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Microarray Analysis of Transposon Insertion Mutations in *Bacillus anthracis*: Global Identification of Genes Required for Sporulation and Germination▽†

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A transposon site hybridization (TraSH) assay was developed for functional analysis of the *Bacillus anthracis* genome using a mini-Tn*10* transposon which permitted analysis of 82% of this pathogen's genes. The system, used to identify genes required for generation of infectious anthrax spores, spore germination, and optimal growth on rich medium, was predictive of the contributions of two conserved hypothetical genes for the phenotypes examined.

The goal of obtaining a detailed understanding of *Bacillus anthracis* physiology has not been realized due to limited information concerning factors required for growth, sporulation, and virulence. For example, few factors or processes required for generation of the anthrax spore, which develops to enable long-term survival outside the host, have been described (28). Such information would be very valuable as the spore is the infectious form of the pathogen and has been the focus of several virulence and vaccine studies (1, 5, 15, 16). Recently, a functional genomics assay termed the transposon site hybridization (TraSH) assay was described, and this assay has the potential to significantly increase our understanding of microbial physiology by directly identifying genes required for growth and survival in specific environments (18). The TraSH assay uses microarray technology to globally determine the locations of transposon insertions in a population of mutants and to compare the effects of each insertion on the representation of clones before and after exposure to an environmental stress (Fig. 1) (19, 20). Comprehensive TraSH screens depend on thorough distribution of transposon insertion mutations in the genome. Several investigators have noted that transposons commonly used in gram-positive bacteria (Tn*916* and Tn*917*) appear to target preferentially one or both of the *B. anthracis* virulence plasmids and that few insertions are observed in the chromosome (9, 11, 24, 26). Recently, a *mariner*-based transposon mutagenesis system which shows promise was described for *B. anthracis*; however, the utility of this system in global mutagenesis has not been demonstrated (23). Thus, such mutagenesis systems may not permit global genome-wide analysis of *B. anthracis* physiological systems in strains harboring one or both of the virulence plasmids. This presents a significant hurdle since strains that lack both virulence plasmids are completely attenuated and of limited use in genetic screens for virulence traits in animal models of disease (26). In this study we sought to adapt TraSH to functional genomic analyses of the *B. anthracis* genome using a mini-Tn*10* transposon and whole-genome *B. anthracis* microarrays.

Mini-Tn*10* permits global mutagenesis of the *B. anthracis* genome. Plasmid pIC333, a mini-Tn*10* vector encoding spectinomycin resistance and containing a *tnpA* allele with relaxed target specificity to increase the randomness of transposition (21), was used to mutagenize two *B. anthracis* strains, *B. anthracis* ΔAmes harboring the capsule plasmid (pXO1$^-$ pXO2$^+$) and a derivative of this strain, *B. anthracis* ΔAmes *gdh*, modified at the chromosomally encoded *gdh* locus [Δ*gdh* (*atxA* Ω*kan PpagA-lacZ pagR*)], which was used in screens for genes that influence anthrax toxin and capsule expression (data not shown). Mutagenesis was performed as described by Gominet et al. (8). The mutants were selected for growth on brain heart infusion agar plates containing spectinomycin (250 μg/ml) incubated 15 h at 37°C. Pools of mutants of each of the two strains (ΔAmes and ΔAmes *gdh*) were maintained separately to generate two mutant populations, which were designated WAD205 and WAD272, respectively. The use of two distinct mutant populations enhanced analyses of the coverage of each population with transposon insertions and allowed detection of insertional biases (see below). Differential plating and sequence analysis of the insertion sites of several clones demonstrated that roughly 10% of the mutants harbored mutations in which, following transposition of elements encoded inside the inverted repeats, the plasmid had integrated into the chromosome via Campbell-type recombination (data not shown). No evidence of multiple insertions was observed using

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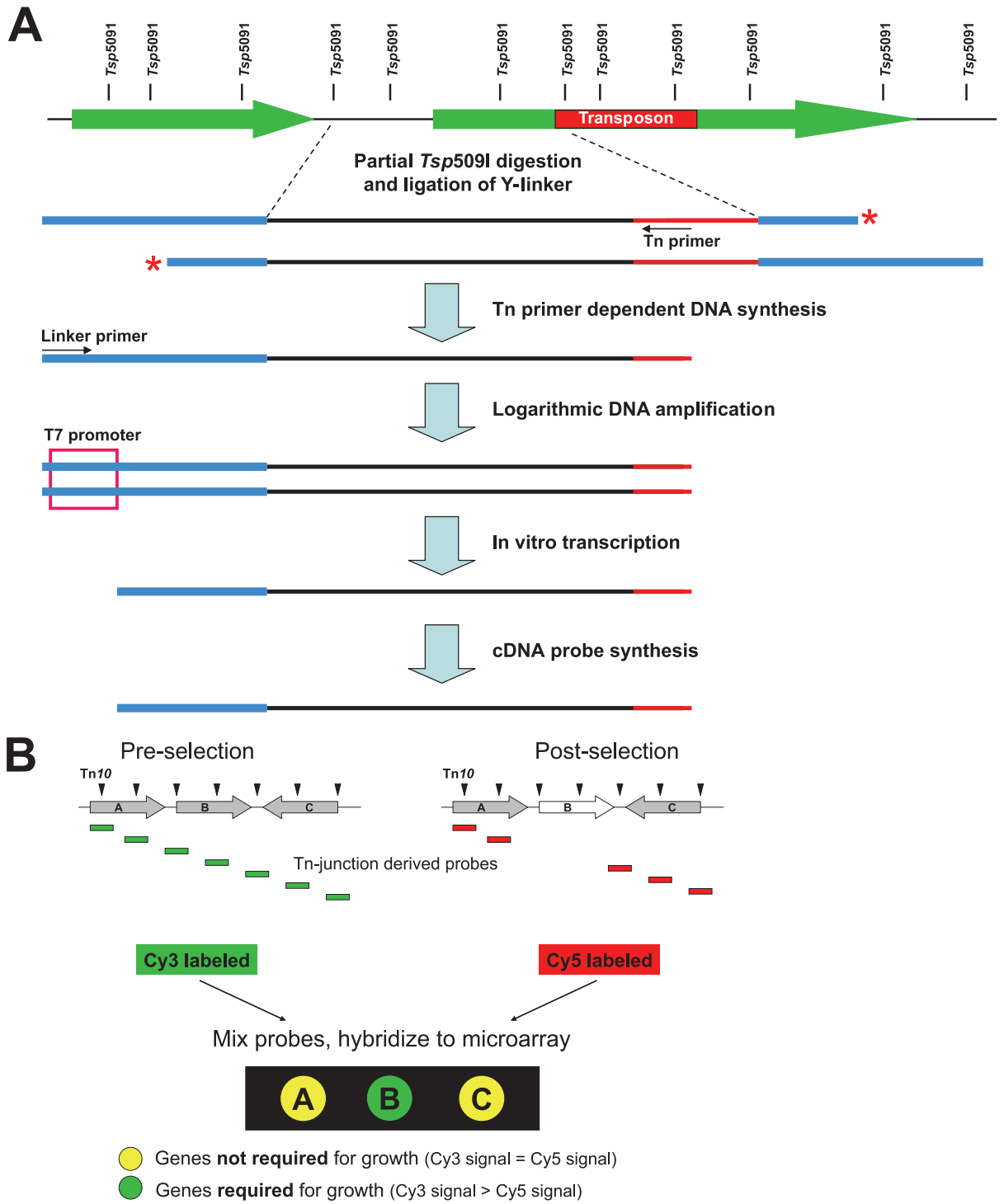

FIG. 1. Schematic diagram of the TraSH screening strategy. (A) TraSH probe generation. Genomic DNA from a population containing transposon insertions (red) is partially digested with the four-base cutter Tsp509I and size fractionated; fragments are ligated to Y-shaped linker DNA (blue) which encodes an inactive T7 promoter but not the complementary strand. Ligation products are subjected to PCR amplification using a transposon-derived primer and a linker-derived primer which encodes a functional T7 promoter; logarithmic amplification of PCR products is dependent upon DNA synthesis from the transposon-derived primer, which generates the linker-derived primer binding site (extension of the linker, which would generate a binding site for the linker-derived primer, is prevented by a 3′ amino modification [red asterisk]). As a result, PCR products are generated only from sequences adjacent to transposon insertions. PCR products are transcribed in vitro from the T7 promoter, and amino-allyl-labeled cDNA probes are reverse transcribed from the RNA. (B) Microarray hybridization of TraSH probes. A population of mutants containing multiple transposon insertion mutations (indicated by arrowheads above genes A, B, and C) grown on rich medium (preselection) is subjected to growth under selective pressure (postselection). TraSH probes, PCR amplified from the mutant populations before and after growth selection and differentially labeled with fluorescent dyes, are cohybridized to microarrays. Clones harboring mutations in genes required for fitness under the selective pressure (gene B) are reduced or eliminated from the postselection population, resulting in reduced microarray hybridization signals compared to those of the parental preselection population. The figure was adapted from the study of Sassetti and Rubin (20).

probes derived from mini-Tn*10* in Southern hybridization analyses of several mutant genomes (data not shown).

To determine the location and distribution of transposon insertions in each mutant population, TraSH probes were generated from each population as described by Sassetti and Rubin (20), with the following modifications. *B. anthracis* genomic DNA that was partially digested with Tsp509I and ranged from 700 to 2,000 bp long was ligated to linker DNA generated by annealing two oligonucleotides, BD154 (5′-A ATTCGACCACGACCA-C7 amine modification-3′) and BD155 (5′-CCGTTTAATACTACTCACTATAGGGAGAT GGTCGTGGTCG-3′), which contained an inactive version of the consensus T7 promoter (10), as well as a 5′ overhang complementary to the Tsp509I sticky end. Products of the ligation reaction (2 μl) were used as a template in a PCR (40 μl) performed with transposon-derived primer E1 (5′-CGTT GGCCGATTCATTAATGC-3′) or E3 (5′-CGATATTCACG GTTTACCCAC-3′) and linker-derived primer BD156 (5′-CC GTTTAATACGACTCACTATAG-3′), which encodes a mismatch resulting in a functional consensus T7 promoter in the amplified products (10). The PCR products were cleaned and transcribed in vitro using a T7-Megashortscript kit (Ambion, Austin, TX), and aminoallyl-dUTP cDNA was synthesized as described by Bourgogne et al. (3). The cDNA derived from both mutant populations was coupled to either Cy3 or Cy5, and the differentially labeled probes were cohybridized to a whole-genome *B. anthracis* microarray containing both amplicons and oligonucleotides (referred to as spots) printed in duplicate as described previously (3). Microarray signals were analyzed using the GenePix software (Molecular Devices, Sunnyvale, CA). The spots associated with transposon insertions in each population that had sufficient signal intensity for further analysis were those for which the intensity of at least 50% of the pixels was greater than the local background intensity plus two standard deviations for both spots corresponding to a gene, as defined by Badarinarayana et al. (2). Spots that did not meet these criteria were excluded from further analyses.

Transposons were associated with 82% of the WAD205 genes and 68% of the WAD272 genes, including virulence plasmid pX02 and chromosomal loci (Table 1). A comparison of the WAD205 and WAD272 hybridization signals showed that the intensities of the signals for a majority of the genes were equivalent in the two populations; moreover, the signal patterns suggested that transposon insertion in each population was unbiased and of high density (Fig. 2). Although a majority of the genes associated with transposons were shared between both mutant populations, a significant portion of the signals were observed in only one of the populations (Table 1). This observation indicated that neither of the mutant populations harbored transposon insertions in all nonessential genes. Therefore, any list of genes identified in screens using either of these mutant populations would likely be incomplete. Several genes with high-intensity hybridization signals were observed in each mutant population (Fig. 2). However, the intensities of these genes varied widely between the two populations, and few genes had high-intensity signals in both populations. These observations suggested that the intense hybridization signals resulted from overrepresentation of clones sustaining transposon insertions early in the mutagenesis culture (resulting in siblings) and not from repeatedly targeted sequences in the *B. anthracis* genome (hot spots) or sequence characteristics of the spots arrayed on the microarray slides (G+C content or nucleotide length). Collectively, these results suggested that transposon insertion was unbiased and of sufficient density, targeting both virulence plasmid and chromosomal genes, to permit global TraSH analyses of a majority of the pathogen's genome.

TABLE 1. Tn*10* distribution in two distinct *B. anthracis* ΔAmes (pXO1$^-$ pXO2$^+$) populations[a]

Population	% Genes::Tn*10*[b,c]	No. of shared genes[b]	No. of unique genes[b]	No. of pXO1 genes[b]	No. of pXO2 genes[b]
WAD205	82	2,881	1,304	0/87	42/49
WAD272	68	2,881	596	1/87[d]	44/49

[a] The distribution of transposon insertions in both populations was determined in triplicate using TraSH; the data were derived from a single representative experiment.

[b] Significant hybridization signals were defined as follows: the intensity of at least 50% of the pixels was greater than the local background intensity plus two standard deviations (2).

[c] The values were calculated by dividing the number of genes associated with Tn*10* insertions by the number of genes with hybridization signals when the microarrays were probed with *B. anthracis* Ames genomic DNA (typically 85% of the genes on the array) minus the number of pXO1 genes (87 genes).

[d] Hybridization signals were observed for pXO1 gene *atxA* encoded on the WAD272 chromosome [*B. anthracis* ΔAmes Δ*gdh* (*atxA* Ω*kan PpagA-lacZ pagR*)].

Several observations indicated that the probes generated were derived from regions immediately adjacent to transposon insertions. Repeated attempts to synthesize TraSH probes from a nonmutagenized *B. anthracis* genome failed to generate PCR products, and no RNA was synthesized following the linker transposon-based PCR (data not shown). In contrast, complex populations of PCR products 100 to 600 bp long were routinely amplified from the genomic DNA of populations containing Tn*10* insertions, and abundant RNA (70 to 100 μg per in vitro transcription reaction) was synthesized from the PCR products (data not shown).

TraSH assay permits global identification of genes required for growth, sporulation, and germination. To demonstrate that the adapted mutagenesis and probe generation methods could be effectively used in a TraSH screen, we applied them to genetic analysis of the development of heat-resistant *B. anthracis* spores and spore germination. These are developmental pathways that have been studied extensively in *Bacillus subtilis*, and bioinformatic analyses have identified putative sporulation and germination orthologs in the *B. anthracis* genome (17). ΔAmes mutant population WAD205 was cultured in triplicate by spreading 10^6 mutant bacilli on tryptic soy agar plates containing spectinomycin and allowing the cells to sporulate. The resultant spore populations were heat shocked (40 min at 65°C) to kill clones containing transposon mutations in genes required for generation of heat-resistant spores, which would likely exist as heat-sensitive bacilli. The three populations of heat-resistant spores were inoculated into separate flasks containing brain heart infusion broth with spectinomycin and incubated for 15 h to germinate the spores and to amplify viable clones. As a result, clones harboring mutations in genes required for optimal growth in rich medium, development of heat-resistant spores, and spore germination in rich medium were eliminated from the populations recovered or their num-

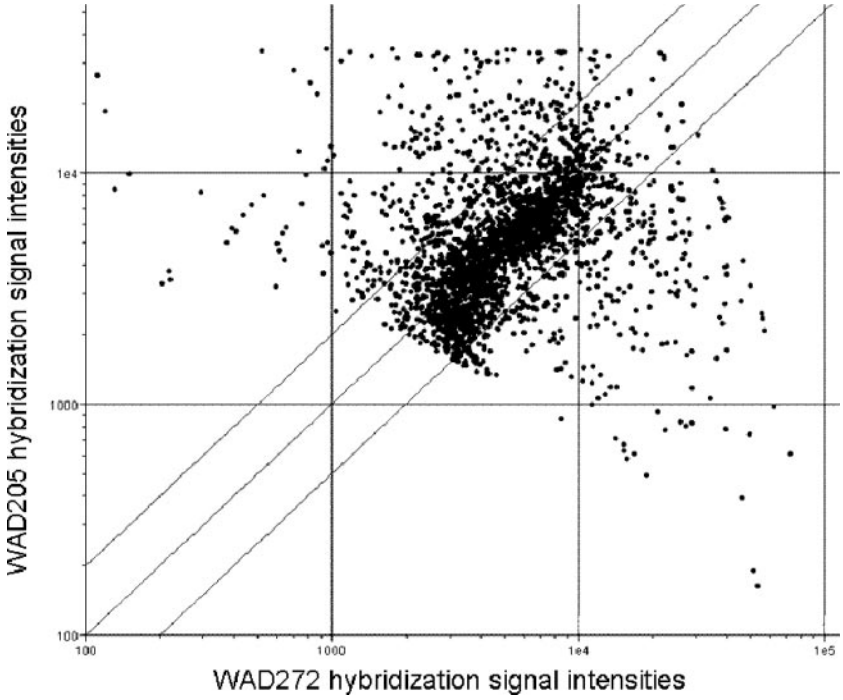

FIG. 2. Hybridization signals for two distinct *B. anthracis* ΔAmes mutant populations. Each gene's hybridization signal intensity for WAD205 and WAD272 populations following recovery of each population on rich medium with antibiotics was plotted. Genes with low signal intensities (as defined in the text) in either population were not included. Hybridizations were performed four times. The data were derived from a single representative experiment.

bers were diminished. TraSH probes were synthesized from each of the three sporulated populations (postselection), combined with differentially labeled TraSH probes synthesized from the presporulated parent population (preselection), and hybridized to the *B. anthracis* microarray in duplicate. Data were filtered as described above to include only spots with significant presporulation hybridization signals on all six arrays using the GenePix software. The data for all six hybridizations were then normalized using average spot intensity (Lowess) (6), combined, averaged, and filtered to identify genes required for optimal growth, sporulation, or germination with preselection mutant population/postselection population hybridization signal ratios significantly greater than 1 ($P < 0.05$, as determined by a *t* test; Benjamini and Hoch FDR filter) using the GeneSpring software. An additional filter excluded genes which resulted in subtle defects in growth, sporulation, or germination (less than threefold change in median preselection/postselection hybridization signal ratios). Genes required for optimal growth, sporulation, or germination in all three selected populations (253 genes) are described in Table S1 in the supplemental material.

The TraSH screen identified orthologs of 31 genes required for spore generation or germination in other *Bacillus* species, suggesting that these genes have similar, if not identical, roles in *B. anthracis* spore physiology (Table 2). These studies provided the first experimental evidence for the role of many of these elements in the development of the infectious anthrax spore. Approximately 50 genes essential for *B. subtilis* sporulation have been identified; orthologs of most of these genes have been identified in the *B. anthracis* genome by bioinformatic analyses (12). Roughly one-half of these predicted sporulation genes were identified in our TraSH screen. The failure to identify genes in our screen may have resulted from a lack of transposon insertions in the open reading frames or from the use of high-stringency screening conditions involving a single selective sweep for generation of heat-resistant spores. Genes encoding several germinant receptors that respond to specific environmental cues, such as inosine, histidine, and alanine, were identified, which is consistent with the idea that responsiveness to multiple germinants is required for optimal *B. anthracis* spore germination in vitro (25). The screen also identified several genes encoding elements of ABC transport systems, oligopeptide permeases, cytochrome synthesis proteins, and factors required for efficient spore cortex lysis and germination (Table 2). The essential roles of these factors in the development of heat-resistant spores and spore germination have been demonstrated in other *Bacillus* species (7, 8, 13, 14, 22, 27). Identification of these factors validated the adapted TraSH methods and demonstrated the assay's usefulness for functional analysis of the *B. anthracis* genome.

The TraSH screen also identified genes involved in several distinct biochemical pathways and, in many cases, multiple genes in a specific biochemical pathway, indicating that these pathways are required for optimal expression of the selected phenotypes. The pathways identified include those required for synthesis of several cofactors, as well as pathways required for transport and catabolism of different carbon sources. An additional one-third of the genes identified in the screen (85 genes) encode factors with unknown functions (see Table S1 in the supplemental material). These genes include conserved

TABLE 2. *B. anthracis* orthologs of *B. subtilis* sporulation and germination genes identified in the TraSH screen

Gene	Description[a]	*P* value[b]
BA1048	ABC transporter, ATP-binding protein EcsA	0.0193
BA4151	Cytochrome *c* oxidase, subunit IVB	0.0191
BA5050	Cytochrome *d* ubiquinol oxidase, subunit I	0.0123
BA3938	Dipicolinate synthase, B subunit	0.04
BA4637	GTP pyrophosphokinase, RelA	0.0439
BA1510	Negative regulator of competence MecA, putative	0.0107
BA0424	Polysaccharide deacetylase, PdaA	0.0175
BA0819	Germination protein GerN	0.00571
BA1639	Germination protein GerN	0.0267
BA4986	Spore germination protein GerHC	0.0444
BA0634	Spore germination protein GerKB	0.00841
BA0709	Spore germination protein GerLA	0.000711
BA0710	Spore germination protein GerLB	0.00452
BA0763	Spore germination protein GerYA	0.0328
BA1492	Spore maturation protein	0.00565
BA4546	Spore protease	0.00295
BA5730	Sporulation initiation inhibitor protein Soj	0.0399
BA2644	Sporulation kinase B	0.00153
BA4530	Sporulation protein	0.00715
BA4394	Stage 0 sporulation protein A	0.0335
BA5581	Stage 0 sporulation protein F	5.28E-05
BA4688	Stage II sporulation protein B, putative	0.00279
BA5528	Stage II sporulation protein D	0.024
BA0061	Stage II sporulation protein E	0.00533
BA2068	Stage II sporulation protein P	0.0365
BA5263	Stage III sporulation protein J	0.0352
BA1530	Stage IV sporulation protein A	3.02E-05
BA4396	Stage IV sporulation protein B	0.00333
BA4643	Stage V sporulation protein B	0.00298
BA0054	Stage V sporulation protein B, putative	0.00863
BA4050	Stage V sporulation protein E	0.00438

[a] Gene descriptions were obtained from The Institute for Genome Research genome annotation (17).

[b] Values were calculated by comparing the mean hybridization signal ratio (preselection/postselection) to 1 using the GeneSpring software as described in the text.

hypothetical genes widely found in the genomes of organisms that do not form spores. Identification of these elements may reflect our lack of knowledge of factors and biochemical pathways required for optimal growth of organisms belonging to different genera on rich media. Interestingly, nine hypothetical genes, currently known to be present only in genomes of bacteria in the *Bacillus cereus* group, were identified in the screen (see Table S1 in the supplemental material). Identification of such narrowly distributed genes may reflect reworking of conserved ancestral phenotypes (optimal growth, sporulation, or germination) in these organisms. The presence of such genes raises the possibility that there may be unique anti-*B. anthracis* targets that affect phenotypes that can be measured in vitro. Similar insights have been provided by TraSH analyses of *Mycobacterium tuberculosis* cultured in vitro (19). These observations suggest that competitive negative selection-based studies, such as TraSH, are invaluable for identification of factors required for optimal growth in an environment.

TraSH assay is predictive. To independently demonstrate that the TraSH assay could be used to predict a gene's role in fitness, strains were constructed with insertion mutations in two conserved hypothetical genes identified in the screen (BA4451 and BA5728) that are present exclusively in the genomes of spore-forming bacteria (*Bacillus* and *Clostridium* spp.). Mutagenesis was performed as described previously (4) using shuttle plasmid pASD2 that harbored both ColE1 and pE194ts origins of replication and *aphA-3* and *aad-9* genes encoding resistance to kanamycin and spectinomycin, respectively. Heat-resistant spores of normal appearance were recovered from each strain, and no germination defects were observed for either strain using a spectrophotometric assay (data not shown). The TraSH assay is a competitive growth assay in which a limited number of growth-impaired mutants compete with growth-competent mutants. Therefore, a competitive growth assay was used to broadly measure the growth of the two mutant strains compared to the growth of the parent under selective conditions similar to those used in the TraSH screen. One million parental and mutant *B. anthracis* ΔAmes heat-activated spores were combined, spread on the surfaces of tryptic soy agar plates in triplicate, and allowed to germinate, grow, and resporulate. Spores were harvested from each culture and heat shocked to kill the bacilli. The ratios of parental bacteria to mutant bacteria before and after growth selection were determined by spreading appropriate dilutions of heat-shocked spores on tryptic soy agar plates with and without antibiotics and counting the resultant colonies. Following growth selection the ratios of the mutant strains to the parent were significantly reduced (Fig. 3). These observations demonstrated that the TraSH functional genomics screen is predictive of a gene's contribution to fitness in the environment selected.

Functional genomics studies such as TraSH have the potential to rapidly expand our knowledge of the factors required for *B. anthracis* fitness in various environments and to enhance our understanding of the selective pressures encountered by the pathogen in specific niches. Insights generated by such studies provide important foundations for the study of organisms, such as *B. anthracis*, for which few genes associated with conserved phenotypes (such as sporulation) or novel phenotypes (such as

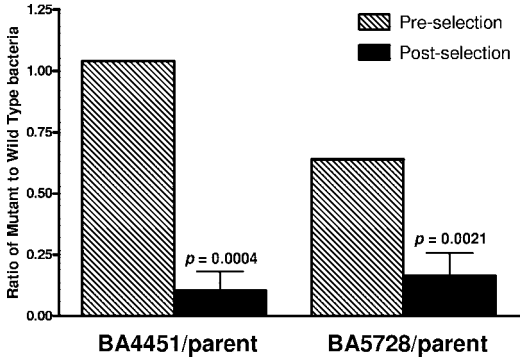

FIG. 3. Genes identified in the TraSH screen are required for optimal *B. anthracis* ΔAmes fitness under the growth conditions used. Competitive growth, sporulation, and germination cultures were established in triplicate by mixing approximately equal numbers of parent and mutant bacterial spores. The ratios of mutant organisms to parent organisms before and after competitive culture were determined by plating dilutions of the mixed populations on medium with and without antibiotics and counting the resultant colonies. The error bars indicate the 95% confidence levels; *P* values were calculated by comparing the input and output parent bacterium/mutant bacterium ratios for each mutant strain.

virulence) have been identified. Collectively, our results demonstrated that TraSH was successfully adapted for analysis of the *B. anthracis* genome. We showed that the combination of global transposon mutagenesis and novel TraSH probe synthesis methods with microarray analysis provided a rapid functional genomics assay. This assay should be useful for identifying *B. anthracis* genes required for expression of a wide variety of conserved and novel traits and may reveal new therapeutic and diagnostic targets to combat this important pathogen.

Nucleotide sequence accession numbers. The sequences of the *B. anthracis* ΔAmes *gdh* strain allele [Δ*gdh* (*atxA* Ω*kan PpagA-lacZ pagR*)] and shuttle vector pASD2 have been deposited in the GenBank database under accession numbers DQ898554 and DQ898555, respectively.

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