Type III Secretion: a Virulence Factor Delivery System Essential for the Pathogenicity of *Burkholderia mallei*

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By creating mutations in the *Burkholderia mallei* ATCC 23344 animal pathogen-like type III secretion system (TTSS), this study analyzes the correlation between type III secretion and the pathogenicity of ATCC 23344 in vivo. Mutagenesis demonstrated that a functional TTSS was required for the full pathogenicity of ATCC 23344 in the BALB/c mouse and Syrian hamster models of infection. However, vaccination with each mutant failed to elicit a protective immunity against challenge with wild-type ATCC 23344.

Burkholderia mallei, the causative agent of glanders disease, is a gram-negative, oxidase-positive, nonmotile bacillus that normally infects equines (horses, mules, and donkeys). *B. mallei* is an obligate animal pathogen that can infect various solipeds, mice, guinea pigs, rabbits, monkeys, hamsters, and even lions and dogs that consume contaminated meat (2, 6, 8). Humans are considered an incidental host for *B. mallei*, and individuals whose occupation subjects them to contact with *B. mallei*, particularly laboratory workers, are at high risk for contracting glanders disease (2). Occupational infection with *B. mallei* can occur via the cutaneous and inhalation routes.

Numerous gram-negative bacteria, including both animal and plant pathogens, encode specialized type III secretion systems (TTSS) that deliver bacterial proteins (effector molecules) into the plasma membrane and cytoplasm of eukaryotic cells (4). Several investigations recently identified the presence of animal and plant pathogen-like TTSS in *Burkholderia pseudomallei* (9, 13, 15). Of particular interest, and the scope of this investigation, is the animal pathogen-like TTSS encoded by *B. mallei* ATCC 23344. To evaluate the role of TTS in the virulence of ATCC 23344, specific mutations were created within the *bsaQ* and *bsaZ* alleles, which are homologues of the *Salmonella enterica* serovar Typhimurium *invA* and *spaS* genes and have been shown to encode proteins required for bacterial pathogenicity both in vivo and in vitro (4). In *B. pseudomallei*, mutation of the *bsaZ* (strain RD02 in this study) gene caused a drastic reduction in intracellular replication within J774.2 murine macrophages, failure of endocytic vacuole escape, and inhibition of actin polymerization (13). Considering the history and highly infective nature of *B. mallei*, as well as the potential for weaponization, there is a significant need to identify virulence determinants encoded by this bacterium.

Analysis of the ATCC 23344 genome (http://www.tigr.org/) revealed the presence of a TTSS genetically similar (Fig. 1) to the *B. pseudomallei* animal pathogen-like TTSS locus reported by Stevens et al. (13). The ATCC 23344 TTSS encodes 30 open reading frames (ORFs) and shares genetic similarity to the *Salmonella inv/spa/prg* and *Shigella ipa/mxi/spa* systems (thoroughly reviewed in reference 4). Interestingly, multiple sequence gaps ranging from 1 to 90 bp were identified when the *B. pseudomallei* K96243 (http://www.sanger.ac.uk/) and ATCC 23344 TTSS loci were aligned with each other and compared at the nucleotide level (Fig. 1). Many of the sequence gaps are flanked by simple sequence repeats, which may provide a mechanism for the addition or removal of amino acids from the encoded proteins. A majority of the sequence gaps (99%) occurring within putative ORFs did not induce frameshift mutations because they occurred in multiples of 3 bp. In contrast, sequence gaps not divisible by 3, which generate frameshifts, were primarily present within intergenic regions. However, located in the 5′ region of the *bsaX* allele (Fig. 1), a 1-bp deletion was identified, which conceivably causes a frameshift resulting in a truncated protein in *B. mallei*. These results suggest the BsaX protein may not be an essential component of the *Burkholderia* TTSS.

Bacterial strains, plasmids, and PCR primers used in this study are listed in Table 1. Primers for the *bsaQ* and *bsaZ* (13) genes were designed in silico using the nucleotide sequence of the *B. mallei* ATCC 23344 TTSS. Disruption of these alleles, which are essential components of the secretion apparatus, would likely exert a polar effect on the ATCC 23344 TTSS and disrupt effector molecule secretion. Genomic DNA for PCR amplification was purified using the MasterPure DNA purification kit according to the manufacturer's instructions (Epicentre Technologies, Madison, Wis.). Internal gene fragments for creating disruption cassettes were PCR amplified using the following conditions: one cycle at 94°C for 5 min; 30 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 30 s; followed by a final 7-min extension at 72°C. Internal gene amplicons were subcloned into pCR2.1-TOPO (Invitrogen, Carlsbad, Calif.) and transformed into One Shot chemically competent *Escherichia coli* cells (Invitrogen). Integration cassettes were made by digesting pCR2.1-TOPO containing the *bsaQ* and *bsaZ* internal gene amplicons with *Eco*RI (New England Biolabs, Beverly, Mass.) followed by subcloning (Fast-Link DNA ligation kit; Epicentre Technologies) into the suicide vector pTSV3 digested with *Eco*RI. Ligations were electroporated into *E. coli* SM10 (11) and mobilized into ATCC 23344 using methods described by DeShazer et al. (3). To confirm site-

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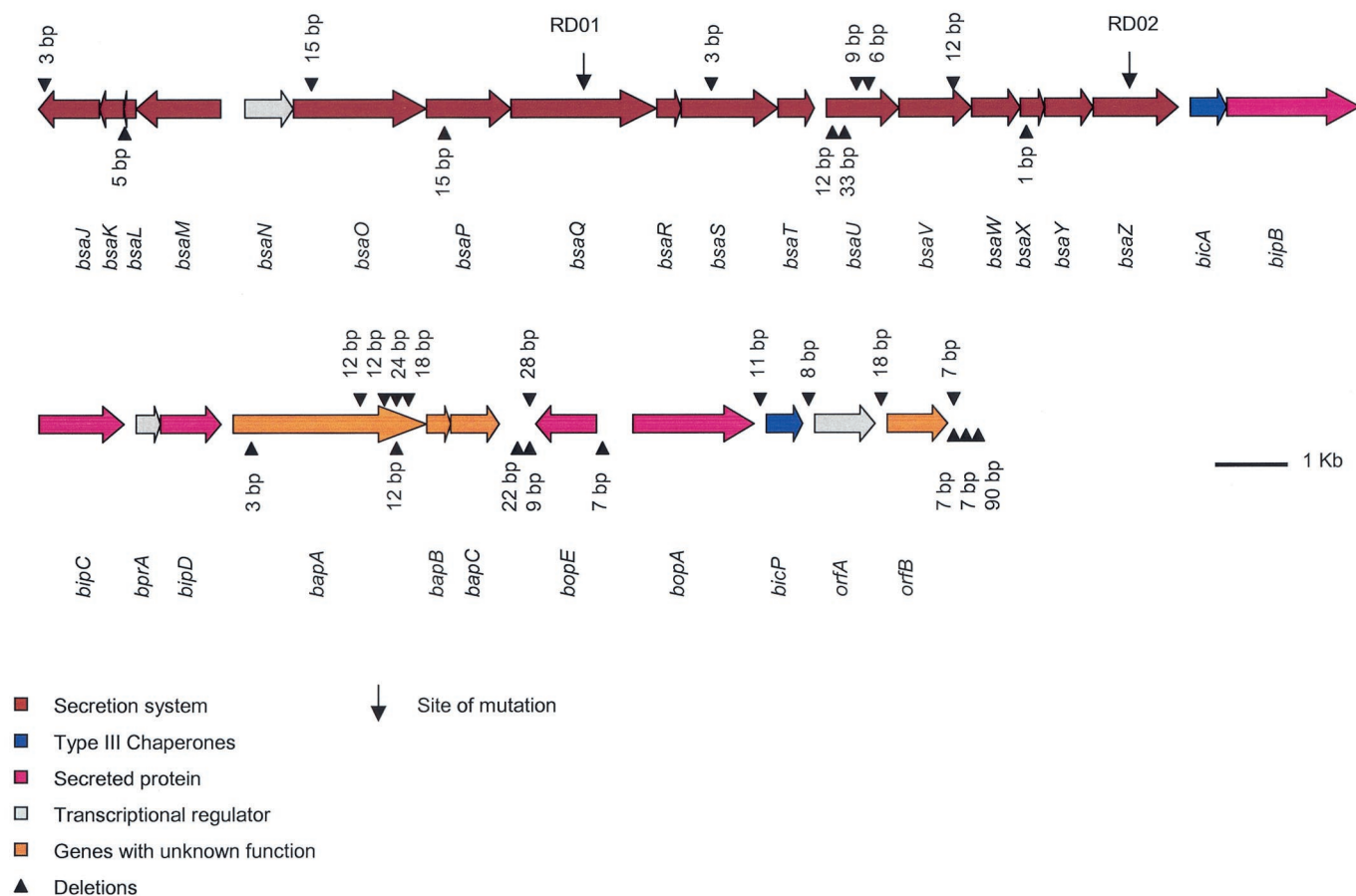
FIG. 1. Schematic organization of the genes encoding the *B. mallei* ATCC 23344 animal pathogen-like TTS apparatus. The nomenclature of the putative ORFs has been kept consistent with that of the *B. pseudomallei* TTSS. The *B. mallei* TTS locus is approximately 32,000 bp, has a relative G+C content of 69.9%, which is consistent with the ATCC 23344 genome, and is located on chromosome 2 beginning at nucleotide 1,201,133 and ending at nucleotide 1,233,132. Inverted triangles (▼) located above the ORFs represent sequence gaps present in the *B. pseudomallei* K96243 TTSS, while triangles (▲) depict sequence gaps identified in the ATCC 23344 TTSS. Mutation sites are indicated with arrows.

specific integration (single-crossover mutants), PCR parameters modified from those described above, specifically increasing the extension to 4 min, were employed using gene-specific primers flanking the *bsaQ* and *bsaZ* alleles (Table 1). To confirm the stability of our merodiploids in vitro, organ extracts 30 days postexposure were plated on Luria-Bertani plates (Fisher Scientific, Pittsburgh, Pa.) containing 4% glycerol (vol/vol) (LBG) with and without antibiotic. Whole-body aerosol exposures of 6- to 8-week-old female BALB/c mice (National Cancer Institute, Frederick, Md.) using ATCC 23344, RD01 (*bsaQ*::pTSV3), and RD02 (*bsaZ*::pTSV3) were performed as described by Jeddeloh et al. (5). A relative dose of 1.0×10^4 CFU was targeted for each strain, representing approximately 10 times the 50% lethal dose (LD_{50}) (5).

Figure 2A depicts the survival pattern of BALB/c mice (10 mice for each bacterial strain) infected via aerosol with ATCC 23344, RD01, or RD02. Mice infected with approximately 1.2×10^4 CFU of ATCC 23344 began to die at day 4, and the remaining animals died at day 5. All mice exposed to RD01 and RD02 survived an aerosol challenge of 1.8×10^4 and 1.5×10^4 CFU, respectively, and exhibited no clinical symptoms 42 days postexposure.

To analyze the pathogenicity of ATCC 23344, RD01, and RD02, bacterial loads within the spleen, liver, and lungs of BALB/c mice challenged with each strain (doses were identical to those previously described) were assayed over a 5-day period postexposure. Briefly, one mouse from each experimental group was euthanized (CO_2 chamber) on each of days 1 to 5, and the organs were extracted, homogenized in sterile phosphate-buffered saline, serially diluted, and plated on LBG. In addition, to determine if animals were chronically infected with RD01 and RD02, a single animal was euthanized 30 days postchallenge and organs were processed as described above. Figure 2B shows the bacterial loads of ATCC 23344, RD01, and RD02 within the lungs of BALB/c mice. Bacterial counts for ATCC 23344 progressively increased over the 5-day course of analysis and peaked at day 4, with an average load of 2.4×10^6 CFU/g of homogenized tissue. Organ loads within the liver and spleen spiked at day 5 with an enumerated concentration of 5.8×10^5 and 3.5×10^5 CFU/g of organ, respectively (Fig. 2C and D). Due to the animal mortality of groups infected with wild-type ATCC 23344, organ assays were not possible at day 30. In comparison, recovery of RD01 and RD02 within the lungs of challenged mice was markedly reduced in contrast to

TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Description[c]	Reference or source
E. coli strains		
TOP10	Used for cloning and blue-white screening	Invitrogen
SM10	Mobilizing strain, RP4 chromosomally integrated, Km^r	12
B. mallei strains		
ATCC 23344	Human isolate	USAMRIID
RD01	ATCC 23344 derivative, *bsaQ*::pTSV3, Tp^r	This study
RD02	ATCC 23344 derivative, *bsaZ*::pTSV3, Tp^r	This study
Plasmids		
pPCR2.1-TOPO	TA cloning vector, Km^r Ap^r	Invitrogen
pTSV3	pTn*Mod*-OTp lacking the 1.5-kb *Bgl*II *tnp* fragment; mobilizable Tp^r suicide vector	This study
pRD01	Contains a 489-bp PCR amplicon from the ATCC 23344 *bsaQ* gene; Tp^r	This study
pRD02	Contains a 313-bp PCR amplicon from the ATCC 23344 *bsaZ* gene; Tp^r	This study
pTn*Mod*-OTp	Modular mini-transposon; pMB1 oriR, Tp^r	1
Primers		
bsaQiF[a]	5′-ACGCGATGCCCGGCAAGCAGATGA-3′	This study
bsaQiR	5′-GCGCGAGCGCGATCAGCAGGAAGA-3′	This study
bsaQgF[b]	5′-CGCTCGGCGGCGTGCTGCTCA-3′	This study
bsaQgR	5′-AGCGGGGTCATCGCATGGCTCCTG-3′	This study
bsaZiF	5′-GATGAATCTCGCGCCGCTTGCTCT-3′	This study
bsaZiR	5′-GACGCGGTGAAGGCGCTGCTCTAT-3′	This study
bsaZgF	5′-CGCCGCCGCCATTTCCTG-3′	This study
bsaZgR	5′-GCGCCGTTCGTGAGCCTGTTCTAT-3′	This study

[a] Within the primer nomenclature, *i* specifies internal gene primers.
[b] Gene-specific primers are designated with the letter *g*.
[c] Km^r, kanamycin resistance; Tp^r, trimethoprim resistance; Ap^r, ampicillin resistance.

that of ATCC 23344 (Fig. 2B). Both RD01 and RD02 were cleared from the lungs at days 2 and 3 postexposure, respectively. Interestingly, animals challenged with RD01 contained no recoverable organisms from the liver or spleen at days 2 to 30 (Fig. 2C and D). In contrast, at days 2 and 5, the livers of the experimental group receiving RD02 contained 2.9×10^1 CFU/g of organ tissue. Unlike ATCC 23344 and RD01, RD02 caused a biphasic course of infection and trophism for the liver and spleen was observed. At days 3 and 30, the livers of mice infected with RD02 were sterile compared to the spleen, which contained 7.3×10^3 CFU/g of homogenized tissue at day 30 (Fig. 2D). The stability of each merodiploid was analyzed from lung, liver, and spleen samples (30 days postexposure) by plating extracts on nonselective LBG. As anticipated, vector integration in vivo was stable without the presence of trimethoprim (data not shown).

To determine if RD01 and RD02 provided protection against wild-type ATCC 23344, experimental groups exposed to RD01 and RD02 were rechallenged by aerosol with each corresponding mutant at day 21. Following this booster, on day 42 mice were aerosolized with 1.7×10^5 CFU of ATCC 23344 (100 times the LD$_{50}$). Unfortunately, neither RD01 nor RD02 conferred protection against challenge with wild-type ATCC 23344. These results could have been the consequence of the dose of ATCC 23344 delivered. For RD01, these findings were consistent with the observed rapid clearance from the lungs of infected animals (Fig. 2B). However, these results were surprising for RD02, which persisted in the liver for several days postchallenge at concentrations conceivably sufficient to generate an adequate immunological response (Fig. 2C).

Clinical manifestations, such as fur ruffling and animal hud-

dling, of mice challenged with ATCC 23344 began 2 days postexposure. Animals receiving RD01 and RD02 did not exhibit any clinical symptoms throughout the 42-day course of infection. However, gross pathological analysis of spleen extracts from animals challenged with RD02 (30 days after exposure) revealed extensive splenic abscesses (Fig. 2E), indicating a chronic form of infection.

Previous investigations demonstrated the extreme sensitivity (<10 CFU) of Syrian golden hamsters to ATCC 23344 (3), which makes this animal model ideal for analyzing the pathogenicity of mutant derivatives of *B. mallei*. Using the methods described by DeShazer et al. (3), male Syrian golden hamsters were infected intraperitoneally with 1.0×10^1 to 1.0×10^3 CFU of ATCC 23344, 1.0×10^1 to 1.0×10^6 CFU of RD01, or 1.0×10^1 to 1.0×10^5 CFU of RD02. Five animals for each 10-fold dilution series were used, and the LD$_{50}$ was determined at day 4 as described by Reed and Muench (10). The relative LD$_{50}$ for ATCC 23344 was <13 CFU, in contrast to >10^6 CFU and >10^5 CFU for RD01 and RD02, respectively. These findings definitively illustrate that TTS is crucial for the pathogenicity of ATCC 23344.

In addition to analyzing the organ loads of infected animals and determining the LD$_{50}$s, the immunoglobulin G (IgG) titer against RD01 and RD02 was assayed 30 days postchallenge. IgG titers for each strain, in one animal from the ATCC 23344, RD01, and RD02 groups used for organ load analysis, were determined using an enzyme-linked immunosorbent assay-based technique described by Waag et al. (14). Positive control ATCC 23344 antigen was prepared by harvesting an overnight culture of ATCC 23344 grown in glycerol (4% [vol/vol])–tryptone broth (Fisher Scientific). The cellular pellet was resus-
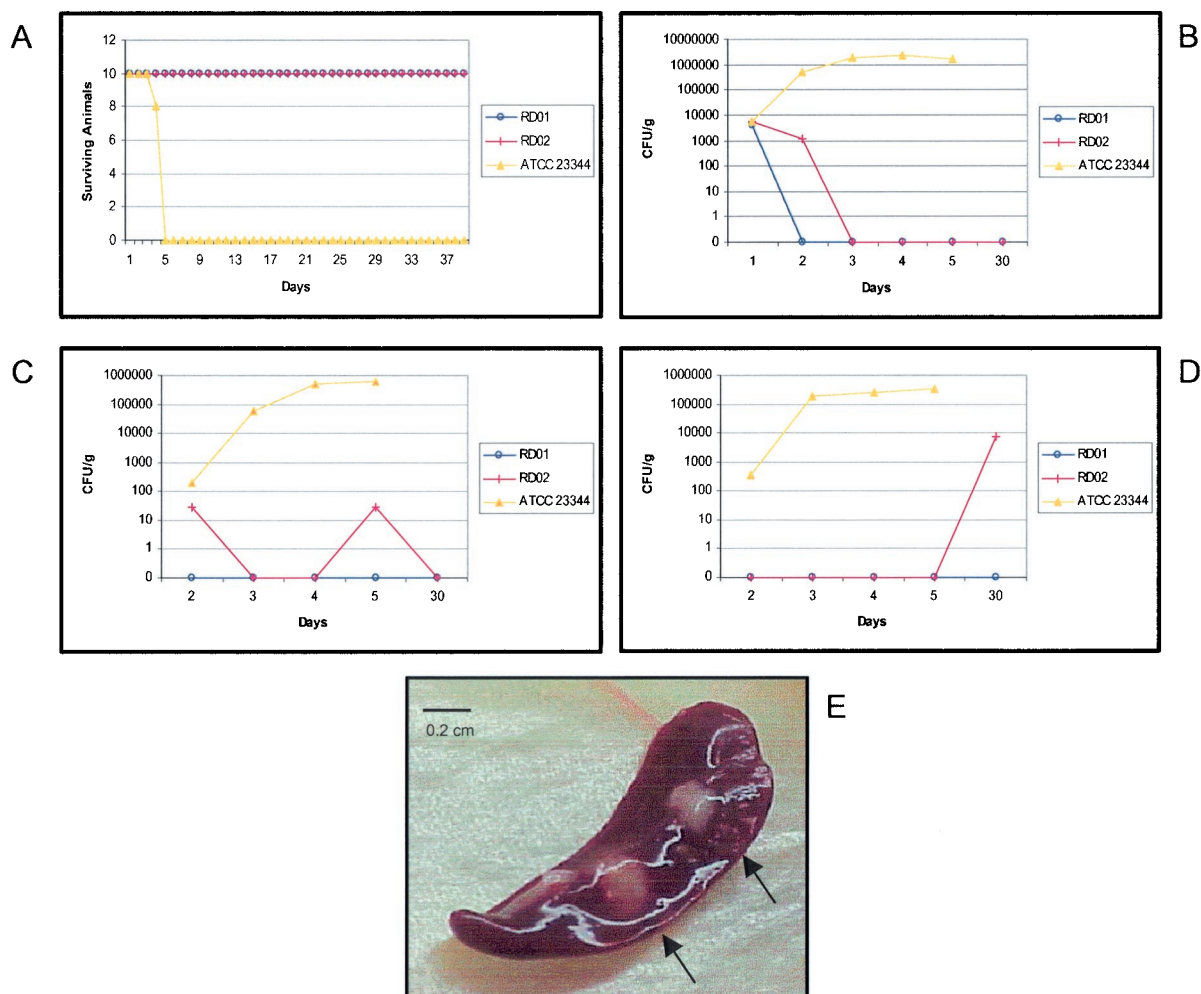
FIG. 2. Mutations in the *B. mallei* ATCC 23344 TTSS locus drastically reduce virulence in a BALB/c animal model. (A) Survival of BALB/c mice exposed to ATCC 23344, RD01 (*bsaQ*::pTSV3), and RD02 (*bsaZ*::pTSV3). (B to D) Relative bacterial loads within the lungs, liver, and spleen of BALB/c mice challenged via aerosol with ATCC 23344, RD01, and RD02. (E) Multiple splenic abscesses (arrows) observed after 30 days postexposure to RD02.

pended in Hanks balanced salt solution, irradiated at 2.1 Mrads, washed three times in Hanks balanced salt solution, and resuspended in sterile water to yield a 1-mg/ml stock. An assayable immunological response, expressed as the reciprocal of the highest dilution yielding a positive reaction, was observed for RD02 (800) and the ATCC 23344 positive control (12,500). However, the IgG titer of animals infected with RD01 was undetectable. These results are consistent with the observed rapid clearance, 48 h postexposure, of RD01 from the lungs (Fig. 2B) of infected mice.

The only virulence factor definitively shown to be essential for the pathogenicity of *B. mallei* is an extracellular capsule (3). Mutational analysis of the *B. pseudomallei* animal pathogen-like TTSS, using in vitro techniques, demonstrated that the TTSS is required for intracellular replication within murine macrophage-like cells (J774.2), escape from endocytic vacuoles, and actin polymerization (13). By creating specific mutations located within the *bsaQ* and *bsaZ* genes (*Salmonella invA* and *spaS* homologues), this investigation, despite only being able to analyze the organ loads from a single animal for each

time point, suggests that TTS plays an essential role in the virulence of *B. mallei* ATCC 23344 in vivo. Further studies will be needed to identify putative effector molecules and decipher the complex molecular mechanisms associated with this animal pathogen-like TTSS.

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