Quorum sensing has an unexpected role in virulence in the model pathogen *Citrobacter rodentium*

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The bacterial mouse pathogen *Citrobacter rodentium* causes attaching and effacing (AE) lesions in the same manner as pathogenic *Escherichia coli*, and is an important model for this mode of pathogenesis. Quorum sensing (QS) involves chemical signalling by bacteria to regulate gene expression in response to cell density. *E. coli* has never been reported to have *N*-acylhomoserine lactone (AHL) QS, but it does utilize *luxS*-dependent signalling. We found production of AHL QS signalling molecules by an AE pathogen, *C. rodentium*. AHL QS is directed by the *croIR* locus and a *croI* mutant is affected in its surface attachment, although not in Type III secretion. AHL QS has an important role in virulence in the mouse as, unexpectedly, the QS mutant is hypervirulent; by contrast, we detected no impact of *luxS* inactivation. Further study of QS in *Citrobacter* should provide new insights into AE pathogenesis. As the *croIR* locus might have been horizontally acquired, AHL QS might exist in some strains of pathogenic *E. coli*.

Keywords: *Citrobacter rodentium*; quorum sensing; *N*-acylhomoserine lactone; pathogenesis; attaching and effacing pathogen

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INTRODUCTION

Citrobacter rodentium is an enteric bacterium that naturally infects mice, causing transmissible murine colonic hyperplasia (Mundy *et al*, 2005). Enteropathogenic *Escherichia coli* (EPEC) and enterohaemorrhagic *E. coli* (EHEC) are significant gastrointestinal pathogens of humans. EPEC is a major cause of infantile diarrhoea in developing countries, and EHEC causes outbreaks of haemorrhagic colitis and haemolytic uraemic syndrome in developed countries (Nataro & Kaper, 1998). EPEC and EHEC infection induces the formation of attaching and effacing (AE) lesions, which are characterized by the intimate attachment of bacteria

to the intestinal epithelial cells of the host, involving a host cytoskeletal rearrangement to 'cup' the bacteria individually and effacement (destruction) of the epithelial microvilli (Garmendia *et al*, 2005). The genes directing AE lesion formation are found on a pathogenicity island called the locus of enterocyte effacement (LEE). The LEE encodes a Type III secretion system (T3SS), an outer membrane adhesin (intimin) plus its translocated receptor (Tir), secreted translocators and effectors (Map, EspABDFGH), and regulatory genes (for example, Ler), with the T3SS injecting various effector proteins directly into the host cell to subvert host signalling processes (Garmendia *et al*, 2005). *C. rodentium* is also an AE pathogen, carrying the same 41-gene LEE as EPEC and EHEC, and causing identical AE lesions (Luperchio & Schauer, 2001; Deng *et al*, 2001, 2004). Therefore, *C. rodentium* provides an excellent model system to study the molecular basis of AE pathogenesis in a natural host (Luperchio & Schauer, 2001).

Quorum sensing (QS) is a process of intercellular communication by which bacteria detect their population cell density by using diffusible signal molecules and regulate gene expression accordingly. Many important processes, in diverse bacterial species, are regulated by QS; for example, virulence, secondary metabolite production, symbiosis, sporulation and biofilm formation (Whitehead *et al*, 2001). The most extensively studied QS systems in Gram-negative bacteria are those using *N*-acylhomoserine lactone (AHL) signal molecules, in which LuxI homologues synthesize various AHL signals and LuxR-type transcriptional regulatory proteins bind to their cognate signal at high cell densities and alter gene expression (Whitehead *et al*, 2001; Lazdunski *et al*, 2004). Production of AHLs has never been reported in *E. coli* or *Salmonella*. However, both have a LuxR homologue, SdiA, which responds to exogenously produced AHLs and might be used to 'tune into' QS signals from other intestinal bacteria (Michael *et al*, 2001; Ahmer, 2004; Van Houdt *et al*, 2006). Another QS system has also been described in Gram-negative bacteria in which LuxS produces a signal known as autoinducer-2 (AI-2). As reviewed by Vendeville *et al* (2005), AI-2 comprises a number of interconverting furanone-like molecules and seems to be used as a QS signal in some, but certainly not all, of the bacteria that produce it; LuxS also has a role in methionine metabolism. In EPEC and EHEC, a LuxS-dependent

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extracellular signal is required for full expression of the LEE genes and motility genes; however, this signal does not seem to be AI-2 (Sperandio *et al*, 2003).

Here, we show for the first time to our knowledge that, unlike the closely related *E. coli*, *C. rodentium* has an AHL QS system. Furthermore, by using its natural host, we show that AHL QS has an important role in the virulence of this AE pathogen.

RESULTS AND DISCUSSION

C. rodentium DBS100 (wild-type strain) was tested for AHL production using the LIS and CV026 AHL biosensor strains—AHL non-producing mutants of *Serratia* ATCC39006 and *Chromobacterium violacein*, respectively, which produce haloes of pigment in response to exogenous AHLs (McClellan *et al*, 1997; Thomson *et al*, 2000). We found that *C. rodentium* produces AHL signal molecules (Fig 1A). This was an unexpected result as its close relative *E. coli* has never been reported to produce AHLs. Transposon mutagenesis and screening on CV026 was then used to isolate an AHL nonproducing mutant, strain IF232. AHL production was growth phase dependent and was undetectable in IF232 (Fig 1B). A comparison of sequences around the transposon insertion point with the ongoing *C. rodentium* genome project (www.sanger.ac.uk/Projects/C_rodentium/) showed that the transposon had inserted into a gene encoding a LuxI homologue, hereafter named *croI*. The *croI* gene was cloned and introduced into IF232, where it restored AHL production to the *croI* mutant, confirming that CroI is required for AHL production (Fig 1A).

Next, the sequence of *croI* and its flanking regions was examined (Fig 2). Adjacent to *croI* and convergently transcribed is a *luxR* homologue presumed to encode the cognate receptor for *croI*, hereafter named *croR*. The predicted CroI protein is most similar to the AHL synthase, Smal, from *Serratia* ATCC39006 (44% identity in 179 amino acids), and CroR is most similar to Smar from the same organism (42% identity in 244 amino acids). Upstream of *croI* is an open reading frame (orf), orf1, encoding a putative protein Tyr/Ser phosphatase (conserved domain COG2365). Upstream of *croR* is orf2, which is predicted to encode a transposase of the IS4 family (99% identity with Transposase 11 from *Salmonella typhimurium* pSB101), followed by orf3, encoding a conserved hypothetical protein. The presence of a transposase sequence adjacent to the *croIR* locus, together with the presence of a partial integrase sequence further upstream of *croR* (data not shown), suggests that the *croIR* locus might have been horizontally acquired, as has been suggested in another enteric genus, *Serratia* (Coulthurst *et al*, 2006). In support of this idea, the GC content of the *croIR* region shown in Fig 2 is 44.7%, which deviates from the current estimated GC content of the total genome of 50% (data not shown). A homologue of *E. coli* SdiA was also detected elsewhere in the genome (76% identity with SdiA from EHEC O157:H7), but no other LuxR homologues were detected (data not shown). SdiA can respond to exogenous AHLs in *E. coli* and *Salmonella* (Ahmer, 2004; Van Houdt *et al*, 2006); therefore it will be of interest to determine the nature of any crosstalk between SdiA and the CroIR system.

Wild-type *C. rodentium* produces molecules that strongly activate the LIS AHL biosensor, which primarily responds to *N*-butanoyl-L-homoserine lactone (BHL), and less strongly activate the CV026 biosensor, which primarily responds to

Fig 1 | Wild-type *Citrobacter rodentium*, but not a *croI* mutant, produces *N*-acylhomoserine lactone signalling molecules. (A) AHL production by strains of *C. rodentium*: (i) DBS100 (wild type); (ii) IF232 (*croI*); (iii) DBS100 (pBluescript; vector control); (iv) IF232 (pBluescript); (v) DBS100 (pSJC45; *croI* in trans); (vi) IF232 (pSJC45), as detected using LIS and CV026 biosensor strains. (B) Production of AHL activity throughout growth by DBS100 (WT) and IF232 (*croI*). AHL activity was detected using LIS or CV026 (reported as area of pigmentation induced in the sensor) and growth was measured as OD₆₀₀. Results for one representative culture are shown; those from duplicate cultures were essentially identical. (C) Analysis of different AHL-like signalling molecules produced by *C. rodentium*. Cell-free supernatant samples from DBS100 (wild type), IF232 (*croI*), *Escherichia coli* DH5α (pBluescript) and *E. coli* DH5α (pSJC45) were subjected to ethyl acetate extraction and separation by thin-layer chromatography. AHL-like signalling molecules were detected using LIS and CV026 overlays. The 'standards' sample contained 50 μM BHL, HHL and OHHL, and the positions of these standards are indicated. (Note that twice the amount of sample was loaded for the DBS100 and IF232 samples on the CV026 plate compared with the other samples and the LIS plate.) AHL, *N*-acylhomoserine lactone; BHL, *N*-butanoyl-L-homoserine lactone; HHL, *N*-hexanoyl-L-homoserine lactone; OD, optical density; OHHL, *N*-3-oxohexanoyl-L-homoserine lactone.

Fig 2 | Schematic representation of the *croIR* locus of *Citrobacter rodentium* DBS100. Numbers refer to lengths in base pairs, and the position of the transposon insertion in IF232 (after base pair 280 of *croI*) is indicated with an asterisk. Orf, open reading frame.

N-hexanoyl-L-homoserine lactone (HHL; McClean *et al*, 1997; Thomson *et al*, 2000), suggesting that *C. rodentium* produces BHL as the major AHL. Further analysis of the AHLs produced by *C. rodentium* was carried out by using thin-layer chromatography (TLC) of supernatant extracts, in combination with LIS and CV026 overlays to detect bioactive molecules (Fig 1C). The use of LIS showed a large spot comigrating with the BHL standard, confirming that *C. rodentium* produces BHL as a primary product. Using CV026, three distinct spots able to activate the biosensor were observed. One spot comigrated with HHL, suggesting that HHL is produced as a minor product, but the identities of the other two remain unknown—we cannot exclude the possibility that the highest spot was BHL, as its R_f was similar to that of BHL on the LIS plate. All spots were absent in the *croI* mutant, confirming that *croI* was required for their synthesis. It remained possible that CroI was regulating a second AHL synthase, although no other predicted AHL synthases were detectable in the genome (data not shown). We confirmed that CroI was producing all of these distinct AHL-like activities by overexpressing *croI* in *E. coli*, where the same pattern of AHL-like molecules was observed as in the native background (Fig 1C). The closest homologue of CroI, Smal, also produces BHL as a major, and HHL as a minor, AHL. However, only these two products produced by Smal are detectable by bioassay (Thomson *et al*, 2000), whereas CroI produces three or four products, in a defined ratio, irrespective of host background, making it a mechanistically interesting enzyme.

Although *E. coli* has never been reported to have an AHL QS system, the role of *luxS*-mediated signalling in regulating traits such as LEE gene expression in *E. coli* is well documented (Walters & Sperandio, 2006). Therefore, we were interested to ascertain whether *luxS* had a similar role in *C. rodentium* and whether it was linked with the AHL QS system. A *luxS* gene was identified from the *C. rodentium* genome by sequence homology with *E. coli*, and a *luxS* mutant was constructed by allelic exchange. As shown in supplementary Fig 1 online, wild-type *C. rodentium*, but not the *luxS* mutant, produced extracellular AI-2 activity in a typical growth-phase-dependent manner. Production of AHL was not affected by *luxS* mutation and, conversely, production of AI-2 was not affected in the *croI* mutant (data not shown). Hence, *C. rodentium* produces *luxS*-dependent AI-2 activity, in addition to AHL QS, but the two potential signalling systems operate independently of each other, at least at the level of signal production.

Next, the biological role of the novel AHL QS system was investigated. An important phenotype of *C. rodentium* is T3S, as this organism is currently attracting much interest as a model for

Fig 3 | Surface attachment of *Citrobacter rodentium* is modulated by quorum sensing. (A) The ability of *C. rodentium* strains DBS100 (wild type, WT), IF232 (*croI* mutant) and TJE2 (*luxS* mutant) to adhere to the wells of a 96-well microtitre plate was determined in the presence and absence of 2 μ M BHL, after incubation for either 40 h at 25 °C (grey bars) or 20 h at 37 °C (black bars). (B) Attachment of DBS100 (WT), KJR025 (*croR* mutant), IF232 (*croI*) and KJR026 (*croI*, *croR*) after incubation for 20 h at 37 °C. Attachment was quantified using crystal violet staining and is expressed as A₅₉₅; bars show mean $\pm$ s.d. ($n = 3$). BHL, *N*-butanoyl-L-homoserine lactone.

AE pathogenesis, for which T3S is essential. In addition, T3S has been reported to be under the control of AHL QS in *Pseudomonas aeruginosa* (Blevins *et al*, 2005). However, no significant impact on the levels of the most abundant Type III-secreted proteins was detected in the *croI* mutant (supplementary Fig 2 online). Hence, the role of AHL QS in *C. rodentium* does not include the control of T3S, which might be expected as T3S operates early in infection, rather than at high cell densities. Furthermore, mutation of *luxS* had no discernible impact on T3S (data not shown). This is in contrast with the situation in EHEC, in which T3S is markedly reduced in the *luxS* mutant (Sperandio *et al*, 2003). Hence, regulation of T3S is clearly not identical between *C. rodentium* and *E. coli*.

Another phenotype that has been reported to be QS dependent in a range of bacteria, including human pathogens, is surface attachment and/or biofilm formation (Parsek & Greenberg, 2005). In *C. rodentium*, by using a simple attachment assay, the *croI* mutant was observed to attach less well to an abiotic surface than the wild type (Fig 3). This phenotype could be complemented by

the addition of exogenous BHL (Fig 3A), confirming that it was due to a lack of AHL QS, and also by the expression of *crol* *in trans* (supplementary Fig 3 online). By contrast, the *luxS* mutant was indistinguishable from the wild type. Although this assay does not accurately represent an *in vivo* or environmental attachment scenario, this phenotype indicates a role for AHL QS in modulating the surface properties and/or adhesion of *C. rodentium*, traits likely to influence the outcome of a host infection.

Next, we used the attachment phenotype to confirm whether the Crol-dependent AHL QS system operates through CroR. A *croR* mutant, KJR025, and a *crol*, *croR* double mutant, KJR026, were constructed by allelic exchange. The *croR* mutant showed attachment similar to the wild type. However, crucially, introduction of the *croR* mutation into a *crol* background was able to restore wild-type levels of attachment to the *crol* mutant (Fig 3B). This indicates that *croR* behaves genetically as a repressor in the absence of AHL and that this repression is relieved in the presence of AHL. It also confirms that the AHL QS system is operating through CroR rather than SdiA. Typically, LuxR family regulators act as AHL-dependent activators; however, a small number of LuxR homologues acting through a similar derepression mechanism have been reported, such as SmaR of *Serratia* ATCC39006 (Slater *et al*, 2003). It seems that CroR represents another example of this alternative mechanism, at least in the regulation of surface attachment.

Finally, we examined the role of the novel AHL QS system in the virulence of *C. rodentium* *in vivo*. Although QS has been reported to be important for the virulence of other human and animal pathogens, it is rare to be able to carry out such analyses using the natural host of the pathogen. In the case of *C. rodentium*, however, we were able to examine the impact of *crol* mutation on its virulence in the natural mammalian host—truly '*in vivo*'. A marked increase in morbidity was observed in the *crol* mutant compared with the wild type (Fig 4). On day 7, mice infected with the *crol* mutant began to succumb and none were alive by day 14. By contrast, only one of the mice infected with the wild type failed to survive the course of the experiment. The experiment was repeated with male mice and a similar trend was observed, although with different kinetics (data not shown). Hence, *crol*, and by implication the *croIR* AHL QS system, has an important role in pathogenesis—the first example of a role for AHL QS in an AE pathogen. Furthermore, the QS mutant is hypervirulent. Although loss of AHL QS through inactivation of the AHL synthase can lead to reduced virulence in other pathogens, for example, *P. aeruginosa* (Whitehead *et al*, 2001), this is the first example, to our knowledge, where virulence *in vivo* has increased in such a mutant, broadening our view of the role of QS systems. The mechanism by which AHL QS affects the host–pathogen interaction is not yet clear, but it does not seem to be acting through the T3S system. However, T3S is not the only virulence determinant or means of host–pathogen interaction in AE pathogens; others include adhesins and non-Type III secreted proteins (Spears *et al*, 2006). Defining the targets of the QS system should provide new information on the mechanisms and regulation of AE pathogenicity. We were unable to detect any difference in the ability of the *C. rodentium* *luxS* mutant to infect mice compared with the wild type, which is consistent with a lack of significant phenotypic differences *in vitro* (data not shown). Hence, *C. rodentium* has an AHL QS system that has an important role in virulence, whereas

Fig 4 | Morbidity curves of C3H/HeJ mice infected with wild-type *Citrobacter rodentium* (DBS100) or the *crol* mutant (IF232).

luxS seems to have a minor role, if any. In *E. coli*, in sharp contrast, AHL QS is not present, but *luxS* signalling has an important regulatory role (Walters & Sperandio, 2006). Hence, although the mechanics of AE lesion formation are conserved between *E. coli* and *Citrobacter*, we have shown that not all aspects of regulation of virulence are similarly conserved.

Speculation

Why should loss of AHL QS in *C. rodentium* cause increased virulence? Perhaps QS acts to promote normal organ colonization and microcolony formation *in vivo*. Evidence from *in vitro* attachment assays (Fig 3) suggests that the *crol* mutant has a reduced ability to adhere to surfaces. Indeed, preliminary *in vivo* experiments suggest that the *crol* mutant might spread to the colon more rapidly than the wild type (data not shown). If, *in vivo*, the *crol* mutant spreads more freely to the surrounding tissue, rather than remaining in a microcolony, then this might cause more host cell damage and lead to the premature death of the animal.

The presence of transposase and integrase-like sequences in the vicinity of the *croIR* locus is consistent with our recent observations of facile acquisition and imposition of AHL QS control by horizontal gene transfer of QS loci (Coulthurst *et al*, 2006). Therefore, the *croIR* locus might have been acquired by horizontal transfer, suggesting that AHL QS might also be used by some isolates of pathogenic *E. coli* yet to be discovered.

METHODS

Bacterial strains and culture conditions. Wild-type *C. rodentium* DBS100 (Schauer & Falkow, 1993) and *E. coli* DH5 α (Invitrogen, Paisley, UK) were cultured at 37°C in Luria broth (LB; 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) unless stated otherwise. DMEM was from Sigma-Aldrich (Dorset, UK). Where necessary, the following antibiotics were added: 100 μ g/ml ampicillin, 25 μ g/ml chloramphenicol (Cm) and 50 μ g/ml nalidixic acid (Nx).

Detection and analysis of AHL production. Production of AHLs was detected using the LIS and CV026 biosensor strains, and that of AI-2 using the BB170 biosensor strain, as described previously (Coulthurst *et al*, 2006). TLC analysis of AHL species was carried out as described previously by Shaw *et al* (1997). Culture

supernatants, after 6 h growth, were extracted with ethyl acetate and the residue from approximately 2 ml of culture was resuspended in 20 µl acetonitrile. A volume of 2–4 µl of extract, together with synthetic AHL standards, was separated on C₁₈ reversed-phase TLC plates (Whatman) using 60:40 methanol:water and AHLs were visualized by overlay with LIS or CV026.

Generation and complementation of an AHL mutant. Random transposon mutagenesis of DBS100 was carried out using a modified version of the plasposon Tn5-RL27, which is delivered on pRL27 (Larsen *et al*, 2002). pRL27 was modified by replacing the transposon kanamycin-resistance determinant with Cm resistance and introducing tetracycline resistance into the nonmobile vector backbone, generating pDS1028 (D.S. Smith, unpublished data). Following conjugal mating between DBS100 and *E. coli* BW20767 (pDS1028), transposon insertion mutants were selected on Cm + Nx and then visually screened for loss of a purple halo on CV026. The AHL-deficient mutant IF232 was selected and the transposon insertion point was identified by random primed PCR as described previously (Fineran *et al*, 2005), using the transposon-specific primers CTAGAGTCGACCTG CAGGCATGCAAGC and GCGGATGAATGGCAG, and confirmed by PCR using these primers and SC92, SC93 (below) and sequencing. The presence of a single transposon insertion in IF232 was confirmed by showing 100% co-transduction of Cm resistance with loss of AHL production (data not shown). Construction of TJE2 (*luxS*), KJR025 (*croR*) and KJR026 (*crol*, *croR*) is described in supplementary information online. To generate pSJC45, the *crol* gene was amplified using primers SC92 (CATA GAATTCAGACCTTAACCAGAGCTATCC) and SC93 (TATAG GATCCGCATGGAGCTCAGATTACTGG) and cloned into pBluescript KSII⁺ (Stratagene, Amsterdam, the Netherlands) on an *EcoRI*–*Bam*HI fragment.

In vitro phenotypic assays. Attachment assays were carried out as described previously (Coulthurst *et al*, 2006). Synthetic BHL was added at a final concentration of 2 µM in DMSO (DMSO alone was added to control cultures). For preparation of secreted proteins, 30 ml DMEM was inoculated to a starting OD₆₀₀ of 0.08 and incubated statically for 6 h at 37 °C. Following removal of the cells by centrifugation, secreted proteins were precipitated using an equal volume of 50:50 chloroform:methanol, washed with methanol, resuspended in SDS gel sample buffer, separated by 12% SDS–polyacrylamide gel electrophoresis and visualized by staining with Coomassie blue G250.

In vivo virulence assay. Female 6- to 8-week-old C3H/HeJ mice were purchased from Bantin & Kingman (Hull, UK) and were from colonies free of specific pathogens. Animals were housed individually in HEPA-filtered cages with sterile bedding and free access to sterilized food and water. Bacterial inocula were prepared by culturing bacteria overnight in 100 ml LB + Nx (100 µg/ml). Cultures were collected by centrifugation and resuspended in 1/10 volume of PBS. Mice were orally inoculated with 200 µl of the bacterial suspension using a gavage needle. Viable counts of inocula were determined by retrospective plating. Animals were monitored in accordance with the Home Office licence and, when individual mice crossed preset guidelines, they were killed by an approved schedule 1 method.

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

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