

## Efflux-Mediated Aminoglycoside and Macrolide Resistance in *Burkholderia pseudomallei*

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*Burkholderia pseudomallei*, the causative agent of melioidosis, is intrinsically resistant to a wide range of antimicrobial agents including  $\beta$ -lactams, aminoglycosides, macrolides, and polymyxins. We used Tn5-OT182 to mutagenize *B. pseudomallei* to identify the genes involved in aminoglycoside resistance. We report here on the identification of AmrAB-OprA, a multidrug efflux system in *B. pseudomallei* which is specific for both aminoglycoside and macrolide antibiotics. We isolated two transposon mutants, RM101 and RM102, which had 8- to 128-fold increases in their susceptibilities to the aminoglycosides streptomycin, gentamicin, neomycin, tobramycin, kanamycin, and spectinomycin. In addition, both mutants, in contrast to the parent, were susceptible to the macrolides erythromycin and clarithromycin but not to the lincosamide clindamycin. Sequencing of the DNA flanking the transposon insertions revealed a putative operon consisting of a resistance, nodulation, division-type transporter, a membrane fusion protein, an outer membrane protein, and a divergently transcribed regulator protein. Consistent with the presence of an efflux system, both mutants accumulated [ $^3$ H] dihydrostreptomycin, whereas the parent strain did not. We constructed an *amr* deletion strain, *B. pseudomallei* DD503, which was hypersusceptible to aminoglycosides and macrolides and which was used successfully in allelic exchange experiments. These results suggest that an efflux system is a major contributor to the inherent high-level aminoglycoside and macrolide resistance found in *B. pseudomallei*.

*Burkholderia pseudomallei* is a motile, gram-negative bacillus found in tropical regions of the world including Southeast Asia, northern Australia, and temperate regions bordering the equator (5, 14). It is the causative agent of melioidosis, a disease which can manifest itself as an acute, subacute, or chronic form of illness (7, 38). The acute form of the disease is a septicemic illness which is often rapidly fatal, despite antibiotic treatment, and which is responsible for considerable morbidity and mortality, especially in northeastern Thailand (7). Subacute melioidosis is described as a prolonged febrile illness with multiorgan involvement, systemic abscess formation, and bacteremia (38). The existence of chronic melioidosis, perhaps the most common form of the disease, becomes evident by the postmortem examination of infected tissues or by activation of other forms of the disease as a result of a traumatic event (35). Infection likely occurs as a result of contact with the organism in soil or water, with entrance into the body via inhalation, via aspiration, or through cuts and abrasions (14, 44). Successful treatment of melioidosis patients is difficult because *B. pseudomallei* is inherently resistant to a variety of antibiotics including  $\beta$ -lactams, aminoglycosides, macrolides, and polymyxins (14, 17). We initiated the studies described here in an effort to understand the antibiotic resistance mechanisms present in this organism. We report here on the identification of AmrAB-OprA, a unique multidrug efflux system in *B. pseudomallei* which is specific for both aminoglycoside and macrolide antibiotics. This system shows substantial homology to multidrug efflux systems found in *Pseudomonas aeruginosa* (16, 30–32), *Neisseria gonorrhoeae* (13), and *Escherichia coli* (21, 23), al-

though in terms of antibiotic specificity, the multidrug efflux system in *B. pseudomallei* appears to have little if any similarity to those in the other organisms.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in these studies are listed in Table 1. Unless noted otherwise, cultures were grown at 37°C on Luria broth (LB) agar (Becton Dickinson, Cockeysville, Md.) or in LB. Antibiotics were used as described previously (15).

**Tn5-OT182 mutagenesis and isolation of aminoglycoside-susceptible strains of *B. pseudomallei*.** Mutagenesis of *B. pseudomallei* was performed as described previously (11) with slight modifications in the selection of mutants. Briefly, pOT182 (Table 1) was introduced into *B. pseudomallei* 1026b via conjugation with *E. coli* SM10(pOT182). Transposon mutants of *B. pseudomallei* were selected by plating the conjugation mixtures onto *Pseudomonas* isolation agar (Difco) containing 50  $\mu$ g of tetracycline per ml. The colonies that arose on this medium were picked and transferred to LB agar containing tobramycin (50  $\mu$ g/ml) or streptomycin (50  $\mu$ g/ml) and to a new *Pseudomonas* isolation agar-tetracycline plate. Colonies which did not grow on tobramycin- and streptomycin-containing media were characterized further.

**Streptomycin uptake assays.** Uptake assays were performed essentially as described by Bryan and Van den Elzen (4). Broth cultures of *B. pseudomallei* were grown overnight at 37°C at 250 rpm in 3 ml of nutrient broth. When required, the medium contained 50  $\mu$ g of tetracycline per ml. Overnight cultures were diluted 1:100 in 20 ml of nutrient broth in a 250-ml flask without antibiotic and were allowed to grow to an optical density at 600 nm of 0.4 to 0.6. When the culture was at the proper optical density, 10 ml of the culture was transferred to another flask and was allowed to incubate for another 5 min. Assays were begun by the addition of a mixture of [ $^3$ H]dihydrostreptomycin (10 Ci/mmol; American Radiolabeled Chemicals Inc., St. Louis, Mo.) and unlabeled streptomycin (Sigma) to a final concentration of 50  $\mu$ g of streptomycin per ml. The specific activity of streptomycin in the assay was 15 to 20 dpm of streptomycin per ng. Control experiments showed that there was no difference in uptake if dihydrostreptomycin rather than streptomycin was used as a carrier. Samples (1 ml) were removed at various time points and were filtered through a 0.45- $\mu$ m-pore-size cellulose acetate membrane filter (Sartorius) held in a Millipore filter apparatus. The filters were soaked in a 2.5-mg/ml solution of streptomycin prior to the assay to prevent nonspecific binding of labeled antibiotic. Samples were washed with 15 ml of 3% NaCl. The filters were allowed to air dry and were placed in scintillation vials, and 4 ml of ReadySafe liquid scintillation fluid (Beckman) was added. The radioactivity in the samples was counted in a Beckman LS6500 scintillation counter.

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TABLE 1. Strains and plasmids used in this study

| Strain or plasmid       | Description <sup>a</sup>   | Reference  |
|-------------------------|--|------------|
| <b>Strains</b>          |  |            |
| <i>E. coli</i> SM10λpir | SM10 with a λ prophage carrying the gene encoding the π protein; Km <sup>r</sup>   | 26         |
| <i>B. pseudomallei</i>  |  |            |
| 1026b                   | Clinical isolate; AG <sup>r</sup> Tc <sup>s</sup>  | 11         |
| C21                     | 1026b derivative; <i>gspD</i> ::Tn5-OT182 AG <sup>r</sup> Tc <sup>s</sup>  | 10         |
| RM101                   | 1026b derivative; <i>amrA</i> ::Tn5-OT182 AG <sup>s</sup> Tc <sup>r</sup>  | This study |
| RM102                   | 1026b derivative; <i>amrB</i> ::Tn5-OT182 AG <sup>s</sup> Tc <sup>r</sup>  | This study |
| RM104                   | 1026b derivative; 1026b::pSK240 Tc <sup>r</sup>  | This study |
| RM102 Sm <sup>r</sup>   | RM102 derivative; <i>rpsL</i> (Sm <sup>r</sup> )   | This study |
| DD502                   | RM102 Sm <sup>r</sup> ::pDD502 Sm <sup>s</sup> Km <sup>r</sup> Tc <sup>r</sup>   | This study |
| DD503                   | DD502 derivative; Δ( <i>amrR-oprA</i> ) <i>rpsL</i> (Sm <sup>r</sup> ) AG <sup>s</sup> Tc <sup>s</sup>   | This study |
| <b>Plasmids</b>         |  |            |
| pOT182                  | PSUP102(Gm)::Tn5-OT182 Cm <sup>r</sup> Gm <sup>r</sup> Ap <sup>r</sup> Tc <sup>r</sup>   | 24         |
| pUCP28T                 | Broad-host-range vector; IncP OriT; pRO1600 ori; Tp <sup>r</sup>   | 36         |
| p34E                    | Cassette cloning vector; Ap <sup>r</sup>   | 42         |
| pKAS46                  | Mobilizable allelic exchange vector; π-dependent R6K replicon; Ap <sup>r</sup> Km <sup>r</sup> <i>rpsL</i> <sup>+</sup> (Sm <sup>s</sup> )       | 37         |
| pSKM11                  | Mobilizable allelic exchange vector; Tc <sup>r</sup> Ap <sup>r</sup>   | 27         |
| pSKM240                 | Mobilizable allelic exchange vector; pSKM11 containing a 240-bp internal <i>Ssr1</i> fragment from <i>amrR</i> ; Tc <sup>r</sup> Ap <sup>r</sup> | This study |
| pBluescript SK          | General cloning vector; Ap <sup>r</sup>  | Stratagene |
| pAW22                   | 14.0-kb <i>NotI</i> fragment from RM101 obtained by self-cloning; Ap <sup>r</sup> Tc <sup>r</sup>  | This study |
| pAW14                   | 1.0-kb <i>SalI</i> fragment from RM101 obtained by self-cloning; Ap <sup>r</sup> Tc <sup>r</sup>   | This study |
| pAW31                   | 12.1-kb <i>BamHI</i> fragment from RM102 obtained by self-cloning; Ap <sup>r</sup> Tc <sup>r</sup>   | This study |
| pAW32                   | 19.3-kb <i>BamHI</i> fragment from RM101 obtained by self-cloning; Ap <sup>r</sup> Tc <sup>r</sup>   | This study |
| pRM105                  | pUCP28T containing a 14.7-kb <i>EcoRI</i> fragment from pAW32  | This study |
| pSR20                   | pSK-Bluescript containing a 4.0-kb <i>SacI</i> fragment from pAW32   | This study |
| pDD500                  | pKAS46 derivative; 2.4-kb <i>SacI-EcoRI</i> fragment from pSR20 containing 3' end of <i>oprA</i> and 1.4 kb of downstream DNA                    | This study |
| pDD501                  | p34E derivative; 2.1-kb <i>BamHI</i> fragment from pAW22 containing the 3' end of <i>amrR</i> and 1.5 kb of downstream DNA                       | This study |
| pDD502                  | pDD500 containing a 2.11-kb <i>XbaI</i> fragment from pDD501 ( <i>XbaI</i> sites flank the <i>BamHI</i> sites in pDD501)                         | This study |

<sup>a</sup> r, resistant; s, susceptible; AG, aminoglycoside; Tc, tetracycline; Ap, ampicillin; Sm, streptomycin; Km, kanamycin.

**DNA sequencing.** DNA was sequenced by the University Core DNA Services (University of Calgary, Calgary, Alberta, Canada) and by ACGT, Inc. (Northbrook, Ill.). The DNA flanking the Tn5-OT182 insertions was isolated by self-cloning (24) and was subcloned into p-Bluescript SK (Stratagene). DNA alignments were performed with Gene Jockey, version 1.2, for the Macintosh, and homology searches were performed with the BLASTX and BLASTP programs, which were provided by the National Center for Biological Information (1).

**Antimicrobial agent susceptibility testing.** The susceptibilities of *B. pseudomallei* strains to antimicrobial agents were determined via the agar dilution method (28) with a multiwell replicator and Mueller-Hinton II agar (Becton Dickinson Microbiology Systems) or by E tests (AB Biodisk, Solna, Sweden) according to the manufacturer's instructions. The bacteria used for MIC determinations were resuspended in phosphate-buffered saline to a 0.5 McFarland standard.

**Animal studies.** The hamster model of acute *B. pseudomallei* infection has been described elsewhere (3). The 50% lethal dose (LD<sub>50</sub>) for each of various *B. pseudomallei* strains was calculated 48 h after infection by the method of Reed and Muench (34).

**Construction of pRM105.** To attempt complementation of the aminoglycoside-macrolide-susceptible phenotype of RM102, plasmid pRM105 was constructed by ligating a 14.7-kb *EcoRI* fragment from pAW32 into the *EcoRI* site of pUCP28T. The 14.7-kb fragment contained 7.9 kb of Tn5-OT182 DNA originating at the *EcoRI* site in Tn5-OT182 and 6.8 kb of DNA downstream of the transposon insertion terminating at a *EcoRI* site located downstream of the *oprA* gene. The fragment contains all of *amrB*. The resulting construct was electroporated into *E. coli* SM10 and was conjugated into RM102 as described previously (11). A single transconjugant, RM102(pRM105), was selected, and the aminoglycoside and macrolide MICs for the transconjugant were determined.

**Construction of strain DD503.** *B. pseudomallei* DD503 (Table 1) was constructed for use in allelic exchange experiments with the vector pKAS46 (37). An Sm<sup>r</sup> derivative of RM102 was selected by plating 100-μl aliquots of an overnight LB culture onto LB agar plates containing 100 μg of streptomycin per ml and 50 μg of tetracycline per ml. One Sm<sup>r</sup> Tc<sup>r</sup> colony was selected for further studies and was named RM102-Sm<sup>r</sup>. With the exception of Sm<sup>r</sup>, this strain exhibited the same antibiotic susceptibility profile as RM102, which strongly suggested that Sm<sup>r</sup> was due to an *rpsL* mutation. Plasmid pDD502 (Table 1) was conjugated from SM10λpir to RM102-Sm<sup>r</sup>, and transconjugants were selected on LB agar plates containing 50 μg of tetracycline per ml and 50 μg of kanamycin per ml.

One Tc<sup>r</sup> Km<sup>r</sup> colony (RM102-Sm<sup>r</sup>::pDD502) was selected for further studies and was named DD502. Several isolated colonies of DD502 were spread onto separate LB agar plates containing 100 μg of streptomycin per ml to select for transconjugants that excised pKAS46 DNA. A Sm<sup>r</sup> Tc<sup>s</sup> Km<sup>r</sup> colony was selected and was named DD503.

**PCR amplification of the Δ(*amrR-oprA*) locus of DD503.** Oligodeoxyribonucleotide primers DD503L (5'-CAGATAGTGCGACGCGCAA-3' [nucleotides 311 to 330 of the submitted nucleotide sequence]) and DD503R (5'-TTCCGGC GAGCCCCAGTTGAT-3' [within *oprA*]) were used to amplify a 575-bp product from *B. pseudomallei* DD503 chromosomal DNA by PCR with *Taq* DNA polymerase (Gibco BRL). Dimethyl sulfoxide was added to the PCR mixture at a final concentration of 10%. The conditions for PCR involved a 2-min denaturation step at 97°C, followed by 30 cycles of 97°C for 30 s, 60°C for 30 s, and 72°C for 30 s. The 575-bp PCR product was purified with the Magic PCR Preps DNA Purification System (Promega, Madison, Wis.), and DNA sequencing reactions were initiated with DD503R and DD503L.

**β-Galactosidase assays.** β-Galactosidase activity was measured as described by Miller (25). Overnight cultures were diluted in LB and were grown to the early logarithmic phase. Aliquots (1 ml) were removed over the course of several hours, and the β-galactosidase activity was determined.

**Nucleotide sequence accession number.** The nucleotide sequence reported in this paper has been deposited in the GenBank database under accession no. AF072887.

## RESULTS

**Isolation of aminoglycoside- and macrolide-susceptible mutants.** Prior studies of the antibiotic susceptibility of *B. pseudomallei* have demonstrated high levels of resistance to aminoglycoside antibiotics (14, 17). On the basis of these findings, it was hypothesized that an efflux system might be responsible for aminoglycoside resistance in *B. pseudomallei*. To identify proteins associated with a putative efflux system, *B. pseudomallei* 1026b was mutagenized with Tn5-OT182, and aminoglycoside-susceptible mutants were isolated and analyzed. Approx-

TABLE 2. Susceptibilities of *B. pseudomallei* strains to antimicrobial agents

| Antimicrobial agent | MIC ( $\mu\text{g/ml}$ ) |       |       |                |                 |
|---------------------|--------------------------|-------|-------|----------------|-----------------|
|                     | 1026b                    | RM101 | RM102 | RM102 (pRM105) | DD503           |
| Gentamicin          | 256                      | 2     | 2     | 96             | 0.75            |
| Kanamycin           | 16                       | <1    | <1    | 24             | ND <sup>a</sup> |
| Neomycin            | 32                       | <4    | <4    | ND             | <4              |
| Spectinomycin       | >1,064                   | 64    | 64    | ND             | 64              |
| Streptomycin        | 1,064                    | 16    | 16    | 512            | >1,024          |
| Tobramycin          | 48                       | 0.75  | 1.5   | 16             | 0.75            |
| Clarithromycin      | >256                     | 12    | 16    | 192            | 8               |
| Erythromycin        | >256                     | 8     | 16    | >256           | 8               |
| Clindamycin         | >256                     | >256  | >256  | ND             | >256            |

<sup>a</sup> ND, not determined.

imately 3,000 transposon mutants were screened for their susceptibilities to streptomycin and tobramycin. We identified two mutants, RM101 and RM102, which were sensitive to both antibiotics. Further examination revealed that both RM101 and RM102 were susceptible to a variety of aminoglycoside antibiotics, with a 64-fold reduction in the MIC of streptomycin, a greater than 16-fold reduction in the MIC of kanamycin, a 32- to 64-fold reduction in the MIC of tobramycin, and a greater than 128-fold reduction in the MIC of gentamicin (Table 2). In addition, both mutants showed increased susceptibility to the macrolide antibiotics erythromycin and clarithromycin but not to clindamycin. The MICs of ampicillin, benzalkonium chloride, ceftazidime, cetrime, chloramphenicol, ciprofloxacin, nalidixic acid, protomine sulfate, rifampin, sodium dodecyl sulfate, trimethoprim, and polymyxin B for the mutants were not different from those for the parent strain,

strain 1026b (data not shown). In addition, strain C21 had the same MIC profile as strain 1026b, indicating that Tn5-OT182 had no effect on susceptibility to antibiotics (data not shown).

**Streptomycin uptake in RM101 and RM102.** Uptake assays demonstrated that both transposon mutants rapidly accumulated streptomycin, while the parent strain, strain 1026b, did not (Fig. 1). Strain C21, a *B. pseudomallei* 1026b derivative with a Tn5-OT182 insertion in a type II secretion pathway gene (*gspD*) (10), did not accumulate streptomycin, indicating that streptomycin accumulation in strains RM101 and RM102 was not a function of the transposon. These results are consistent with the presence of an efflux system which recognizes aminoglycosides. Uptake of streptomycin in parent strain 1026b was not enhanced by the addition of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) (100  $\mu\text{M}$  to 5 mM) or arsenate (10 mM) (data not shown).

**Analysis of DNA flanking transposon insertions in RM101 and RM102.** The DNA flanking the transposon insertions in both mutants was isolated by self-cloning (11, 15, 24). Three of the resulting plasmids, pAW14, pAW31, and pAW32, were sequenced, and nucleic acid and protein databases were analyzed for homologous sequences. These analyses revealed that the transposon insertions in RM101 and RM102 were located in two genes whose products showed homology to proteins belonging to the resistance, nodulation, division-type (RND-type), multidrug resistance proteins. We have given the genes involved in aminoglycoside and macrolide resistance in *B. pseudomallei* the prefix *amr* (aminoglycoside and macrolide resistance). The plasmids, the locations of the transposon insertions, and the arrangements of the *amr* genes are presented in Fig. 2. The transposon insertion in RM101 was in an open reading frame 1,199 nucleotides in length. This open reading frame was named *amrA*. The product of this open reading

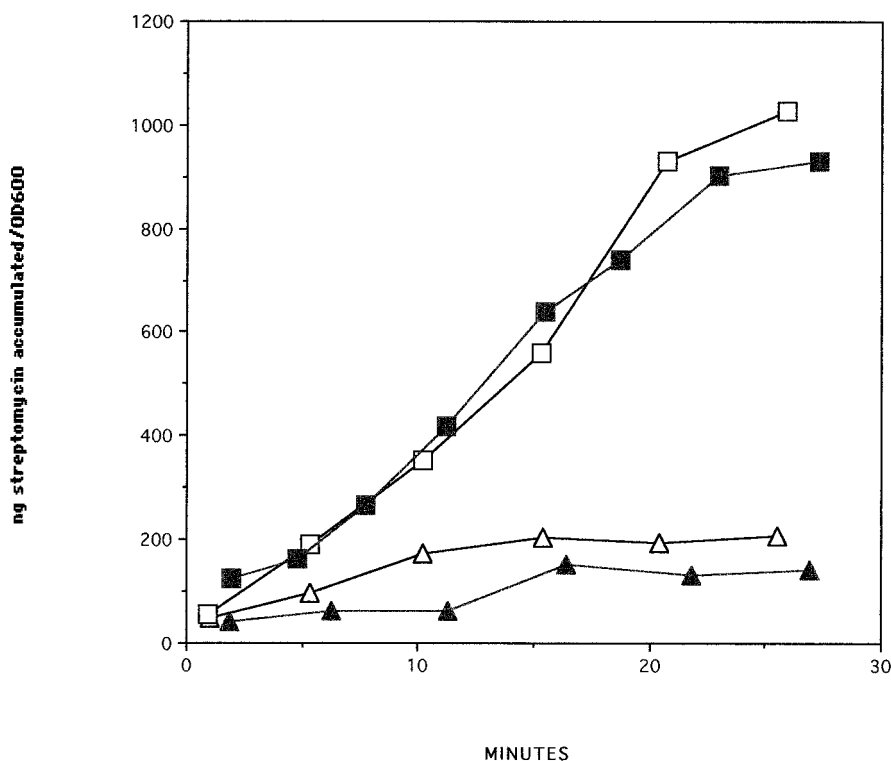


FIG. 1. [<sup>3</sup>H]dihydrostreptomycin (streptomycin) uptake by *B. pseudomallei* 1026b (open triangles), *B. pseudomallei* C21 (closed triangles), *B. pseudomallei* RM101 (open squares), and *B. pseudomallei* RM102 (closed squares) in nutrient broth. OD600, optical density at 600 nm.

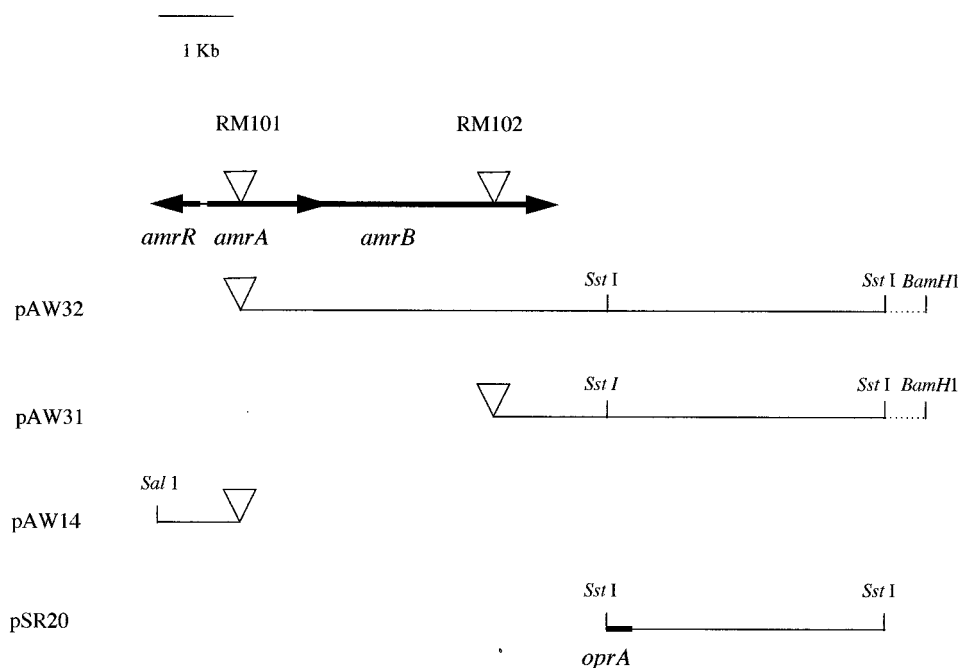


FIG. 2. Arrangement of the *amr* locus in *B. pseudonallei* 1026b. Triangles indicate Tn5-OT182 insertions within the *amr* locus and the transposon location relative to the location of the cloned flanking DNA in pAW32, pAW31, and pAW14.

frame showed strong homology to the *P. aeruginosa* membrane fusion protein MexC (50% identity). A putative ribosome-binding sequence, GAG, was located 8 to 10 bp upstream of the potential ATG start codon. Analysis of the Tn5-OT182 insertion in RM102 revealed an open reading frame 3,131 nucleotides in length (*amrB*). The product of this open reading frame showed strong homology to the *E. coli* AcrD protein (57% identity) and the *P. aeruginosa* RND-type efflux protein MexB (54% identity). Analysis of the sequence downstream of *amrB* in pAW32 subclones revealed a gene encoding a potential outer membrane protein (Fig. 2). The partial sequence of this gene indicates a protein with strong similarity to amino acids 90 to 216 of OprJ, an outer membrane protein associated with the *mexC-mexD-oprJ* efflux operon in *P. aeruginosa* (31). We were unable to fully sequence the outer membrane gene (tentatively named *oprA*), presumably because of a secondary structure resulting from the high GC content (68%) of *B. pseudomallei* DNA (46). In addition, a fourth open reading frame immediately upstream and divergently transcribed from *amrA* was identified. The product of this gene, named *amrR*, showed strong homology (41% identity) to MtrR, a protein in *N. gonorrhoeae* which acts as a transcriptional repressor of the *mtrRCDE* efflux system and which is arranged in a similar, divergent manner (13). The *amrR* gene was 564 bp in length and had a putative ribosome-binding sequence, GGAG, located 10 to 13 bp upstream of the ATG start codon.

**Complementation of RM102.** Plasmid pRM105, which contains 6.8 kb of DNA downstream of the transposon insertion in RM101, was able to restore aminoglycoside and macrolide resistance in susceptible mutant RM102. These results strongly suggest that the aminoglycoside-macrolide-susceptible phenotype in RM102 is due to the transposon insertion in this gene and that the *amr* operon is responsible for aminoglycoside and macrolide resistance in this organism.

***lacZ* transcriptional fusion analysis of RM101 and RM102.** Both mutants RM101 and RM102 were *lacZ* transcriptional fusions, as indicated by sequence analysis and by their high

levels of  $\beta$ -galactosidase activity.  $\beta$ -Galactosidase activity increased during growth in liquid cultures for both RM101 and RM102 but was not inducible by the addition of 2  $\mu$ M streptomycin. Induction of  $\beta$ -galactosidase activity was also examined in RM102 Sm<sup>r</sup>, an *rpsL* derivative of RM102; however, streptomycin (100  $\mu$ g/ml) did not induce  $\beta$ -galactosidase activity beyond the level seen in control cultures which did not receive streptomycin (data not shown). In addition, stress conditions (4% ethanol or 0.5% NaCl [22]) did not result in the induction of  $\beta$ -galactosidase activity in these strains (data not shown).

**Characterization of *B. pseudomallei* DD503, an *amr* locus deletion mutant suitable for allelic exchange.** An *amr* locus deletion mutant, DD503, was constructed (Table 1). As a result of the *amr* locus deletion, DD503 displays the same antibiotic resistance and susceptibility profile as RM101 and RM102, thus confirming the effect of the transposon insertions in these strains. The increased susceptibility makes DD503 a useful recipient for allelic exchange experiments and allows the use of many currently available allelic exchange vectors containing the conditionally counterselectable Sm<sup>s</sup> marker (*rpsL*<sup>+</sup>), such as pSS1129 and pKAS46 (39, 40). The antibiotic susceptibility of DD503 is similar to those of RM101 and RM102, with the exception that DD503 is Sm<sup>r</sup> Tc<sup>s</sup> and lacks *lacZ* because Tn5-OT182 was deleted during construction of the deletion. The nucleotide sequence at the junction of the  $\Delta$ (*amrR-oprA*) mutation in DD503 was determined by amplification and sequencing of the DNA of a PCR product generated with an *amrR*-specific oligodeoxynucleotide primer (DD503L) and an *oprA*-specific primer (DD503R). Although we have not determined the entire sequence of *oprA*, the sequence of primer DD503R was determined from the partial sequence of a subclone of pAW32 (pSR20) which contained a portion of *oprA*. The nucleotide sequence of the PCR product confirmed the predicted junction of the  $\Delta$ (*amrR-oprA*) mutation extending from the 5' end of *amrR* (nucleotide 550) to the *oprA* sequence



determined from pSR20, including a 136-bp portion of the pKAS46 polylinker from *Xba*I to *Sac*I.

**Relative virulence of *amr* mutants in Syrian hamsters.** The virulence of *amr* mutants was assessed in a Syrian hamster model of acute *B. pseudomallei* infection (3). There was no significant difference in the LD<sub>50</sub> for parent strain 1026b, RM101, RM102, and DD503. The LD<sub>50</sub>s for all strains were <10 bacteria at 2 days. In addition, all of the infected hamsters were bacteremic at the time of death. This demonstrates that transposon mutant strains RM101 and RM102 and deletion mutant DD503 remain highly virulent and suggests that the *amr* locus is probably not a virulence determinant in this animal model of acute *B. pseudomallei* infection. Similarly, the *rpsL* mutation (Sm<sup>r</sup>) in DD503 has no effect on virulence in hamsters.

## DISCUSSION

Classical mechanisms of drug resistance include enzyme modification of antibiotics, target modification, and active efflux of specific drugs (e.g., tetracycline). Somewhat recently, it has been recognized that bacteria, in addition to the methods mentioned above, use multidrug efflux systems capable of recognizing a wide range of seemingly unrelated compounds. In *P. aeruginosa*, at least three multidrug efflux systems which contribute to the intrinsic antibiotic resistance found in this organism exist. These systems include the MexAB-OprM system (30), the MexCD-OprJ system (31), and the MexEF-OprN system (16). The three systems overlap in their ability to efflux compounds such as tetracycline, chloramphenicol, and fluoroquinolones but show specificity toward the efflux of  $\beta$ -lactams and newer cepheims such as cefepime and ceftipime. In other bacteria, such as *N. gonorrhoeae* and *E. coli*, multidrug efflux systems confer resistance to hydrophobic antibiotics, detergents, and dyes (13, 21, 23).

In this study we report on the identification of a multidrug efflux system in *B. pseudomallei* which is specific for both aminoglycoside and macrolide antibiotics. To our knowledge, this is a unique mechanism of high-level aminoglycoside resistance (9). Inactivation of either *amrA* or *amrB* in *B. pseudomallei* resulted in enhanced susceptibility to aminoglycoside and macrolide antibiotics. Further analysis revealed the presence of genes encoding an outer membrane protein and a putative regulatory protein. Despite the similarity between the system described here and the products of the *mex* genes in *P. aeruginosa*, there was no apparent similarity in substrate specificity. Whereas *mexAB-oprM* and *mexCD-oprJ* are associated with enhanced resistance to tetracycline, chloramphenicol, fluoroquinolones, and  $\beta$ -lactams, the *amr* locus in the *B. pseudomallei* system did not confer resistance to these compounds. That these genes served as an efflux pump was made evident by the accumulation of [<sup>3</sup>H]dihydrostreptomycin in RM101 and RM102. The *amr* locus also confers resistance to macrolides such as erythromycin but not to lincosamides such as clindamycin. Efflux systems which recognize macrolides but not lincosamides have been described in *Streptococcus* and *Staphylococcus* (6, 41, 45). In addition, efflux-mediated macrolide resistance has been reported in gonococci (13), although in gonococci, the system described was not specific for macrolides and the level of resistance mediated by the efflux system was increased only fourfold. In addition, MdfA, a multidrug resistance protein in *E. coli*, is reported to recognize erythromycin and certain aminoglycosides, although for the *E. coli* strains that expressed MdfA from multicopy plasmids, only two- to fourfold increases in the MICs of these antibiotics were detected (12).

A hallmark of multidrug efflux systems in gram-negative bac-

teria is energy-dependent drug exclusion (29). Thus, collapse of a proton gradient across the cell membrane by the addition of CCCP should result in a marked increase in drug accumulation. Surprisingly, however, efflux of streptomycin was not inhibited by the addition of CCCP. However, we have recently sequenced a region of the *B. pseudomallei* chromosome that encodes a product with strong homology to *emrA* from *E. coli* (20), a locus reported to confer resistance to CCCP. We are examining this homolog in *B. pseudomallei* to determine if this may account for the inability of CCCP to inhibit the efflux capacity of the *amr* locus.

The homology of the *amrR* sequence with the sequences of other multidrug efflux system-negative regulators would suggest that the *amrR* gene product functions in this capacity as well. Construction of *amrR* knockout mutants should confirm this hypothesis.

At least one substrate for the *amr* system, streptomycin, was not able to induce  $\beta$ -galactosidase activity in *amr-lacZ* fusion strains. In addition, stress conditions (4% ethanol or 0.5% NaCl) which have been shown to increase the level of transcription of *acrAB* (22) did not result in the induction of  $\beta$ -galactosidase activity in these strains, indicating some other means of mediating *amr* regulation. Further work is required to identify inducers of the *amr* system.

We have constructed and characterized *B. pseudomallei* DD503, a strain that should greatly enhance the study of genetic determinants of this organism. The results of the studies with animals reported here demonstrate that DD503 exhibits a virulence capacity that is indistinguishable from that of the parent strain, strain 1026b, which is an essential characteristic for a strain that will be used in allelic exchange experiments involving virulence determinants. We have used DD503, in conjunction with pKAS46, in allelic exchange experiments to introduce defined mutations (antibiotic resistance cassettes and deletions) into the flagellin structural gene (*fliC*) and into a genetic locus encoding resistance to serum (10). In addition, many broad-host-range cloning vectors and antibiotic resistance cassettes encode resistance to antibiotics such as kanamycin, gentamicin, and erythromycin as selective markers. These vectors and cassettes are not useful as selective markers in *B. pseudomallei* because it is inherently resistant to these antibiotics by the multidrug resistance pump encoded by the *amrAB oprA* operon. These selective markers may be used in *B. pseudomallei* DD503, however, and should further facilitate the genetic analysis of this organism.

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