INFECTION AND IMMUNITY, Dec. 1997, p. 4972–4977 Vol. 65, No. 12
0019-9567/97/$04.00+0

Identification and Characterization of a Two-Component Regulatory System Involved in Invasion of Eukaryotic Cells and Heavy-Metal Resistance in *Burkholderia pseudomallei*

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Received 13 June 1997/Returned for modification 19 September 1997/Accepted 29 September 1997

***Burkholderia pseudomallei* is the causative agent of melioidosis, a disease increasingly recognized as an important cause of morbidity and mortality in many regions of the world. *B. pseudomallei* is a facultative intracellular pathogen capable of invading eukaryotic cells. We used Tn*5*-OT182 mutagenesis to generate mutants deficient in the ability to invade a human type II pneumocyte cell line (A549 cells). One of these mutants, AJ1D8, exhibited approximately 10% of the ability of the parental strain, 1026b, to invade A549 cells. There was no difference in the abilities of 1026b and AJ1D8 to resist killing by RAW macrophages or the human defensin HNP-1. The nucleotide sequence flanking the Tn*5*-OT182 integration in AJ1D8 was determined, and two open reading frames were identified. The predicted proteins shared considerable homology with two-component regulatory systems involved in the regulation of heavy-metal resistance in other organisms. AJ1D8 was 16-fold more sensitive to Cd^{2+} and twofold more sensitive to Zn^{2+} than was 1026b but was not sensitive to any of the other heavy metals examined. The *B. pseudomallei* two-component regulatory system, termed *irlRS*, complemented the invasion-deficient and heavy-metal-sensitive phenotype of AJ1D8 in *trans*. There was no significant difference between the virulence of AJ1D8 and that of 1026b in infant diabetic rats and Syrian hamsters, suggesting that the *irlRS* locus is probably not a virulence determinant in these animal models of acute *B. pseudomallei* infection.**

Burkholderia pseudomallei is a motile, non-spore-forming, gram-negative bacillus (18, 45). It is the causative agent of melioidosis, a glanders-like disease (43). Melioidosis can be seen as an inapparent infection, an asymptomatic pulmonary infiltration, an acute localized suppurative infection, an acute pulmonary infection (most common), an acute septicemic infection, or a chronic suppurative infection (11, 43). *B. pseudomallei* can disseminate from sites of localized infection, such as the lungs or skin, to virtually any other organ of the body (12). The incubation period is not well defined and has ranged from 2 days to 26 years (27). The disease has the potential for a prolonged latency period, with recrudescence into an acute, fulminating, and fatal infection (22). The latent form of melioidosis is of considerable diagnostic and epidemiological importance. The activation of the latent infection is often associated with concurrent disease or injury. This often leads to difficulty in prompt diagnosis, and in conjunction with the resistance to antimicrobial therapy encountered in chronic forms of melioidosis, the infection often results in significant morbidity and mortality (25, 31).

B. pseudomallei is a natural inhabitant of soil, stagnant water, and rice paddies in areas where it is endemic (37). The main areas of endemicity are Southeast Asia, particularly northeast Thailand, and northern Australia (9). This bacterium is a common cause of opportunistic infections in areas of endemicity (41). The protean manifestations of melioidosis contribute to the difficulty of diagnosis in rural Southeast Asia, where infection is an occupational risk for families working in flooded rice paddies (10). Individuals particularly susceptible to infection include diabetics and those with renal disease (9).

The pathogenesis of melioidosis has not been well defined. Several putative virulence factors have been identified although they have not been well characterized. Putative extracellular virulence factors include a thermolabile toxin, a protease, a lipase, and a lecithinase (1, 39). Cell-associated virulence determinants include lipopolysaccharide, pili, extracellular polysaccharide, and flagella (5, 6, 8, 41). *B. pseudomallei* produces a water-soluble siderophore for iron acquisition from the host, which contributes to its survival and maintenance (46, 47).

B. pseudomallei is resistant to the bactericidal action of normal human serum, which provides a significant survival advantage to the organism, since it may be directly introduced into the blood via cuts or skin abrasions (15, 19). *B. pseudomallei* is a facultative intracellular pathogen capable of the invasion of nonprofessional phagocytes and of intracellular survival and replication in professional phagocytes in vitro (20, 33, 34).

Here we describe the identification and characterization of AJ1D8, an invasion-deficient transposon mutant of *B. pseudomallei* 1026b.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in these studies are described in Table 1. *B. pseudomallei* and *Escherichia coli* were grown at 37°C on Luria broth (LB) agar (Becton Dickinson, Cockeysville, Md.), tryptic soy agar (Boehringer Mannheim, Mannheim, Germany), or in LB. When appropriate, antibiotics were added at the following concentrations: 100 μg of ampicillin (Ap)/ml, 25 μg of kanamycin (Km)/ml, 25 μg of chloramphenicol (Cm)/ml, 12.5 μg of tetracycline (Tc)/ml, 100 μg of streptomycin (Sm)/ml, and 1.5 mg of trimethoprim (Tp)/ml for *E. coli* and 50 μg of tetracycline/ml, 100 μg of streptomycin/ml, and 100 μg of trimethoprim/ml for *B. pseudomallei*. A 100 mg/ml stock solution of trimethoprim was prepared in

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference
Strains		
E. coli		
SM10	Mobilizing strain; transfer genes of RP4 integrated in chromosome; Km^r Sm^s	40
SURE	e14$^-$(*mcrA*) Δ(*mcrCB-hsdSMR-mrr*)*171 endA1 supE44 thi-1 gyrA96 relA1 lac recB recJ sbcC umuC*::Tn*5 uvrC* (F′ *proAB lacI*^{q}*ZΔM15* Tn*10*); Km^r Tc^r	Stratagene
DH5α	F$^-$ ϕ80d *lacZ ΔM15* Δ(*lacZYA-argF*)*U169 endA1 recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*	Gibco BRL
HB101	F$^-$ Δ(*gpt-proA*)62 *leu supE44 ara14 galK2 lacY1* Δ(*mcrC-mrr*) *rpsL20* (Sm^r) *xyl-5 mtl-1 recA13*	3, 36
B. pseudomallei		
1026b	Clinical isolate; Sm^r Tc^s	13
AJ1D8	1026b derivative; *irlR*::Tn*5*-OT182	This study
Plasmids		
pUCP28T	Broad-host-range vector; IncP OriT; pRO1600 ori; Tp^r	38
pOT182	pSUP102(Gm)::Tn*5*-OT182; Cm^r Gm^r Ap^r Tc^r	28
pBluescript SK	General cloning vector; ColE1; Ap^r	Stratagene
pZErO-2.1	Positive selection cloning vector; P_{lac} *lacZ-ccd*; ColE1; Km^r	Invitrogen
pAJ1D8B	13.5-kb *Bam*HI fragment from AJ1D8 obtained by self-cloning; Ap^r Tc^r	This study
pAJ1D8H	19-kb *Hin*dIII fragment from AJ1D8 obtained by self-cloning; Ap^r Tc^r	This study
pALJZ10	pZErO-2.1 containing 2.2-kb PCR product; *irlR irlS*	This study
pALJRS	pUCP28T containing 2.2-kb *Kpn*I-*Xba*I fragment from pALJZ10; Tp^r; *irlR irlS*	This study

N,*N*-dimethylacetamide. Plasmids were isolated from overnight cultures with Wizard Plus minipreps (Promega, Madison, Wis.).

Transposon mutagenesis and plasmid conjugations. The procedures for Tn*5*-OT182 mutagenesis and plasmid conjugations have been previously described (13).

Screen for Tn*5*-OT182 mutants deficient in invasion of A549 cells. Individual transposon mutants were screened for their ability to invade A549 cells. Bacterial mutants were centrifuged onto confluent monolayers of A549 cells and incubated for 2 h. Extracellular bacteria were removed by a 2-h incubation with 150 μg of kanamycin/ml. Monolayers were then lysed with 0.1% Triton X-100 (BDH Chemicals, Toronto, Canada), and the lysate was spread onto one-fourth of an LB agar plate containing streptomycin and tetracycline. Mutants of *B. pseudomallei* deficient in the ability to invade A549 cells remained extracellular and were preferentially killed by the kanamycin. Invasion-deficient mutants were thus recovered in far lower numbers than the parent strain. Quantitative invasion assays were performed on putative invasion-deficient mutants, using previously described methods (20). Briefly, 5×10^5 A549 cells were incubated with 25 μl of bacteria diluted to give a multiplicity of infection of approximately 10 bacteria per A549 cell. After a 2-h incubation to allow for bacterial entry, extracellular bacteria were killed by an additional 2-h incubation in medium containing 150 μg of kanamycin/ml. A549 cells were then lysed, and intracellular bacteria were quantitated by plating serial dilutions of the lysate. All quantitative invasion assays were performed in triplicate and repeated on at least three occasions.

Survival of *B. pseudomallei* in RAW macrophages. The ability of *B. pseudomallei* AJ1D8 to survive intracellularly and replicate in RAW cells was assessed by using a modification of the methods of Bowe and Heffron (2). RAW cells were obtained from the American Type Culture Collection (Rockville, Md.) and maintained in Dulbecco's modified Eagle's medium (DMEM) (Bio Whittaker, Walkersville, Md.) containing a standard antibiotic mixture (100 U of penicillin, 0.1 mg of streptomycin, and 0.25 μg of amphotericin B per ml; Sigma Chemical Co., St. Louis, Mo.) plus 10% fetal bovine serum (Gibco Canada Inc., Mississauga, Canada) at 37°C in 5% CO_2.

RAW cells were seeded on Thermanox 15-mm-diameter round plastic coverslips (Nunc, Inc., Napersville, Ill.) in a petri dish. Nine coverslips were placed aseptically in a sterile 60- by 20-mm petri dish. RAW cells were adjusted to a concentration of 2×10^6 in 6 ml of DMEM with 10% fetal calf serum without antibiotics and were added to the petri dish. Macrophages were allowed to grow to confluency overnight. Five milliliters of the medium was removed from the petri dish containing the coverslips and added to appropriately diluted overnight cultures of bacteria. A total of 2×10^7 bacteria were added to each petri dish. After a 1-h incubation, the medium was removed and the coverslips were washed three times with phosphate-buffered saline. The macrophages were incubated for 2 h in fresh medium containing kanamycin (150 μg/ml) to kill extracellular bacteria. Coverslips were removed in triplicate, and the macrophage monolayer was lysed by vortexing the coverslips in 5 ml of sterile distilled H_2O. Viable intracellular bacteria were quantitated by plating serial dilutions of the lysate. To assess intracellular survival and growth, the media in duplicate petri dishes were replaced with DMEM containing 20 μg of kanamycin/ml. The number of viable intracellular bacteria at 3 and 20 h postinfection was determined by plating serial dilutions of the lysate. Macrophage viability was monitored throughout the experiment by trypan blue dye exclusion. The ability of the RAW macrophages to undergo a respiratory burst was confirmed by the methods of Bowe and Heffron (2).

Activity of HNP-1 against *B. pseudomallei*. The bactericidal activity of human neutrophil peptide 1 (HNP-1) was tested against *B. pseudomallei* strains and *E. coli* HB101 by the methods of Harwig et al. (17). HNP-1 was generously provided by M. Selsted of the University of California, Irvine. HNP-1 was stored as a 1 mg/ml stock solution in 0.01% acetic acid at −20°C. Briefly, mid-logarithmic-phase cells were washed in 10 mM phosphate and adjusted to a concentration of 5×10^6 CFU/ml. Bacteria were incubated in 10 mM phosphate buffer containing 50 μg of HNP-1/ml or an equivalent volume of 0.01% acetic acid as a control. After 2 h at 37°C, the reactions were stopped by the addition of 0.15 M NaCl and the CFU were measured by plating serial dilutions on tryptic soy agar plates. Each incubation was performed in triplicate.

DNA manipulation and sequencing. DNA manipulation and general molecular biology procedures were described previously (13). Automated DNA sequencing was performed by the University Core DNA Services (University of Calgary, Calgary, Canada) with an ABI PRISM DyeDeoxy termination cycle sequencing system and AmpliTaq DNA polymerase (Perkin-Elmer, Norwalk, Conn.). DNA sequencing reactions were analyzed with an ABI 373A DNA sequencer. DNA sequence reactions were initiated with previously described primers (13). Custom oligodeoxyribonucleotide primers were synthesized at University Core DNA Services. DNA sequence analysis of *irlR* and *irlS* was aided by sequencing exonuclease III-generated deletions of subclones (Erase-a-Base; Promega) and by using a primer-walking strategy.

Sequence analysis. DNA and protein sequences were analyzed with GeneJockey version 1.20 software for the Macintosh and the University of Wisconsin Genetics Computer Group package (14). The BLASTX and BLASTP programs were used to search the nonredundant sequence database for homologous proteins (26). Alignment of amino acid sequences was performed with the Genetics Computer Group programs PileUp and BestFit.

β-Galactosidase assays. Overnight *B. pseudomallei* cultures were diluted 1:100 in LB and grown for an additional 5 h to reach mid-logarithmic phase, and 1-ml aliquots were removed for determination of β-galactosidase activity as previously described (29). The optical density at 600 nm was determined for each aliquot.

MICs of metal cations for *B. pseudomallei*. *B. pseudomallei* was grown overnight in Mueller-Hinton broth, diluted 1/1,000, and used to inoculate tubes containing twofold serial dilutions of metal cations in Mueller-Hinton broth. The cultures were incubated at 37°C overnight, and the MIC of each cation was determined as the lowest concentration of cation that inhibited the growth of *B. pseudomallei.*

PCR amplification of the wild-type *irlRS* locus. PCR amplification of a 2.2-kb product containing *irlR* and *irlS* was accomplished by using the GeneAmp PCR system 9600 (Perkin-Elmer). The oligodeoxyribonucleotides irl-lt (5′-ATCATC GAGGTGAATCCAGA-3′) and irl-rt (5′-CTCGATCAGCACGATCAAAC-3′) were used to amplify the 2.2-kb fragment from *B. pseudomallei* 1026b chromosomal DNA by PCR with *Taq* DNA polymerase (Gibco-BRL) and the PCR optimizer kit (Invitrogen, Carlsbad, Calif.). Amplification of the desired product required the addition of dimethyl sulfoxide at a final concentration of 10% to the PCRs. The conditions for PCR involved a 4-min denaturation step at 97°C followed by 30 cycles of 97°C for 10 s, 55°C for 45 s, and 72°C for 60 s. The PCR was then held at 72°C for 5 min. The PCR product contained 19 bp upstream and 136 bp downstream of the *irlRS* locus.

Cloning of the wild-type *irlRS* locus and complementation of the invasion-deficient phenotype and metal sensitivity of AJ1D8. The 2.2-kb PCR product containing *irlR* and *irlS* was cloned into the *Eco*RV site of the positive selection vector pZErO-2.1, creating the plasmid pALJZ10. This plasmid was used to transform *E. coli* DH5α, and kanamycin-resistant clones were selected. The 2.2-kb fragment from pALJZ10, containing *irlR* and *irlS*, was cloned into the broad-host-range plasmid pUCP28T. The pUCP28T derivative pALJRS was delivered to *B. pseudomallei* AJ1D8 via conjugation with *E. coli* SM10(pALJRS).

TABLE 2. Invasion of A549 cells by *B. pseudomallei* 1026b and AJ1D8[a]

Strain	Expt 1	Expt 2	Expt 3
1026b	$1.9 \times 10^4 \pm 2.8 \times 10^3$	$1.7 \times 10^4 \pm 2.1 \times 10^3$	$2.3 \times 10^4 \pm 9.2 \times 10^3$
AJ1D8	$1.3 \times 10^3 \pm 9.0 \times 10^{2b}$ (6.7)	$1.9 \times 10^3 \pm 1.8 \times 10^{2b}$ (11.0)	$1.8 \times 10^2 \pm 5.0 \times 10^{1b}$ (0.8)

[a] Data are expressed as mean number of intracellular organisms ± standard error of the means of three wells. Numbers in parentheses represent the relative percent invasion compared to that of 1026b.
[b] $P < 0.05$ compared to 1026b.

The invasion ability and heavy-metal sensitivity of one transconjugant, AJ1D8 (pALJRS), were determined as described above.

Animal studies. The virulence of the invasion-deficient mutant AJ1D8 was assessed in two animal models of acute *B. pseudomallei* infection. By the methods of Brett et al. (4), female Syrian hamsters were inoculated intraperitoneally with 100 μl of one of a number of serial dilutions of exponential-phase cultures adjusted appropriately with sterile phosphate-buffered saline. Five animals were used per dilution. The 50% lethal doses (LD_{50}s) for AJ1D8 and the parent strain, 1026b, were calculated 2 days after inoculation according to the methods of Reed and Muench (35).

By the methods of Woods et al. (44), the virulence of *B. pseudomallei* AJ1D8 was compared to the parent strain, 1026b, in infant diabetic rats. Infant Sprague-Dawley rats (average weight, 30 g) were made diabetic with streptozocin and inoculated intraperitoneally with 100 μl of a dilution of *B. pseudomallei* 1026b or AJ1D8. Five animals were used per dilution, and LD_{50}s were calculated at 7 days (35).

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been deposited in the GenBank database under the accession no. AF005358.

RESULTS AND DISCUSSION

Identification of the invasion-deficient mutant AJ1D8. The human pulmonary carcinoma cell line A549 possesses the morphological and biochemical characteristics of type II pneumocytes of the intact lung (24). The interaction of *B. pseudomallei* with A549 cells, an established pulmonary epithelial cell line, provides a means to study the early phase of human respiratory tract infection by this organism. Tn*5*-OT182 mutants of *B. pseudomallei* 1026b were screened for their ability to invade A549 cells as described in Materials and Methods. Approximately 1,000 Tn*5*-OT182 mutants were screened, and one invasion-deficient mutant, AJ1D8, was identified and used in further studies. The ability of AJ1D8 to invade A549 cells in a quantitative invasion assay is shown in Table 2. In three separate experiments, AJ1D8 exhibited invasion levels that were 11% or less of the invasion level of the parental strain, 1026b (Table 2). The ability of AJ1D8 to invade epithelial cell lines other than A549 was also tested. AJ1D8 exhibited invasion levels that were approximately 10% of those of 1026b in HeLa and CHO cells (data not shown). Thus, the inability of AJ1D8 to invade eukaryotic cells appeared to be a general phenomenon and not specific to A549 cells.

Survival of AJ1D8 in RAW macrophages. We have previously demonstrated the intracellular survival of *B. pseudomallei* in professional phagocytic cells (20). Intracellular survival of AJ1D8 in RAW macrophages did not differ significantly from that of 1026b. Equivalent numbers of 1026b and AJ1D8 organisms were present intracellularly in the macrophages after the initial incubation. Lysates of macrophages contained approximately 2×10^7 AJ1D8 or 1026b organisms per ml. Equivalent numbers of AJ1D8 (2.8×10^5 CFU/ml) and 1026b (4.4×10^5 CFU/ml) organisms were present intracellularly in the macrophages after a 20-h incubation. Macrophage viability at all time points exceeded 95% as measured by trypan blue dye exclusion. In addition, the cultured RAW macrophages were found to be capable of undergoing a respiratory burst (data not shown). These results demonstrate that the locus disrupted by Tn*5*-OT182 in AJ1D8 is not important for survival intracellularly in cultured macrophages. The defect in AJ1D8 appears to be specific to invasion of nonprofessional phagocytes.

Activity of HNP-1 against AJ1D8. *B. pseudomallei* is highly resistant to killing mediated by cationic peptides such as polymyxin B, protamine, and the human defensin HNP-1 (16, 20). The activity of HNP-1 against *B. pseudomallei* AJ1D8 was determined. AJ1D8 did not differ significantly from 1026b in its ability to resist the antimicrobial effects of HNP-1. *B. pseudomallei* AJ1D8 was unaffected by HNP-1, exhibiting a 0.4 $\log_{10}$ increase in CFU during the 2-h incubation with HNP-1. Similarly, *B. pseudomallei* 1026b exhibited a 0.54 $\log_{10}$ increase in CFU during the incubation with HNP-1. Viable CFU of *E. coli* HB101 decreased a statistically significant 0.74 $\log_{10}$ ($P < 0.05$ by Student's *t* test) during the 2-h incubation. These results demonstrate that the locus disrupted by Tn*5*-OT182 in AJ1D8 is not essential for resistance to the human defensin HNP-1.

Nucleotide sequence analysis of *irlR* and *irlS*. The DNA flanking Tn*5*-OT182 in AJ1D8 was isolated by self-cloning (28). Chromosomal DNA was digested with *Hin*dIII or *Bam*HI, ligated, and used to transform *E. coli* SURE. The resulting plasmids were named pAJ1D8H and pAJ1D8B, respectively (Table 1). The nucleotide sequence of a 2,650-bp region from these plasmids indicated that AJ1D8 contains a Tn*5*-OT182 integration within a gene encoding a putative two-component response regulator homolog, which was named *irlR* for invasion-related locus (Fig. 1). Sequencing of the DNA downstream of *irlR* revealed the presence of *irlS*, a gene encoding a putative two-component sensor protein homolog (Fig. 1).

The nucleotide sequences of *irlR* and *irlS* were determined on both strands. The G+C content of this region was 69%, which was similar to the 68% G+C content of the *B. pseudomallei* ATCC 23343 genome (45). *irlR* was 689 nucleotides in length, and a putative ribosome binding sequence, GAG, was located 11 to 13 bp upstream of the *irlR* start codon. The Tn*5*-OT182 integration in AJ1D8 was 62 bp downstream of the ATG start codon of *irlR* (Fig. 1). *irlS* was 1,394 nucleotides in length, and the ATG start codon for *irlS* overlapped the TGA stop codon for *irlR*. A putative ribosome binding sequence, GAG, was located 4 to 6 bp upstream of the *irlS* start codon. The nucleotide sequences upstream of *irlR* (160 bp) and downstream of *irlS* (409 bp) were also determined.

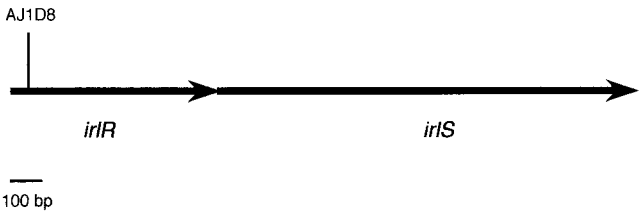

FIG. 1. Schematic representation of the *irlRS* locus. The arrows represent the locations and directions of transcription of *irlR* and *irlS*. The location of the Tn*5*-OT182 integration in AJ1D8 is also shown.

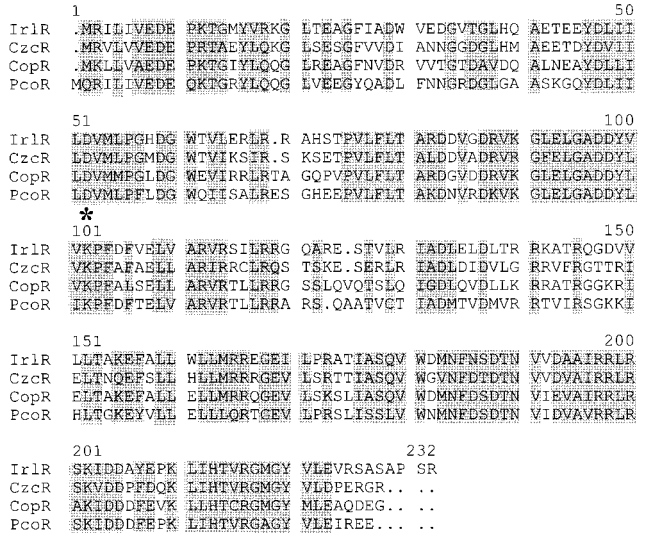

FIG. 2. Alignment of IrlR with regulatory proteins from other two-component systems. The proteins are *A. eutrophus* CzcR (42), *P. syringae* CopR (30), and *E. coli* PcoR (7). Residues conserved in at least three sequences are shaded. The putative phosphorylation site (aspartic acid residue, position 52) is indicated by an asterisk. Periods indicate gaps inserted in the amino acid sequence to optimize alignment.

Tn*5*-OT182 contains a promoterless *lacZ* reporter gene that allows for the formation of promoter fusions when the transposon integrates downstream of a functional promoter (13, 28). The nucleotide sequence data suggested that the Tn*5*-OT182 integration in AJ1D8 did not form a *irlR-lacZ* transcriptional fusion. We found that AJ1D8 produced only background levels of β-galactosidase (14 Miller units), and therefore it does not contain a *lacZ* transcriptional fusion. Thus, the orientation of the predicted gene with regard to Tn*5*-OT182 was confirmed by the β-galactosidase assay.

IrlR and IrlS are homologous to two-component sensor-responder proteins involved in regulating resistance to heavy metals. The protein encoded by *irlR* was predicted to be 229 amino acids in length. Figure 2 depicts the alignment of IrlR with regulatory proteins from other two-component systems involved in regulating resistance to heavy metals. Residues conserved in at least three of the sequences are indicated. The proteins are *Alcaligenes eutrophus* CzcR (42), *Pseudomonas syringae* CopR (30), and *E. coli* PcoR (7). *A. eutrophus* CzcR is a two-component response activator of genes that mediate resistance to Cd^{2+}, Zn^{2+}, and Co^{2+} (42). *P. syringae* CopR and *E. coli* PcoR are two-component response activators of genes that mediate resistance to Cu^{2+} (7, 30). The conserved aspartic acid residue present in all four proteins at amino acid position 52 is thought to be the phosphorylation site important for regulatory activation (Fig. 2). IrlR exhibits 61, 64, and 62% identity with CzcR, CopR, and PcoR, respectively.

The protein encoded by *irlS* was predicted to be 464 amino acids in length. Figure 3 depicts the alignment of IrlS with sensor proteins from other two-component systems involved in regulating resistance to heavy metals. The proteins are *A. eutrophus* CzcS (42), *P. syringae* CopS (30) and *E. coli* PcoS (7). *A. eutrophus* CzcS is likely to be a two-component sensor kinase that regulates resistance to Cd^{2+}, Zn^{2+}, and Co^{2+} (42). Similarly, *P. syringae* CopS and *E. coli* PcoS are two-component sensor kinases that regulate resistance to Cu^{2+} (7, 30). There are three conserved regions in sensors of the two-component family of regulatory proteins, and these are indicated in Fig. 3. All four proteins contain the putative histidine autophosphorylation residue at position 262 (Fig. 3). IrlS exhibits 35, 31, and 28% identity with CzcS, CopS, and PcoS, respectively. The hydropathicity profile of the predicted amino acid sequence of IrlS was generated by using the algorithm of Kyte and Doolittle (23). Hydrophobic regions of 15 or more amino acids were present from amino acids 9 to 35 and 167 to 193, suggesting the presence of two membrane-spanning regions in IrlS.

The *P. syringae* CopR-CopS and *E. coli* PcoR-PcoS two-component systems regulate the transcription of *copABCD* and *pcoABCD*, respectively (7, 30). The proteins encoded by these genes are highly homologous, and they mediate resistance to

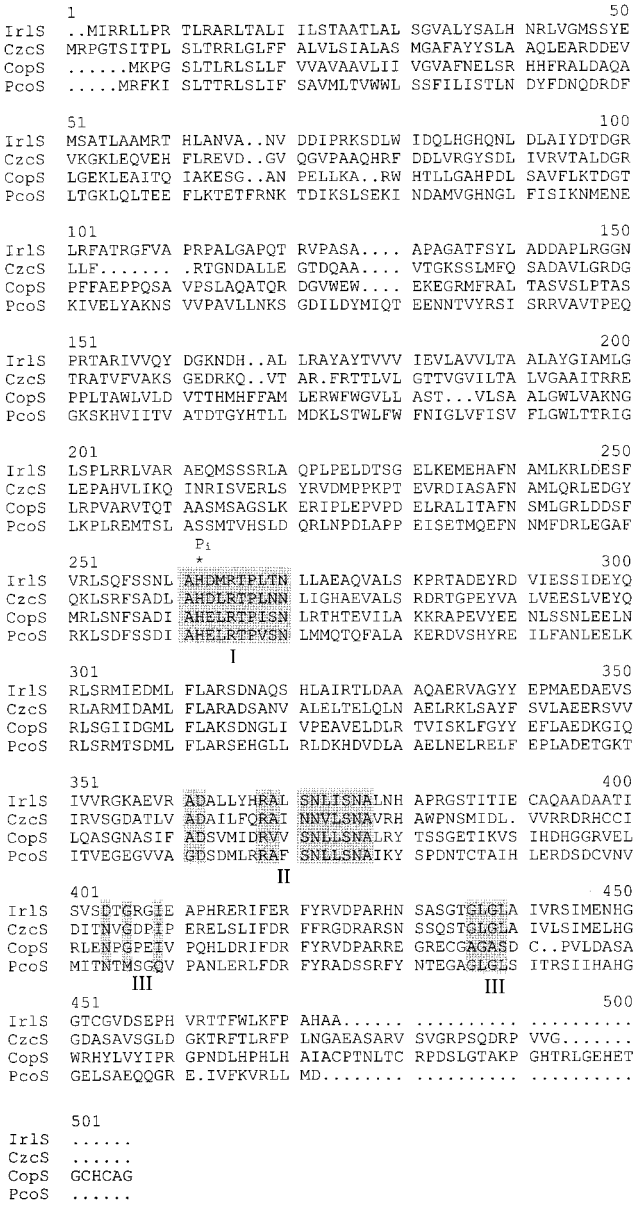

FIG. 3. Alignment of IrlS with sensor proteins from other two-component systems. The proteins are *A. eutrophus* CzcS (42), *P. syringae* CopS (30), and *E. coli* PcoS (7). Three conserved regions present in the primary amino acid sequences of two-component sensor proteins are shaded and labeled I, II, and III. The putative autophosphorylation residue (histidine) at position 262 is indicated (P_i). Periods indicate gaps inserted in the amino acid sequence to optimize alignment.

Cu^{2+} by sequestering it in the periplasm and/or exporting it out of the cell. The *A. eutrophus* CzcR-CzcS two-component system regulates the transcription of *czcCBA*, which encodes a cation-proton antiporter that confers resistance to Co^{2+}, Zn^{2+}, and Cd^{2+} (32, 42). In all three systems, the genes encoding the two-component systems are found downstream of the genes the systems regulate (7, 30, 42). We have recently identified an open reading frame immediately upstream of *irlRS* that encodes a protein with considerable homology to *A. eutrophus* CzcA (21).

MICs of metal cations. On the basis of the similarities between IrlS and IrlR and other two-component sensor-responder proteins involved in regulating resistance to heavy metals, the effects of various ions on the growth of 1026b and AJ1D8 were examined. The metal ions tested included Cu^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, and Mg^{2+}. There was no difference between the MIC for the invasion-deficient mutant AJ1D8 and that for 1026b of Cu^{2+} (9.6 mM), Co^{2+} (1.2 mM), Ni^{2+} (4.8 mM), or Mg^{2+} (>38.4 mM). There was a 16-fold difference between the MICs of Cd^{2+} for 1026b and AJ1D8. The MICs of Cd^{2+} were determined to be 9.6 mM for 1026b and 0.6 mM for AJ1D8. There was also a twofold difference between the MICs of Zn^{2+} for 1026b and AJ1D8. The MICs of Zn^{2+} were determined to be 4.8 mM for 1026b and 2.4 mM for AJ1D8. These results suggest that IrlR and IrlS may be involved in the regulation of two distinct phenotypes, invasion and heavy-metal (Cd^{2+} and Zn^{2+}) resistance. The ability of *B. pseudomallei* to invade cultured epithelial cell lines has been described previously (20), but to our knowledge this is the first report describing heavy-metal resistance in this organism.

Complementation of the invasion-deficient phenotype of AJ1D8. The plasmid pALJRS was assessed for its ability to complement the invasion-deficient phenotype of AJ1D8 in *trans*. Approximately 1.5×10^3 AJ1D8(pUCP28T) organisms entered A549 cells compared to 4.0×10^4 AJ1D8(pALJRS) organisms and 1.5×10^4 1026b(pUCP28T) organisms. The relative percent invasion of AJ1D8(pUCP28T) was 10% of that of 1026b(pUCP28T). This was similar to the results obtained with 1026b and AJ1D8 without the vector pUCP28T (Table 2). In comparison, the relative percent invasion of AJ1D8(pALJRS) was 267% of that of 1026b(pUCP28T). Thus, pALJRS restored the ability of AJ1D8 to invade A549 cells to a level greater than that of the parental strain. Invasion was unaffected by the presence of pUCP28T, as AJ1D8 containing pUCP28T alone was invasion deficient. Additionally, 1026b containing pUCP28T exhibited wild-type invasion levels. These results strongly suggest that *irlR* and *irlS* are integral *B. pseudomallei* invasion determinants. Furthermore, the mutation in AJ1D8 does not have a polar effect on genes downstream of the *irlRS* locus. The insert containing *irlR* and *irlS* was cloned into pUCP28T in an orientation such that the expression of *irlR* and *irlS* would be driven by the *lacZ* promoter. Additionally, the putative ribosome binding site was included on the cloned insert. It is likely that the *irl* gene products are overexpressed due to the fact that their expression was driven by the *lacZ* promoter and that pUCP28T is a high-copy-number plasmid. Thus the invasion level of AJ1D8 (pALJRS) was greater than that of 1026b(pUCP28T).

Complementation of the Cd^{2+} sensitivity of AJ1D8. The plasmid pALJRS was also tested for its ability to complement the Cd^{2+} sensitivity of *B. pseudomallei* AJ1D8. The MICs of Cd^{2+} were 2.4 mM and 0.3 mM for 1026b(pUCP28T) and AJ1D8(pUCP28T), respectively. In comparison, the MIC of Cd^{2+} was 2.4 mM for AJ1D8(pALJRS). Thus pALJRS was able to complement the Cd^{2+} sensitivity of AJ1D8 in *trans*. It should be noted that the Cd^{2+} MICs for 1026b and AJ1D8 were 9.6 and 0.6 mM, respectively (Table 2). The lower MICs obtained for 1026b and AJ1D8 containing pUCP28T could be a result of the presence of trimethoprim in the assay, or the vector itself could affect the sensitivity of *B. pseudomallei* to Cd^{2+}. It is interesting that the putative overexpression of *irlRS* from pALJRS did not result in an increase in resistance to Cd^{2+} in AJ1D8(pALJRS) compared to that of 1026b(pUCP28T). Taken together, the results of the complementation assays clearly demonstrate the importance of the *irlRS* locus in heavy-metal resistance and invasion.

Studies are in progress to characterize the gene(s) upstream of *irlRS* and determine if it is regulated by IrlR-IrlS and if it is important in mediating Cd^{2+} and Zn^{2+} resistance and invasion. It is also possible that *B. pseudomallei* heavy-metal resistance and invasion are encoded by separate genes that are both regulated by the IrlR-IrlS two-component system. Further studies are required to fully understand the role of IrlR-IrlS in heavy-metal resistance and invasion.

Relative virulence of AJ1D8 in Syrian hamsters and diabetic infant rats. The virulence of the invasion-deficient mutant AJ1D8 was assessed in two animal models of acute *B. pseudomallei* infection, Syrian hamsters (4) and diabetic infant rats (44). In hamsters, there was no difference between the LD_{50} of the invasion-deficient mutant AJ1D8 (<10 organisms) and that of the parent strain, 1026b (<10 organisms). The hamsters progressed to death very rapidly (48 h), and it was not surprising that the ability to invade eukaryotic cells did not appear to play a role in this acute *B. pseudomallei* infection model. Additionally, in the studies performed with diabetic infant rats, also an acute *B. pseudomallei* infection model, there was no difference detected between the LD_{50} of AJ1D8 (1.9×10^4 organisms) and that of 1026b (1.9×10^4 organisms) after 7 days. These results suggest that the *irlRS* locus is not required for virulence in experimental animal models of acute infection.

Currently, our laboratory is developing an animal model of chronic *B. pseudomallei* infection. It will be of significant interest to test invasion-deficient mutants in this model, where one could predict that a pronounced difference in virulence between the mutant and parent strains would be observed.

ACKNOWLEDGMENTS

This work was supported by the Canadian Bacterial Diseases Network of Centers of Excellence and by the Canadian Department of National Defense contract number W7702-5-R512/01-XSG. D.D. is the recipient of an Alberta Heritage Foundation for Medical Research Fellowship award.

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Editor: J. G. Cannon