LuxS-dependent quorum sensing in *Porphyromonas gingivalis* modulates protease and haemagglutinin activities but is not essential for virulence

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***Porphyromonas gingivalis* is a Gram-negative black-pigmented obligate anaerobe implicated in the aetiology of human periodontal disease. The virulence of *P. gingivalis* is associated with the elaboration of the cysteine proteases Arg-gingipain (Rgp) and Lys-gingipain (Kgp), which are produced at high bacterial cell densities. To determine whether quorum sensing plays a role in the regulation of Rgp and Kgp, biosensors capable of detecting either *N*-acylhomoserine lactone (AHLs) or the *luxS*-dependent autoinducer (AI-2) quorum-sensing signalling molecules in spent culture supernatants were first employed. While no AHLs could be detected, the *Vibrio harveyi* BB170 biosensor was activated by spent *P. gingivalis* W50 culture supernatants. The *P. gingivalis luxS* gene was cloned and demonstrated to restore AI-2 production in the *Escherichia coli luxS* mutant DH5 α . Mutation of *luxS* abolished AI-2 production in *P. gingivalis*. Western blotting using antibodies raised against the recombinant protein revealed that LuxS levels increased throughout growth even though AI-2 activity was only maximally detected at the mid-exponential phase of growth and disappeared by the onset of stationary phase. Similar results were obtained with *E. coli* DH5 α transformed with *luxS*, suggesting that AI-2 production is not limited by a lack of LuxS protein. Analysis of Rgp and Kgp protease activities revealed that the *P. gingivalis luxS* mutant produced around 45% less Rgp and 30% less Kgp activity than the parent strain. In addition, the *luxS* mutant exhibited a fourfold reduction in haemagglutinin titre. However, these reductions in virulence determinant levels were insufficient to attenuate the *luxS* mutant in a murine lesion model of *P. gingivalis* infection.**

Keywords: *Porphyromonas gingivalis*, *luxS*, quorum sensing, gingipains, haemagglutinins

INTRODUCTION

Porphyromonas gingivalis is found in the subgingival plaques of patients with progressive periodontitis. This black-pigmented Gram-negative anaerobe has been strongly implicated in the aetiology of this chronic

inflammatory disease which ultimately leads to the loss of tooth-supporting tissues and periodontal bone resorption. *P. gingivalis* has also been implicated as a risk factor for other conditions including cardiovascular disease and pre-term delivery of low birth weight infants (Beck *et al.*, 1996; Offenbacher *et al.*, 1996; Lamont & Jenkinson, 1998).

In the periodontal pocket, *P. gingivalis* is found predominantly as a component of complex biofilms containing multiple bacterial species. As well as a capacity

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Abbreviations: AHLs, *N*-acylhomoserine lactones; AI-2, autoinducer 2.

to adhere to a variety of host cell surfaces including the teeth, gingiva, cheek and tongue, *P. gingivalis* also adheres to other oral bacteria, presumably to facilitate colonization and survival within the oral cavity. *P. gingivalis* produces multiple cell envelope and exoproduct virulence determinants capable of promoting attachment, degrading host cell tissues and immune effector molecules, acquiring nutrients and facilitating epithelial cell invasion (Holt *et al.*, 1999; Kinder & Holt, 1989; Lamont & Jenkinson, 1998; Yoneda & Kuramitsu, 1996). Probably the most intensively investigated *P. gingivalis* virulence determinants are the exoproteases (Aduse-Opoku *et al.*, 1995; Kuramitsu *et al.*, 1997; Nakayama *et al.*, 1995; Okamoto *et al.*, 1996). Two major classes of cysteine proteinases specific for Arg-X and Lys-X bonds have been characterized and termed Arg-gingipain (Rgp) and Lys-gingipain (Kgp), respectively (Curtis *et al.*, 1999). The multiple protease forms present on the *P. gingivalis* cell surface and in spent culture supernatants arise from the products of three genes: *rgpA* and *rgpB* (which encode the Rgp activity) and *kgp* (which encodes the Kgp activity). The different forms appear to arise from the proteolytic post-translational processing of larger polyprotein precursors (Curtis *et al.*, 1999). For example, for RgpA, the initial precursor consisting of a pro-region, α -catalytic domain, a β -adhesin/haemagglutinin domain and a C-terminal γ domain is cleaved to generate three mature isoenzyme forms, HRgpA (an $\alpha\beta$ -heterodimer of around 110 kDa), RgpA_{cat} (α monomer of approx. 55 kDa) and mt-RgpA_{cat} (a highly post-translationally modified α monomer of 70–80 kDa). These proteases degrade a variety of host proteins including immunoglobulins, complement cascade components, collagen, fibrinogen and fibronectin. As a consequence, they not only promote resistance to host defence mechanisms but also generate free amino acids and small peptides for use as nutrients (Shah & Gharbia, 1989). Furthermore, the haemagglutinin/adhesin domains of RgpA and Kgp share significant amino acid sequence homology with haemagglutinin domain of HagA, a protein containing four contiguous direct repeats each of which contains a functional haemagglutinin domain (Han *et al.*, 1996). *P. gingivalis* also possesses four further haemagglutinin genes (*hagB*, *hagC*, *hagD* and *hagE*; Lépine *et al.*, 1996), and the ability to agglutinate erythrocytes strongly is a feature which distinguishes the organism from other black-pigmented anaerobes (Mayrand & Holt, 1988). Although the functions and contribution of these multiple haemagglutinins to virulence are not well understood, they clearly promote adherence and colonization of tissue surfaces and, in conjunction with the exoproteases, promote attachment and detachment to target human cells (Chen *et al.*, 2001).

To facilitate adaptation to life within the oral cavity, *P. gingivalis* must be capable of sensing and responding to the prevailing environmental conditions, including variations in temperature, oxygen tension, pH, nutrient availability and the presence of other cells. While there is relatively little information on the mechanisms by

which gene expression is regulated in this strict anaerobe, it is clear that the levels of virulence factors such as the proteases, fimbriae and haemagglutinins are controlled by temperature and haemin availability and affected by the presence of serum and saliva (Genco *et al.*, 1995; Tokuda *et al.*, 1998; Xie *et al.*, 2000). Regulation has been shown to occur at both transcriptional and post-transcriptional levels and intriguingly the fimbrillin gene (*fimA*) has recently been suggested to be controlled by both the fimbrillin protein itself and by Rgp and Kgp (Xie *et al.*, 2000). Given that Rgp and Kgp are produced maximally in the late stationary phase of growth (Rangarajan *et al.*, 1997), it is possible that they are regulated in a cell-density-dependent manner via quorum sensing (Williams *et al.*, 2000; Withers *et al.*, 2001). This is a cell-to-cell communication mechanism which enables an individual bacterial cell to sense other bacterial cells and, in response, differentially express specific sets of genes. Quorum sensing involves the activation of a sensor or response regulator by a small diffusible signal molecule, and several chemically distinct families of such molecules have been identified. In Gram-negative bacteria, *N*-acylhomoserine lactones (AHLs) represent the most intensively investigated family of signal molecules, although a number of non-AHL molecules have also been described (Williams *et al.*, 2000; Withers *et al.*, 2001). These include a polar molecule termed AI-2, originally discovered in *Vibrio harveyi* as a second diffusible signal that could activate bioluminescence in the absence of *N*-(3-hydroxybutanoyl)homoserine lactone (3-hydroxy-C4-HSL; Bassler *et al.*, 1994). Although the structure of this diffusible signalling molecule has not yet been elucidated, a gene product (LuxS) involved in the production of AI-2 has been identified not only in *V. harveyi*, but also in a number of human pathogens including *Escherichia coli* and *Salmonella* (Surette *et al.*, 1999; Surette & Bassler, 1998). However, the genes regulated directly via AI-2 in these Gram-negative pathogens have not yet been identified, although AI-2 has been reported to influence type III secretion in enterovirulent *E. coli* (Sperandio *et al.*, 1999) and to modulate expression of *virB* in *Shigella flexneri* (Day & Maurelli, 2001).

In the present paper, we have sought to determine whether *P. gingivalis* employs AHL- and/or AI-2/LuxS-mediated quorum sensing. While we were unable to detect any AHLs, *P. gingivalis* was found to produce a signalling molecule(s) capable of activating a *V. harveyi*-based AI-2 biosensor and to possess an orthologue of LuxS. Mutation of this gene resulted in the loss of AI-2 production and a reduction in protease and haemagglutinating activity which was insufficient to attenuate the *luxS* mutant in a murine lesion model of *P. gingivalis* infection.

METHODS

Bacteria, growth conditions and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. *P. gingivalis* W50 was maintained on Fastidious Anaerobic Agar (FAA; LabM) plates containing 5% horse blood (TCS) at

Table 1. Bacterial strains and plasmids used in this study

Strain/plasmid	Description	Source
Strains		
<i>P. gingivalis</i> W50	Wild-type strain	ATCC 53978
<i>P. gingivalis</i> NBS1	<i>luxS</i> mutant derived from W50	This study
<i>P. gingivalis</i> K1A	<i>kgp</i> mutant derived from W50	Aduse-Opoku <i>et al.</i> (2000)
<i>V. harveyi</i> BB120	Wild-type strain	Bassler <i>et al.</i> (1994)
<i>V. harveyi</i> BB170	Mutant strain which responds to AI-2	Bassler <i>et al.</i> (1994)
<i>C. violaceum</i> CV026	Double Tn5 mutant derived from <i>C. violaceum</i> ATCC 31532; AHL sensor, Km ^R Hg ^R	McClean <i>et al.</i> (1997)
<i>E. coli</i> JM109(pSB401)	AHL biosensor containing a <i>luxRI'</i> :: <i>CDABE</i> fusion in pACYC184, Tet ^R	Winson <i>et al.</i> (1998)
<i>E. coli</i> JM109(pSB536)	AHL biosensor containing an <i>abyRI'</i> :: <i>luxCDABE</i> fusion in pRK415, Amp ^R	Winson <i>et al.</i> (1998)
<i>E. coli</i> JM109(pSB1075)	AHL biosensor containing a <i>lasRI'</i> :: <i>luxCDABE</i> fusion in pUC18, Tet ^R	Winson <i>et al.</i> (1998)
Plasmids		
pBluescript SK (+/-)	A phagemid derived from pUC19, Amp ^R	Stratagene
pHG327	A pUC8 derivative with the multiple cloning site of pUC18, Amp ^R	Stewart <i>et al.</i> (1986)
pMal-c2	High-level expression plasmid containing <i>malE</i> fused to <i>lacZα</i> , Amp ^R	New England BioLabs
pUC18 <i>Not</i>	pUC18 containing <i>NotI</i> restriction sites flanking the multicloning region, Amp ^R	Herrero <i>et al.</i> (1990)
pVA2198	Plasmid containing <i>ermF-ermAM</i> for selection in both <i>P. gingivalis</i> and <i>E. coli</i>	Fletcher <i>et al.</i> (1995)
pNB1	A 989 bp fragment containing <i>luxS</i> and flanking regions ligated into pHG327	This study
pNB2	Deletion of 231 bp from <i>luxS</i> ligated into pHG327	This study
pNB3	A 545 bp fragment containing <i>luxS</i> ligated into pMal-c2	This study
pHGin18	Deletion of 231 bp from <i>luxS</i> ligated into pUC18	This study
pGinerm	pHGin18 containing <i>ermF-ermAM</i>	This study

37 °C in an anaerobic cabinet equilibrated in 80% nitrogen/10% hydrogen/10% carbon dioxide. Liquid cultures of *P. gingivalis* W50 were grown anaerobically at 37 °C in Brain Heart Infusion (BHI) broth (Oxoid) containing 5 µg haemin ml⁻¹. For detection of AI-2 in *P. gingivalis* culture supernatants, the organism was grown in the chemically defined medium described by Milner *et al.* (1996).

E. coli was grown at 37 °C in Luria-Bertani (LB) medium and *V. harveyi* strains were grown at 30 °C in Autoinducer Bioassay (AB) medium (Greenberg *et al.*, 1979). Antibiotics, where required, were used at the following concentrations: ampicillin, 50 µg ml⁻¹; clindamycin, 5 µg ml⁻¹; tetracycline, 10 µg ml⁻¹; erythromycin, 5 µg ml⁻¹; and kanamycin, 10 µg ml⁻¹.

Bioassays for quorum-sensing signal molecules. Assays to detect the presence of AHLs were carried out as previously described (Cámara *et al.*, 1998). Cell-free culture supernatants were extracted with dichloromethane and the extracts were subjected to TLC on reverse phase aluminium backed RP18 F_{254S} TLC plates (20 cm × 20 cm; Merck) with a mobile phase of 60% (v/v) methanol in water. After chromatography, TLC plates were overlaid with soft top agar seeded with an AHL biosensor as described by McClean *et al.* (1997). The AHL biosensors used were the Tn5-generated *Chromobacterium violaceum* mutant CV026, which responds to a range of AHLs (with from C₄ to C₈ acyl side chains) by inducing the synthesis of the purple pigment violacein, and the *lux*-based biosensors (pSB401, pSB536 and pSB1075; Table 1) described by Winson *et al.* (1998), which are capable of detecting AHLs with acyl side chains from C₄ to C₁₄. Bioluminescence was detected using a Luminograph LB980 photon imaging camera (EG&G Berthold).

The AI-2 bioassay, using the *V. harveyi* biosensor BB170

(*luxN* negative mutant), was carried out as described by Greenberg *et al.* (1979). Bacterial culture supernatants were added to the BB170 biosensor strain and AI-2 activity was measured in an EG&G Wallac Victor² luminometer as Relative Luminescence Units (RLU).

DNA manipulation techniques. Plasmid isolation, agarose electrophoresis, restriction digests and transformation of *E. coli* were performed as described by Sambrook *et al.* (1989). PCR amplifications were performed according to Ausubel *et al.* (1989). Bacterial chromosomal DNA was purified using the hexadecyltrimethylammonium bromide (CTAB) method (Murray & Thompson, 1980). Purification of DNA fragments from agarose gels was carried out using Qiaquick DNA purification columns (Qiagen). Probes for Southern blot analysis were labelled using a DIG-labelling system (Boehringer Mannheim). Oligonucleotide synthesis and DNA sequencing was carried out at the Biopolymer Synthesis and Analysis Unit, Queen's Medical Centre, University of Nottingham.

Construction of a *P. gingivalis* genomic library in *E. coli* containing *lux*-based AHL reporters. To clone any putative *P. gingivalis* AHL synthases, the functional complementation assay described by Swift *et al.* (1993) was used. Genomic DNA from *P. gingivalis* W50 was digested with *HindIII*, *KpnI*, *PstI*, *BamHI* or *EcoRI*, purified from agarose gels and ligated into pBluescript SK(+) previously digested with the appropriate restriction enzyme and dephosphorylated. The ligation mixtures were electroporated into *E. coli* JM109 containing either the *lux*-based AHL reporter pSB401 or pSB1075 (Winson *et al.*, 1998; Laue *et al.*, 2000) and plated on antibiotic selective media. Bioluminescent transformants were sought by viewing colonies with a Luminograph LB980 photon imaging camera (EG&G Berthold).

Cloning of *luxS*. For cloning of the *P. gingivalis luxS* gene, preliminary sequence data were obtained from The Institute for Genomic Research through the website at <http://www.tigr.org>. PCR amplification of *luxS* from *P. gingivalis* W50 chromosomal DNA was carried out using primers Por11 (5'-GTATTATCAGCGGAATTC^{CGG}CGGAAGGTCG-3') and Por12 (5'-GATACCGCTCCGGATCCAATAATCCATC-CGG-3'), which were designed using the published information on the *P. gingivalis* W83 genome sequence and contained *EcoRI* and *BamHI* restriction sites, respectively (see underlined sequences above). The PCR product was purified from an agarose gel and ligated into pHG327 (Table 1), previously digested with *EcoRI* and *BamHI*, to generate pNB1 (Table 1).

Generation of a *P. gingivalis luxS* mutant. A deletion mutant of *luxS* lacking 231 internal nucleotides and containing an erythromycin (*erm*)-resistance cassette was constructed as follows. Two separate pairs of primers introducing *NotI* restriction sites (see underlined) were used: Por11 (see above) with Por13B (5'-GCGGCCGCCACCAAATGCTCGATC-GTATGCCAG-3') (to amplify the 5'-end of *luxS*) and Por14B (5'-TGGCGGCCGCGCGTGAGGTACTCGATGT-AGCG-3') with Por12 (see above) (to amplify the 3'-end of *luxS*). *P. gingivalis* W50 chromosomal DNA was used as a template. Subsequently, each of the purified PCR products served as a template in a second PCR amplification using primers Por11 and Por12. The PCR product was digested with *BamHI* and *EcoRI* and ligated into similarly digested pHG327 to generate pNB2 (Table 1). This plasmid was then digested with *EcoRI* and *BamHI* to release the truncated *luxS* and ligated into pUC18, to generate pHGin18. To insert an *erm* cassette in the *NotI* site of the truncated *luxS*, the *erm* cassette from pVA2198 (Rangarajan *et al.*, 1997) was excised using *EcoRI* and *BamHI* digestion and cloned into pUC18*Not*, similarly digested. The *erm* cassette was then isolated from pUC18*Not* by digestion with *NotI* and subsequently cloned into the *NotI* site of pHGin18, thus creating pGinerm. This construct was electroporated into *P. gingivalis* W50 as described by Rangarajan *et al.* (1997), except that the plasmid was not linearized prior to electroporation. Transformants were selected on FAA containing clindamycin.

Overexpression, purification and detection of the *P. gingivalis LuxS*. *luxS* was amplified from *P. gingivalis* W50 chromosomal DNA, ligated into the *EcoRI/BamHI* sites of pMal-c2 (New England BioLabs) to generate pNB3, and electroporated into *E. coli* DH5 α . The soluble recombinant MalE-LuxS fusion obtained was purified on an amylose resin column and bound protein was eluted with 10 mM maltose. The purified MalE-LuxS fusion protein was cleaved using Factor Xa protease (New England BioLabs) and, following SDS-PAGE, the protein was electro-eluted and used to raise polyclonal antibodies in rabbits.

For the detection of recombinant or native LuxS protein, cell lysates from *E. coli* DH5 α , *P. gingivalis* W50 and the *luxS* mutant were obtained by harvesting cells throughout the growth curve. The protease inhibitor *N* α -*p*-tosyl-L-lysine chloromethyl ketone (TLCK) was added, at a concentration of 50 mM, to *P. gingivalis* cells prior to lysis. Samples were standardized for protein content, boiled, resolved by SDS-PAGE and transferred to nitrocellulose membranes. LuxS was detected by incubation with the LuxS polyclonal antiserum followed by a protein A-peroxidase conjugate and developed with an Amersham ECL kit (Amersham International).

Rgp and Kgp protease assays. Protease activity was measured as described by Rangarajan *et al.* (1997). *P. gingivalis* whole

cultures, cell lysates (prepared by sonication of cells in PBS, pH 7.4) or cell-free supernatants prepared from cells grown to the same optical density (OD₆₀₀) were each added to a solution of 10 mM L-cysteine, 10 mM CaCl₂, 100 mM Tris/HCl buffer (pH 8.1) containing 0.5 mM *N*- α -benzoyl-DL-arginine-*p*-nitroanilide (DL-BAPNA) or 0.5 mM *N*- α -benzoylcarbonyl-L-lysine-*p*-nitroanilide (Ac-Lys-*p*NA). Samples were incubated for 1 h at 30 °C and the reaction was monitored by following the increase in A₄₀₅ due to release of *p*-nitroaniline. Experiments were repeated three times in duplicate.

Haemagglutination assay. The haemagglutination assay was performed as described by Dusek *et al.* (1993). Essentially, sheep red blood cells (SRBCs) were harvested and washed twice in PBS (pH 7.4) at 4 °C by centrifugation at 2000 *g* for 3 min and diluted to 0.5% (v/v) in PBS. *P. gingivalis* W50 wild-type and *luxS* mutant were grown to an approximate OD₅₈₀ of 0.8, washed once in PBS and resuspended to an OD₅₈₀ of 1.0. Doubling dilutions of bacterial cells (50 μ l) were performed in V-shaped 96-well plates. The SRBCs (50 μ l) were added to the plates followed by incubation at 4 °C overnight and scored for haemagglutination.

***P. gingivalis* murine infection model.** The model described by Kesavalu *et al.* (1992) was used to determine whether the *luxS* mutant NBS1 exhibited reduced virulence. BALB/c mice (eight mice per group) were challenged s.c. for each strain with either 2.5 $\times$ 10¹⁰, 5 $\times$ 10¹⁰ or 1 $\times$ 10¹¹ c.f.u. of *P. gingivalis* W50, the isogenic *luxS* mutant or the *kgp* mutant K1A (Aduse-Opoku *et al.*, 2000). K1A is known to be attenuated in this animal model (M. A. Curtis, unpublished data). For each strain, the mean survival time was determined for each of three challenge doses and the entire experiment was repeated twice. The data obtained were analysed statistically using standard methods for survival analysis allowing for right-censoring of time to death when animals survived to the scheduled end of the experiment. Life-table methods were used to display cumulative survival probabilities during experiments, while Cox proportional hazards models were used to estimate hazard ratios for comparisons of interest controlling for the effects of bacterial dose and for between-experiment variation in cumulative survival (Cox & Oakes, 1984).

RESULTS

AHL-dependent quorum sensing and *P. gingivalis*

Solvent-extracted spent culture supernatants from *P. gingivalis* W50 were examined for the presence of AHLs by TLC assays employing four different biosensors capable of detecting a wide range of AHLs. No active spots were observed. Furthermore, interrogation of the *P. gingivalis* W83 genome sequence database (www.tigr.org) for homologues of (a) the LuxR transcriptional regulator family and (b) AHL synthases belonging to either the LuxI or LuxM family failed to identify any candidates. Since a putative third family of AHL synthases (HdtS) was recently described by Laue *et al.* (2000), the possible existence of a novel AHL synthase was investigated by introducing genomic libraries of *P. gingivalis* W50 DNA into *E. coli* strains containing either the *lux*-based AHL reporter pSB401 or pSB1075. Examination of each of the transformants obtained for bioluminescence and the characteristic accompanying satelliteism (where surrounding colonies emit light as a consequence of cross-feeding from a positive clone) proved negative.

Fig. 1. (a) Diagrammatic representation of the *P. gingivalis luxS* locus showing upstream the presence of *pfs* and *pg97*, which encode a putative 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase and the *P. gingivalis* W50 47 kDa immunodominant antigen, respectively. Downstream of *luxS* is *tgt*, which is predicted to encode a tRNA guanine transglycosylase. (b) SDS-polyacrylamide gel showing the purification of the *P. gingivalis* LuxS protein. Lanes: 1, molecular mass markers; 2, MalE-LuxS fusion purified by affinity chromatography eluted from an amylose resin column with maltose; 3, purified LuxS protein cleaved from the MalE-LuxS fusion as described in Methods. The weak upper band in lane 3 is a dimer of LuxS.

Characterization of a *P. gingivalis* LuxS orthologue

To determine whether *P. gingivalis* possesses an AI-2/LuxS-based quorum-sensing system, the *P. gingivalis* genome database was searched using the predicted amino acid sequence of the *V. harveyi* LuxS protein. A 159 amino acid residue (18.5 kDa) LuxS orthologue was identified. When compared with other LuxS orthologues present in the databases, the *P. gingivalis* LuxS protein is most closely related to the LuxS of *Borrelia burgdorferi* (similarity 68%; identity 50%) and shares 33% identity (53% similarity) with the *E. coli* LuxS protein. Examination of the DNA sequence surrounding *P. gingivalis luxS* (Fig. 1a) revealed the presence of an adjacent ORF, the translated amino acid sequence of which shares significant homology (37% identity; 54% similarity) with the *E. coli pfs* gene product, a 5'-methylthioadenosine/S-adenosylhomocysteine nucleosidase (Cornell & Riscoe, 1998). This *pfs* gene is separated from *luxS* by only 24 bp, suggesting that the two genes constitute an operon. Homologues of the *P. gingivalis* W50 47 kDa immunodominant antigen (GenBank accession no. AF153770) and a tRNA guanine transglycosylase (Tgt; Reuter *et al.*, 1991) were located approximately 700 bp upstream and downstream of the *pfs/luxS* locus, respectively (Fig. 1a).

To determine whether *luxS* encodes a functional protein, it was expressed in *E. coli* DH5 α , which is AI-2 negative and carries a frameshift mutation in the *luxS* gene (Surette *et al.*, 1999). This was achieved by amplification of *luxS* from *P. gingivalis* W50 by PCR, followed by

cloning into pHG327 to generate pNB1. In contrast to *E. coli* DH5 α , spent culture supernatants from *E. coli* DH5 α (pNB1) stimulated light production in the AI-2 biosensor strain *V. harveyi* BB170 (data not shown), suggesting that the recombinant *P. gingivalis* protein is functional. To confirm that the AI-2 activity detected was due to *luxS* expression, an internal fragment of 231 bp was deleted and the truncated *luxS* gene was cloned into pHG237 to generate pNB2. Spent culture supernatants from *E. coli* DH5 α (pNB2) failed to stimulate bioluminescence in *V. harveyi* BB170 above background (data not shown).

AI-2 levels in both *P. gingivalis* and *E. coli* do not correlate with LuxS levels

To investigate the relationship between AI-2 levels and LuxS production as a function of growth in both *E. coli* and *P. gingivalis*, antibodies were first raised against the recombinant *P. gingivalis* LuxS protein expressed from pNB3 (Table 1) as a MalE-LuxS fusion. The 62 kDa fusion protein was purified by amylose affinity chromatography, cleaved using Factor Xa and the recombinant LuxS protein obtained for antibody production via electro-elution of the protein from SDS-polyacrylamide gels used to separate LuxS from MalE (Fig. 1b).

E. coli DH5 α transformed with pNB3 (Table 1) produced AI-2, indicating either that the MalE-LuxS fusion protein was functional or that sufficient LuxS for AI-2 synthesis was cleaved by endogenous *E. coli* proteases.

Fig. 2. (a) Growth and AI-2 production by *P. gingivalis* W50. *P. gingivalis* was grown anaerobically in defined medium for 48 h at 37 °C. At 2, 4, 6, 8, 10, 24, 30 and 48 h, cell-free culture supernatants were assayed for AI-2 activity using *V. harveyi* BB170. (b, c) Western blot analysis of whole-cell lysates of *P. gingivalis* W50 and the isogenic *luxS* mutant throughout the growth curve. Lane C in each panel contains the purified recombinant LuxS fusion protein as a control and the remaining lanes contain lysates of W50 (b) or the *luxS* mutant (c) harvested throughout growth at 6, 12, 24, 48, 72 and 96 h of growth and adjusted to standardized protein content. Following Western blotting, membranes were probed with antibodies raised against the recombinant *P. gingivalis* LuxS.

Western blotting of *E. coli* DH5 α (pNB3) whole-cell extracts using antibodies raised against LuxS revealed that the LuxS protein accumulates throughout growth (data not shown). In addition, analysis of culture supernatants from *E. coli* DH5 α (pNB3) taken throughout the growth curve showed increasing levels of AI-2 activity from mid-exponential phase disappearing by early-stationary phase when LuxS levels are still increasing (data not shown). The levels of AI-2 production were higher when cells were grown in the presence of glucose (0.4%, w/v) than when this sugar was omitted (data not shown).

Bioluminescence assays using *V. harveyi* BB170 failed to reveal the presence of AI-2 in culture supernatants taken throughout the growth curve of *P. gingivalis* W50 grown in BHI medium. However, when this organism was grown in a chemically defined medium (DM), AI-2 was maximally detected at mid-exponential phase of growth (Fig. 2a), disappearing at the beginning of the stationary phase, while Western blots still detected increasing levels of LuxS (Fig. 2b). These results are consistent with those obtained in *E. coli* DH5 α (pNB3), suggesting that AI-2 production is not limited by LuxS protein levels.

LuxS modulates protease levels and haemagglutination

To determine whether LuxS contributes to the regulation of virulence determinants in *P. gingivalis*, a *luxS* null mutant (NBS1) was first constructed as described in Methods. Southern blot (data not shown) and Western blot analysis (Fig. 2c) confirmed the absence of LuxS protein and no AI-2 activity could be detected in spent culture supernatants of the *luxS* mutant using the *V. harveyi* BB170 AI-2 biosensor (data not shown).

Fig. 3. Protease production by *P. gingivalis* W50 and the isogenic *luxS* mutant. (a) Kgp and (b) Rgp levels were determined using the substrates Ac-Lys-pNA and BApNA, respectively, in whole cultures (tot), cell-free supernatants (sup) and lysates (sol) of *P. gingivalis* W50 wild-type (WT) and the *luxS* null mutant NBS1 (M). Fractions were prepared as described in Methods and enzyme activity was followed by incubation at 30 °C for 1 h. The reaction was monitored at 405 nm.

To quantify the levels of protease activity produced by *P. gingivalis* W50 and the isogenic *luxS* mutant, whole culture, cell lysates and cell-free culture supernatants were incubated with either the Kgp substrate Ac-Lys-pNA or the Rgp substrate DL-BApNA. Fig. 3(a) shows

Table 2. Virulence of *P. gingivalis* in a murine soft tissue model

Values are the mean survival time (h). Mice were challenged s.c. with either the *P. gingivalis* wild-type, *luxS* mutant (NBS1) or the *kgp* mutant K1A and examined up to 190 h for lesion formation, weight and lethality. The numbers of animals surviving are in parentheses.

Strain	Challenge dose (c.f.u.)		
	2.5×10^{10}	5×10^{10}	1×10^{11}
W50	190 (8/8)	31 (0/8)	22 (0/8)
NBS1	116 (3/8)*	39 (0/8)	25 (0/8)
K1A	190 (8/8)	113 (2/8)	64 (0/8)

* Reduced survival time for the *luxS* mutant at this challenge dose is not statistically significant.

that after 48 h growth, each fraction prepared from the *luxS* mutant contained around 30% less Kgp activity than the parent strain. For Rgp, each fraction prepared from the *luxS* mutant contained around 45% less Rgp protease activity (Fig. 3b).

Since the C-terminal region of the RgpA family of proteinases shares extensive sequence homology with the haemagglutinins of *P. gingivalis* (Lamont & Jenkinson, 1998), we compared the capacity of the wild-type and *luxS* mutant to agglutinate sheep erythrocytes. The haemagglutination titre of the *luxS* mutant (1 in 8) was fourfold lower than the wild-type (1 in 32; data not shown).

luxS and the virulence of *P. gingivalis*

BALB/c mice were challenged s.c. with the wild-type, *luxS* mutant NBS1 or *kgp* mutant K1A. Table 2 shows that there were no differences in the mean survival times of mice infected with either the wild-type strain or *luxS* mutant. These data contrast with the *kgp* mutant, which was clearly attenuated as revealed by the extended survival times of mice inoculated with 5×10^{10} or 1×10^{11} c.f.u.

DISCUSSION

As a determinant of cell population density, quorum sensing is emerging as an integral component of bacterial global gene regulatory networks responsible for facilitating environmental adaptation. Several chemically distinct classes of quorum-sensing signal molecules have emerged, of which the AHL family in Gram-negative bacteria has been the most intensively investigated (Williams *et al.*, 2000; Withers *et al.*, 2001). Although we were unable to obtain any evidence to suggest that the Gram-negative anaerobe *P. gingivalis* employs AHL-mediated quorum sensing, we have demonstrated that this periodontal pathogen produces a diffusible signal molecule capable of activating the *V. harveyi* AI-2

biosensor. During the preparation of this manuscript, two other groups also reported that *P. gingivalis* as well as other Gram-negative oral bacteria could activate the AI-2 biosensor (Frias *et al.*, 2001; Chung *et al.*, 2001). Production of AI-2 in *P. gingivalis* as determined using the *V. harveyi* biosensor BB170 is abolished following mutation of *luxS* (this study and Chung *et al.*, 2001). Mutation of *luxS* in *E. coli* (Surette *et al.*, 1999; Sperandio *et al.*, 1999), *Helicobacter pylori* (Forsyth & Cover, 2000; Joyce *et al.*, 2000) and *Shig. flexneri* (Day & Maurelli, 2001) also results in the loss of AI-2 synthesis.

AI-2 appears to represent a new class of quorum-sensing signal molecule involved in cross-communication between different bacteria because Gram-negative and Gram-positive organisms both contain LuxS orthologues (Bassler *et al.*, 1997; Bassler, 1999). However, at present, the chemical identity of the *V. harveyi* AI-2 molecule is not known although Schauder *et al.* (2001) have very recently reported that it is probably a furanone derived from 4,5-dihydroxy-2,3-pentadione and that the function of LuxS is to generate 4,5-dihydroxy-2,3-pentadione from *S*-adenosylhomocysteine. Thus, in contrast to the AHLs, where a family of signal molecules with different *N*-linked acyl chains has been identified (Withers *et al.*, 2001), it has been suggested that AI-2 is structurally invariant, i.e. that despite their sequence divergence, LuxS orthologues all drive the synthesis of the same molecule (Schauder *et al.*, 2001).

In *P. gingivalis*, the *luxS* gene is located adjacent to *pfs*; RT-PCR analysis suggests that they constitute an operon (N. Burgess, unpublished data; Chung *et al.*, 2001). The *pfs* gene product is a 5'-methylthioadenosine/*S*-adenosylhomocysteine nucleosidase which can cleave the glycosidic bond releasing adenine and the corresponding thiopentose (either methylthioribose or *S*-ribosylhomocysteine; Cornell & Riscoe, 1998). Indeed, during the preparation of this manuscript, Schauder *et al.* (2001) reported that AI-2 can be generated *in vitro* from the purified LuxS proteins of *E. coli*, *V. harveyi*, *Vibrio cholerae*, *Salmonella typhimurium* and *Enterococcus faecalis* in conjunction with Pfs and *S*-adenosylhomocysteine. We have also shown that the production of AI-2 *in vitro* via the *P. gingivalis* LuxS protein and *S*-adenosylhomocysteine depends on the presence of the *P. gingivalis* *pfs* gene product (Winzer *et al.*, 2002).

By growing *P. gingivalis* in a chemically defined medium, we were able to show that AI-2 is produced during mid-exponential growth but appears to have been degraded by the stationary phase. These data are consistent with results obtained for *E. coli* DH5 α harbouring the *P. gingivalis luxS* gene (this paper) and with those of Surette *et al.* (1999) for *Sal. typhimurium*. Similarly, in *H. pylori*, AI-2 activity is maximal in early-exponential phase and rapidly decreases as the organism enters stationary phase (Joyce *et al.*, 2000). These data suggest that LuxS-mediated quorum sensing is only operational during periods of rapid, nutrient-rich growth. Interestingly, Western blot analysis of LuxS production in *P.*

gingivalis and *E. coli* throughout the growth curve revealed that despite the LuxS protein being present, AI-2 activity is absent from stationary phase samples. In both *E. coli* and *Sal. typhimurium*, the existence of a degradation pathway has been proposed, given the rapid loss of AI-2 when carbohydrate is depleted from the medium or when the cells enter stationary phase (Surette & Bassler, 1998). This implies that the substrates necessary for AI-2 synthesis are not available in stationary phase and/or that the levels of LuxS are maintained to enable the cells to take immediate advantage of any changes in environmental parameters (e.g. nutrient availability) without the need for transcription (Surette & Bassler, 1999). Alternatively, the retention of the LuxS protein may be required to prevent accumulation of S-ribosylhomocysteine, which is an inhibitor of all S-adenosylmethionine-dependent methylation reactions (Cornell & Riscoe, 1998), or to function as a component of a salvaging pathway for adenine and methionine synthesis.

Apart from modulation of bioluminescence in *V. harveyi* (Bassler *et al.*, 1994), the identification and characterization of the signal transduction pathways and target genes of the AI-2 quorum-sensing system in other bacteria has met with limited success. For *H. pylori*, mutation of *luxS* did not influence expression of any of the established virulence factors (e.g. *vacA*) and analysis of the two-dimensional protein profiles of wild-type and mutant failed to reveal any differences (Joyce *et al.*, 2000; Forsyth & Cover, 2000). In enteropathogenic *E. coli*, Sperandio *et al.* (1999) reported that LuxS weakly regulates operons encoding the type III secretion apparatus, intimin receptor and intimin. Although the virulence of pathogenic *E. coli luxS* mutants was not evaluated, an *Shig. flexneri luxS* mutant was discovered to be as invasive and virulent as the parental strain despite the contribution of LuxS in enhancing expression of *virB* in the late-exponential phase (Day & Maurelli, 2001). In *P. gingivalis*, Chung *et al.* (2001) have very recently used the qualitative techniques of differential display (DD)-PCR and RT-PCR to show that mutation of *luxS* influenced the transcript levels of several genes involved in haemin acquisition and an exonuclease ABC homologue. Although their analysis of *rgpA* expression gave equivocal results depending on which technique was used, Chung *et al.* (2001) reported that *rgpA* transcript levels were increased in the *luxS* mutant. However, no data were shown and no direct quantitative assays of Rgp protease activity were undertaken. In the present paper, we have established that mutation of *luxS* in *P. gingivalis* results in a 30–45% reduction in the levels of the Rgp and Kgp proteases. While this may at first appear to conflict with the results of Chung *et al.* (2001), a 30% reduction in protein is unlikely to be accompanied by a reduction in transcript level of sufficient magnitude to be detectable by DD-PCR or by RT-PCR. It is also possible that the regulation is at the post-transcriptional level. Both RgpA and Kgp are subject to significant post-translational processing (Curtis *et al.*, 1999) which may also be influenced by the

luxS mutation. Indeed, preliminary analysis of the Rgp isoforms in the *luxS* mutant NBS1 revealed a reduction in the level of the membrane-type RgpB isoform (mt-RgpB; unpublished data), the main vesicle-associated form of the Arg-gingipains (Aduse-Opoku *et al.*, 1998). This suggests that LuxS may influence the post-translational modification pathway of these enzymes.

Furthermore, the *luxS* mutant exhibits reduced whole-cell haemagglutinin activity compared with the parent. This finding is consistent with previous reports that cysteine proteases and haemagglutinin activities of *P. gingivalis* appear to be structurally related (Pavloff *et al.*, 1995; Yoneda & Kuramitsu, 1996). This phenotype may therefore be associated with RgpA and Kgp, which contain haemagglutinin/adhesion domains (Curtis *et al.*, 1999). Alternatively, it may be a consequence of reduced expression of haemagglutinins such as *hagA*, which encodes a surface protein haemagglutinin containing domains homologous to the adhesion domains of Rgp and Kgp. Thus given the central role of proteases in the pathogenicity of *P. gingivalis*, we sought to determine whether the reduced production of Rgp and Kgp by the *luxS* mutant influenced virulence. In an acute mouse model of soft tissue infection, there were no obvious differences in the virulence of the *P. gingivalis luxS* mutant when compared with the parent strain. However, although frequently used to assess the virulence of, and immunity to, *P. gingivalis*, the infection model used (Kesavalu *et al.*, 1992) depends on a high bacterial challenge dose and does not follow the natural course of periodontal disease. In addition, *P. gingivalis* is generally found only in low numbers within the oral cavity such that under these conditions the expression of proteases/haemagglutinins may be reduced. In disease, *P. gingivalis* can represent a significant proportion of the total flora and, under these conditions, it remains possible that AI-2/LuxS may play a role in up-regulation of these virulence properties and hence the development of disease.

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