INFECTION AND IMMUNITY, July 2004, p. 4172–4187 Vol. 72, No. 7
0019-9567/04/$08.00+0 DOI: 10.1128/IAI.72.7.4172–4187.2004

Contribution of Gene Loss to the Pathogenic Evolution of *Burkholderia pseudomallei* and *Burkholderia mallei*

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Received 25 February 2004/Returned for modification 9 March 2004/Accepted 12 March 2004

***Burkholderia pseudomallei* is the causative agent of melioidosis. *Burkholderia thailandensis* is a closely related species that can readily utilize L-arabinose as a sole carbon source, whereas *B. pseudomallei* cannot. We used Tn*5*-OT182 mutagenesis to isolate an arabinose-negative mutant of *B. thailandensis*. Sequence analysis of regions flanking the transposon insertion revealed the presence of an arabinose assimilation operon consisting of nine genes. Analysis of the *B. pseudomallei* chromosome showed a deletion of the operon from this organism. This deletion was detected in all *B. pseudomallei* and *Burkholderia mallei* strains investigated. We cloned the *B. thailandensis* E264 arabinose assimilation operon and introduced the entire operon into the chromosome of *B. pseudomallei* 406e via homologous recombination. The resultant strain, *B. pseudomallei* SZ5028, was able to utilize L-arabinose as a sole carbon source. Strain SZ5028 had a significantly higher 50% lethal dose for Syrian hamsters compared to the parent strain 406e. Microarray analysis revealed that a number of genes in a type III secretion system were down-regulated in strain SZ5028 when cells were grown in L-arabinose, suggesting a regulatory role for L-arabinose or a metabolite of L-arabinose. These results suggest that the ability to metabolize L-arabinose reduces the virulence of *B. pseudomallei* and that the genes encoding arabinose assimilation may be considered antivirulence genes. The increase in virulence associated with the loss of these genes may have provided a selective advantage for *B. pseudomallei* as these organisms adapted to survival in animal hosts.**

Burkholderia pseudomallei is an environmental saprophyte that has been isolated widely from soil in Southeast Asia, and the relationship between environmental contamination and clinical melioidosis has been established. White and colleagues reported that two distinct types of *B. pseudomallei*, differentiated by the ability to assimilate L-arabinose but with similar morphologies and antigenicities, could be isolated from soil in Thailand and that the arabinose assimilation property of *B. pseudomallei* was one of the determinants indicating virulence of this organism (31). Subsequently, our investigators designated the arabinose-positive *B. pseudomallei* biotype as a separate species, *Burkholderia thailandensis*, based upon a number of significant genetic and phenotypic dissimilarities between *B. pseudomallei* and *B. thailandensis*, including differences in 16S rRNA, arabinose assimilation, ethanol assimilation, and secreted protein profiles (8).

Ongoing discussions surround the evolutionary relationships among the *Burkholderia* spp., particularly regarding the origins of *B. thailandensis*, *B. pseudomallei*, and *B. mallei* and their relationships to one another. The most prevalent view is that *B. pseudomallei* and *B. thailandensis* evolved from a common ancestor, probably a soil saprophyte. Spratt and colleagues have recently proposed that *B. mallei* is a clone of *B. pseudomallei*, based upon results from multilocus sequence typing of a large number of *B. pseudomallei* strains but only a small number of *B. mallei* strains (17). Thus, it is conceivable that *B. mallei* could have evolved from *B. pseudomallei*. What is clear, however, is that *B. pseudomallei* and *B. mallei* are significant pathogens while *B. thailandensis* is not, and neither *B. pseudomallei* nor *B. mallei* assimilates arabinose.

In the present studies, we provide experimental data that arabinose assimilation is an antivirulence property, and the genes encoding arabinose assimilation should be termed antivirulence genes. Maurelli and colleagues initially defined the concept of gene loss in the evolution of bacterial pathogens from commensals as a mechanism of fine-tuning pathogen genomes for maximal fitness in new host environments (22). In our studies, we have examined the nature of the genes comprising the *B. thailandensis* arabinose assimilation operon and how deletion of these genes has contributed to the evolution of *B. pseudomallei* and *B. mallei* as pathogens.

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MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are described in Table 1. In addition to those listed in Table 1, the following *B. pseudomallei* strains were used: K96243, 1026b, 576, E8, E12, E13,

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
E. coli strains		
HB101	General cloning	6
TOP10	General cloning and blue-white screening	Invitrogen
SM10λ*pir*	SM10 with a λ prophage carrying the π protein; Km^r	30
B. thailandensis strains		
E264	Soil isolate from central Thailand	7
DW503	E264 derivative; Δ(*amr-oprA*) *rpsL* (Sm^r) Tc^s	9
DD5026	E264::pDD157; Tc^r	This study
RM600	DW503 derivative; *araC*::Tn*5*OT182 Ara^- Tc^r	This study
RM601	DW503 derivative; *araC*::pGSV3-*lux* Gm^r	This study
RM602	DW503 derivative; *araE*::pGSV3-*lux* Gm^r	This study
RM603	DW503 derivative; *araI*::pGSV3 Gm^r	This study
RM604	DW503 derivative; *araA*::pGSV3 Gm^r	This study
B. pseudomallei strains		
1026b	Clinical isolate; AG^r Tc^s	12
K96243		
DD503	1026b derivative; Δ(*amrR-oprA*) *rpsL* (Sm^r) AG^s Tc^s	24
406e	Tc^s	Clinical isolate, Thailand
316c	Tc^s	Clinical isolate, Thailand
SZ5026	406e::pDD157; Tc^r	This study
SZ5028	406e::pDD5026H; Tc^r	This study
Plasmids		
pDD157	1,136-bp *araA* cloned into pSKM11; Ap^r Tc^r	This study
pDD5026H	13.5-kb arabinose assimilation operon from DD5026 in pSKM11 via self-cloning; Ap^r Tc^r	This study
pCR2.1-TOPO	3.9-kb TA cloning vector; pMB1 oriR; Ap^r Km^r	Invitrogen
pGSV3	Mobilizable suicide vector; OriT Gm^r	10
pGSV3-*lux*	Mobilizable suicide vector containing *lux* operon from pSC26; OriT Gm^r	This study
pCS26-Pac	Reporter plasmid containing *luxCDABE* of *Photorhabdus luminescens*	4
pRM601Int	pGSV3-*lux* containing 465-bp internal fragment from *araC*, used to construct RM601	This study
pRM602Int	pGSV3-*lux* containing 676-bp internal fragment from *araE*, used to construct RM602	This study
pRK2013	Self-transmissible helper plasmid; Km^r	16
pSKM11	Mobilizable suicide vector; Tc^r Amp^r	23

112c, 238, 295, 296, 713, and 730. Additional *B. thailandensis* strains included E27, E30, E32, E96, E100, E105, E111, E120, E125, E132, and E135. Additional *B. mallei* strains included NCTC 10248, NCTC 10229, NCTC 10260, NCTC 10247, NCTC 120, and ATCC 23344. Bacterial strains were routinely grown at 37°C in Luria-Bertani broth (LB) or on LB agar plates. In some cases M9 broth (Difco) or M9 agar was used with filter-sterilized carbon sources added to a final concentration of 0.4 or 1.0%. Bacteria used for microarray analysis were grown in M9 supplemented with the appropriate carbon source at 1.0% and harvested during exponential growth. Antibiotics were used at the following concentrations for *B. pseudomallei* and *B. thailandensis*: polymyxin B (PXB), 100 μg/ml; gentamicin (GEN) and kanamycin, 25 μg/ml; and tetracycline (TET), 50 μg/ml. For *Escherichia coli* the following antibiotics were used: ampicillin and carbenicillin, 100 μg/ml; GEN and kanamycin, 25 μg/ml; and TET, 15 μg/ml.

Tn*5*-OT182 mutagenesis and isolation of an L-arabinose utilization mutant. To identify genes involved in arabinose utilization, *B. thailandensis* E264 was mutagenized with Tn*5*-OT182 (12), and mutants unable to grow using L-arabinose as a sole carbon source were identified. Tn*5*-OT182 mutagenesis was performed as previously described, except that transposon mutants were selected on LB agar containing PXB and TET. Mutants were subsequently replica plated to LB-TET and M9-TET-arabinose agar plates. Colonies unable to grow on arabinose plates were tested for growth on M9-glucose to eliminate mutants which were generally defective in carbohydrate metabolism. One mutant, RM600, was isolated that was able to grow on LB and M9-glucose but not on M9-arabinose.

DNA sequencing and analysis. DNA flanking the Tn*5*-OT182 integration in RM600 was isolated by self-cloning and was sequenced by the University Core DNA Services (University of Calgary, Calgary, Alberta, Canada) and by ACGT, Inc. (Northbrook, Ill.). BLAST analysis (1) of the sequenced DNA was performed using programs provided by the National Center for Biotechnology Information.

Construction of pGSV3 insertional inactivation mutants. Specific arabinose assimilation operon genes were insertionally inactivated using the GEN-resistant suicide vector pGSV3 (Table 1) (10). Typically, an approximately 500-bp internal region of the target gene was PCR amplified and cloned into pCR2.1-TOPO (Invitrogen). The cloned fragment was excised using EcoRI, gel purified, and ligated into EcoRI-restricted, calf intestinal phosphatase (CIP)-treated pGSV3. A portion of the ligation was transformed into chemically competent *E. coli* TOP10 cells, and cells were plated onto LB-GEN selective medium. Transformants were screened for the pGSV3-containing insert, and plasmid DNA from positive clones was then used to transform *E. coli* SM10λ*pir*. Plasmids were then conjugated into *B. thailandensis* DW503, and transconjugants with pGSV3 integrated in the chromosome via homologous recombination were selected on LB-GEN-PXB medium. Confirmation of the proper insertional inactivation event was performed by PCR with one of the primers originally used to amplify the internal fragment and a primer internal to pGSV3.

Construction of pGSV3-*lux* and *lux* fusions. The *lux*-based suicide vector pGSV3-*lux* was constructed by excising the *lux* operon from pCS26-Pac and ligating it into the NotI Site in pGSV3. pCS26-Pac was digested with NotI and electrophoresed on a 1% agarose gel. A 5.8-kb band corresponding to the *lux* operon was excised from the gel, purified, and ligated into NotI-digested, CIP-treated pGSV3. The ligation mix was used to transform chemically competent *E. coli* TOP10 cells, and transformants were subsequently screened for *lux*-mediated light production to identify clones containing a functional *lux* operon. Light production was measured from 200 μl of cells using a SystemSure luminometer

(Nova Biomedical, Waltham, Mass.). The orientation of the *lux* operon was determined using PCR.

lux fusions were constructed in a similar manner as the pGSV3 insertional inactivation mutants. An internal region of approximately 500 bp was PCR amplified from the sequence encoding arabinose dehydrogenase (*araE*) and the sequence encoding α-ketoglutarate semialdehyde dehydrogenase (α-KSAD; *araC*) and cloned into pCR2.1-TOPO. The PCR products were excised from pCR2.1-TOPO and ligated into EcoRI-digested, CIP-treated pGSV3-*lux* as described above and transformed into *E. coli* SM10λ*pir*. Conjugations with *E. coli* SM10λ*pir*(pRM602Int) and *E. coli* SM10λ*pir*(pRM601Int) with *B. thailandensis* DW503 were performed, and transconjugants were selected on LB-GEN-PXB agar. Transconjugants were screened for *lux*-mediated light production by assaying 200 μl of overnight broth cultures of individual colonies. Cultures which displayed light production were then tested for insertion of pGSV3-*lux* into the target gene using PCR as described above. Representative mutants RM601 and RM602 were chosen for further study.

Induction of arabinose dehydrogenase and α-KSAD in *B. thailandensis*. Five-hundred-milliliter flasks containing 50 ml of LB broth were inoculated with 1 ml of an overnight culture of RM601 or RM602 grown in LB broth. The cultures were allowed to grow for 3 h, at which point glucose alone, glucose and arabinose, or arabinose alone was added to a final concentration of 0.2%. Optical density at 600 nm and *lux*-mediated light production were measured hourly for 6 h.

Construction of *B. pseudomallei* SZ5026 and SZ5028. The construction of *B. pseudomallei* strains harboring arabinose genes from *B. thailandensis* E264 DNA integrated into their chromosomes is shown schematically in Fig. 5, below. The transcriptional regulator gene (*araA*) from the *B. thailandensis* arabinose assimilation operon was PCR amplified. A 1,133-bp PCR product was generated with primers ARA2 and ARA6 and E264 chromosomal DNA. This PCR product was cloned into pCR2.1-TOPO (Invitrogen) and then cloned as an EcoRI fragment into the mobilizable suicide vector pSKM11 to produce the plasmid pDD157. *E. coli* TOP10(pDD157) was conjugated to *B. thailandensis* E264 using the triparental mating protocol described below. The resulting *B. thailandensis* strain DD5026 was generated by integrating pDD157 into the E264 chromosome. The plasmid pDD5026H containing pSKM11 and the entire arabinose assimilation operon from *B. thailandensis* was obtained by self-cloning with the restriction enzyme HindIII, which acts outside of the arabinose assimilation operon (see Fig. 5). This plasmid was transformed into *E. coli* TOP10 and conjugated into *B. pseudomallei* 406e to produce an arabinose-utilizing strain of *B. pseudomallei*. The resulting arabinose-utilizing strain, *B. pseudomallei* SZ5028, was obtained after integration of pDD5026H into a homologous region of the 406e chromosome (see Fig. 5).

Conjugal transfer of plasmids pDD157 and pDD5026H into *B. pseudomallei*. The strains *E. coli* TOP10(pDD157) and *E. coli* TOP10(pDD5026H) were mated with *B. pseudomallei* using a triparental mating procedure. Briefly, 200 μl of an overnight culture of *E. coli* HB101(pRK2013), *B. pseudomallei* 406e, and either *E. coli* TOP10(pDD157) or *E. coli* TOP10(pDD5026H) was centrifuged separately at 1,600 × *g* for 1 min and washed two times with sterile phosphate-buffered saline (PBS) (13). Each pellet was resuspended in a final volume of 100 μl of PBS, and the three resuspended pellets, corresponding to the mating being performed, were combined into one microcentrifuge tube and mixed well. The entire mixture was spread onto an LB–10 mM $MgSO_4$ plate, and the plate was incubated at 37°C for 24 h. The next day, bacteria were scraped off the plate and resuspended in 1 ml of PBS, and 100-μl aliquots were plated onto LB plates containing PXB (100 μg/ml) and TET (50 μg/ml) and incubated at 37°C for 48 h. The Px^r Tc^r transconjugants were then plated on M9 medium containing 0.4% arabinose as the sole carbon source to determine if the conjugations resulted in an arabinose-utilizing strain of *B. pseudomallei*.

Utilization of arabinose by *B. pseudomallei* and *B. thailandensis*. Bacteria were inoculated from LB plates into M9 medium containing 0.4% arabinose as the sole carbon source or onto M9 plus 0.4% arabinose plates. After 24 and 48 h of growth at 37°C with shaking, the absorbance values (optical density at 600 nm [OD_{600}]) of the M9 plus 0.4% arabinose cultures were determined. Growth on arabinose plates was determined following incubation of the plates at 37°C for 48 h.

PCR amplification. The *araA* gene was amplified from *B. thailandensis* E264 chromosomal DNA via PCR. The oligodeoxyribonucleotide primers used for the amplification of *araA* were ARA6 (5′-GAATCTGAGCGCAGCTATTG-3′) and ARA2 (5′-CATGGGTGCCGAACCATTGG-3′). PCR amplification was performed in a 50-μl reaction mixture containing 1× HotStarTaq PCR Master mix (QIAGEN, Missiauga, Ontario, Canada), 1× Q-solution (QIAGEN), a 0.5 μM concentration of each primer, and 100 ng of genomic DNA. The mixture was placed in a GeneAmp PCR system 9600 (Perkin-Elmer Cetus) thermal cycler and subjected to a 5-min denaturation step at 95°C followed by 30 cycles of 95°C for 45 s, 56°C for 30 s, and 72°C for 90 s. The reaction mixture was held at 72°C for 10 min and then placed at 4°C until analyzed on a 1% agarose gel. ARA1 (5′-ACGACGACGTCGAGCTTGTC-3′) and ARA3 (5′-CGACGAAGCCGA CGATGTTG-3′) were used to amplify the deletion termini of the arabinose utilization genes of *B. pseudomallei* K96243 and *B. mallei* ATCC 23344. A PCR experiment was performed to confirm the integration of pDD5026H (arabinose assimilation operon) into the *B. pseudomallei* SZ5028 chromosome. ARA6 and ARA2 primers were used to amplify the 1,136-bp *araA* gene from *B. pseudomallei* SZ5026 and SZ5028 and *B. thailandensis* E264. *B. pseudomallei* 406e was used as a negative control. The primers ARA-F (5′-GCGGTACCGAATCTGAGCG CAGCTATTG-3′) and ARA-R2 (5′-GCGGTACCTGCCATCGGCGATTTGT AAT-3′) were used to amplify the entire arabinose operon from *B. pseudomallei* SZ5028 and *B. thailandensis* E264. *B. pseudomallei* SZ5028 and 406e were used as negative controls. Long-distance PCR was performed using the FailSafe PCR PreMix selection kit (EpiCenter, Madison, Wis.). Reaction mixtures were cycled in a GeneAmp PCR system 9600 thermal cycler and subjected to a 2-min denaturation step at 95°C followed by 30 cycles of 95°C for 45 s, 59°C for 45 s, and 72°C for 1 min/kb of expected PCR product. The reaction mixture was held at 72°C for 10 min and then held at 4°C until analyzed.

DNA manipulation. Restriction enzymes and T4 DNA ligase were purchased from Invitrogen Life Technologies (Burlington, Ontario, Canada) and New England BioLabs (Mississauga, Ontario, Canada) and used according to the manufacturers' instructions. Chromosomal DNA was isolated using a Wizard genomic DNA purification kit (Promega Corporation, Madison, Wis.). Plasmid DNA was isolated using a QIAprep Spin miniprep kit (QIAGEN). DNA fragments used in cloning procedures were excised from agarose gels and purified using a QIAGEN gel extraction kit. PCR products were cloned into pCR2.1-TOPO using the TOPO TA cloning kit and chemically competent *E. coli* TOP10 cells (Invitrogen Life Technologies) according to the manufacturer's instructions. The self-cloning of DNA from Tn*5*-OT182 mutants and from the insertion of pDD157 in *B. thailandensis* DD5026 was performed as described previously (14).

Animal studies. The animal model of acute *B. pseudomallei* infection has been previously described (11). Bacteria were grown overnight in LB broth and LB plus 0.4% arabinose with 50 μg of TET/ml for *B. pseudomallei* SZ5026 and SZ5028 at 37°C with shaking. The following day, 3 ml of medium was inoculated with 100 μl of the overnight cultures, and the bacteria were grown to logarithmic phase (4 to 5 h). The OD_{600} values were determined, and the cultures were adjusted to an OD_{600} of approximately 0.55 (5×10^8 CFU/ml). Serial dilutions of the cultures were prepared in sterile PBS, and female hamsters 6 to 8 weeks old were inoculated intraperitoneally with 100 μl of bacteria corresponding to inoculum sizes of 10, 50, 100, and 1,000 CFU. Hamsters were also injected intraperitoneally with 1 mg (100 μl of a 10-mg/ml solution) of TET at time zero and at 24 h to ensure the stability of the plasmids pDD157 and pDD5026H integrated in the *B. pseudomallei* chromosome. After 48 h, the 50% lethal dose (LD_{50}) values were calculated (27).

Microarray analysis: total RNA isolation. Total bacterial RNA was isolated from liquid culture using the RNA*wiz* reagent (Ambion Inc.) with some modifications. Prior to harvesting the cells, RNA*later* solution (Ambion Inc.) was added to the broth culture (1:100 [vol/vol]), in order to protect the RNA from degradation. Bacteria were collected by centrifugation at 3,000 × *g* for 5 min at 4°C. One milliliter of RNA*wiz* reagent was added to the cell pellet (approximately 10^{10} cells), mixed thoroughly by pipetting, and incubated at room temperature for 5 min. Chloroform (0.5 ml) was added to the tube, mixed thoroughly, and incubated at room temperature for 10 min. The tube was centrifuged at 13,000 rpm for 15 min at 4°C. After centrifugation, the mixture was separated into three phases. A 350-μl aliquot of the upper aqueous phase was transferred to a new tube and diluted with 150 μl of RNase-free distilled water. The RNA was then precipitated by adding 1 ml of isopropanol and incubated at room temperature for 10 min. The tube was centrifuged at 13,000 rpm for 15 min at 4°C. The RNA pellet was washed once in 1 ml of cold 75% ethanol and centrifuged at 13,000 rpm for 5 min. The RNA pellet was dried at room temperature. The pellet was resuspended in 30 μl of the RNase-free distilled water and treated with DNase I (DNA-*free*; Ambion Inc). The RNA concentration was quantified by using a spectrophotometer, and samples were electrophoresed in a formaldehyde agarose gel to confirm the quantity and quality of RNA. RNA was considered suitable for microarray analysis if the A_{260}/A_{280} ratio was greater than 1.8.

Labeling of the samples. Equal amounts of total RNA (3 to 10 μg) from two RNA samples (under different growth conditions) were mixed separately with an equal amount of the random hexamer, $pd(N)_6$ (Amersham Bioscience), and the total volume was adjusted to 15 μl with RNase-free distilled water. The mixture

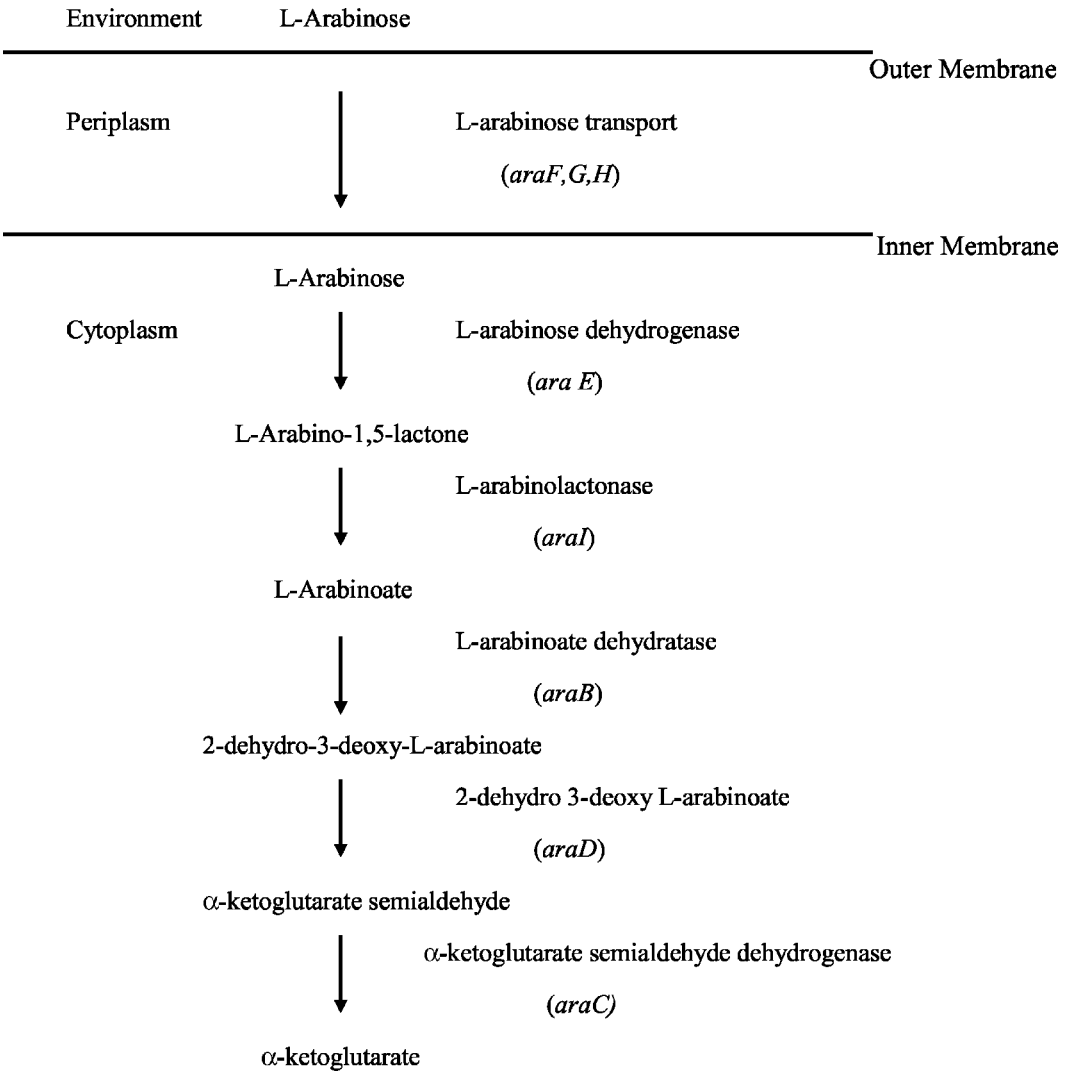

FIG. 1. Arabinose assimilation pathway in *B. thailandensis*.

was incubated at 70°C for 10 min and was maintained on ice. The cDNA was generated in a reverse transcription reaction using 100 U of StrataScript reverse transcriptase, 2 μl of 10× StrataScript reaction buffer, 1 μl of 20× deoxynucleoside triphosphate mix with an amino allyl dUTP, 1.5 μl of 0.1 M dithiothreitol, and 0.5 μl of a 40-U/μl solution of RNase block (Stratagene Inc.). The reaction mixture was incubated at 48°C for 1.5 h. The amino allyl-labeled cDNA was treated with 10 μl of 1 M NaOH and incubated at 70°C for 10 min to hydrolyze the RNA strand. A 10-μl aliquot of 1 M HCl and 4 μl of 3 M sodium acetate (pH 4.5) were added to the mixture to equilibrate the pH. The cDNA was precipitated by adding 1 μl of a 20-mg/ml solution of glycogen and 100 μl of cold 95% ethanol and incubating at −20°C overnight. The tube was centrifuged at 13,000 rpm for 15 min at 4°C. The supernatant was carefully removed, and the cDNA pellet was washed once in 0.5 ml of cold 70% ethanol followed by centrifugation for 5 min at 4°C. The supernatant was carefully removed, and the pellet was left to dry at room temperature. The cDNA was resuspended in 5 μl of 2× coupling buffer (Stratagene Inc.) and incubated at 37°C for 10 min. The two cDNA samples were labeled separately with 5 μl of either Cy3 or Cy5 (Cy^{TM3} and Cy^{TM5} monofunctional dyes; Amersham Bioscience) and incubated in the dark at room temperature for 30 min before further purification. The uncoupled dye was removed from the fluorescent dye-labeled cDNA by using a microspin cup and DNA binding solution (Stratagene Inc.). The eluate from the microspin cup (50 μl) was reduced to approximately 3 μl by vacuum centrifugation.

Hybridization. Each of the Cy3- and Cy5-labeled cDNA samples was diluted with 9 μl of DIG Easy Hyp solution (Roche Inc.) containing 5% (vol/vol) each of yeast tRNA and salmon sperm DNA as the blocking agents. Both hybridization solutions were combined and mixed carefully by pipetting and then heated at 95°C for 3 min. The hybridization solution was left to cool at room temperature and mixed thoroughly by centrifugation.

The hybridization was performed with 35-mer oligo microarray slides. The slides contained 200 genes, and each gene had four replicated spots. A whole-genome *B. mallei* open reading frame array was also used for microarray analysis. To use the array for *B. pseudomallei* samples, we compared the amplicon sequences representing the 5,244 genes in the array to the identified open reading frames in the *B. pseudomallei* genome using Blastn (WU-BLAST; Washington University). We selected a total of 4,375 sequences that matched at over 90% identity (average, ~99%) to a single *B. pseudomallei* gene over its full length. Only the data from the microarray spots of these amplicons were used for this study.

The hybridization solution was applied onto the microarray and covered with a plastic coverslip (Hybri-Slips; Sigma Corp.). The slide was incubated at 42°C in a slide hybridization chamber (Boekel Scientific) for 18 h. Following incubation, the slide was washed with three solutions: solution 1, 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.2% sodium dodecyl sulfate (SDS) for 4 min; solution 2, 0.2× SSC for 1 min; solution 3, 0.1× SSC for 1 min at room temperature. The slide was spin dried in a centrifuge at 550 rpm for 5 min and scanned immediately.

Scanning and data analysis. Scanning of the 200-gene oligonucleotide microarray hybridized slides was performed with a Virtek chip reader (Virtek

TABLE 2. Growth of *B. thailandensis* on M9 agar plates supplemented with 0.4% D-glucose or 0.4% L-arabinose as sole carbon sources

B. thailandensis strain	Growth on carbon source	
	D-Glucose	L-Arabinose
E264 (wild type)	+	+
DW503	+	+
RM600 (DW503 derivative; *araC*::Tn-*5*OT182 Ara$^-$ Tc^r)	+	−
RM601 (DW503 derivative; *araC*::pGSV3-*lux* Gm^r)	+	−
RM602 (DW503 derivative; *araE*::pGSV3-*lux* Gm^r)	+	−
RM603 (DW503 derivative; *araI*::pGSV3 Gm^r)	+	−
RM604 (DW503 derivative; *araA*::pGSV3 Gm^r)	+	−

Biotech Canada Inc.). The fluorescence signals were quantified using the QuantArray microarray analysis software (Packard Bioscience). Normalization and data analysis were performed with the GeneTraffic software (Iobion Informatics).

The whole-genome hybridized slides were scanned using the Axon GenePix 4000B microarray scanner, and the independent TIFF images from each channel were analyzed using TIGR Spotfinder (The Institute for Genomic Research [TIGR], Rockville, Md. [http://www.tigr.org/software/]) to obtain relative expression levels. Data from TIGR Spotfinder (28) were stored in AGED, a relational database designed to effectively capture and store microarray data.

Data were normalized using LOWESS local regression implemented in the MIDAS (28) software tool (TIGR [http://www.tigr.org/software/]). All calculated gene expression ratios were $\log_2$ transformed and averaged over dye-swapped arrays and again averaged over the biological replicate sets. Differentially expressed genes at the 95% confidence level were determined using intensity-dependent *Z*-scores (with $Z = 1.96$) as implemented in MIDAS.

Northern blot analysis. Equal amounts (between 3 and 10 μg) of total RNA from different growth conditions used in the microarray studies were denatured at 95°C and electrophoresed onto a 1.2% formaldehyde agarose gel in 1× MOPS buffer (0.4 M morpholinopropanesulfonic acid, 0.1 M sodium acetate, 10 mM EDTA; pH 7.2) at 100 V for 45 min. RNA was transferred to GeneScreen hybridization membranes (NEN Life Science Products) using a vacuum blotter (Bio-Rad) as per the manufacturer's instructions. Following transfer, the membrane was baked at 80°C for 2 h.

Seven DNA probes (two genes of the type III secretion system [TTSS], *bsaN* and *bsaP*; the *araC* gene of the arabinose operon; and four selected genes, *bpss0766*, *bpss0769*, *bpss0780*, and *bpss0782*) located in the flanking regions of the inserted arabinose operon were obtained by PCR amplification of the following primers: *bsaN* primers, 5′-CGCGATGTCCCATGCCGTTT-3′ and 5′-GACGCGAAGCCGTTGCTCAT-3′; *bsaP* primers, 5′-TGCGCGATTTCATCCGGCAG-3′ and 5′-CCACAGCGACAGCTCGATCT-3′; *araC* primers, 5′-TGCGACCTCGACTTC-3′ and 5′-TCGTCAGTGCGTCCT-3′; *bpss0766* primers, 5′-GCGTGAGGGCTTTCTCGAA-3′ and 5′-CTGCTCCATGTGATCGCGAT-3′; *bpss0769* primers, 5′-TTCCCGACGAAACGATTCCG-3′ and 5′-ATCGGATCGTCGAACGCATG-3′; *bpss0780* primers, 5′-TCGACAGACGGATGCTGATC-3′ and 5′-ACGATTCCTCGCGATAGAGC-3′; *bpss0782* primers, 5′-TCGAGTTTCGCGATGCTCGT-3′ and 5′-AGCGCATAGAGCACCGATTG-3′. All PCR products were purified with QIAquick Spin (QIAGEN). For ^{32}P labeling, 1 μg of DNA was denatured by heating for 3 min at 95 to 100°C and immediately placed on ice for 2 min before mixing with 50 μCi of [α-^{32}P]dCTP (Amersham Pharmacia Biotech). The mixture was incubated at 37°C for 40 min. The probe was heated to 95 to 100°C and immediately placed on ice before adding to the hybridization solution.

Membranes blotted with RNA were soaked in 2× SSC for 1 min before prehybridizing in the hybridization buffer (1% SDS, 10% [wt/vol] dextran sulfate) for 1 h at 65°C. The denatured DNA probe solution was added to the hybridization buffer. Hybridization was performed for 16 h at 65°C with rotation in a hybridization oven (Robbins Scientific Inc.). Following hybridization, the membrane was washed twice with an excess amount of 2× SSC for 5 min at room temperature, three times in a mixture of 2× SSC and 1% SDS at 60°C for 15 to

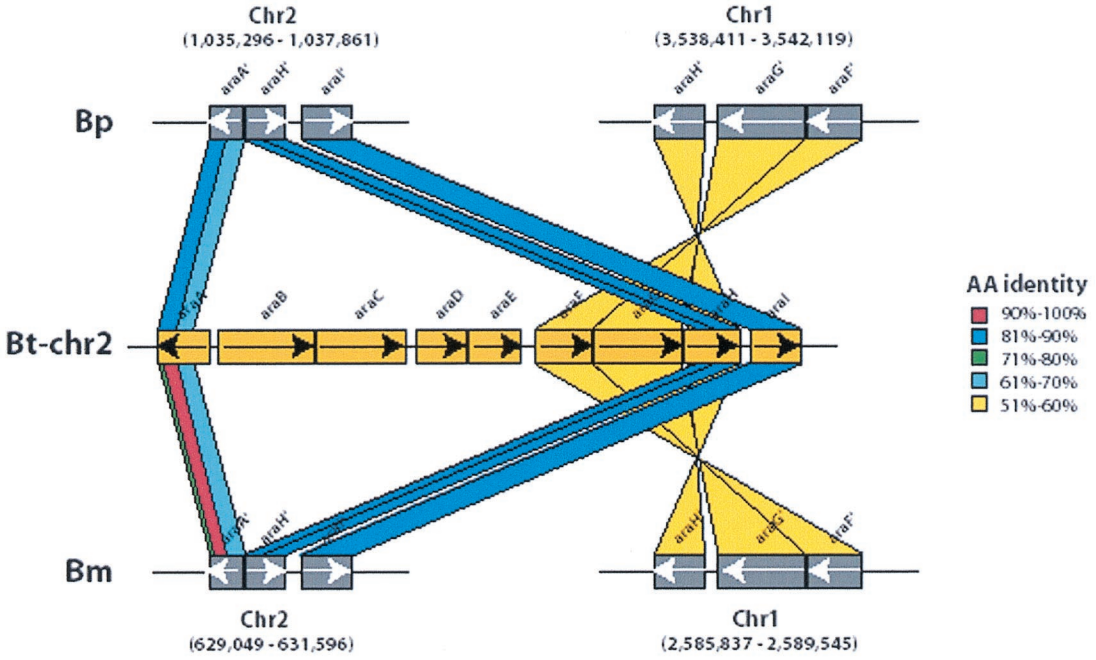

FIG. 2. Arrangement of arabinose assimilation genes on chromosome 2 (Chr2) in *B. thailandensis* (Bt). The corresponding regions on *B. pseudomallei* (Bp) Chr2 and *B. mallei* (Bm) Chr2 are also shown, including the location of the *araA-araH* deletion. The percent amino acid (AA) identity was determined using tBlastn, and the results are color coded. The figure also depicts the duplication and movement of *araF*, *araG*, and *araH* to chromosome 1 in both *B. pseudomallei* and *B. mallei*. The gene order is conserved, but the identity has decayed to lower than 60%. The exact chromosomal locations of all genes are shown in parentheses.

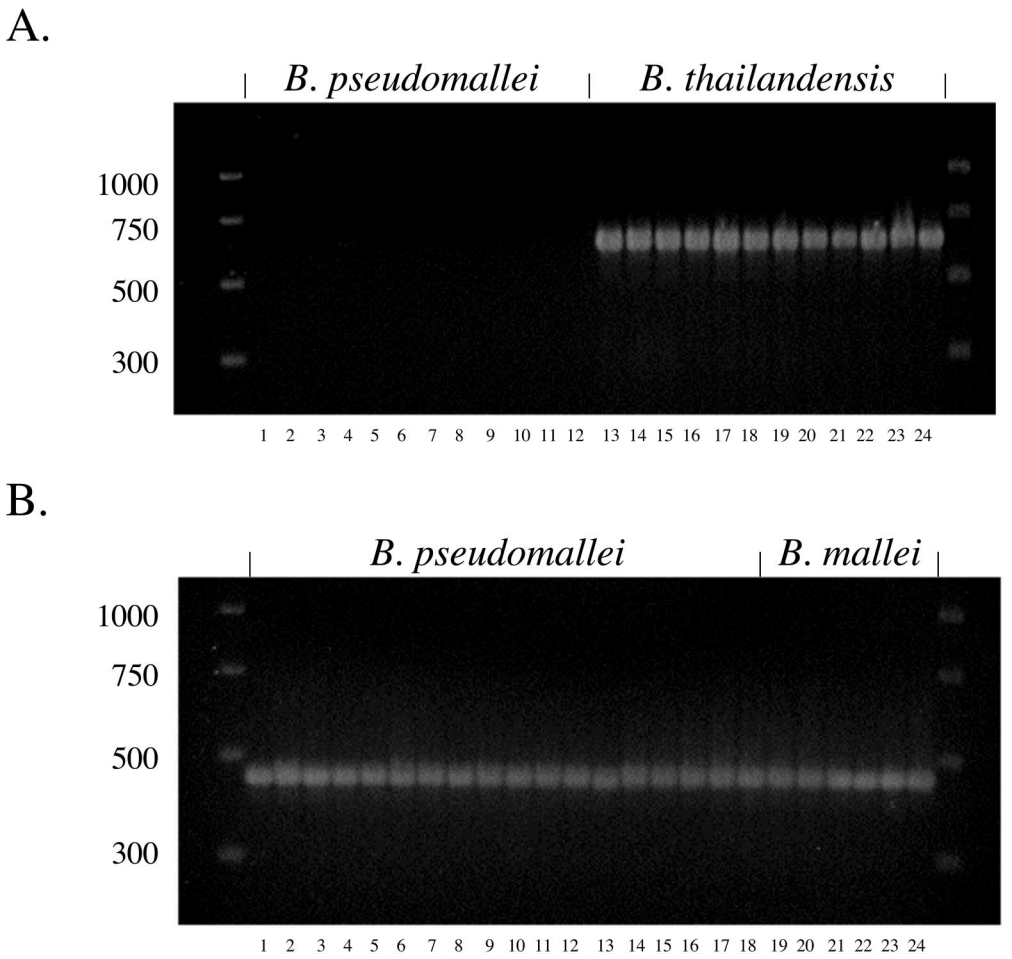

FIG. 3. PCR amplification of *ara* genes. (A) PCR survey for the presence of *ara* genes in *B. pseudomallei* and *B. thailandensis* isolates. PCR primers ARA1 and ARA2 were used to amplify a 638-bp product spanning the *araA-araB* intergenic region of the arabinose utilization locus. Lanes: 1, K96243; 2, 1026b; 3, 576; 4, E8; 5, E12; 6, E13; 7, 112c; 8, 238; 9, 295; 10, 296; 11, 713; 12, 730; 13, E27; 14, E30; 15, E32; 16, E96; 17, E100; 18, E105; 19, E111; 20, E120; 21, E125; 22, E132; 23, E135; 24, E264. (B) All *B. pseudomallei* and *B. mallei* strains examined contained the Δ(*araA-araH*) mutation. The PCR primer pair ARA1 and ARA3 was used to amplify a 454-bp product flanking the Δ(*araA-araH*) mutation in *B. pseudomallei* and *B. mallei* strains. Lanes: 1, K96243; 2, 1026b; 3, 576; 4, E8; 5, E12; 6, E13; 7, 112c; 8, 238; 9, 295; 10, 296; 11, 713; 12, 730; 13, 423; 14, 439a; 15, 465a; 16, 487; 17, 503; 18, 644; 19, NCTC 10248; 20, NCTC 10229; 21, NCTC 10260; 22, NCTC 10247; 23, ATCC 23344; 24, NCTC 120.

30 min, and three times with 0.1× SSC at room temperature. After the final wash, the damp membrane was wrapped securely in plastic wrap and exposed to X-ray film for 1 to 3 h.

RESULTS

Tn*5*-OT182 mutagenesis and isolation of an L-arabinose utilization mutant. To identify genes involved in arabinose utilization, *B. thailandensis* E264 was mutagenized with Tn*5*-OT182, and mutants unable to grow using L-arabinose as a sole carbon source were identified. One mutant, RM600, was isolated and studied further. DNA flanking the transposon insertion in RM600 was isolated via self-cloning and sequenced. BLAST analysis revealed that the transposon integration was located in a gene with strong homology to α-KSAD from several bacterial species. The enzyme expressed from this gene is part of an L-arabinose assimilation pathway found in a variety of soil bacteria, including *Herbaspirillum seropedicae* (21), *Rhizobium* spp. (15), *Azospirillum brasiliense* (25), and *Pseudomonas saccharophilia* (33).

Identification of the arabinose assimilation operon in *B. thailandensis*. We identified a total of nine genes which are likely to be involved in L-arabinose metabolism and suggest a probable assimilation pathway (Fig. 1) based on known L-arabinose assimilation pathways in other bacteria (15, 21, 25, 33). pGSV3 or pGSV3-*lux* knockout mutants were constructed in the *B. thailandensis* DW503 L-arabinose assimilation genes, *araA*, *araC, araI*, and *araE*. None of these mutants was able to grow on M9-arabinose agar (Table 2). The inability of RM604 (*araA* mutant) to grow on L-arabinose suggests that the regulatory protein encoded by this gene acts as a positive regulator for the arabinose assimilation operon. In silico analysis of the genomes of *B. pseudomallei* K96243 (http://www.sanger.ac.uk/) and *B. mallei* ATCC 23344 (http://www.tigr.org/) did not reveal an intact arabinose assimilation operon. However, both con-

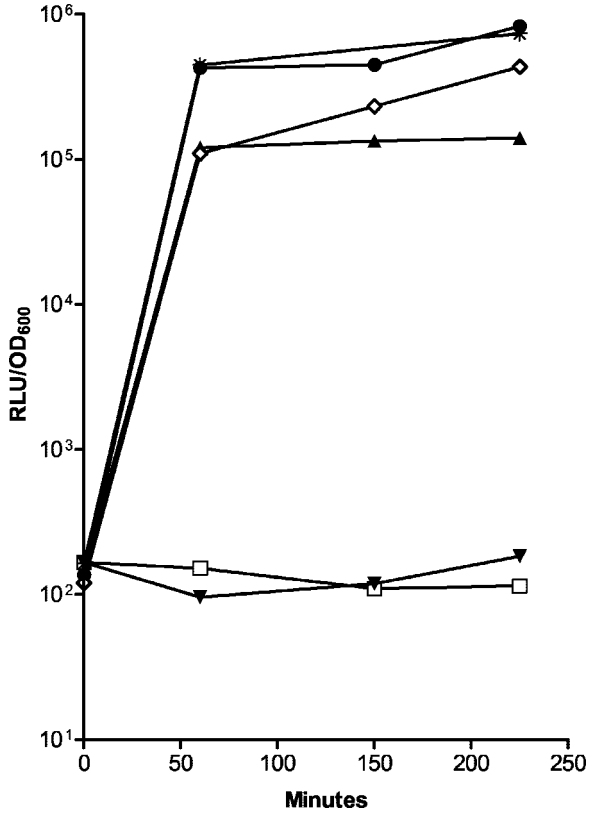

FIG. 4. Induction of arabinose assimilation genes in *B. thailandensis* RM601 (DW503 derivative; *araC*::pGSV3-*lux* Gm^r)and RM602 (DW503 derivative; *araE*::pGSV3-*lux* Gm^r). ▼, RM601 in glucose; □, RM602 in glucose; ◇, RM601 in arabinose; ★, RM602 in arabinose; ▲, RM601 in glucose plus arabinose; ●, RM602 in glucose plus arabinose. RLU, relative light units (luminescence).

tained DNA flanking the arabinose assimilation operon, suggesting that a deletion occurred which removed *araA-araH* from these genomes. The L-arabinose assimilation operon in *B. thailandensis* and the corresponding deleted regions in *B. pseudomallei* and *B. mallei* are shown schematically in Fig. 2.

The *B. pseudomallei* and *B. mallei araA* remnants (5′ truncated) have several frameshift mutations relative to *B. thailandensis araA*. Likewise, the *araH* remnants (5′ truncated) have frameshift mutations in both *B. pseudomallei* and *B. mallei* relative to *B. thailandensis araH*. Interestingly, *araF*, *araG*, and *araH* have duplicated and moved from chromosome 2 to chromosome 1 in both *B. pseudomallei* and *B. mallei* (Fig. 2). The gene order is conserved, but the amino acid identity of the encoded proteins has dropped to lower than 60%. The similarity of the *araFGH* genes on chromosome 1 of *B. pseudomallei* and *B. mallei* suggests that this duplication occurred before *B. mallei* diverged from *B. pseudomallei*.

PCR survey for the presence of *ara* genes in *B. pseudomallei* and *B. thailandensis* isolates. PCR primers ARA1 and ARA2 were designed to amplify a 638-bp product spanning the *araA-araB* intergenic region of the arabinose utilization locus. Twelve *B. thailandensis* strains yielded a PCR product of the correct size when tested with this primer pair (Fig. 3A, lanes 13 to 24). On the other hand, no *B. pseudomallei* strains yielded a positive PCR result with ARA1 and ARA2 (Fig. 3A, lanes 1 to 12). Thus, there is a correlation with the presence of the *araA-araI* genes and the ability to assimilate arabinose in these species. The ARA1 and ARA2 primer pair may be useful for differentiating *B. pseudomallei* and *B. thailandensis* in future studies.

All *B. pseudomallei* and *B. mallei* strains examined contained the Δ(*araA-araH*) mutation. The PCR primer pair ARA1 and ARA3 was designed to amplify a 454-bp product flanking the deletion termini of the arabinose utilization genes of *B. pseudomallei* K96243 and *B. mallei* ATCC 23344. Eighteen clinical and environmental *B. pseudomallei* isolates from Australia and Thailand yielded PCR products of the predicted size with this primer pair (Fig. 3B, lanes 1 to 18). Furthermore, six *B. mallei* strains also yielded a positive PCR result with the ARA1 and ARA3 primer pair (Fig. 3B, lanes 19 to 24). The nucleotide sequences of all of the PCR products were determined, and all contained the same deletion termini that are present in *B. pseudomallei* K96243 and *B. mallei* ATCC 23344. The fact that geographically diverse strains all contain the same Δ(*araA-araH*) mutation suggests that the loss of arabinose assimilation genes occurred early in the evolution of *B. pseudomallei*. Since *B. mallei* is a host-adapted clone of *B. pseudomallei*, it is not surprising that it also harbors this deletion. Although not shown, *B. pseudomallei* 406e gave identical results as those obtained for all other *B. pseudomallei* strains tested.

Induction of genes involved in arabinose metabolism. The expression levels of arabinose dehydrogenase and α-KSAD were examined using the pGSV3-*lux* knockout mutants *B. thailandensis* RM601 and *B. thailandensis* RM602. Growing cultures of each mutant received either arabinose, glucose, or both, and induction of arabinose utilization genes was followed via *lux*-mediated light production. The results of these experiments are shown in Fig. 4 and demonstrate that both arabinose dehydrogenase and α-KSAD were rapidly induced by the addition of arabinose or arabinose in the presence of glucose, but not by glucose alone.

The arabinose assimilation operon is present in the chromosome of *B. pseudomallei* SZ5028. An L-arabinose-utilizing *B. pseudomallei* strain, SZ5028, was constructed as outlined in Fig. 5. Two PCR experiments were performed to confirm the integration of pDD5026H and the presence of the arabinose assimilation operon in the chromosome of *B. pseudomallei* SZ5028. In the first set of reactions, the ARA6 and ARA2 primers were used in an attempt to amplify the 1,136-bp *araA* gene from *B. pseudomallei* 1026b, 406e, SZ5026, and SZ5028 and *B. thailandensis* E264. In the second set of reactions, the ARA-F and ARA-R2 primers were used in an attempt to amplify the entire arabinose assimilation operon from these strains. The *araA* gene was amplified only from *B. pseudomallei* SZ5026 and SZ5028 and *B. thailandensis* E264, as indicated by a band corresponding to the predicted size of 1,136 bp (Fig. 6). As expected, the *araA* gene could not be amplified from wild-type *B. pseudomallei* strains 1026b or 406e due to the deletion of the arabinose assimilation operon, including part of this gene, from the chromosome (Fig. 2). The presence of the entire *araA* gene in *B. pseudomallei* SZ5026 and SZ5028 confirms the integration of pDD157 into the chromosome of SZ5026 and the integration of pDD5026H into the chromosome of SZ5028. Using the primers ARA-F and ARA-R2, the

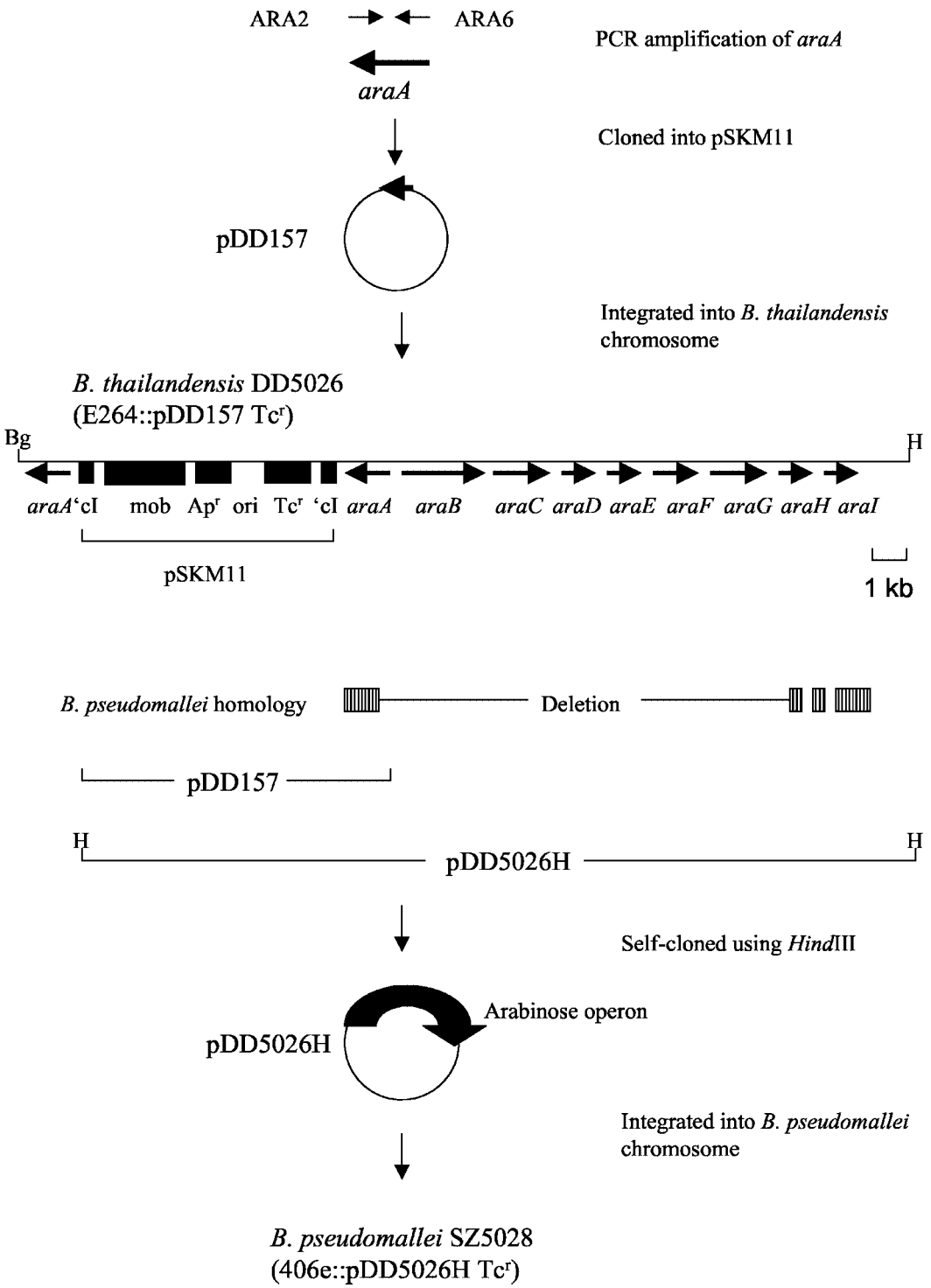

FIG. 5. Construction of the arabinose-utilizing *B. pseudomallei* strain SZ5028. The transcriptional regulator gene (*araA*) from the *B. thailandensis* arabinose assimilation operon was amplified using PCR. A 1,133-bp PCR product was generated with primers ARA6 and ARA2 and E264 chromosomal DNA. This PCR product was cloned into the suicide vector pSKM11 to produce the plasmid pDD157. The *B. thailandensis* strain DD5026 was generated by integrating pDD157 into the E264 chromosome. The plasmid pDD5026H was obtained by self-cloning with the restriction enzyme HindIII. This plasmid contains pSKM11 and the entire arabinose assimilation operon from *B. thailandensis*. pDD5026H was transformed and conjugated to *B. pseudomallei* 406e to produce an arabinose-utilizing strain of *B. pseudomallei*. The resulting strain, *B. pseudomallei* SZ5028, was obtained by integrating pDD5026H into the 406e chromosome. The locations and names of the arabinose genes from *B. thailandensis* are indicated, and the direction of transcription is represented by arrows. The region of homology between *B. thailandensis* and *B. pseudomallei* is indicated. The plasmids pDD157 and pDD5026H are illustrated. The locations of relevant restriction endonuclease recognition sites (Bg, BglII; H, HindIII) are shown.

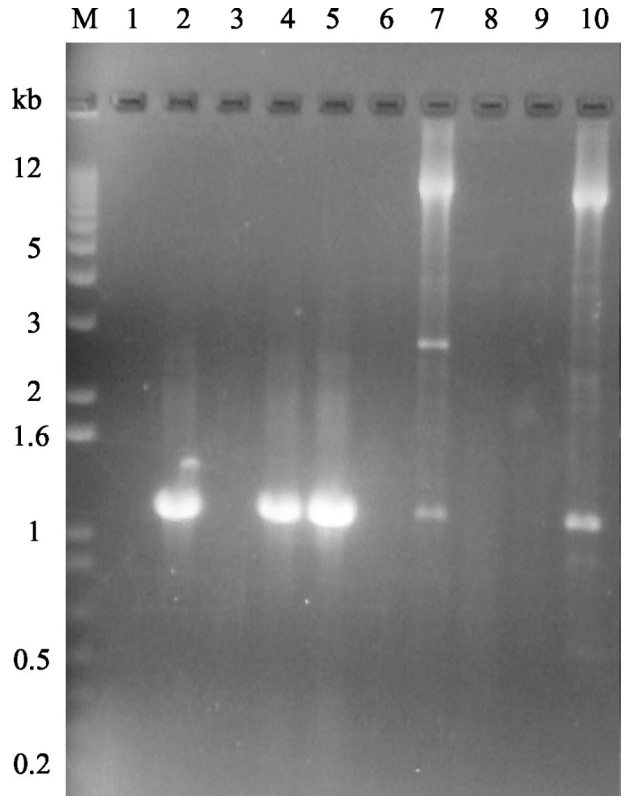

FIG. 6. PCR amplification of the *araA* gene and the arabinose assimilation operon from *B. thailandensis* and *B. pseudomallei* chromosomal DNA. Lanes 1 to 5, PCR amplification of the *araA* gene using primers ARA6 and ARA2. Lanes 6 to 10, PCR amplification of the arabinose assimilation operon using primers ARA-F and ARA-R2. Lane M, 1-kb Plus DNA ladder (Invitrogen Life Technologies); lanes 1 and 6, *B. pseudomallei* 1026b; lanes 2 and 7, *B. thailandensis* E264; lanes 3 and 8, *B. pseudomallei* 406e; lanes 4 and 9, *B. pseudomallei* SZ5026; lanes 5 and 10, *B. pseudomallei* SZ5028.

arabinose assimilation operon was successfully amplified from the positive control *B. thailandensis* E264 as well as from *B. pseudomallei* SZ5028. The presence of an approximately 11-kb band on the gel corresponding to the expected 11,789-bp PCR product indicated that the arabinose assimilation operon is present in SZ5028 (Fig. 6). There was no amplification of the arabinose assimilation operon from wild-type *B. pseudomallei* 406e or 1026b, nor could the operon be amplified from *B. pseudomallei* SZ5026, which only contains the *araA* gene inserted into the chromosome (Fig. 6).

TABLE 3. Virulence of arabinose-utilizing *B. pseudomallei* strain SZ5028 in the Syrian hamster model of infection

Strain	Growth medium	LD_{50} (CFU)
B. pseudomallei 406e	LB	<10
	LB + 0.4% arabinose	<10
B. thailandensis E264	LB	>1,000
	LB + 0.4% arabinose	>1,000
B. pseudomallei SZ5026	LB	<10
	LB + 0.4% arabinose	<10
B. pseudomallei SZ5028	LB	198
	LB + 0.4% arabinose	94

Amplification of the arabinose assimilation operon also produced two nonspecific PCR products from E264 and SZ5028 DNA, indicated by a 2,700-bp band and a 1,000-bp band in the E264 sample and a 1,000-bp band in the SZ5028 sample (Fig. 5). The sequence identity of these PCR products is unknown, but these bands were consistently present in previous PCR amplifications of the arabinose assimilation operon from *B. thailandensis*.

Utilization of L-arabinose by *B. pseudomallei* SZ5028. *B. pseudomallei* 406e, SZ5026, and SZ5028 and *B. thailandensis* E264 were grown in M9 medium containing 0.4% L-arabinose as the sole carbon source or on M9 plus 0.4% arabinose plates. After 24 and 48 h of growth with shaking, the absorbance values (OD_{600}) of the M9 plus 0.4% arabinose cultures were determined. Growth on arabinose plates was determined following incubation of the plates at 37°C for 48 h. As expected, *B. pseudomallei* 406e was not able to utilize arabinose. The OD_{600} values for this strain cultured in M9 plus 0.4% arabinose were 0.016 at 24 h and 0.030 at 48 h, and there was no detectable growth of this strain when grown on M9 plus 0.4% arabinose agar plates. The positive control, *B. thailandensis* E264, grew on the M9 plus 0.4% arabinose agar plates and in the M9 plus 0.4% arabinose medium, demonstrating OD_{600} values of 0.289 at 24 h and 1.374 at 48 h. The strain resulting from the integration of pDD5026H into the 406e chromosome, SZ5028, was found to utilize arabinose at a level comparable to that of *B. thailandensis* E264. The OD_{600} values for SZ5028 grown in M9 plus 0.4% arabinose medium were 0.528 at 24 h and 0.901 at 48 h. In addition, SZ5028 was able to grow on M9 plus 0.4% L-arabinose agar plates, confirming that this strain was capable of arabinose assimilation. The growth of *B. pseudomallei* SZ5026, the vector control strain, was greater in M9 plus 0.4% arabinose medium compared to growth of 406e, but the growth did not increase between 24 and 48 h compared to that of SZ5028 and E264, the arabinose-assimilating strains. In addition, SZ5026 did not grow on the M9 plus 0.4% L-arabinose agar.

***B. pseudomallei* SZ5028 is attenuated for virulence in the Syrian hamster model of melioidosis.** *B. pseudomallei* 406e, SZ5026, and SZ5028 and *B. thailandensis* E264 were grown in the presence of LB and LB plus 0.4% L-arabinose and tested for virulence in the Syrian hamster model of melioidosis. The LD_{50} for SZ5028 after 48 h was 198 CFU when grown in the presence of LB and 94 CFU when grown in LB plus 0.4% L-arabinose medium, compared to LD_{50} values with 406e and SZ5026 of <10 CFU when grown in either medium, representing an approximately 25- to 50-fold decrease in virulence (Table 3). In these experiments, *B. thailandensis* E264 had an LD_{50} value of >1,000, although the actual LD_{50} value for this strain was previously calculated to be 1.8×10^6 CFU (7). Although SZ5028 was more virulent than *B. thailandensis* E264, it was attenuated for virulence compared to wild-type *B. pseudomallei* 406e. This suggests that the presence of the arabinose assimilation operon in *B. pseudomallei* may affect the expression of virulence genes, contributing to decreased virulence of the organism. The difference in virulence between 406e and SZ5028 cannot be attributed to differences in growth rates, since the growth curves of 406e, SZ5026, and SZ5028 were found to be similar (data not shown). When the mutant strain *B. thailandensis* RM600 (able to grow on LB and M9-glucose

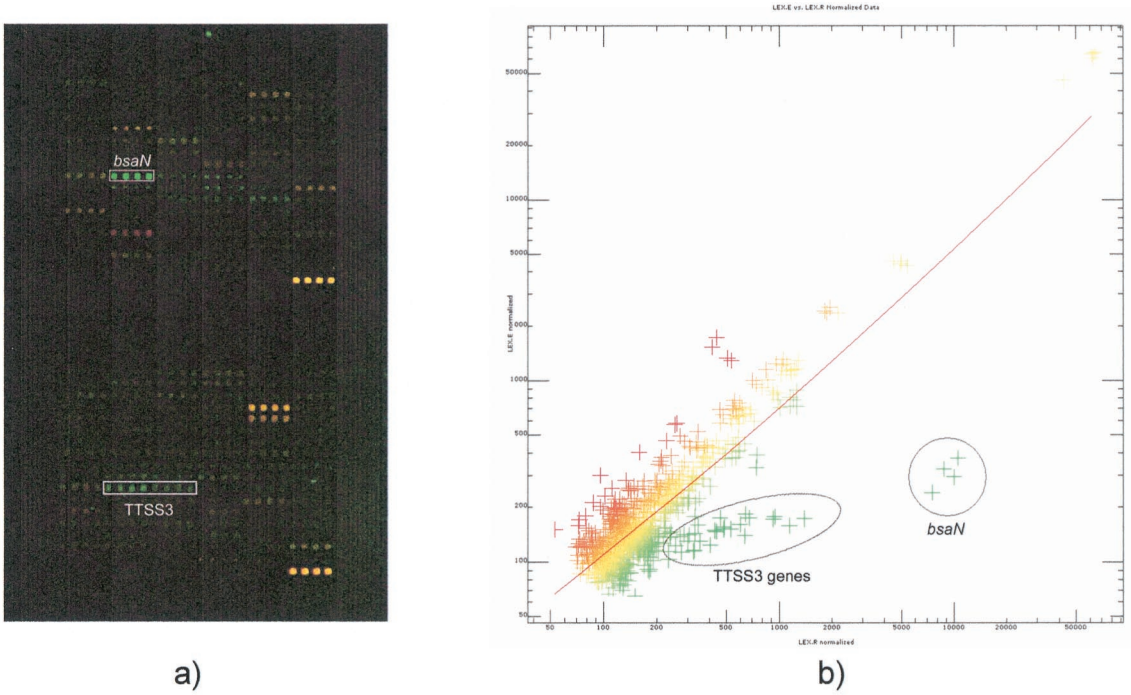

FIG. 7. Gene expression profiles of the arabinose-assimilating *B. pseudomallei* strain SZ5028 grown in M9 medium containing 1% arabinose versus that with 1% glucose. (a) Microarray image showing up-regulated (red) and down-regulated (green) genes. (b) Scatter plot of the normalized data, showing genes involved in TTSS3 that were significantly down-regulated. LEX.R, intensities of the reference sample (cells grown in 1% glucose); LEX.E, intensities of the evaluated sample (cells grown in 1% arabinose). The plotted data were normalized using the LOWESS smoothing (subgrid) method based on the GeneTraffic software, and the regression line shows the 1:1 ratio of 100% LOWESS smoothing factor.

but not on M9-arabinose) was tested for virulence in the hamster model, it was found not to be significantly altered in its virulence properties relative to E264 (data not shown).

Growth on L-arabinose down-regulates type III secretion genes in *B. pseudomallei* SZ5028. Microarray analysis of gene regulation in *B. pseudomallei* SZ5028 grown in the presence of arabinose or glucose using 200-gene microarrays demonstrated that a number of genes involved in one of the TTSSs were down-regulated during growth in M9 plus 1.0% arabinose compared to growth in M9 plus 1.0% glucose (Fig. 7). Of the total of 200 genes examined, 4 genes showed decreased expression. All four genes belonged to a TTSS (TTSS3) homologous with the *Salmonella* SPI-1 pathogenicity island (3), with the greatest difference (7.7-fold) being noted for a gene encoding an AraC-like transcriptional regulator (28) (Table 4). The putative regulator is a homologue of the TTSS positive regulator InvF in *Salmonella*, MxiE in *Shigella*, and VirF in *Yersinia* and was recently named *bsaN* (32). Furthermore, other TTSS genes encoding structural proteins were significantly down-regulated (2.0- to 3.1-fold) (Table 4), although they were not reduced to the same extent as the *bsaN* regulator gene. This result suggests that *bsaN* is a positive transcriptional regulator of the *B. pseudomallei* TTSS3 cluster whose activity is decreased directly or indirectly by the presence of arabinose or the absence of glucose.

Interestingly, results from analysis of the whole-genome microarrays were similar to those obtained from the 200-gene microarrays. Of a total of 4,375 genes examined using the whole-genome arrays, 211 genes showed differentiated expression. We found that 24 out of the 32 linked genes located in chromosome 2 (coordinates from 2073665 to 2105918) were significantly down-regulated, with 95% confidence, in M9 plus 1.0% arabinose compared to M9 plus 1.0% glucose (Table 5), and as seen with the results from the 200-gene microarray

TABLE 4. Comparative expression of *B. pseudomallei* strain SZ5028 type III secretion genes in response to growth in M9 media containing 1.0% L-arabinose or D-glucose from analysis of 200-gene microarrays

Gene	Putative function	Mean $\log_2(I_{Ara}/I_{Glu})$ ± SD
bsaN	Possible TTSS transcriptional regulator, similar to the AraC family regulatory protein	−2.94 ± 1.30
bsaP	TTSS secreted protein similar to *mxiC* gene in *Shigella* spp.	−1.61 ± 0.91
bsaM	Similar to *prgH* gene in *Chromobacteria violaceum*; TTSS inner membrane protein	−1.02 ± 0.65
bprA	Possible TTSS transcriptional regulator, similar to the H-NS DNA binding protein	−1.29 ± 0.49

[a] Mean $\log_2(I_{Ara}/I_{Glu})$ = average value of the $\log_2$-based ratio of intensities from two channels of the microarray: growth in arabinose versus growth in glucose. A negative number denotes down- regulation in arabinose. Only genes which displayed at least a twofold decrease [$\log_2(I_{Ara}/I_{Glu}) \leq -1$] in expression are listed. Results are the average of two independent RNA isolations with reverse labeling of each sample (dye swap), resulting in a total of four analyses.

TABLE 5. Comparative expression of *B. pseudomallei* strain SZ5028 genes in response to growth in M9 media containing 1.0% L-arabinose or D-glucose from analysis of whole-genome microarrays

Mean $\log_2(I_{Ara}/I_{Glu})$[d]	Gene	Common name	Other name
3.660567716	BPSL2188	Isocitrate lyase	
2.872528528	BPSS1799	Conserved hypothetical protein	
2.396046588	BPSL0638	Putative membrane protein	
2.30341532	BPSS1802	Putative membrane protein	
2.214457543	BPSL0688	Putative glycerol-3-phosphate dehydrogenase	
2.166956925	BPSS0772	MarR family regulatory protein	
2.134310112	BPSL0687	Putative glycerol kinase	
1.892752921	BPSS1801	Hypothetical transposon protein	
1.876541084	BPSS1800	Putative dehydrogenase	
1.82906867	BPSS1575	α-Ketoglutarate-dependent taurine dioxygenase	
1.709388586	BPSS1485[b]	Conserved hypothetical protein	
1.704217668	BPSS0310	Hypothetical protein	
1.604675546	BPSL3396	ATP synthase beta chain	
1.492801878	BPSS0190	Putative *O*-acetylhomoserine phosphate transport system, substrate binding exported	
1.491778582	BPSL1359	Periplasmic protein	
1.485378831	BPSS0153	Glutamate-aspartate periplasmic binding protein	
1.479761295	BPSS1571	Probable NADH oxidoreductase	
1.447509585	BPSS1553[a]	Putative membrane protein	
1.41337407	BPSS1415	Putative dehydrogenase-oxidoreductase protein	
1.334014629	BPSS1417	Putative L-fuculose phosphate aldolase	
1.284925131	BPSS0973	Mg^{2+} transport ATPase, P-type 2	
1.277778343	BPSL2336	Putative glutamine synthetase	
1.274610271	BPSS0031	Putative anaerobic growth regulatory protein	
1.256584401	BPSS0257	Putative ribose ABC transport system, substrate binding exported protein	
1.255907584	BPSL3401	ATP synthase c chain	
1.177272863	BPSL2500	Electron transfer flavoprotein beta-subunit	
1.157434208	BPSS0260	Putative kinase	
1.131859522	BPSS0885	*N*-Acylhomoserine lactone synthesis protein	
1.084088768	BPSS1572	Permease component of taurine ABC transporter	
1.072310657	BPSL1363	Phosphate transport system-related protein	
1.065265887	BPSL1362	Phosphate transport system ATP-binding protein	
1.053551864	BPSS0519	Conserved hypothetical protein	
1.051120355	BPSS0524	Conserved hypothetical protein	
1.049706097	BPSS0771	Hypothetical protein	
1.038876362	BPSS0977	Putative cytochrome c subunit II	
1.012236689	BPSS0316	Putative ACB transport system, membrane protein	
0.993355473	BPSL2611	Maltose-binding protein	
0.975444214	BPSL2925	Glutamate dehydrogenase	
0.973999816	BPSL1489	Putative membrane protein	
0.947238343	BPSL2068	Putative two-component system, response regulator	
0.939612293	BPSS0259	Putative dehydrogenase	
0.928566139	BPSS0329	Putative fatty aldehyde dehydrogenase	
0.909462198	BPSS0528	Conserved hypothetical protein	
0.905799795	BPSS1638	Putative hydratase	
0.898099586	BPSL3104	Conserved hypothetical protein	
0.888963775	BPSL3399	ATP synthase delta chain	
0.88606119	BPSS0034	Putative fatty aldehyde dehydrogenase	
0.875598331	BPSL2970	KDPG/KHG aldolase, putative	
0.86945211	BPSL2972	Transcriptional regulator, IclR family	
0.83562524	BPSL2967	L-arabinose ABC transporter, ATP-binding protein	
0.810672159	BPSL1364	Phosphate regulon two-component system response regulator	
0.80527425	BPSL3416	Putative branched-chain amino acid ABC transporter, periplasmic substrate binding protein	
0.799179699	BPSS0156	Putative dehydrogenase	
0.794669183	BPSL1961	Putative electron transport protein	
0.777504798	BPSS0265	Putative porin-related exported protein	
0.755403028	BPSS1356	Hypothetical protein	
0.750869748	BPSS0526	Hypothetical protein	
0.747026223	BPSS0154	D-Amino acid dehydrogenase small subunit	
0.743456855	BPSL2028	Putative fimbriae assembly chaperone	
0.73331917	BPSL2177	Hypothetical protein	
0.711866447	BPSL3021	Cell division protein FtsA	
0.695228684	BPSL2024	Putative two-component regulatory system, response regulator	
0.668360942	BPSS0308	Putative aminotransferase	
0.655842301	BPSS1637	4-Hydroxybutyrate coenzyme A transferase	
0.637379273	BPSS0262	Putative aminoglycoside 6′-*N*-acetyltransferase	

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TABLE 5—*Continued*

Mean $\log_2(I_{Ara}/I_{Glu})$[d]	Gene	Common name	Other name
0.628790939	BPSS0303	Putative diaminopimelate decarboxylase	
0.625501197	BPSS0307	Putative aldehyde dehydrogenase	
0.596306364	BPSL2309	Respiratory nitrate reductase alpha chain	
0.590151581	BPSL2066	Putative exported protein	
0.587354108	BPSL0373	Putative isocitrate dehydrogenase	
0.583575109	BPSL2189	Conserved hypothetical protein	
0.561185706	BPSL1361	Phosphate transport system permease protein	
0.535335317	BPSS1428	Hypothetical protein	
0.528600905	BPSS0799	Hypothetical protein	
0.502223307	BPSL0415	Zinc-binding dehydrogenase (partial)	
0.48860634	BPSL1029	Putative exported outer membrane porin protein	
0.451071772	BPSL1345	Putative exported transglycosylase protein	
0.447920038	BPSS1911	Putative membrane protein	
0.43439967	BPSL1876	Putative phospholipase	
0.415997942	BPSS0945	Putative lipoprotein	
0.414734465	BPSS0880	Putative transcription elongation factor	
0.39974228	BPSL2315	Endonuclease/exonuclease/phosphatase family	
0.392807392	BPSL1278	Putative ABC transport system, iron-binding exported protein	
0.386719733	BPSL2027	Putative fimbriae-related protein	
0.37758332	BPSS0974	Subtilase family protease	
0.376154287	BPSL0812	TetR family regulatory protein	
0.372701224	BPSL2840	Putative flavohemoglobin like protein	
0.369640959	BPSS0944	LysR family regulatory protein	
0.350896899	BPSL1904	Putative exported protein	
−0.265546681	BPSL2397	Hypothetical protein	
−0.375599667	BPSL1062	Translation initiation factor IF-1	
−0.385629165	BPSS0648	AraC family regulatory protein	
−0.401476867	BPSS2146	Putative LysR family transcriptional regulator	
−0.410106054	BPSL2368	Putative membrane protein	
−0.418904592	BPSL0600	Hypothetical protein	
−0.42521041	BPSL0474	Putative transporter protein	
−0.461122926	BPSS1508[b]	Hypothetical protein	
−0.470627995	BPSL3368	Putative AraC family transcriptional regulator	
−0.477031398	BPSL0491	Putative acyl carrier protein	
−0.489581077	BPSL2946	C4-dicarboxylate transport protein	
−0.500094955	BPSS1867	Putative membrane protein	
−0.508615787	BPSL1762	Precorrin-3b C17-methyltransferase	
−0.534798524	BPSS1510[b]	Putative membrane protein	
−0.540333178	BPSL1442	Putative membrane protein	
−0.551488165	BPSS1004	Putative acyl transferase	
−0.578510311	BPSS1240	Putative penicillin-binding protein	
−0.591347751	BPSL1191	Putative exported protein	
−0.60912439	BPSL0296	Conserved hypothetical protein	
−0.623616771	BPSS1754	Putative membrane protein	
−0.62533172	BPSS1535[a]	Surface presentation of antigens protein	bsaY
−0.626547891	BPSL1174	Two-component system, sensor kinase protein	
−0.634381721	BPSL1170	Putative membrane protein	
−0.659601302	BPSS1614[c]	Putative type III secretion protein	bpscD2
−0.661862496	BPSS0227	Putative membrane protein	
−0.666184002	BPSS1744	Putative cytochrome b_{561}	
−0.666845101	BPSS1620[c]	Putative type III secretion protein	bpscV2
−0.673719979	BPSL2399	Putative glycosyltransferase	
−0.679727251	BPSS1811[c]	Conserved hypothetical protein	hrpV (TTSS2)
−0.682518126	BPSL2034	Putative hydrolase	
−0.684480069	BPSL2540	Putative inner membrane protein	
−0.688717244	BPSL2213	Conserved hypothetical protein (pseudogene)	
−0.692369046	BPSS0008	Putative TetR family regulatory protein	
−0.693886932	BPSS1489[b]	Putative exported protein	
−0.730982339	BPSS1509[b]	Conserved hypothetical protein	
−0.7331972	BPSL2322	Putative membrane protein	
−0.744510147	BPSS1501[b]	Conserved hypothetical protein	
−0.766096437	BPSL3310	Flagellar regulon master regulator subunit FlhC	
−0.770676455	BPSS1504[b]	Putative exported protein	
−0.772200497	BPSL1173	Potassium-transporting ATPase c chain	
−0.775876851	BPSL2657	Urease gamma subunit	
−0.783460065	BPSS2091	Hypothetical protein	
−0.796888021	BPSS1117	Conserved hypothetical protein	
−0.810417894	BPSS1613[c]	Putative type III secretion protein	bpscE2

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TABLE 5—*Continued*

Mean $\log_2(I_{Ara}/I_{Glu})$[d]	Gene	Common name	Other name
−0.826702179	BPSL0192	NADP-dependent alcohol dehydrogenase	
−0.828794009	BPSS1728	Putative hemolysin activator	
−0.852588312	BPSS0692	Fumarylacetoacetate hydrolase family	
−0.856506201	BPSL1591	Conserved hypothetical protein	
−0.881132891	BPSS1118	Putative HlyD family protein	
−0.888650117	BPSL1185	Putative membrane protein	
−0.925094352	BPSL2111	Putative LysR-family transcriptional regulator	
−0.937413375	BPSL2035	Putative regulatory protein	
−0.960146058	BPSS1496[b]	Conserved hypothetical protein	
−0.984330728	BPSS1550[a]	TTSS protein	bsaJ
−0.986561024	BPSS1507[b]	Hypothetical protein	
−0.996980632	BPSS0966	Putative manganese transport protein	
−1.003770427	BPSS1500[b]	Conserved hypothetical protein	
−1.004140376	BPSS1497[b]	Conserved hypothetical protein	
−1.006872551	BPSS1896	Ubiquinol oxidase polypeptide I	
−1.022502983	BPSS1551[a]	TTSS protein	sctKBp3
−1.023479736	BPSS1782	Organic hydroperoxide resistance protein	
−1.035169118	BPSL1322	Putative heat shock protein	
−1.036142443	BPSL2301	Pyruvate dehydrogenase E1 component	
−1.038757904	BPSL3369	Acetaldehyde dehydrogenase	
−1.045056203	BPSL0494	LysR family regulatory protein	
−1.056941766	BPSL2929	Thermoresistant gluconokinase	
−1.071013189	BPSS1612[c]	Hypothetical protein	bpscF2
−1.125942579	BPSL2489	50S ribosomal protein L19	
−1.12671761	BPSS0752	Putative lipoprotein	
−1.135350706	BPSS2051	Putative DNA-binding protein	
−1.164923297	BPSL1172	Potassium-transporting ATPase b chain	
−1.171472171	BPSS1515[b]	Putative transposase (partial)	
−1.206749612	BPSL1323	Putative heat shock protein	
−1.242957236	BPSS1119	Putative RND efflux transporter	
−1.258189419	BPSL2323	Putative exported protein	
−1.296885852	BPSL3427	Putative two-component sensor kinase	
−1.357746981	BPSS1363	Putative exported protein	
−1.36533721	BPSS0910	Conserved hypothetical protein	
−1.42123191	BPSS1534[a]	Surface presentation of antigens protein	bsaZ
−1.467766735	BPSL1171	Potassium-transporting ATPase a chain	
−1.467876647	BPSL0024	LrgA family protein	
−1.507840263	BPSS1491[b]	Conserved hypothetical protein	
−1.558631442	BPSS1503[b]	Hypothetical protein	
−1.638155545	BPSS1514[b]	GTP cyclohydrolase I	
−1.653768643	BPSS0289	Putative exported protein	
−1.670203034	BPSS1917	Putative crp-family transcriptional regulator	
−1.702728184	BPSS0965	Putative oxalate decarboxylase	
−1.704025718	BPSS1536[a]	Surface presentation of antigens protein	bsaX
−1.725537375	BPSS1748	Hypothetical protein	
−1.730257516	BPSS1783	Hypothetical protein	
−1.756482379	BPSL0025	Putative membrane protein	
−1.818503841	BPSS1513[b]	Hypothetical protein	
−1.820198806	BPSS1532[d]	Putative cell invasion protein	bapB
−1.842067577	BPSS1541[a]	Surface presentation of antigens protein	bsaS
−1.917238884	BPSS1519[b]	Transposase	
−2.04912008	BPSS0514	Acetyl coenzyme A hydrolase/transferase	
−2.08878765	BPSS1520[a]	Putative AraC-family transcriptional regulator	bprC
−2.136822097	BPSS1498[b]	Conserved hypothetical protein	
−2.147227432	BPSS1537[a]	Surface presentation of antigens protein	bsaW
−2.147273814	BPSS0756	Putative exported protein	
−2.209090551	BPSS1543[a]	TTSS protein	bsaQ
−2.412295713	BPSS1549[d]	TTSS protein	bsaK
−2.447592658	BPSS0753	Hypothetical protein	
−2.500150544	BPSS1542[a]	Surface presentation of antigens protein	bsaR
−2.772712922	BPSS1526[a]	Putative invasion protein	bapC
−2.80293907	BPSS1523[a]	Putative chaperone	bicP
−2.868102303	BPSS1545[a]	TTSS protein	bsaO
−2.87670536	BPSS1493[a]	Hypothetical protein	
−2.928005448	BPSS0755	LysR family regulatory protein	
−2.99669704	BPSS1547[a]	TTSS protein	bsaM
−3.023822001	BPSS1494[b]	Putative two-component response regulator	
−3.166688057	BPSS1548[a]	TTSS protein	bsaL

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TABLE 5—*Continued*

Mean $\log_2(I_{Ara}/I_{Glu})$[d]	Gene	Common name	Other name
−3.220777282	BPSS1525[a]	Putative G-nucleotide exchange factor	bopE
−3.2758023	BPSS1522[a]	Putative two-component response regulator	bprB
−3.330120749	BPSS1546[a]	Putative AraC-family regulator of type III secretion system	bsaN
−3.42729804	BPSS1524[a]	Putative intercellular spread protein	bopA
−4.034898051	BPSS1521[a]	Hypothetical protein	
−4.307254378	BPSS1530[a]	Putative HNS-like regulatory protein	bprA
−4.349596738	BPSS1529[a]	Putative membrane antigen	bipD
−4.43240846	BPSS1533[a]	Surface presentation of antigens protein	bicA
−5.391462205	BPSS1516[b]	Hypothetical protein	
−5.818423748	BPSS1517[b]	Hypothetical protein	

[a] TTSS3 gene.
[b] Putative TTSS3 additional gene.
[c] TTSS gene.
[d] Mean $\log_2(I_{Ara}/I_{Glu})$ = average value of the $\log_2$-based ratio of intensities from two channels of the microarray: growth in arabinose versus growth in glucose.

experiments, these are homologous with TTSS genes. It was, however, interesting that a significant number of putative TTSS genes were newly identified from this analysis. In particular, the genes corresponding to footnote a in Table 5 are located outside of the projected boundaries for the *B. pseudomallei* and *B. mallei* type III secretion operons. Further, two of the coordinately regulated TTSS genes are annotated as putative exported proteins and may be genes coding for the secreted effector molecules of this TTSS.

Northern blot analysis confirmed the microarray data. Figure 8 represents the results from the Northern blot analyses of specific gene expression. These data confirmed the results from the microarray analyses in that specific type III secretion genes were repressed in *B. pseudomallei* strain SZ5028 when the organisms were grown in arabinose versus glucose. Additionally, the data indicate that strains *B. pseudomallei* 406e and SZ5028 are isogenic and that the difference in virulence between these strains was not due to effects from the integration of a plasmid which is >13 kb in size, as genes flanking the integration site were not altered in their expression.

DISCUSSION

Arabinose metabolism has historically been a primary means of distinguishing between virulent and avirulent strains of *Burkholderia* spp. isolated from the soils of northern Thailand. We now know that two closely related *Burkholderia* species reside in the soil and that the ability to metabolize arabinose distinguishes avirulent *B. thailandensis* from its virulent close relative, *B. pseudomallei* (31).

In this study we characterized arabinose metabolism in *B. thailandensis* and examined the role of the L-arabinose assimilation operon in the virulence of *B. pseudomallei*. We were able to generate a transposon mutant in *B. thailandensis* that was unable to utilize L-arabinose as a sole carbon source. Using this mutant, we identified an L-arabinose assimilation operon consisting of nine genes, including a transcriptional regulator. The presence of these genes suggested a metabolic pathway similar to that found in a variety of soil bacteria. Using *lux*-fusion constructs, we were able to demonstrate that the operon is inducible by L-arabinose but not by D-glucose. Although unable to utilize arabinose as a sole carbon source, sequence data have shown that the *B. pseudomallei* chromosome contains at least two regions of the arabinose assimilation operon found in *B. thailandensis*. These regions include a portion of *araA*, which encodes the arabinose assimilation operon regulator, and a portion of *araH* (involved in L-arabinose transport) and *araI* (L-arabinolactonase). These observations suggest that at one time, the *B. pseudomallei* chromosome contained an intact L-arabinose assimilation operon.

To examine what effect L-arabinose assimilation genes might have on the virulence of *B. pseudomallei*, we introduced the L-arabinose assimilation operon from *B. thailandensis* into *B. pseudomallei* 406e, generating the strain SZ5028. *B. pseudomallei* SZ5028 was able to grow in minimal medium containing L-arabinose as the sole carbon source. The presence of the L-arabinose assimilation operon in SZ5028 reduced virulence in this strain. Using the Syrian hamster model of *B. pseudomallei* infection, we found that SZ5028 had a significantly higher LD_{50} than did the parent strain, *B. pseudomallei* 406e (198 CFU versus <10 CFU), although SZ5028 remained much more virulent than *B. thailandensis* ($LD_{50} = 1.8 \times 10^6$ CFU). A similar increase in LD_{50} values has also been observed in mutant strains of *B. pseudomallei* which were singly deficient in other virulence factors, including serum resistance (13), motility (14), or type III secretion (J. Warawa and D. E. Woods, submitted for publication). Given the detrimental effects of arabinose upon *B. pseudomallei* virulence, we found it interesting that the growth of *B. pseudomallei* with arabinose as the sole carbon source resulted in the down-regulation of genes of TTSS3—the *Salmonella*-like gene cluster required for virulence in hamsters. The microarray results have provided a direct link between the attenuation of virulence associated with arabinose utilization and down-regulation of TTSS3 functional activity. Further, our microarray data demonstrated that the transcription of the gene for a putative positive regulator of TTSS3, *bsaN*, was reduced during growth with arabinose. BsaN is predicted to be a member of the AraC family of transcriptional regulators, which appear to be common in mammalian-targeting TTSS clusters, such as VirF, PerA, ExsA, MxiE, and InvF in *Yersinia*, enteropathogenic *E. coli*, *Pseudomonas*, *Shigella*, and *Salmonella*, respectively. AraC, in *E. coli*, acts as either a positive or negative regulator and is encoded within the arabinose utilization operon, and its activity is affected by the direct binding of arabinose (for review, see reference 29). We hypothesize that arabinose directly binds the AraC homo-

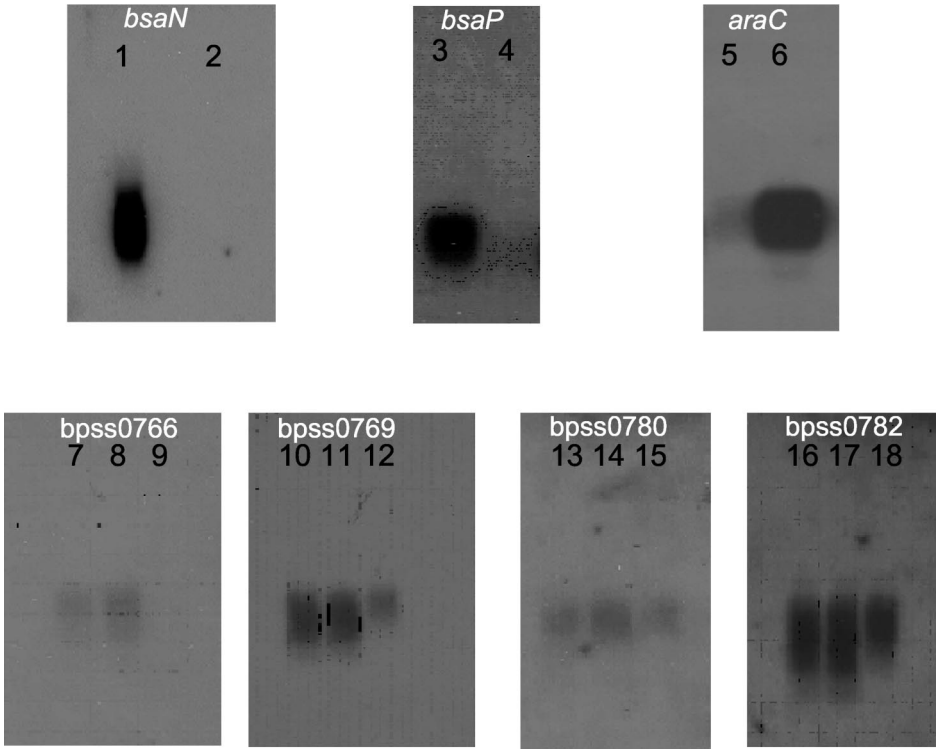

FIG. 8. Northern blot analysis of gene expression. Lanes 1, 3, 5, 8, 11, 14, and 17, total RNA from strain SZ5028 grown in M9 medium supplemented with 1% glucose; lanes 2, 4, 6, 9, 12, 15, and 18, total RNA from strain SZ5028 grown in M9 medium supplemented with 1% arabinose; lanes 7, 10, 13, and 16, total RNA from strain 406e grown in M9 medium supplemented with 1% glucose. The RNA blots were probed with the genes *bsaN*, *bsaP*, *araC*, *bpss0766*, *bpss0769*, *bpss0780*, and *bpss0782*.

logue, BsaN, and thereby inactivates the positive transcriptional regulation of TTSS3 genes in *B. pseudomallei*. The studies to test this hypothesis are ongoing, and the results from these studies could have significant implications for a wide range of TTSSs in mammalian pathogens whereby arabinose may act as an inhibitor of TTSS-mediated pathogenesis.

The abundance of L-arabinose in the environment presumably provides *B. thailandensis* a means of easily acquiring necessary carbon as well as the ability to compete with other soil microorganisms. However, arabinose metabolism may have been detrimental to an organism shifting from a soil environment to the environment of an animal host, resulting in negative selection for arabinose metabolism genes. As such, L-arabinose assimilation genes may be classified as antivirulence genes, as put forth by Maurelli et al. (22). Loss of this function may have had little consequence in regard to survival in tropical soils, where a variety of other carbohydrates might be readily available, but this loss may have been critical for success as an animal pathogen. Loss of an antivirulence metabolic pathway combined with the acquisition of virulence genes, such as those responsible for capsule synthesis (26) and TTSSs, may have allowed *B. pseudomallei* a means of thriving in both soil and animal environments. It has been proposed that *B. mallei* evolved from *B. pseudomallei* (17) and, thus, it is likely that the advantages that loss of arabinose assimilation genes provided were shared with *B. mallei* as it adapted to the more restrictive environment presented to a host-adapted pathogen unable to persist outside of an equine host.

It was interesting that the ability of the *B. thailandensis* arabinose utilization locus to affect expression of virulence genes in *B. pseudomallei* and *B. mallei* using whole-genome DNA microarray analysis provided results similar to those obtained using significantly smaller 200-gene microarrays. Most certainly, small gene arrays have been used in other studies with different organisms and have yielded important and significant results (2, 18–20). In our studies, we have provided additional documentation for the utility of small gene arrays in the analysis of gene expression, and it is clear that intuitive selection of specific genes for the construction of small gene arrays can lead to important conclusions regarding gene expression under various environmental conditions.

ACKNOWLEDGMENTS

This work was funded by the Department of Defense contract no. DAMD 17-03-C0062 and the Canadian Institutes of Health Research (MOP-36343). D.E.W. is a Canada Research Chair in Microbiology.

We thank Marina Tom and Patricia Baker for excellent technical assistance throughout various stages of this work.

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Editor: J. T. Barbieri