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The PmlI-PmlR Quorum-Sensing System in *Burkholderia pseudomallei* Plays a Key Role in Virulence and Modulates Production of the MprA Protease

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***Burkholderia pseudomallei* is the causative agent of melioidosis, an often fatal infection of humans and animals. The virulence of this pathogen is thought to depend on a number of secreted proteins, including the MprA metalloprotease. We observed that MprA is produced upon entry into the stationary phase, when the cell density is high, and this prompted us to study cell density-dependent regulation in *B. pseudomallei*. A search of the *B. pseudomallei* genome led to identification of a quorum-sensing system involving the LuxI-LuxR homologs PmlI-PmlR. PmlI directed the synthesis of an *N*-acylhomoserine lactone identified as *N*-decanoylhomoserine lactone. A *B. pseudomallei pmlI* mutant was significantly less virulent than the parental strain in a murine model of infection by the intraperitoneal, subcutaneous, and intranasal routes. Inactivation of *pmlI* resulted in overproduction of MprA at the onset of the stationary phase. A wild-type phenotype was restored following complementation with *pmlI* or addition of cell-free culture supernatant. In contrast, there was no significant difference between the virulence of a *B. pseudomallei mprA* mutant and the virulence of the wild-type strain. These results suggest that the PmlI-PmlR quorum-sensing system of *B. pseudomallei* is essential for full virulence in a mouse model and downregulates the production of MprA at a high cell density.**

Burkholderia pseudomallei, a gram-negative bacterium, is the causative agent of melioidosis in humans and animals (9, 13, 45). Melioidosis is endemic in tropical areas of Southeast Asia and northern Australia, but it is also sporadically found in many other countries. This highly pathogenic microorganism is deemed a potential agent of bioterrorism (listed as category B by the Centers for Disease Control and Prevention). The clinical manifestations of melioidosis vary greatly, ranging from an asymptomatic state to acute septicemia, pulmonary forms, and chronic granulomatous lesions (34). The latency period of the disease is between 2 days and 26 years (21, 34). Successful treatment is difficult because *B. pseudomallei* is inherently resistant to a wide range of antibiotics and relapse is common (43). Although melioidosis was first described in 1912 (44), the virulence determinants of *B. pseudomallei* have not been well characterized. Studies of *B. pseudomallei* pathogenicity have mostly concentrated on exoproducts, which include a protease, a lipase, a phospholipase C, and a hemolysin (3, 34, 45). The regulatory circuits governing the production of exoproducts in *B. pseudomallei* remain unknown. However, many gram-negative pathogens regulate the production of extracellular virulence factors by quorum sensing, a cell-density-dependent mechanism. Quorum sensing is the process of producing and responding to high intracellular concentrations of *N*-acylhomoserine lactone (AHL) autoinducers and relies on two proteins: (i) an AHL synthase belonging to the LuxI family, which directs the synthesis of AHL; and (ii) a transcriptional regulator belonging to the LuxR family, which, after binding of AHL, is thought to activate or repress transcription of targeted genes (11, 22, 29). In *Burkholderia cepacia*, which is phylogenetically related to *B. pseudomallei* (13), the CepI-CepR quorum-sensing system positively regulates protease production and represses synthesis of the siderophore ornibactin (19).

There has been much interest in the contribution of the *B. pseudomallei* protease to virulence. This enzyme was purified from culture supernatants as a 36-kDa metalloenzyme and appeared to be necessary for full virulence in a rat model of lung infection (32). In contrast, we found no correlation between virulence and the level of protease activity after intraperitoneal infection of mice with *B. pseudomallei* (12). These results suggested that the role played by the protease in pathogenesis could be dependent on the route of infection. Recently, the *mprA* gene of *B. pseudomallei* was cloned and was found to encode a 50-kDa serine metalloprotease, the only protease produced by *B. pseudomallei* (17). However, the discrepancy with the size determined by Sexton et al. (32) has not been explained yet; perhaps the difference is due to proteolysis or the use of different techniques (17). Our previous investigations on the *B. pseudomallei* protease (12, 25) led us to examine whether enzyme production is regulated by quorum sensing.

The role of quorum-sensing systems in the pathogenicity of different gram-negative bacteria has been demonstrated previously (28, 35). The existence of such a system in *B. pseudomallei* could provide new data on the virulence determinants of this organism.

Here we describe identification of the PmlI-PmlR quorum-sensing system of *B. pseudomallei*. This system is crucial for full virulence of *B. pseudomallei* in a murine model of infection and modulates the production of the MprA protease.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains used in this study were obtained from the collection of the Centre de Recherches du Service de Santé des Armées Emile Pardé (La Tronche, France). The prototype strain *B. pseudomallei* 008 was isolated in 1993 from a patient at the Grenoble-La Tronche Hospital in La Tronche, France. All experiments involving live bacteria were conducted in a biosafety level 3 facility. *Escherichia coli* S17-1 (*pro thi recA hsdR* chromosomal RP4-2; Tn*1*::ISR*1* Tc::Mu Km::Tn7) (33) and *E. coli* Sm10 (*thi thr leu* chromosomal RP4-2; Tc::Mu) (33) carried the transfer genes of plasmid RP4 integrated into the chromosome and allowed mobilization of cloning vectors in which the Mob (*oriT*) region of pRP4 was cloned. Strains were routinely grown at 37°C in tryptic soy broth (TSB) or on tryptic soy agar. Cell density was monitored at 600 nm with a colorimeter (model 6061; Jenway, Dunmow, Essex, United Kingdom) and was expressed as optical density at 600 nm (OD_{600}). When necessary, antibiotics were added at the following concentrations for *E. coli* and *B. pseudomallei*: tetracycline, 20 μg ml^{-1}; trimethoprim, 100 μg ml^{-1}; and carbenicillin, 100 μg ml^{-1}. Kanamycin was added at a concentration of 50 μg ml^{-1} to *E. coli* cultures and at a concentration of 200 μg ml^{-1} to *B. pseudomallei* cultures.

The mobilizable plasmid pSUP401 (33) was used as a cloning vector in *E. coli* and as a suicide vector in *B. pseudomallei*. The mobilizable plasmid pUCP28T (31) was used as a cloning vector in *E. coli* and *B. pseudomallei*. The tetracycline cassette, used for gene disruption, was PCR amplified from pACYC184 (6) with primers TC-3 (AATTTATCTCTTCAAATGTAGCAGCTGAAGTCAGCCCC) and TC-4 (ATGCGCCGCGTGCGGCAGCTGGAGATGGCGGAC) and was used to construct *B. pseudomallei* 008 (*mprA*::Tc) and *B. pseudomallei* 008 (*pmlI*::Tc) mutants (see below).

DNA manipulations. The methods employed for making constructs and manipulating recombinant DNA were essentially the methods described by Sambrook et al. (30). Plasmid DNA was prepared as described by Birnboim and Doly (4). Transformation was performed in *E. coli* S17-1 and in *E. coli* Sm10 by the $CaCl_2$ method (30). When required, homologous recombination between the disrupted gene in pSUP401 derivatives and the corresponding wild-type gene on the *B. pseudomallei* 008 chromosome was performed by allelic exchange as described previously (40). Double recombination was further confirmed by PCR analysis by using the appropriate primers.

PCR was performed with a PTC 200 thermocycler (MJ Research, Waltham, Mass.) by using Ready To Go PCR beads (Amersham Pharmacia Biotech Inc, Piscataway, N.J.) according to the manufacturer's instructions.

Construction of a *B. pseudomallei mprA* mutant. A fragment containing the *mprA* gene and promoter (17) was amplified from *B. pseudomallei* 008 DNA by using primers MprA-1 (ACGGAAGACGAATTCTCCGGCTCGCGCAGC CGG) and MprA-2 (GCAACGCCCGAATTCGCTCACTGCGCGGCGGCG), both of which were designed to contain an EcoRI restriction site at the 5′ end. The 1.8-kb amplified fragment was cloned into pSUP401, a suicide vector in *B. pseudomallei*. To insertionally inactivate the *mprA* gene in pSUP401, a tetracycline cassette was inserted into the internal EcoRV site of the gene. This construct was used to transform *E. coli* S17-1, and the resulting strain was mated with *B. pseudomallei* 008. Transconjugants that had undergone homologous recombination between the inactivated gene on pSUP401 and the corresponding wild-type gene on the *B. pseudomallei* chromosome were first selected on plates containing carbenicillin and tetracycline. The clones were then tested for kanamycin sensitivity and loss of the vector. As expected, most clones were kanamycin sensitive. The double recombination event was confirmed by PCR with primers MprA-1 and MprA-2. One of the mutants, designated *B. pseudomallei* 008 (*mprA*::Tc), was selected for further study. For complementation analysis, the amplified *mprA* gene was cloned into pUCP28T, yielding pMprA, and this recombinant plasmid was transferred by conjugation from *E. coli* Sm10 to *B. pseudomallei* 008 (*mprA*::Tc).

Construction of a *B. pseudomallei pmlI* mutant. The *pmlI* gene was amplified by PCR with primers PmlI-1 (GGCGCTAAATTGAAGCTTGGCGTCTTGCC AGCG) and PmlI-8 (ATCGAACGTAGGAAGCTTCGCGCGAAATACCG), cloned into pSUP401, and then interrupted by the tetracycline cassette inserted into the NruI site of the *pmlI* open reading frame (ORF). This recombinant plasmid was transferred by conjugation from *E. coli* S17-1 into *B. pseudomallei* 008. The double recombination event in carbenicillin- and tetracycline-resistant but kanamycin-sensitive transformants was confirmed by PCR analysis by using primers PmlI-1 and PmlI-8. One of the transformants, *B. pseudomallei* 008 (*pmlI*::Tc), was selected for further analysis. In parallel, the amplified *pmlI* gene was cloned into pUCP28T, yielding pPmlI, which was transferred into *B. pseudomallei* 008 (*pmlI*::Tc) for complementation analysis.

Measurement of protease activity. Strains were grown at 37°C in 100 ml of TSB shaken at 180 rpm in 250-ml glass culture flasks. Media were inoculated with cell pellets from 5-ml cultures shaken overnight in TSB at 37°C. At selected times, 2 ml of culture was removed, and the bacteria were harvested by centrifugation (10,000 × *g* for 10 min). Culture supernatants were carefully collected, filtered through 0.2-μm-pore-size filters, and supplemented with 10% (final concentration) sodium dodecyl sulfate (SDS). The bacterial pellets were washed twice in TSB and resuspended in 2 ml of TSB. The cells were lysed with 10% SDS as described previously (25) and filtered through 0.2-μm-pore-size filters, which yielded cell lysates. Total extracts were obtained by adding 10% (final concentration) SDS to 2 ml of total culture. Protease activity was assessed by using an EnzChek protease assay kit (Interchim, Rockford, Ill.) as recommended by the manufacturer. This activity was determined at different stages of the growth curve. Briefly, samples were diluted 50-fold in 1× digestion buffer. Then 100-μl portions of a BODIPY FL casein solution were added to 100-μl portions of diluted samples in microplates with black walls. After the microplates were incubated in the dark at 37°C for 18 h, fluorescence was measured with a microplate reader (FL800; Bio-Tek Instruments, Winooski, Vt.). One protease unit was defined as the activity that increased the fluorescence by 1 relative fluorescence unit per OD_{600} unit. Mean values were calculated by using the results of at least three independent assays, and the standard deviations were less than 10% of the reported values.

Preparation and use of cell-free conditioned supernatants. Cell-free supernatants were prepared as described previously (7). Briefly, *B. pseudomallei* 008 (*mprA*::Tc) and *E. coli* TG1 (30) were shaken in TSB for 18 h at 37°C. Cell-free supernatants were prepared by centrifugation, sterilized with 0.2-μm-pore-size filters, and stored at 4°C. To support growth, 10% (vol/vol) fresh TSB was added to the sterile conditioned supernatant. *B. pseudomallei* 008 or *B. pseudomallei* 008 (*pmlI*::Tc) was first grown overnight in TSB, and then 1 ml of the culture was removed and the bacteria were harvested by centrifugation. The pellet was used to inoculate 100 ml of conditioned medium.

Identification of the *B. pseudomallei* AHL. Spent supernatants (500 ml) from stationary-phase cultures of *B. pseudomallei* grown in minimal medium M9 were passed through a 0.2-μm-pore-size filter and extracted with 200 ml of dichloromethane. The extract was concentrated by rotary evaporation at room temperature. The residue was reconstituted in 1.5 ml of acetonitrile and applied to a C_8 reverse-phase analytical high-performance liquid chromatography (HPLC) column (Spheri 5 RP8; 220 by 4.6 mm; Perkin-Elmer, Norwalk, Conn.). It was then eluted with a 5 to 70% acetonitrile gradient in water at a flow rate of 1.5 ml per min. The autoinducer-containing fractions, as determined by the *E. coli* VJS533(pHV200I$^-$), *E. coli* MG4(pVKDT17) (24), and *Ralstonia solanacearum* (p395B) bioassays (10), were pooled and subjected to chromatography again by using a 20 to 100% acetonitrile gradient in water. Synthetic AHLs (C_8-AHL, C_{10}-AHL, and C_{12}-AHL) (Fluka, Saint Quentin Fallavier, France), used as standards, were subjected to chromatography on the same column as described above.

Mice and experimental infection. Female Swiss mice were purchased from the Centre d'Elevage R. Janvier (Le Genest St. Isle, France). Animals were given sterilized U.A.R. chow (U.A.R., Villemoisson sur Orge, France) and sterile water ad libitum. For all experiments, animals were used when they were 8 weeks old. The Centre de Recherches du Service de Santé des Armées Emile Pardé Animal Care Committee approved all in vivo studies described below (file numbers 34-2001 and 26-2002). Bacteria were streaked on tryptic soy agar plates and incubated overnight at 37°C. TSB was inoculated with single colonies and shaken for 18 h at 37°C. Mice were inoculated with 10-fold dilutions of each culture in sterile saline. The numbers of viable bacteria in the dilutions used were determined by plate counting. To establish the 50% lethal dose (LD_{50}), groups of six mice were inoculated with 200-μl aliquots of the various dilutions via the intraperitoneal or subcutaneous route or with 50-μl aliquots via the intranasal route. Death was recorded over the following 6 weeks. The LD_{50} was calculated as described by Reed and Muench (27). In another experiment, we assessed the survival rates of mice infected by the intraperitoneal, subcutaneous, and intranasal routes. To do this, groups of 20 mice were inoculated with ca. 1 LD_{50} of *B. pseudomallei* 008, as determined for each challenge route. Death was recorded daily for 6 weeks. Kaplan-Meier survival curves were constructed, and the significance of differences between groups was assessed by the log-rank test (26).

RESULTS

Activity of MprA in relation to the growth phase. We first measured the activity of the MprA protease in supernatants of *B. pseudomallei* 008 grown in TSB. The MprA activity was low during the exponential phase (7,429 U after 4 h) and then

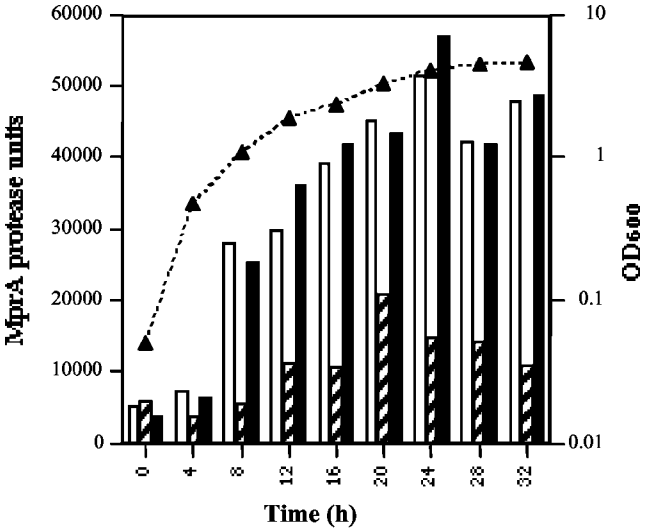

FIG. 1. Growth phase modulation of MprA protease activity. *B. pseudomallei* 008 was grown at 37°C in TSB. The OD_{600} (dashed line) and the MprA activity in culture supernatants (open bars), cell lysates (striped bars), and total extracts (solid bars) were measured every 4 h.

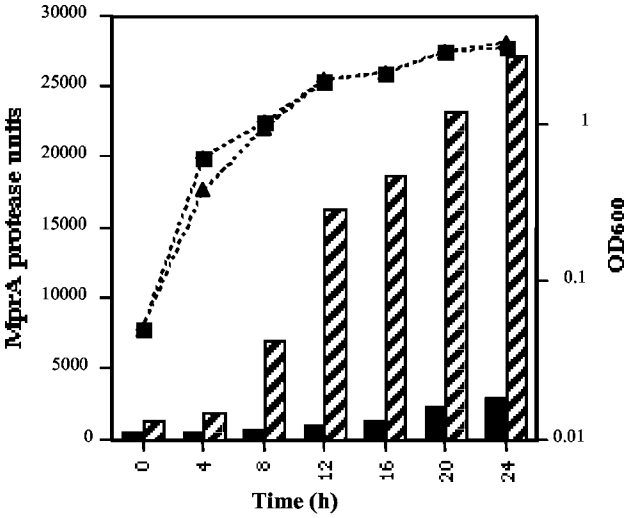

FIG. 2. Effect of *mprA* mutation on protease production in *B. pseudomallei*. Bacterial strains were grown at 37°C in TSB. The OD_{600} (dashed lines) and the MprA activity (bars) in total extracts were measured every 4 h. ▲ and solid bars, *B. pseudomallei* 008 (*mprA*::Tc) mutant; ■ and striped bars, *B. pseudomallei* 008 (*mprA*::Tc) mutant harboring pMprA.

drastically increased upon entry into the stationary phase (27,936 U after 8 h) (Fig. 1). To examine whether MprA was produced but not secreted during the exponential phase, we measured the MprA activity in cell lysates and total extracts during growth (Fig. 1). Like the protease activities in culture supernatants, the protease activities in cell lysates and total extracts were low during the exponential phase. Thus, the MrpA protease appeared to be poorly produced during the exponential growth phase. The protease activity in total extracts increased at the beginning of the stationary phase, whereas it remained nearly constant in cell lysates. After 24 h, 85% of the MprA activity was present in the culture supernatant and the remaining 15% was cell associated (Fig. 1). Thus, production and secretion of MprA are growth phase dependent, increasing markedly upon entry into the stationary phase.

Characterization of a *B. pseudomallei mprA* mutant. MprA activity was determined in total extracts during growth of the *B. pseudomallei* 008 (*mprA*::Tc) mutant and of its derivative harboring pMprA. Total extracts of the *mprA*::Tc mutant contained very low levels of protease activity throughout growth (Fig. 2). Complementation of *B. pseudomallei* 008 (*mprA*::Tc) with pMprA restored production of the protease, and moreover, this production was growth phase dependent (Fig. 2), as observed for the parental strain (Fig. 1). These results demonstrated that the protease activity detected in culture supernatants of *B. pseudomallei* 008 requires an intact *mprA* gene and that the MprA protein is probably the only protease produced by *B. pseudomallei* 008 in our experimental conditions.

Identification of the PmlI-PmlR quorum-sensing system in *B. pseudomallei*. To identify a putative quorum-sensing system in *B. pseudomallei*, we carried out a BLAST search of the *B. pseudomallei* genome using the assembly contigs generated by the *B. pseudomallei* genome sequencing project (www.sanger.ac.uk/Projects/B_pseudomallei). This search allowed us to identify two ORFs, which we designated *pmlI* and *pmlR* and which exhibited high levels of sequence identity to *B. cepacia* *cepI* and *cepR*, respectively. The *pmlI* and *pmlR* sequences were analyzed further with the programs available at the www.ncbi.nlm.nih.gov/BLAST/ website. The *pmlI* ORF is predicted to encode a 203-amino-acid protein with a calculated molecular mass of 22,156 Da. The PmlI protein exhibited 98% sequence identity to the BpsI protein described by Lumjiaktase et al. (accession no. AF501236), 97% sequence identity to the AHL synthase of *Burkholderia mallei*, and 78% sequence identity to the *B. cepacia* CepI protein. The characteristic domain of the LuxI family members (accession no. pfam00765) is highly conserved in PmlI. The *pmlR* ORF is predicted to encode a 239-amino-acid protein with a calculated molecular mass of 26,668 Da. The PmlR protein exhibited 98% sequence identity to the AHL-binding regulator of *B. mallei* and 79% sequence identity to the CepR protein of *B. cepacia*. Both the AHL-binding domain and the helix-turn-helix motif of the LuxR family members (accession no. pfam03472) are present in PmlR. *pmlR* is separated from *pmlI* by 741 bp and is divergently transcribed. This genetic organization is similar to that found in *B. cepacia* (19) and in *Burkholderia vietnamiensis* (8). Together, all these data strongly suggest that *pmlI* and *pmlR* constitute a quorum-sensing system in *B. pseudomallei*.

Construction and characterization of a *B. pseudomallei pmlI* mutant. We constructed a *pmlI* mutant of *B. pseudomallei* 008 as described in Materials and Methods. We then measured the MprA activities in culture supernatants and total extracts of the parental strain, the *pmlI*::Tc mutant, and the *pmlI*::Tc mutant complemented with pPmlI throughout growth. Inactivation of the *pmlI* gene had no obvious effect on protease activity during the exponential phase (Fig. 3). In contrast, the protease activity in the culture supernatant was 1.5- to 2-fold higher upon entry into the stationary phase for the *pmlI*::Tc mutant than for the parental strain (Fig. 3). Consistent with the results obtained with the wild-type strain, approximately 90% of the total activity was present in the culture supernatant of the

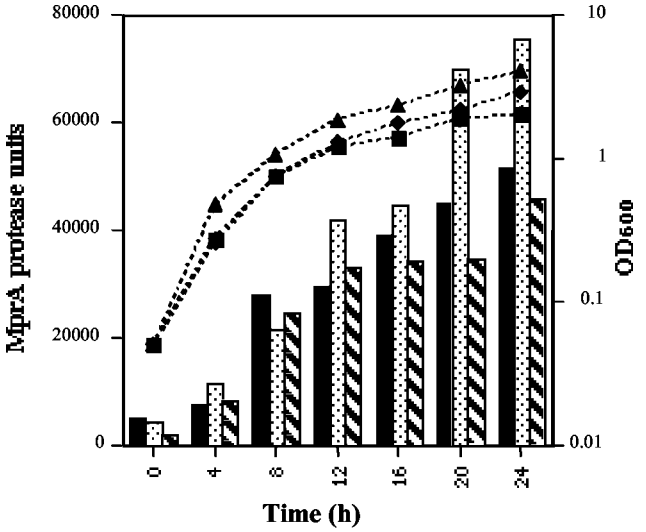

FIG. 3. Modulation of MprA protease activity by the PmlI-PmlR quorum-sensing system. Bacterial strains were grown at 37°C in TSB. The OD_{600} (dashed lines) and MprA activity (bars) in culture supernatants were determined every 4 h. ▲ and solid bars, parental strain *B. pseudomallei* 008; ■ and stippled bars, *B. pseudomallei* 008 (*pmlI*::Tc) mutant; ♦ and striped bars, *B. pseudomallei* 008 (*pmlI*::Tc) mutant harboring pPmlI.

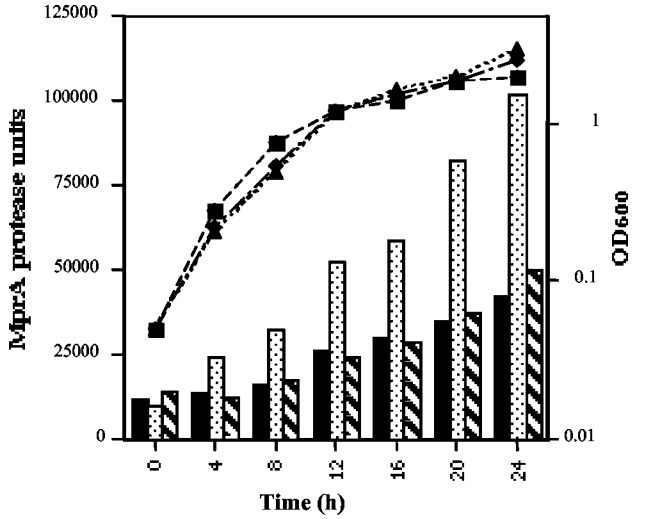

FIG. 4. Effects of cell-free culture supernatants on MprA protease activity. Bacterial strains were grown at 37°C in normal or conditioned TSB. OD_{600} (dashed lines) and MprA activity (bars) of total extracts were measured every 4 h. ▲ and solid bars, parental strain *B. pseudomallei* 008 grown in conditioned TSB; ■ and spotted bars, *B. pseudomallei* 008 (*pmlI*::Tc) mutant grown in normal TSB; ♦ and striped bars, *B. pseudomallei* 008 (*pmlI*::Tc) mutant grown in conditioned TSB.

pmlI::Tc mutant (data not shown), indicating that MprA was efficiently secreted by this mutant. When the mutant strain was complemented with pPmlI, the MprA protease activity of this strain was restored to a level similar to that of the parental strain (Fig. 3). These results strongly suggested that *B. pseudomallei* MprA production is limited by the PmlI-PmlR quorum-sensing system at a high cell density.

Characterization of the *B. pseudomallei* AHL. Assuming that PmlI is an AHL synthase, we looked for autoinducer activity in culture supernatants of *B. pseudomallei*. Cell-free supernatant of *E. coli* TG1 or *B. pseudomallei* 008 (*mprA*::Tc), which contained very low levels of protease activity (Fig. 2), was added prior to inoculation of *B. pseudomallei* 008 and *B. pseudomallei* 008 (*pmlI*::Tc), the latter of which overproduced the MprA protease (Fig. 3). As a control, the *E. coli* TG1 cell-free supernatant did not affect bacterial growth or the protease activity of total extracts from *B. pseudomallei* 008 (*pmlI*::Tc) (data not shown). Additionally, growth of *B. pseudomallei* 008 (*pmlI*::Tc) was not modified by *B. pseudomallei* 008 (*mprA*::Tc) supernatant (Fig. 4). Therefore, the presence of *B. pseudomallei* 008 (*mprA*::Tc) culture supernatant in the growth medium did not affect the protease activity in total extracts of *B. pseudomallei* 008 (Fig. 1 and 4). In contrast, addition of *B. pseudomallei* 008 (*mprA*::Tc) cell-free supernatant greatly reduced the MprA activity in total extracts of *B. pseudomallei* 008 (*pmlI*::Tc) and restored the parental phenotype to the *pmlI*::Tc mutant (Fig. 4). Thus, soluble signaling molecules absent from the *E. coli* TG1 supernatant are involved in negative control of MprA protease production by the PmlI-PmlR quorum-sensing system in *B. pseudomallei*.

To identify these signaling molecules, an acetonitrile extract of *B. pseudomallei* 008 culture supernatant was fractionated by C_8 reverse-phase HPLC. Only one peak of AHL activity was found when each fraction was assayed by using three AHL bioassays. The *E. coli* VJS533(pHV200I$^-$) bioassay, which can detect AHL molecules with acyl groups containing five to eight carbons (24), responded very weakly to the compound produced by *B. pseudomallei* 008. Both the *E. coli* MG4 (pVKDT17) and *R. solanacearum*(p395B) bioassays, which can detect AHL with acyl groups containing eight or more carbons (10, 24), gave positive responses (data not shown). This suggested that *B. pseudomallei* 008 produces an AHL with an acyl group that is longer than eight carbon atoms. As expected, the acetonitrile extract of the *B. pseudomallei* 008 (*pmlI*::Tc) mutant did not contain detectable levels of autoinducer activity, as shown by the *E. coli* MG4(pVKDT17) and *R. solanacearum*(p395B) bioassays. Fractions containing the active compound were pooled and further separated by HPLC as described in Materials and Methods. *B. pseudomallei* autoinducer was eluted at a position very similar to that of synthetic *N*-decanoylhomoserine lactone (data not shown). These results were consistent with the hypothesis that *pmlI* encodes an autoinducer synthase that is required for the production of homoserine lactone in *B. pseudomallei*.

Virulence of *B. pseudomallei pmlI* and *mprA* mutants in mice. As quorum sensing regulates the production of virulence factors in several gram-negative species (11, 22, 29), we investigated the role of the PmlI-PmlR system in *B. pseudomallei* virulence using a murine model of infection. To do this, Swiss mice were infected with the wild-type *B. pseudomallei* 008 strain and its isogenic *pmlI*::Tc mutant, and the LD_{50}s were determined. The LD_{50}s were $\log_{10}$ 5.6, 6.8, and 2.2 for *B. pseudomallei* 008 and 6.3, 7.9, and 3.6 for *B. pseudomallei* 008 (*pmlI*::Tc) after intraperitoneal, subcutaneous, and nasal infection, respectively. Thus, the LD_{50} of the *pmlI* mutant was about 5- to 25-fold higher than that of the parental strain. In agree-

ment, there was a significant difference ($P < 0.05$, as determined by a log rank test) between the percentage of survival for the group of mice infected with *B. pseudomallei* 008 and the percentage of survival for the group of mice infected with *B. pseudomallei* 008 (*pmlI*::Tc) (Fig. 5). Only one, four, and three of the mice inoculated with *B. pseudomallei* 008 by the intraperitoneal, subcutaneous, and intranasal routes, respectively, survived the challenge. In contrast, 17, 13, and 10 mice survived after inoculation with *B. pseudomallei* 008 (*pmlI*::Tc). Thus, the PmlI-PmlR quorum-sensing system is essential for full virulence of *B. pseudomallei* in this mouse model.

To examine the contribution of MprA to virulence, we studied the survival of mice infected with parental strain *B. pseudomallei* 008 and its isogenic *mprA*::Tc mutant via the intraperitoneal, subcutaneous, and intranasal routes. Neither the percentage of survival nor the median survival time of mice infected by either of the strains was significantly affected by the infection route (data not shown). Thus, MprA is probably not a virulence determinant in this infection model.

DISCUSSION

Many gram-negative bacteria, including pathogens, sense population density and control the expression of many phenotypes by using members of the LuxI-LuxR family of quorum-sensing components (11, 22, 29). To date, two quorum-sensing systems have been characterized in the genus *Burkholderia*: the CepI-CepR system in *B. cepacia* (19) and the BviI-BviR system in *B. vietnamiensis* (8). Here, we identified a third LuxI-LuxR homolog, the PmlI-PmlR quorum-sensing system in *B. pseudomallei*. The *pmlI* gene appears to encode an autoinducer synthase as the PmlI protein is a member of the LuxI protein family and as a *pmlI* mutant did not produce detectable levels of AHL. HPLC analysis strongly suggested that the PmlI synthase directs the synthesis of *N*-decanoylhomoserine lactone, an AHL also produced by *B. vietnamiensis* (8). On the basis of a sequence comparison, we speculated that *pmlR* encodes a transcriptional regulatory protein belonging to the LuxR family, but further studies are required to confirm this hypothesis. The large region between *pmlI* and *pmlR* (741 bp) does not contain any putative ORF with significant similarity to any known gene. A similar observation was reported for the *cepI-cepR* intergenic region (727 bp) in *B. cepacia* (19).

The PmlI-PmlR system appears to negatively regulate MprA production in *B. pseudomallei* during the stationary phase. In the *pmlI* mutant, which did not produce detectable amounts of AHL signal molecules, MprA was overproduced upon entry into the stationary phase. Indeed, addition of cell-free culture supernatant of *B. pseudomallei*, which produced the autoinducer molecule, restored a wild-type phenotype to the *pmlI* mutant. Assuming that PmlR is a transcriptional regulator, it is tempting to speculate that PmlR represses *mprA* expression at high autoinducer concentrations.

Other quorum-sensing systems also repress the expression of their target genes. The CepI-CepR system negatively controls the production of ornibactin, a siderophore of *B. cepacia*, when the cell density is high (19). EsaR from *Pantoea stewartii* represses exopolysaccharide production at a low cell density, and derepression requires micromolar amounts of AHL produced by the EsaI synthase (41, 42).

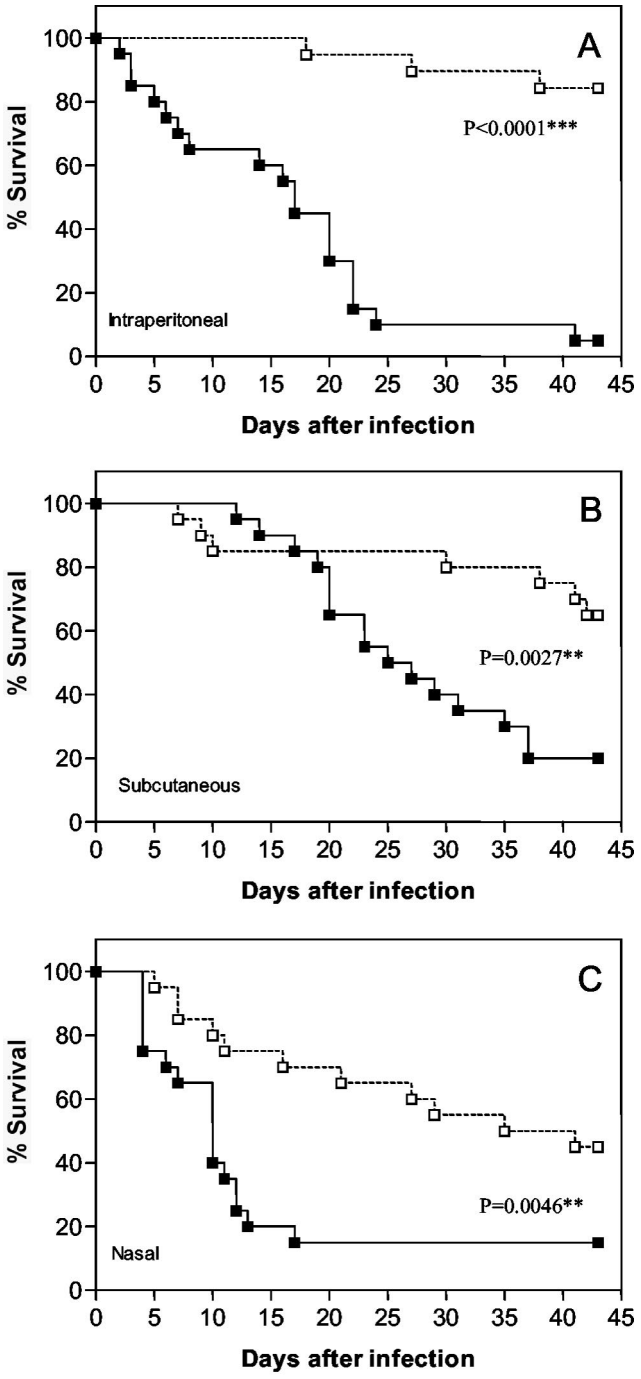

FIG. 5. Percentages of survival of Swiss mice after intraperitoneal (A), subcutaneous (B), and intranasal (C) inoculation with the parental strain *B. pseudomallei* 008 (■) and the isogenic *pmlI*::Tc mutant (□). Twenty mice in each group were infected with ca. 1 LD_{50} of *B. pseudomallei* 008; the number of bacteria was dependent on the infection route. The *P* value is significant if it is <0.05.

Quorum sensing regulates not only exoproducts but also other types of virulence factors in pathogenic bacteria (11, 22, 29). For example, recent work has demonstrated that LuxO, a quorum-sensing regulator in *Vibrio cholerae*, is pivotal for biofilm formation, cholera toxin production, and protease secretion (39).

In the human opportunistic pathogen *Pseudomonas aeruginosa*, the secreted virulence factors are under control of two quorum-sensing systems, LasI-LasR and RhlI-RhlR (16, 23). The fact that a *lasR* mutant exhibits significantly reduced virulence in a mouse model of pneumonia demonstrates the importance of quorum sensing in the pathogenesis of *P. aeruginosa* infection (18, 38). Consistent with this, our results indicated that the PmlI-PmlR quorum-sensing system is essential for the pathogenesis of melioidosis, as a *pmlI* mutant of *B. pseudomallei* is less virulent than the wild-type strain in a murine model of infection when either the intraperitoneal, subcutaneous, or intranasal route is used. This work clearly demonstrated that the production of MprA is regulated by the PmlI-PmlR system but that this protein plays a minor role in *B. pseudomallei* virulence, unlike the role observed in respiratory infection by *Burkholderia cenocepacia* (36). Further studies are thus specifically needed to identify essential virulence determinants controlled by the PmlI-PmlR quorum-sensing system in *B. pseudomallei* (2). In *P. aeruginosa*, it has been demonstrated that the autoinducer has a direct effect on the host immune response and contributes to the pathogenicity in a pulmonary challenge (28, 35). Thus, the role of the PmlI-PmlR quorum-sensing system in *B. pseudomallei* could be explained by regulated expression of virulence factors and induced immunomodulation in the host. However, Cabrol et al. pointed out that the quorum-sensing gene *lasR* played a minor role in the pathogenesis of 50% of the *P. aeruginosa* strains that they studied (5). Similarly, it will be of interest to determine the role of other regulatory systems, such as alternative sigma factors (1) or posttranscriptional regulators (15), in the pathogenicity of *B. pseudomallei*.

In most of the previous studies quorum sensing of risk group 2 pathogens was examined. Jones and Blaser (14) and Taminiau et al. (37) described in vitro AHL production in the risk group 3 bacteria *Bacillus anthracis* and *Brucella melitentis*, respectively. To our knowledge, the present study is the first in vivo demonstration that a quorum-sensing system is indispensable for the full virulence of a risk group 3 pathogen.

As the treatment of melioidosis is long and difficult due to resistance to multiple antibiotics and the lack of an efficacious vaccine, the results described here indicate that inactivation of AHL cell-cell signaling might represent a novel strategy for therapy (20).

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