Identification of a Quorum-Sensing Signal Molecule in the Facultative Intracellular Pathogen *Brucella melitensis*

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*Brucella melitensis* is a gram-negative alpha2-proteobacterium responsible for abortion in goats and for Malta fever in humans. This facultative intracellular pathogen invades and survives within both professional and nonprofessional phagocytes. A dichloromethane extract of spent culture supernatant from *B. melitensis* induces bioluminescence in an *Escherichia coli* acyl-homoserine lactone (acyl-HSL) biosensor strain based upon the activity of the LasR protein of *Pseudomonas aeruginosa*. HPLC fractionation of the extract, followed by mass spectrometry, identified the major active molecule as N-dodecanoylhomoserine lactone (C12-HSL). This is the first report of the production of an acyl-HSL by an intracellular pathogen. The addition of synthetic C12-HSL to an early log phase culture of either *B. melitensis* or *Brucella suis* 1330 reduces the transcription of the virB operon, which contains virulence genes known to be required for intracellular survival. This mimics events seen during the stationary phase of growth and suggests that quorum sensing may play a role in the control of virulence in *Brucella*.

*Brucella* is the causative agent of brucellosis, a widely distributed zoonosis affecting a broad range of mammals and causing a chronic undulant fever in humans (reviewed by Smith and Ficht [53]). Brucellosis remains a health problem and a source of major economic losses in developing countries, where it is endemic. *Brucella* are nonmotile gram-negative bacteria and facultative intracellular pathogens that are able to invade and replicate in macrophages and nonprofessional phagocytes (12). In HeLa cells, virulent *Brucella* cells alter the intracellular trafficking of membrane-bound intracellular compartments to create a novel vacuolar niche in which they replicate (41). The mechanisms and the factors involved in this process are not well understood, with knowledge about the interactions between *Brucella* and the mammalian cell being mainly descriptive.

Molecular genetic analyses have identified several types of factors that are important for *Brucella*-host cell interactions: structural components like lipopolysaccharide (1, 16, 32) and cyclic β(1-2) glucan (19), stress response proteins like DnaK (23) and Hfq, a homologue of *Escherichia coli* host factor I (HF-I) (43), and also a type IV secretion system (14, 34, 51). The VirB type IV secretion system is homologous to the VirB system of *Agrobacterium tumefaciens* and to the PII system of * Bordetella pertussis* (4). DNA sequence analysis of the *Brucella suis* virB operon has revealed 12 open reading frames (ORFs) encoding homologues of the 11 VirB proteins encoded by the pTi plasmid of *Agrobacterium* and a 12th ORF encoding a putative lipoprotein (34). Independent mutants of *virB5*, *virB9*, or *virB10* are highly attenuated in an in vitro infection model of *Brucella abortus* (54), and a second system, homologous to VsrB/VsrC of *Ralstonia solanacearum*, has been identified in *Brucella melitensis* in a signature-tagged mutagenesis screening in the mouse infection model (27). If two-component regulation systems allow each single bacterium within the population to react in face of environmental changes, other systems, such as the quorum sensing, respond to signals produced by the bacteria themselves and control virulence factors at the whole-population level.

Quorum sensing (QS) is a regulatory system for controlling gene expression in response to increasing cell density (reviewed in references 13, 15, 58, and 66). This cell-to-cell signaling is achieved through the production and release of a small signaling molecule or pheromone. With the growth of the bacterial population, there is an accumulation of this pheromone until a threshold concentration is reached, indicating that a quorum of bacteria is present. At this point, the pheromone activates a transcriptional regulator, leading to the activation or, in some cases, the repression of target genes (49, 55, 64, 65, 70). In a large number of gram-negative bacterial...
species, the signal molecule is an N-acyl homoserine lactone (acyl-HSL). In most cases, the acyl-HSL synthase is a member of the LuxI family and the response regulator is a member of the LuxR family (reviewed by Swift [59]). Here, the concentration of the signal molecule reflects the numbers of bacterial cells and the perception of a threshold level of the signal indicates a quorate population, ready to employ multicellular adaptive responses involved in, for example, virulence (21, 37, 38), adaptive responses involved in, for example, virulence (21, 37, 38), and in this paper, we report the identification and purification of N-dodecanoyl-homoserine lactone (C12-HSL) and its role in the control of the transcription of the virB operon, thus suggesting a putative link between QS and virulence in *Brucella*.

**Materials and Methods**

**Table 1** describes the bacterial strains and plasmids used in this study. *E. coli* and *Chromobacterium violaceum* were routinely cultured at 30°C in Luria-Bertani (LB) medium (NaCl, 10 g liter⁻¹; yeast extract, 5 g liter⁻¹; peptone, 10 g liter⁻¹) supplemented with tetracycline (20 μg ml⁻¹) where appropriate. *Brucella* strains were routinely cultured in 2YT medium (NaCl, 5 g liter⁻¹; yeast extract, 10 g liter⁻¹; peptone, 10 g liter⁻¹). For acyl-HSL extraction, a liquid culture of *Brucella* sp. was grown at 37°C in RPMI 1640 medium (Life Technologies, Merelbeke, Belgium) supplemented with 0.1% glucose (RPMI-glucose). 2YT medium, yeast extract solution, and peptone solution used in competition assays with HSL were not autoclaved but were sterile filtered. As high temperature may promote the formation of cyclic dipeptides (diketopiperazines) (17), this ensured that inhibitory compounds found in these solutions were not the result of such treatment.

To examine the influence of acyl-HSLs upon virB expression, *B. melitensis* 16M Na⁺ and *B. suis* were grown in modified minimal E medium (24).

**Extraction, analysis, and detection of acyl-HSLs.** *B. melitensis* 16M Na⁺ was grown to stationary phase (96 h). Bacteria were removed from the culture by centrifugation at 3,000 × g for 10 min followed by filtration (0.2-μm pore size). The spent, cell-free supernatant from stationary phase cultures of *B. melitensis* 16M Na⁺ grown in RPMI glucose was extracted three times with dichloromethane (supemantant-to-dichloromethane ratio of 70:300). The dried extract was reconstituted in acetonitrile, and samples were subjected to analytical thin-layer chromatography (TLC) and preparative high-performance liquid chromatography (HPLC). TLC analysis was carried out on normal phase Silica gel 60F254 (Merck) using a solvent system of 45% hexane in acetone. Once resolved, the plate was dried and overlaid with a thin film of the biosensor *E. coli* (pSB1075) in 0.3% LB agar (31, 50, 68). After incubation at 30°C, acyl-HSLs were visualized either by autoradiography or photon camera imaging (Berthold LB980 photon video camera; EG&G Berthold U.K. Ltd., Milton Keynes, United Kingdom). A tentative identification of an acyl-HSL can be made by comparing the *Rₐ* values of the unknown with that of synthetic acyl-HSL standards. For preparative HPLC, samples were separated using a Kromasil KR100-5C5 (250 by 8 mm) reverse-phase column (Highchrom, Reading, United Kingdom) with a linear gradient of acetonitrile in water (20 to 100%) at a flow rate of 2 ml min⁻¹ over a 37-min period. Seven fractions (F1 to F7) were collected, based on the retention time of the known acyl-HSLs and assayed using the acyl-HSL biosensor strains.

Positive fractions were identified in agar plate assays and microplate assays as described by McClean et al. and Winson et al., respectively (31, 68). Microplate assays were counted in a Microtiter LB96P microplate luminometer (EG&G Berthold). Positive fractions were further fractionated with an isocratic mobile phase of 60 or 70% of acetonitrile in water for fractions 5 or 6, respectively. Active fractions were analyzed by LC mass spectrometry (Micromass Instruments). Samples were ionized by positive-ion fast-atom bombardment (FAB), and the molecular ion (M + H) peaks recorded by FAB-MS were further analyzed by tandem mass spectrometry (MS-MS).

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

<table>
<thead>
<tr>
<th>Strains or plasmid</th>
<th>Description</th>
<th>Reference or source</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>B. melitensis</em> 16M Na⁺</td>
<td>Spontaneous nalidixic acid-resistant mutant of the reference strain <em>B. melitensis</em> 16 M (Brucella Culture Collection of Nouzilly)</td>
<td>62</td>
</tr>
<tr>
<td><em>B. suis</em> 1330</td>
<td><em>B. suis</em> biovar 1; reference strain (Brucella Culture Collection of Nouzilly)</td>
<td>ATCC 23444</td>
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<tr>
<td><em>C. violaceum</em> CV026</td>
<td>Mini-Tn5 mutant derived from <em>C. violaceum</em> ATCC 31532 (H₂, evl::Tn5ADE, Km², plus spontaneous Sm², acyl-HSL biosensor producing a purple pigment)</td>
<td>31</td>
</tr>
<tr>
<td><em>E. coli</em> JM109</td>
<td>F⁻ trdA36, proAB, lac⁰, lacZ/AM15/recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, Δ(lac-proAB), mcrA</td>
<td>Promega</td>
</tr>
<tr>
<td><em>E. coli</em> S17/1 λpir</td>
<td>λ-pir lysogen of S17-1 (thi pro hsdR⁻ hsdM⁻ recA4 RP4 2-Tc::Mu-Km::Tn17(Tp³ Sm⁰))</td>
<td>52, 57</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pSB401</td>
<td>lacR lac⁰ (Photobacterium Fischeri [ATCC 7744]):luxCDABE (Photorhabdus luminescens [ATCC 29999]) fusion; pACYC184-derived, Tc², acyl-HSL biosensor producing bioluminescence</td>
<td>68</td>
</tr>
<tr>
<td>pSB1075</td>
<td>lasR lac⁰ (P. aeruginosa PA01)::luxCDABE (P. luminescens [ATCC 29999]) fusion in pUC18 Ap⁰, acyl-HSL biosensor producing bioluminescence</td>
<td>68</td>
</tr>
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</table>
Synthesis of HSLs. The general method described by Chhabra et al. (5) was used to synthesize N-octanoyl-L-homoserine lactone (C8-HSL), N-(3-oxoheptanoyl)-L-homoserine lactone (3-oxo-C6-HSL), N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL), and N-dodecanoyl-L-homoserine lactone (C12-HSL). Each compound was purified to homogeneity by semi-preparative HPLC, and its structure was confirmed by mass spectrometry and proton NMR spectroscopy.

RNA extraction and slot blot analysis. Total RNA was extracted with the RNAasy total RNA isolation kit (Qiagen, Courtabouef, France) according to the manufacturer’s protocol. Samples were treated with DNase (Roche, Bruxelles, Belgium) to remove contaminating DNA. The preparation was heated to 95 °C for 5 min and extracted with phenol to remove DNase. The RNA was precipitated with ethanol, collected by centrifugation, dried to near completion, and redissolved in an appropriate volume of DEPC double-distilled water, and quantified with a spectrophotometer. Slot blot analysis was performed as described by Ausbel et al. (3). Serial dilutions were immobilized on positively charged nylon membrane (Roche), ranging from 1 μg to 15.625 ng of total RNA. The virB5 and pII12 probes were labeled by PCR, using 0.1 mM digoxigenin-UTP (Roche) according to the manufacturer’s instructions. Probe hybridization, stringency washes, and probe detection by an anti-digoxigenin alkaline phosphatase (AP) conjugate, and visualization using the chemiluminescent AP substrate CSPD (Roche) were as directed by the manufacturer (14).

RESULTS

Acyl-HSL activity is present in *B. melitensis* culture supernatant. T-streak assays of *B. melitensis* 16M NaI against the biosensor strains *C. violaceum* CV026, *E. coli* JM109 (pSB401), and *E. coli* JM109 (pSB1075) (Table 1) on 2YT at 37°C failed to detect any acyl-HSL activity. To obtain a concentrated sample of any putative *Brucella* acyl-HSLs that could be assayed at the recommended temperature of 30°C (31, 68), dichloromethane extracts of spent *Brucella* culture supernatants (2YT [E16M] and RPMI [E16M] grown) were prepared and concentrated 10,000-fold in acetonitrile. A twofold end-point dilution assay was performed for each extract with the biosensors *E. coli* JM109 (pSB401) and *E. coli* JM109 (pSB1075) by using dilutions of purified 3-oxo-C6-HSL and 3-oxo-C12-HSL, respectively, as positive controls. As a negative control for these assays, an equivalent extract of the noninoculated medium (denoted E2YT and E6400, respectively, for 2YT and RPMI 1640). No acyl-HSL activity was detected by *E. coli* JM109 (pSB401) in extracts from 2YT- or RPMI-cultured *B. melitensis* 16M NaI. No acyl-HSL activity was detected by *E. coli* (pSB1075) in extracts from 2YT; however, a clear activation of the LasR-based biosensor was observed in an extract of RPMI-cultured *B. melitensis* 16M NaI (Fig. 1), suggesting that a long-chain acyl-HSL was present. No activation of the biosensors could be seen when incubated with the extract of noninoculated RPMI 1640.

The negative results observed with E16M may be due to inhibitory activities present in the dichloromethane extract, which antagonized activation of the AHL biosensor. To investigate this further, synthetic acyl-HSLs were added to dichloromethane extracts of 2YT, yeast extract (YE), and peptone (Pep) and incubated with biosensors in a twofold dilution series (data not shown). This experiment revealed that extracts of 2YT, YE, and Pep contained compounds that were able to antagonize the acyl-HSL-mediated activation of the AHL biosensors.

*B. melitensis* produces C12-HSL. A dichloromethane extract of 18 liters of *B. melitensis* 16M NaI-spent RPMI culture supernatant was dried and reconstituted in a final volume of 1.2 ml of acetonitrile. The extract was fractionated using reverse-phase HPLC with a linear gradient of acetonitrile in water. HPLC fractions F5 and F6 activated *E. coli* (pSB1075) by using LasR and the *lasI* promoter by acyl-HSL activities are shown in two-fold dilution series of (left to right) synthetic C12-HSL (5 ng in the first column), synthetic C12-HSL plus solvent extract from 2YT medium (**E**2YT) (5 ng of C12-HSL plus 5 μl of E2YT [corresponding to 50 ml of medium] in the first column), solvent extraction from RPMI medium (**E**6400; 5 μl [corresponding to 50 ml of medium] in the first column), and solvent extraction from RPMI-cultured *B. melitensis* 16M NaI (**E**1640; 5 μl [corresponding to 25 ml of medium] in the first column). The expression of lasI:luxCDABE was measured in relative light units (RLU).

![FIG. 1. RPMI-cultured B. melitensis 16M NaI cells produce an acyl-HSL activity detected by E. coli (pSB1075). The activation of LasR and the lasI promoter by acyl-HSL activities are shown in two-fold dilution series of (left to right) synthetic C12-HSL (5 ng in the first column), synthetic C12-HSL plus solvent extract from 2YT medium (E2YT) (5 ng of C12-HSL plus 5 μl of E2YT [corresponding to 50 ml of medium] in the first column), solvent extraction from RPMI medium (E6400; 5 μl [corresponding to 50 ml of medium] in the first column), and solvent extraction from RPMI-cultured B. melitensis 16M NaI (E1640; 5 μl [corresponding to 25 ml of medium] in the first column). The expression of lasI:luxCDABE was measured in relative light units (RLU).](https://www.ncbi.nlm.nih.gov/pmc/articles/PMC139307/jiai0016002001f8.jpg)
The evidence presented here demonstrates, for the first time, that acyl-HSLs are produced by an intracellular pathogen. B. melitensis produces the QS signal C12-HSL when cultured at 37°C in vitro in the tissue culture medium RPMI. C12-HSL was identified by mass spectrometry after solvent extraction of spent B. melitensis culture supernatant and fractionation by HPLC. A second active fraction was detected, but the identity of the molecule present could not be determined by mass spectrometry, probably because the amount of this minor signal was too small. Comparison of the migration profiles of this unknown molecule in HPLC and TLC with synthetic acyl-HSL standards suggests that it is most probably 3-oxo-C12-HSL (data not shown). Up to now, we do not know if this second potential HSL has its own specificity or not (67).

In comparison with other bacteria studied, Brucella appears to produce low levels of acyl-HSLs. To obtain sufficient C12-HSL for mass spectrometry, it was necessary to extract 18 liters of spent culture medium (compared with around 4 to 6 liters reported in the literature for other bacteria [e.g., as seen in references 44 and 56]). One explanation for this observation may be found in the hydrophobic nature of C12-HSL. Pearson et al. have shown that the efficiency of extraction depends upon the hydrophobicity of the acyl-HSL (38). For synthetic [3H]3-oxo-C12-HSL incubated with a P. aeruginosa culture, only 75% of the signal is recovered by the extraction process, with 25% remaining trapped in bacterial membranes. C12-HSL is more hydrophobic than 3-oxo-C12-HSL, so we expected that in our extractions of the Brucella spent supernatant that less than 75% of the total production would have been recovered.

The intracellular lifestyle of Brucella may provide a second explanation for the production of low levels of acyl-HSL. Brucella cells survive intracellularly in mammalian host cells but do not begin to replicate until they have modified intracellular trafficking to produce the replication vacuole. At this point and depending on the type of cell infected, some heterogeneity of behavior has been observed. In placental trophoblasts, for example, the bacteria multiply within membrane-bound intracellular compartments until the infected cell lyses. In chronic infection, however, Brucella cells survive in macrophages and can be seen in low numbers. In either case, however, Brucella will be found within a confined compartment and any C12-
HSL produced by Brucella will accumulate, especially given the slow diffusion of hydrophobic acyl-HSLs across membranes, leading to the activation of QS at a lower cell density than in an open environment (38). Given that Brucella cells are unlikely to encounter any other sources of C12-HSL within their specialized intracellular niche, it is probable that they have evolved a system sensitive to low levels of signal.

An alternative third explanation for low levels of acyl-HSL production in vitro is a possible regulation mechanism controlling the HSL synthesis. A mechanism that could rely on the presence of a typical environment or a key host cell-derived signal to activate acyl-HSL production. This may be particularly relevant given the influence of the host cell-derived signals (like opines and flavonoids) on QS in other alpha subgroup proteobacteria (Agrobacterium and Rhizobium, respectively) (8, 9, 39) and the possibility that C12-HSL may be produced for both its effects upon the bacterial gene regulation and its effects upon the host organism.

Extracellular pathogens, such as P. aeruginosa, Erwinia carotovora, and Aeromonas hydrophila, use QS to control the expression of virulence factors. This was explained by the necessity to prevent an early release of these factors that would elicit an inflammatory or immune response until the population density is high enough to cope successfully with this host.

FIG. 4. MS of F6.3 identifies C12-HSL. (A) The chemical structure of C12-HSL showing the two fragmentation ions having major peaks in electrospray MS (m/z 102 and m/z 183). (B) Comparison of the MS spectrum of synthetic C12-HSL and of the C12-HSL found in subfraction F6.3 of the culture supernatant extract of B. melitensis 16M Nalr.

FIG. 5. Total RNA was extracted from lag (A600 = 0.05), early log (A600 = 0.15), and transition (A600 = 1) phase cultures of B. melitensis 16M Nalr in the presence (+) or absence (−) of 50 ng of synthetic C12-HSL ml−1. A twofold dilution series of total RNA for each treatment was immobilized on a positively charged nylon membrane. Blots were hybridized with digoxigenin-labeled virB5 probe (A) and digoxigenin-labeled f7/f12 probe (B) as controls.
has also been described, e.g., for the regulation of gene expression by QS is most commonly described as a process of activation. However, negative regulation has also been described, e.g., for Burkholderia cepacia, where QS induces protease production but also represses siderophore production (28), and Yersinia pseudotuberculosis, where QS appears to repress flagellin production (and hence flagellar-mediated motility) (2). Analysis of the QS regulon in Brucella may therefore identify genes positively and/or negatively regulated and will give important information regarding the factors required during different phases of the infection process.

Recently, Sieira et al. (51) described the virB operon in B. abortus 2308 and reported that expression is induced in the stationary phase by monitoring β-galactosidase activity in a virB10::lacZ fusion strain. This result, shown for B. abortus, is in contradiction to the data we present here and elsewhere for B. melitensis and B. suis (Boschirol et al., submitted). However, the experimental conditions, and hence the species, used by Sieira et al. are different from those described in this paper. B. abortus cells were cultured in tryptone soy broth, a rich medium similar to 2YT and not to RPMI or E medium. We suggest that compounds present in tryptone soy broth may inhibit the QS regulation of virB expression in Brucella. Indeed, we have not been able to demonstrate any significant effect of C12-HSL upon virB transcription in rich medium (data not shown).

The nature of the inhibitory compound is not known, however; Holden et al. (17) demonstrated the presence of small cyclic dipeptides or diketopiperazines (DKPs) in Pseudomonas culture supernatant that are able to activate or to antagonize QS biosensor systems. They showed that several DKPs are able to antagonize the induction of bioluminescence in E. coli (pSB401) by 3-oxo-C6-HSL without interfering with light emission from E. coli harboring constitutively expressed lux genes. Moreover, DKPs have been isolated from protein digests used as media components, and so the inhibitory effects observed with 2YT could be attributed to these molecules.

The immunosuppressive effect of 3-oxo-C12-HSL upon the host immune system observed by Telford et al. (60) prejudices against antibacterial action and so favors the bacteria. Additionally, the vasorelaxant activity of 3-oxo-C12-HSL observed by Lawrence et al. (26) may contribute to the ability of the bacteria to maintain the supply of key nutrients to the site of infection by increasing local blood flow. This acyl-HSL, produced by P. aeruginosa, can also modulate the expression of membrane receptors of human trabecular gland cells, aggravating Pseudomonas infections in cystic fibrosis patients (47). Given the chemical proximity between 3-oxo-C12-HSL and those produced by Brucella, a possible effect of C12-HSL on the immune system should not be ignored, especially given the biology of Brucella and its ability to deal with cellular actors of the immune system. Indeed, 3-oxo-C12-HSL has been shown to inhibit interleukin-12 and tumor necrosis factor alpha production, thus creating a situation potentially leading to a TH2 immune response (60). As an intracellular pathogen, Brucella has been shown to be more susceptible to a TH1-oriented response. For instance, depletion of IL-12 leads to an exacerbated infection by Brucella (69). Taken together, the possibility that C12-HSL and 3-oxo-C12-HSL could have a similar effect on cytokine production would obviously benefit Brucella.

We plan to extend our investigation of the role of QS in Brucella infections. We benefit now from the availability of the B. melitensis genome sequence for the search of the luxI/luxR homologs. The deletion of these genes will allow us to evaluate their impact upon the virulence of Brucella. Moreover, it will be interesting to identify other genes under the control of QS in Brucella.

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