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Detection and mutation of a *luxS*-encoded autoinducer in *Proteus mirabilis*

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Quorum sensing regulates the expression of virulence factors in a wide variety of pathogenic bacteria. This study has shown that *Proteus mirabilis* harbours a homologue of *luxS*, a gene required for the synthesis of the quorum sensing autoinducer 2 (AI-2). AI-2 activity is expressed during and is correlated with the initiation of swarming migration on agar surfaces. The *P. mirabilis luxS* locus was cloned and a LuxS$^-$ strain constructed by allelic-exchange mutagenesis. While lacking AI-2 activity, a null mutation in *luxS*, however, did not affect swimming or swarming motility, swarmer cell differentiation, or virulence in a mouse model of ascending urinary tract infection.

Keywords: quorum sensing, LuxS, swarming, virulence, urinary tract infection

INTRODUCTION

Urinary tract infections (UTIs) are among the most common bacterial infections of any organ system, accounting for an estimated 8 million visits to physicians each year (Mobley & Warren, 1996). Of the bacteria responsible for causing uncomplicated cystitis or acute pyelonephritis, *Proteus mirabilis* represents only a small percentage of cases, but it is much more frequently isolated from patients with complicated urinary tracts, i.e. those with functional or anatomical abnormalities or with chronic instrumentation (Mobley & Chippendale, 1990). In these cases, *P. mirabilis* is a more frequent uropathogen and its infection may be more severe due to recurring infection and the production of urinary stones in the kidney(s). Struvite (magnesium ammonium phosphate) and apatite (calcium phosphate) stones, formed as a result of the breakdown of urea by *P. mirabilis* urease, are commonly found in the renal pelvis. Stone formation makes *P. mirabilis* important in complicated UTIs, especially since *P. mirabilis* infections appear preferentially localized to the kidneys (Johnson *et al.*, 1993b).

Stickler and his collaborators (McLean *et al.*, 1997; Stickler & Hughes, 1999; Stickler *et al.*, 1998) have demonstrated the importance of *P. mirabilis* biofilms in the formation of struvite crystals during UTI. When grown on nutrient agar, *P. mirabilis* also forms a similar biofilm (or bacterial colony) with a unique pattern of development. These colonies frequently develop a concentric bull's-eye pattern (Fig. 1), underscoring the cyclic nature of this motile behaviour. As shown in Fig. 1, each cycle of biofilm development may be divided into four parts: (i) swarmer cell differentiation, (ii) the lag period prior to active movement, (iii) swarming colony migration, and (iv) consolidation (where the cells stop moving and de-differentiate to swimmer cell morphology). The bull's-eye pattern is itself mirrored in the activity of a set of proteins that are also expressed coordinately with the cycles of swarming. Included in this group of swarmer-cell-dependent proteins are a set of virulence factors, including flagellin, the ZapA protease, urease and haemolysin (Allison *et al.*, 1992). It has been postulated, based in part on the evidence of co-ordinate expression of virulence factors during cellular differentiation, that the swarmer cell and swarming behaviour may be involved in UTI and pathogenesis (Allison *et al.*, 1992, 1993; Chippendale *et al.*, 1994).

The presence of a lag period prior to swarming behaviour and the co-ordination of the swarming colony biofilm to form bull's-eye patterns suggests that cell–cell interaction and communication may control these events. Our previous work demonstrated that the lag period prior to *P. mirabilis* swarming migration is dependent upon the density of cells in the nascent

Abbreviations: AHL, *N*-acylhomoserine lactone; AI, autoinducer; UTI, urinary tract infection.

The GenBank accession number for the sequence reported in this paper is AY044337.

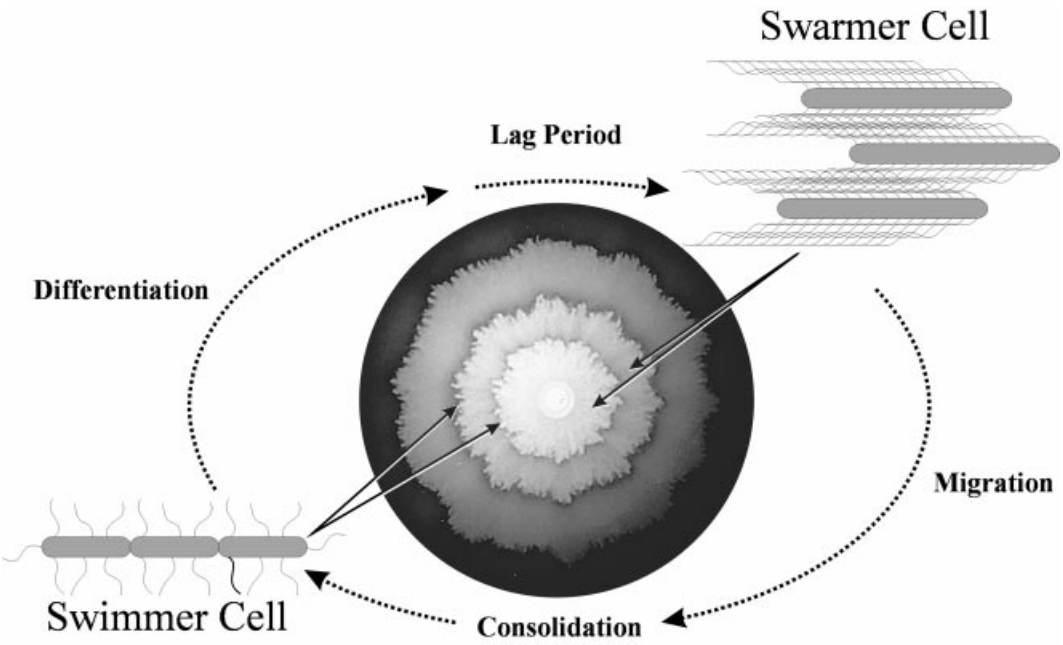

Fig. 1. Spatial and temporal cycling of *P. mirabilis* swarming behaviour and colony formation. Moving clockwise from the lower left, vegetative swimmer cells are induced to differentiate into swarmer cell morphology (elongated, hyperflagellated, polynucleoid cells) upon contact with a surface such as L agar. The microcolony of swarmer cells grows through cell division, but swarming motility is not expressed. This lag period is typically about 3 h on L agar at 37 °C. At this point, an unknown event triggers swarmer cell migration, which is manifested as a seemingly spontaneous uniform movement of swarmer cells outwards from the colony periphery. Migration continues for about 3 h and is followed by a short (30–45 min) consolidation phase where the cells dedifferentiate to swimmer cell morphology and cease to swarm. These events are cyclical, such that on agar media, swarming colony biofilm development is observed as a series of ever-expanding concentric rings that are composed of morphologically and physiologically distinct cell types (arrows indicate areas populated by either swimmer or swarmer cells). This cycle leads to the bull's-eye pattern typical of *P. mirabilis* colonies.

preswarming colony (Belas *et al*., 1998). In this earlier work, mutations in *rsbA*, a gene encoding a putative membrane sensor protein (RsbA) with homology to *Vibrio harveyi* LuxQ (Bassler *et al*., 1994), were shown to have a reduced lag period, presumably due to defects in the ability of RsbA to sense the density of cells. These data suggest that RsbA may function in an analogous role as *V. harveyi* LuxQ to sense the same signal as LuxQ.

Quorum sensing is a common mechanism that acts to control bacterial physiology by regulating gene expression in response to population density. Quorum sensing relies on the accumulation of small extracellular signalling molecules, referred to as autoinducers, to modulate the transcription of target genes and operons. Autoinducer 1 (AI-1) quorum systems, using *N*-acyl-homoserine lactone (AHL) derivatives as the signalling molecules, have been uncovered in many Gram-negative bacteria (Fuqua *et al*., 1996; Swift *et al*., 1996). The genes under the influence of AHL quorum sensing regulation include those encoding virulence factors (Parkins *et al*., 2001; Passador *et al*., 1993; Pirhonen *et al*., 1993; Tang *et al*., 1996; Telford *et al*., 1998), as well as those responsible for biofilm formation (Davies *et al*., 1998; De Kievit *et al*., 2001; Eberl *et al*., 1996). However, despite extensive analysis, AHL AIs do not appear to play a major role in *P. mirabilis* swarming colony biofilm formation (Belas *et al*., 1998).

Quorum sensing can be achieved through molecules other than AHL. Bassler and co-workers (Bassler, 1999; Surette & Bassler, 1998; Surette *et al*., 1999) have described an alternative quorum sensing mechanism that uses an autoinducer molecule, referred to as autoinducer 2 or AI-2, that appears to be highly conserved in both Gram-negative and Gram-positive bacteria (Schauder & Bassler, 2001). In *V. harveyi*, the membrane protein LuxQ, which is a homologue of *P. mirabilis* RsbA, acts as the receptor of AI-2. The gene *luxS* is crucial for AI-2 activity, and *luxS* orthologues have been found in many bacteria (Bassler, 1999; Surette *et al*., 1999). The molecular identity of AI-2 has been recently reported as a derivative of furanone (Schauder *et al*., 2001).

With the exception of its role in the regulation of *V. harveyi* luminescence, the function of AI-2 remains enigmatic for most of the bacterial species found to possess *luxS* orthologues. For example, many pathogenic bacteria possess genes with homology to *luxS*, leading to speculation that AI-2 may function to regulate aspects of bacterial virulence and pathogenicity. Recent

reports have tested this hypothesis using enterohaemorrhagic and enteropathogenic *Escherichia coli* (EHEC and EPEC) (Sperandio *et al*., 1999), *Helicobacter pylori* (Forsyth & Cover, 2000; Joyce *et al*., 2000), *Shigella flexneri* (Day & Maurelli, 2001) and *Porphyromonas gingivalis* (Chung *et al*., 2001; Frias *et al*., 2001). The strongest connection between AI-2 activity and pathogenicity has been seen in *E. coli* O157:H7, where AI-2 controls the expression of type III secretion gene transcription and protein secretion (Sperandio *et al*., 1999). AI-2 activity may also function to control a gene involved in haemin acquisition in *P. gingivalis* (Chung *et al*., 2001) and could, through this interaction, be involved in *P. gingivalis* pathogenicity. However, a *luxS* mutation in *S. flexneri* had no effect on virulence (Day & Maurelli, 2001) and a similar mutation in *H. pylori* had no effect on the expression of any known virulence factor (Joyce *et al*., 2000). Thus, greater knowledge of the role of AI-2 in the regulation of virulence and biofilm formation is needed to understand the function of AI-2 quorum sensing regulation in bacterial pathogenicity.

We are interested in understanding the genetic mechanisms controlling the development of *P. mirabilis* biofilms, the swarming colony pattern, and how swarming behaviour enhances the uropathogenicity of these bacteria. We hypothesized that AI-2 quorum sensing may influence both *P. mirabilis* biofilm and colony pattern formation, as well as virulence. In this report, we show the *luxS*-dependent synthesis of AI-2 by *P. mirabilis* and the coordinate expression of AI-2 during swarming migration, and compare the phenotype and virulence of mutants defective in AI-2 production to those of the wild-type.

METHODS

Strains, plasmids and media. The strains and plasmids used in this study are listed in Table 1. *P. mirabilis* BB2000 is wild-type for swimming and swarming behaviours. *E. coli* and *P. mirabilis* strains were grown in Luria–Bertani broth (LB) or L agar (1·5 %, w/v, agar) at 37 °C. To prevent swarming, *P. mirabilis* strains were grown on LSW^{-} agar as previously described (Belas *et al*., 1991a, b). Swimming behaviour was measured microscopically and macroscopically in Mot agar (Belas *et al*., 1998). Swarming behaviour was assessed using the method of Belas *et al*. (1998). All *V. harveyi* strains were maintained on LM medium (Belas *et al*., 1984). *V. harveyi* was grown aerobically in Autoinducer Bioassay (AB) medium at 25 °C for the AI-2 bioassay (Greenberg *et al*., 1979). Urease activity was measured by using urea agar medium (Difco) according to the recommendations of the manufacturer. Protease activity was measured as a zone of clearing on Skimmed Milk Agar medium, as described by Walker *et al*. (1999). Haemolytic activity was detected as a zone of greenish discoloration surrounding positive colonies on trypticase soy agar with 5 % sheep red blood cells (TSA II, Becton Dickinson Microbiology Systems) after an overnight incubation at 37 °C. Antibiotics were used at the following final concentrations: 40 μg kanamycin ml^{-1}, 100 μg ampicillin ml^{-1}, 40 μg chloramphenicol ml^{-1}, and 100 μg rifampicin ml^{-1}.

Molecular cloning and mutagenesis. Standard methods were used for the manipulation of DNA (Ausubel *et al*., 1987) unless specified otherwise. The *P. mirabilis luxS* gene ($luxS_{PM}$; used to distinguish this gene from the *E. coli* orthologue, $luxS_{EC}$) was identified by Southern blot hybridization of genomic DNA cleaved with *Eco*RI and hybridized to a radioactively labelled DNA fragment obtained by PCR amplification of the *E. coli* MG1655 (Blattner *et al*., 1997) *luxS* gene ($luxS_{EC}$) using oligonucleotides luxSfor (5′-TTGGGATGACGCAACAGCA-3′) and luxSrev (5′-GAACGCCGTCAGCAGGAAA-3′) as primers. A random *Eco*RI library of *P. mirabilis* genomic DNA fragments was then ligated with plasmid pCR2.1 (Invitrogen) and transformed into *E. coli* DH5α. The library of recombinants was screened for AI-2 activity using *V. harveyi* BB170 (described below). A transformant that complemented AI-2 production in DH5α was identified and the nucleotide sequence of the inserted DNA analysed. This plasmid, pRS104, harboured a 1598 bp insert of *P. mirabilis* genomic DNA containing $luxS_{PM}$.

A nonpolar null mutation in $luxS_{PM}$ was constructed by first removing the plasmid-borne *Eco*RI site in pRS104, resulting in pRS107 (Table 1). A 847 bp *Sma*I fragment containing the *aphA-3* gene from pUC18K (Menard *et al*., 1993) was inserted at the *Eco*RI site internal to $luxS_{PM}$ by blunt-end ligation to Klenow-treated plasmid. The resulting plasmid was named pRS110. Plasmid pRS110 was digested with *Sac*I and *Kpn*I, and a 2·5 kb fragment containing *luxS′*::*aphA*::*′luxS* was ligated to pGP704 (Miller & Mekalanos, 1988) also digested with *Sac*I and *Kpn*I, resulting in pRS112. This plasmid was transformed into *E. coli* SM10 λpir and conjugally transferred to *P. mirabilis* BB2000 by filter mating (Belas *et al*., 1991a). Mutation of the chromosomal copy of $luxS_{PM}$ was confirmed by antibiotic resistance spectrum (Km^{R} Ap^{S}), PCR amplification of $luxS_{PM}$ locus from the putative *luxS* strain, and measurement of AI-2 activity using the *V. harveyi* luminescence assay. Several colonies that lacked AI-2 resulting from the introduction of *luxS′*::*aphA*::*′luxS* were found. A representative was chosen and named RS601.

Nucleotide sequencing and analysis. Double-stranded DNA was used as a template for nucleotide sequencing by the recommended procedures of the Prism Ready Reaction Dye Deoxy Termination Kit (Applied Biosystems) in conjunction with *Taq* polymerase and a model 373A DNA sequencer (Applied Biosystems). Nucleotide and deduced amino acid sequences were analysed with Vector NTI V5.0 software (Informax) and the BLAST family of programs (Altschul *et al*., 1990, 1997; Gish & States, 1993; Worley *et al*., 1995). Phylogenetic trees of *P. mirabilis* LuxS relatedness were constructed by aligning the LuxS deduced amino acid sequences obtained from complete and partial genome databases using CLUSTAL W V1.75 [http://www.es.embnet.org/Doc/phylodendron/clustal-form.html] (Thompson *et al*., 1994) and arranged with the computer program 'Phylodendron' [http://iubio.bio.indiana.edu/soft/molbio/java/apps/trees/].

The following nucleotide sequences and accession numbers were used in generating LuxS dendrograms: *Escherichia coli* MG1655, U00096; *Salmonella typhimurium* LT, WUGSC_99287; *Salmonella paratyphi* A, WUGSC_32027; *Salmonella typhi* CT18, WUGSC_573; *Klebsiella pneumoniae*, Contig 1032; *Yersinia pestis* strain CO-92, Sanger_632; *Vibrio harveyi*, AF120098; *Vibrio cholerae* chromosome I, AE003852; *Shewanella putrefaciens*, sputre_7833; *Neisseria meningitidis* serogroup B strain M, AE002098; *Neisseria gonorrhoeae*, OUACGT_485; *Haemophilus influenzae* Rd, U32731; *Pasteurella multocida* PM70, AE004439; *Actinobacillus actinomycetemcomitans*, OUACGT_714; *Campylobacter jejuni*, AL111168; *Deinococcus radiodurans* R1,

Table 1. Strains and plasmids used

Strain or plasmid	Relevant characteristic(s)*	Derivation or description	Source and/or reference
***E. coli* K-12**			
MG1655	Wild-type	Expresses AI-2	Blattner *et al.* (1997)
DH5α	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA–argF*)*U169 endA1 recA1 hsdR17*(r_k^- m_k^+) *deoR thi-1 supE44* λ^- *gyrA96 relA1*	Does not express AI-2	Laboratory stock (Sambrook *et al.*, 1989)
SM10 (*λpir*)	Rec$^-$ RP4-2Tc::Mu λpir	C600	De Lorenzo *et al.* (1990); Simon *et al.* (1982)
INVαF′	F′ *endA1 recA1 hsdR17*(r_k^- m_k^+) *supE44 gyrA96 relA1* ϕ80d*lacZ*ΔM15 Δ(*lacZYA–argF*) U169*deoR thi-1* λ^-		Invitrogen
P. mirabilis			
BB2000	Wild-type, Rf^R Tc^R	Spontaneous from PRM1	Belas *et al.* (1991a)
RS601	*luxS′*::*aphA*::*′luxS*, Km^R		This study
V. harveyi			
BB7	Wild-type		Laboratory stock (Belas *et al.*, 1982)
BB170	*luxN*::Tn*5*	AI-2 sensor strain	Surette & Bassler (1998)
Plasmids			
pBluescriptII	Ap^R	pBluescriptII SK(+) and KS(−)	Stratagene
pCR2.1	Ap^R Km^R	PCR TA cloning vector	Invitrogen
pGP704	Ap^R *ori*R6K	Conjugatable suicide vector	Miller & Mekalanos (1988)
pUC18K	Ap^R Km^R, *aphA-3*		Menard *et al.* (1993)
pRSluxS$_{EC}$	Ap^R Km^R, *luxS$_{EC}$*	*E. coli luxS* cloned in pCR2.1	This study
pRS104	Ap^R *luxS$_{PM}$*	*Eco*RI clone of *luxS$_{PM}$* in pBluescript SK(+)	This study
pRS107	Ap^R *luxS$_{PM}$*	*Sma*I–*Eco*RV deletion of pRS104	This study
pRS110	Ap^R Km^R, *luxS$_{PM}$*::*aphA-3*::*luxS$_{PM}$*	Non-polar mutation of *luxS$_{PM}$* in pRS107	This study
pRS112	Ap^R Km^R, *luxS$_{PM}$*::*aphA-3*::*luxS$_{PM}$*, *ori*R6k, *mob*	*Sac*I and *Kpn*I insert from pRS110 in pGP704	This study

* Ap^R, ampicillin resistance; Km^R, kanamycin resistance; Rf^R, rifampin resistance; Tc^R, tetracycline resistance.

AE000513; *Clostridium perfringens*, AB028629; *Streptococcus mutans*, UOKNOR_1309; *Streptococcus pneumoniae*, S.pneumoniae_3836; *Streptococcus equi*, sequi_Contig243; *Streptococcus pyogenes* strain SF370, AE004092; *Enterococcus faecalis* unfinished, gef_11360; *Clostridium difficile* strain 630 (epidemic type X) unfinished, Sanger Centre unassigned; *Staphylococcus epidermidis*, TIGR_1282; *Staphylococcus aureus*, Sanger_1280_3; *Helicobacter pylori* 26695, AE000511; *Bacillus subtilis*, AL009126; *Bacillus anthracis*, TIGR_1392; *Bacillus halodurans* C-125, BA000004; *Borrelia burgdorferi*, AE000783; *Clostridium acetobutylicum*, C.aceto_gnl; *Porphyromonas gingivalis* W83, P.gingivalis _GPG.con; *Haemophilus ducreyi* strain 35000HP, HTSC_730.

Preparation of cell-free conditioned media for AI-2 assays. Conditioned media from broth cultures were prepared using a modification of the protocol described by Sperandio *et al.* (1999). Overnight cultures grown in LB at 37 °C were diluted 1:100 in fresh LB and cultured in a shaking water bath at 37 °C. When the cultures reached an OD_{600} of 0·3, the bacteria were again diluted 1:100 in fresh LB and incubated in a shaking water bath at 37 °C until an OD_{600} of 1·2 was reached (about 12 h incubation). The bacteria were removed by centrifugation (14000 ***g***, 5 min, 20 °C) and the supernatant filter-sterilized (0·2 μm pore-size cellulose acetate filter; Millipore). Aliquots of the conditioned media were kept on ice to be used immediately in AI-2 assays or stored at −20 °C until needed.

Production of AI-2 during *P. mirabilis* swarming migration and behaviour on L agar medium was assessed as follows. *P. mirabilis* overnight cultures grown in LB were prepared for swarming behaviour assays as described by Belas *et al.* (1998), in which a 5 μl inoculum of about $2·5 \times 10^6$ cells was dispensed

on the L agar surface, incubated at 37 °C, and assayed for swarming motility. To determine AI-2 activity during swarming migration and colony formation, a cylinder of agar 1 cm in diameter and 1 cm in height from the outer edge of the developing swarming colony was removed at 1 h intervals using a sterile brass cork borer (Fisher Scientific). Colony-forming units (c.f.u.) were determined in parallel by removing the cells from the surface of the agar core with vigorous mixing, followed by growth of serial dilutions of the suspension on L agar. AI-2 activity was also measured at discrete points within a preformed 5 h old swarming colony that represents one cycle of swarming behaviour. In this case, agar cores were removed from the point of inoculation, a point one-third of the way to the periphery, a point two-thirds of the distance to the periphery, and at the periphery of the colony. Each agar plug with overlying bacteria was frozen at −20 °C for 18 h and then thawed rapidly by addition of 300 μl AB medium. Insoluble agar and bacterial cells were removed by centrifugation and filtration as described above, and the supernatant assayed immediately or stored at −20 °C. This 'freeze-squeeze' process resulted in 800–900 μl fluid.

***V. harveyi* luminescence assay for AI-2.** The presence of AI-2 in the conditioned media was assayed by using the *V. harveyi* BB170 (*luxN*::Tn*5*) reporter strain, which responds only to AI-2 (Surette & Bassler, 1998). The luminescence assays were performed as described by Surette & Bassler (1998), using an EG&G Berthold Microlumat LB 96P luminometer. The data are reported as relative light units (RLUs) or as the fold stimulation of light emission by *V. harveyi* BB170 compared to the RLU values obtained from the corresponding DH5α (AI-2$^-$) negative control.

Murine UTI virulence assay. A modification (Johnson *et al.*, 1987) of the well-established mouse model of ascending UTI (Hagberg *et al.*, 1983) was used to measure *P. mirabilis* virulence. Female CBA/J (Harlan Sprague–Dawley) were transurethrally challenged with about 3×10^7 c.f.u. of bacteria per mouse. After 7 days, the mice were killed, and bacteria recovered from urine, bladder and kidneys were enumerated on LSW$^-$ agar plates containing the appropriate antibiotics. The range of detection in this assay is 10^2–10^9 c.f.u. (ml urine)$^{-1}$ or c.f.u. (g tissue)$^{-1}$ (Li *et al.*, 1999).

Statistical methods. Mean numbers of c.f.u. ml^{-1} or g^{-1} from cultures of urine or tissue homogenates were compared by the Mann–Whitney and non-parametric ANOVA tests.

Materials and reagents. All reagents were of the highest purity available. Components of bacteriological media were purchased from Difco. Restriction endonucleases and DNA-modifying enzymes were obtained from New England Biolabs, Boehringer Mannheim Biochemicals, Promega or Qiagen and were used according to the supplier's recommendations.

RESULTS AND DISCUSSION

Identification of AI-2 activity in *P. mirabilis*

We have previously reported on the density-dependent nature of *P. mirabilis* swarming, the homology between RsbA and LuxQ, and the lack of identifiable AI-1 activity associated with *P. mirabilis* swarming behaviour (Belas *et al.*, 1998). These led us to speculate that *P. mirabilis* may possess an AI-2 extracellular signalling molecule that might influence swarming behaviour, biofilm formation and other phenotypes, notably expression of virulence factors, during UTI. To test this hypothesis, the *V. harveyi* AI-2 reporter strain, BB170 (a gift from B. Bassler; Table 1) lacking a sensor for AI-1 (AHL) but containing an intact sensor for AI-2, was used to detect AI-2 activity in *P. mirabilis* cell-free conditioned media. Table 2 shows the results from this assay. *V. harveyi* BB170 was inoculated into AB medium containing 10% cell-free conditioned broth from *P. mirabilis* swimmer cells and the luminescence measured after 4–5 h incubation. *V. harveyi* BB7 and *E. coli* MG1655 served as positive controls for AI-2 production, while *E. coli* DH5α, lacking AI-2 activity (Surette *et al.*, 1999), was used as a negative control. We found that *P. mirabilis* swimmer cells produced an extracellular AI-2 activity comparable to that obtained from *V. harveyi* BB7 (Table 2). We conclude that *P. mirabilis* swimmer cells possess an AI-2-like activity.

AI-2 activity coincides with swarming behaviour

We next measured AI-2 activity during swarmer cell differentiation and migration, as a function of both time post-inoculation on L agar, and position in the developing swarmer colony (at a fixed time of 5 h). An inoculum of swimmer cells was applied to the surface of L agar, and AI-2 was measured during swarmer cell differentiation and swarming migration. Our previous work has shown that *P. mirabilis* requires an approximately 3 h lag phase after inoculation on L agar before the onset of swarming migration (Belas *et al.*, 1998). In such experiments, one full cycle of swarming takes about 5 h at 37 °C. As the swarming biofilm developed, agar plugs containing the bacteria and underlying agar matrix were removed from the leading edge of the swarming colony (Methods), and AI-2 activity was measured using the *V. harveyi* BB170 AI-2 assay. As is shown in Fig. 2, AI-2 activity rose to a maximum at 3 h after inoculation, then dropped back to near background level. The peak in AI-2 activity at 3 h corresponds to the time at which the cells start swarming migration and suggests that AI-2 may play a role in orchestrating this behaviour.

AI-2 activity was also assessed at discrete areas of a 5 h swarming colony, i.e. a colony that had completed one cycle of differentiation, migration, dedifferentiation and consolidation (Fig. 3). In this experiment, AI-2 activity was greatest at the site of inoculation and decreased to a low at the periphery of the swarming colony.

Taken together, the data in Figs 2 and 3 suggest that AI-2 activity is correlated with swarming. Specifically, AI-2 activity is maximal at the site of inoculation and peaks at the same time as swarming migration begins. Other factors, such as cell division and growth, may also contribute to the changes in AI-2 activity observed in these experiments. For example, the sharp increase in AI-2 activity at 3 h may be due to an increase in cells cm^{-2}, i.e. a cell density effect due to quorum sensing. The inoculum contained about $2{\cdot}5 \times 10^6$ c.f.u. cm^{-2}, increasing to approximately 4×10^8 c.f.u. cm^{-2} at the 3 h sampling. Thereafter, the cell density

Table 2. Induction of luminescence in *V. harveyi* BB170 by conditioned media

Source of conditioned medium*	Luminescence (RLU)†	Fold induction of luminescence in *V. harveyi* BB170†
V. harveyi BB7‡	247435	1237
E. coli DH5α‡	206	1
E. coli MG1655‡	35141	175
E. coli DH5α(pCR2.1)	174	1
E. coli DH5α(pRSluxS$_{EC}$)	11257	56
E. coli DH5α(pRS104)	35802	179
E. coli DH5α(pRS110)	188	1
P. mirabilis BB2000	687548	3488
P. mirabilis RS601	201	1

* Conditioned media derived from cultures grown in LB at 37 °C to an OD_{600} of 1·2 were prepared as described in Methods.

† Luminescence is recorded in relative light units (RLU). Fold induction represents the mean of three readings of induction of luminescence in *V. harveyi* reporter strain BB170 (sensor 1$^-$, sensor 2$^+$) cultured in conditioned media from the designated sources relative to that of *V. harveyi* BB170 grown in unconditioned LB.

‡ These strains served as positive (BB7 and MG1655) and negative (DH5α) control sources of AI-2 sensed by BB170.

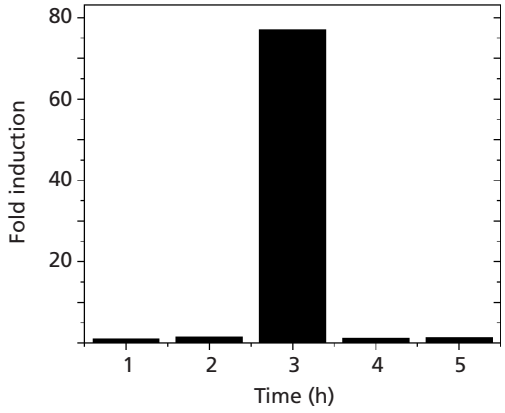

Fig. 2. Temporal expression of AI-2 activity during swarming behaviour and migration on L agar. An inoculum of swimmer cells was pipetted on the surface of L agar, incubated at 37 °C, and AI-2 activity measured as the cells differentiated and began swarming migration and behaviour. At 1 h intervals, a core containing the cells and underlying agar matrix at the periphery of the bacterial growth was removed, and conditioned medium obtained, as described in Methods. AI-2 activity was assayed using the *V. harveyi* BB170 sensor strain. AI-2 activity is expressed as the fold induction of relative light units compared to that of an uninduced control, and represents the mean of at least three experiments.

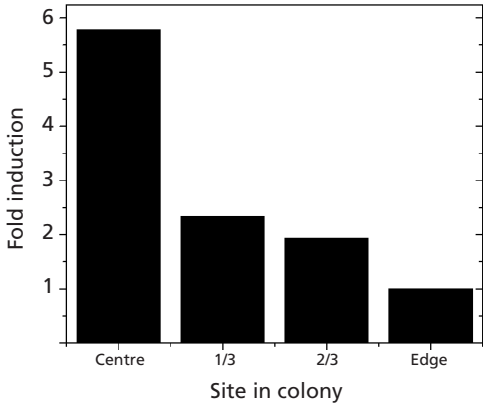

Fig. 3. Spatial expression of AI-2 activity during swarming behaviour and migration on L agar. An inoculum of swimmer cells was pipetted on the surface of L agar and incubated at 37 °C for 5 h, a time at which one complete cycle of swarming behaviour has been completed. AI-2 activity was measured by removing cores of cells plus underlying agar (see Methods) at sections of the colony corresponding to the point of inoculation, one-third of the distance to the colony edge and two-thirds of the distance to the colony edge, and at the periphery of the colony. Conditioned cell-free media were obtained from these cores as described in Methods and AI-2 activity was assayed using the *V. harveyi* BB170 sensor strain. AI-2 activity is expressed as the fold induction of relative light units compared to that of an uninduced control, and represents the mean of at least three experiments.

remained at 1–2 × 10^8 c.f.u. cm^{-2} as the biofilm of cells moved outwards on the agar surface. The increase in cells cm^{-2} at 3 h may be responsible for the increase in AI-2 activity; however, the subsequent drop in AI-2 at 4 and 5 h cannot be solely explained by the slight drop in cell density recorded at those times, and may point to other unknown factors, such as the degradation of AI-2, that control *P. mirabilis* AI-2 expression.

A *luxS* orthologue is responsible for AI-2 activity in *P. mirabilis*

The *luxS* gene is essential for AI-2 production in many bacteria, prompting us to determine whether *P. mirabilis* genomic DNA contained a *luxS* orthologue. Southern

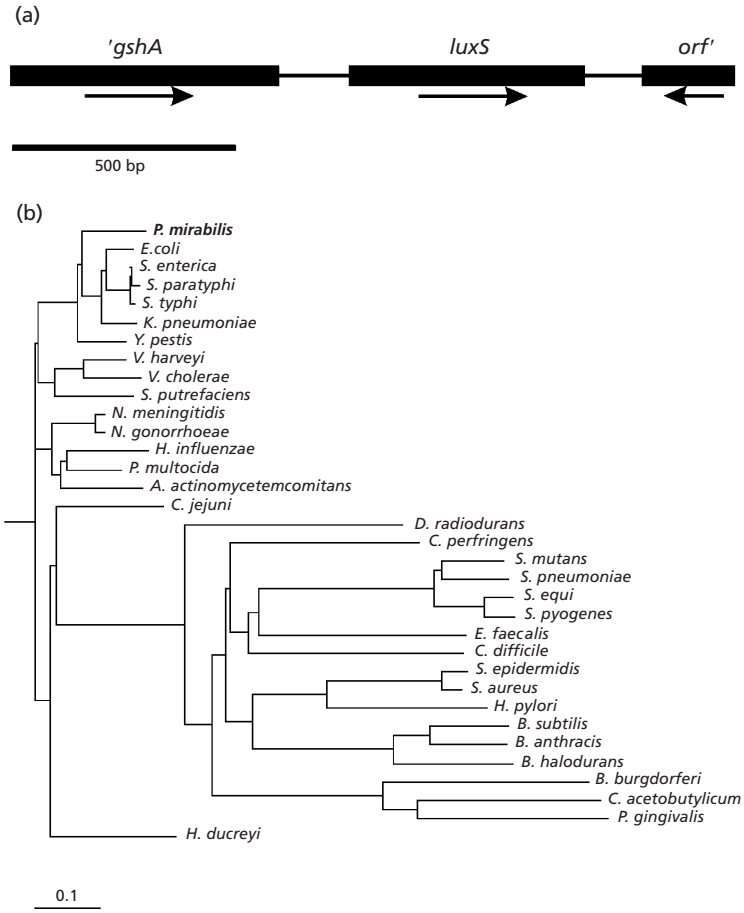

Fig. 4. Organization of the *P. mirabilis luxS* locus and relatedness to LuxS orthologues of other eubacteria. (a) A 1589 bp *Eco*RI fragment from the *P. mirabilis* genome contains, as read from left to right, the 3′ end of a gene homologous to *E. coli gshA*, *luxS*, and the 3′ end of an ORF with partial deduced amino acid homology to antibiotic transport proteins. The direction of the reading frame is indicated by the arrow under each gene. The GenBank accession number for this nucleotide sequence is AY044337. (b) Dendrogram showing the phylogenetic relatedness of *P. mirabilis* LuxS to the deduced amino acid sequences of other LuxS orthologues obtained from computer databases of completed and partial bacterial genome sequences. Alignment of the deduced amino acid sequences was done using CLUSTAL W and the tree was constructed using the associated phylogenetic tree program, Phylodendron (see Methods for details).

blots of *P. mirabilis* genomic DNA separately digested with five restriction endonucleases were hybridized to radioactively labelled pRSluxS$_{\mathrm{EC}}$ containing a PCR fragment of *E. coli luxS*. The analysis of these blots indicated that *Eco*RI digestion resulted in a 1·6 kb DNA fragment that hybridized to *luxS*$_{EC}$. An *Eco*RI digest of genomic DNA was ligated to pBluescript SK(+) and transformed into *E. coli* DH5α, a strain lacking AI-2 activity (Surette *et al*., 1999). The resulting recombinant colonies were screened for AI-2 activity using the *V. harveyi* BB170 AI-2 assay. Five of the cell-free conditioned media supernatants from the recombinant *E. coli* produced AI-2 activity equal to or in excess of conditioned media from DH5α harbouring pRSluxS$_{\mathrm{EC}}$ (Table 2). Restriction mapping of the plasmid carried by these bacteria revealed that each of these recombinant *E. coli* harboured the same foreign DNA, presumably containing *luxS*$_{PM}$, and indicating that *luxS*$_{PM}$ is functional in *E. coli*. A single representative plasmid, pRS104 (Table 1), was used for further work.

The nucleotide sequence of the *Eco*RI fragment of *P. mirabilis* genomic DNA inserted in pRS104 was determined. The analysis of the 1598 bp locus is shown in Fig. 4(a). Three ORFs were found, one of which is highly homologous to other bacterial *luxS* deduced amino acid sequences (Fig. 4b). The homology of ORF2 to other LuxS sequences is greatest with LuxS of *E. coli* (67 % identity) and *Salmonella enterica* serovar Typhimurium (65 % identity). We refer to this ORF as *luxS* and use *luxS*$_{PM}$ to distinguish it from other *luxS* genes. As shown in Fig. 4(a), two partial ORFs were also discovered on either side of *luxS*$_{PM}$. To the left of *luxS*, as drawn in Fig. 4(a), is an ORF whose deduced amino acid sequence is homologous to *E. coli gshA*, encoding γ-glutamylcysteine synthetase, and to the right of *luxS* is an ORF whose deduced amino acid sequence has partial homology to a series of proteins involved in ion or antibiotic transport. The data presented in Fig. 4(a, b) show that the *P. mirabilis luxS* locus is similar in its arrangement of genes to the *luxS* locus of *E. coli* and *S. enterica* serovar Typhimurium. These data also demonstrate that *luxS*$_{PM}$ is homologous to the *luxS* genes of other bacteria (Fig. 4b). The latter point is further strengthened by the ability of *luxS*$_{PM}$ to complement the *luxS* defect in DH5α, thereby producing AI-2 activity in the recombinant strain.

Phenotypic analysis of an AI-2 negative *P. mirabilis*

A nonpolar mutation in *luxS*$_{PM}$ was constructed by inserting the *aphA-3* gene from pUC18K (Menard *et al*., 1993) at an *Eco*RI site internal to *luxS* to produce

pRS110 (Table 1). Plasmid pRS110 failed to produce AI-2 activity in DH5α (Table 2). The 2·5 kb DNA fragment containing $luxS_{PM}'::aphA::'luxS_{PM}$ was then ligated into the suicide vector pGP704 (Miller & Mekalanos, 1988) for conjugal transfer to *P. mirabilis*. This plasmid, pRS112, was used to produce a nonpolar mutation of the chromosomal copy of $luxS_{PM}$ through allelic-exchange mutagenesis. The resulting colonies were screened for loss of plasmid-borne ampillicin resistance, acquisition of kanamycin resistance (conferred by *aphA-3*) and loss of AI-2 activity (measured in cell-free conditioned media). Those strains lacking detectable AI-2 were confirmed to be the result of a double crossover event by a PCR-based analysis of the genomic $luxS_{PM}$. Six colonies were identified that produced no AI-2 activity, resulting from an allelic exchange of *luxS* and the mutated gene. One of these, RS601 (AI-2$^-$, $luxS_{PM}'::aphA::'luxS_{PM}$), was phenotypically characterized.

As anticipated, RS601 did not produce detectable AI-2 activity (Table 2), when assayed using the *V. harveyi* BB170 sensor. We compared RS601 to the parent strain (BB2000) in swimming motility and chemotaxis behaviour, and swarming motility and behaviour. Swimming speed and chemotaxis were measured microscopically as well as during growth in Mot agar. When compared to the wild-type bacteria, RS601 showed no differences in either swimming speed or chemotaxis behaviour. An analysis of swarmer cell morphology, movement and biofilm formation was conducted. Swarmer cell differentiation, the timing of the beginning of swarming migration and the swarming colony pattern formation of RS601 were also indistinguishable from those of the wild-type. Other phenotypes, such as growth rate and the production of urease, protease and haemolysin, were also unaffected by the *luxS* mutation. We conclude that the *luxS* mutation does not lead to an overt change in *P. mirabilis* motility or biofilm phenotypes as determined by this set of assays.

AI-2 activity does not affect *P. mirabilis* virulence during UTI

It may be that defects in AI-2 synthesis are not apparent when the bacteria are grown in nutrient-rich conditions, but are manifested under conditions that challenge the survival of the cells. To determine if loss of AI-2 activity affected *P. mirabilis* virulence, we compared the uropathogenicity of RS601 to that of wild-type *P. mirabilis*, using a modification (Johnson *et al*., 1987) of the mouse model of ascending UTI (Hagberg *et al*., 1983). This mouse model has been successfully used by others to assess the uropathogenicity of *E. coli* (Johnson *et al*., 1993a) and *Providencia stuartii* (Johnson *et al*., 1987), and by our group to assess uropathogenicity of *P. mirabilis* mutants defective in flagellin synthesis (Mobley *et al*., 1996) and strains defective in the production of an extracellular metalloprotease (Walker *et al*. , 1999).

Fig. 5 shows the colonization of mouse urine, bladder and kidneys 7 days after transurethral challenge with either RS601 or wild-type *P. mirabilis*. Ten mice were used per bacterial strain. Two mice infected with wild-type *P. mirabilis* died from stone blockage on day 6 and were not used in the analysis. A pairwise comparison of the mean c.f.u. for each tissue (indicated by the horizontal line in each column in Fig. 5) suggests a noticeable increase in the survival of the AI-2-deficient mutant in each tissue. The trend towards increased survival in the *luxS* strain appears in all samples (urine, bladder and kidney) examined. The *P* values ($> 0{\cdot}05$), however, do not reflect a statistical significance to these differences. Thus, a *luxS* mutant is not attenuated in virulence, suggesting that AI-2 quorum signalling is not required for *P. mirabilis* UTI.

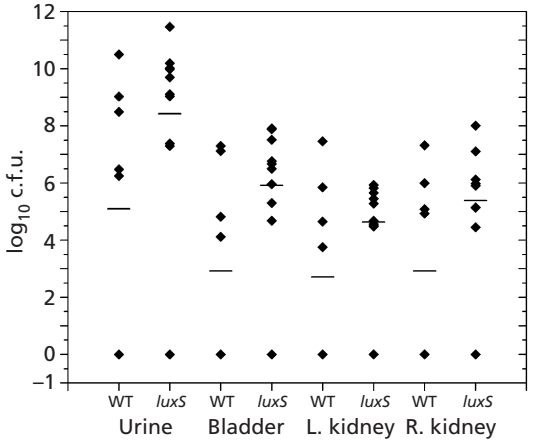

Fig. 5. Mouse urinary tract colonization of the urine, bladder, and kidneys 7 days after transurethral infection with either an AI-2-negative or wild-type *P. mirabilis*. The mean c.f.u. per ml urine or per g tissue of 10 mice is indicated by a horizontal bar in each column. The *P* values, as determined by the Mann–Whitney test, for comparisons of mean numbers of c.f.u. per ml or g of urine or bladder or kidney homogenates for wild-type and LuxS$^-$ strain are: urine, $P = 0{\cdot}055$; bladder, $P = 0{\cdot}059$; left kidney, $P = 0{\cdot}209$; and right kidney, $P = 0{\cdot}106$. The overall *P* value determined by a non-parametric ANOVA comparing the AI-2-negative strain to wild-type is $P = 0{\cdot}061$.

Concluding remarks

In summary, the results presented show that *P. mirabilis luxS* is critical for AI-2 activity. The deduced amino acid sequence of LuxS, as well as the organization of the *luxS* locus, emphasize the homology between $luxS_{PM}$ and *luxS* genes from other bacteria, especially the *Enterobacteriaceae*. A *luxS* mutation in *P. mirabilis*, however, has no demonstrable effects on growth, the production of urease, protease or haemolysin, swimming motility and behaviour, swarmer cell differentiation, swarming behaviour and biofilm formation, or uropathogenicity. The finding that *luxS* does not alter either the swarming phenotype or UTI virulence is in agreement with previous observations that swarming behaviour is essential for virulence (Allison *et al*., 1992, 1993; Chippendale *et al*., 1994). Day & Maurelli (2001) have suggested that the AI-2 quorum sensing is important to those

pathogens that are constantly exposed to high levels of AI-2 derived from normal flora. During UTI, *P. mirabilis* is not likely to encounter high levels of AI-2 activity from other bacteria. However, *P. mirabilis* is also a member of the intestinal flora of many animals, where high AI-2 activity may well exist. Perhaps, because of this dual mode of existence, *P. mirabilis* has adapted to use AI-2 quorum sensing regulation while in the intestine, but exploits other regulatory schemes to control its virulence factors in the urinary tract, which is normally free of resident flora. If true, this possibility may adversely impact those drug therapies design to intervene in *P. mirabilis* UTI through inhibition of AI-2 activity.

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