein molecular marker, Lane 2; crude cell-free lysate, Lane 3; His6-tagged AiiE eluted from the Ni-NTA column, Lane 4; purified AiiE after His6-TEV protease cleavage.
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Conflict of interest. None declared.

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