Novel Role for the yceGH Tellurite Resistance Genes in the Pathogenesis of Bacillus anthracis

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Bacillus anthracis, the causative agent of anthrax, relies on multiple virulence factors to subvert the host immune defense. Using Caenorhabditis elegans as an infection model, we screened approximately 5,000 transposon mutants of B. anthracis Sterne for decreased virulence. One of the attenuated mutants resulted in loss of expression of yceG and yceH, the last two genes in a six-gene cluster of tellurite resistance genes. We generated an analogous insertional mutant to confirm the phenotype and characterize the role of yceGH in resistance to host defenses. Loss of yceGH rendered the mutants more sensitive to tellurite toxicity as well as to host defenses such as reactive oxygen species and the cathelicidin family of antimicrobial peptides. Additionally, we see decreased survival in mammalian models of infection, including human whole blood and in mice. We identify a novel role for the yceGH genes in B. anthracis Sterne virulence and suggest that C. elegans is a useful infection model to study anthrax pathogenesis.

Bacillus anthracis is a Gram-positive, spore-forming bacterium and the etiological agent of the deadly disease anthrax. The most lethal form of the disease, inhalational anthrax, occurs when inhaled spores are phagocytosed by resident macrophages or dendritic cells and are carried to regional lymph nodes (1). En route, the bacteria germinate, escape the phagolysosome, and break out of the cell to replicate extracellularly, eventually leading to massive septicemia, shock, and death (2). The hardy nature of the spores, their relative ease of dissemination, and the high mortality rate make B. anthracis a paramount bioterrorism concern.

B. anthracis must avoid an array of host bacterial defenses during the course of infection. Major plasmid-encoded virulence factors include the lethal and edema toxins and a poly-D-glutamate capsule (2). Although the anthrax toxins and capsule play central roles in disease pathogenesis, increasing evidence indicates that chromosomal genes also contribute to virulence. Sequencing of the B. anthracis genome identified a number of genes that share homologies to known virulence factors of other pathogens (3). Subsequently, studies have coupled targeted mutagenesis with rodent infection models to identify chromosomal virulence genes, including the dltABCD operon for lipoteichoic acid modification (4), the askAB siderophore (5), the ABC transporter mntA (6), the nitric oxide synthase nos (7), the ClpXP protease (8), the purine biosynthesis gene purH (9), and the stress resistance gene hrtA (10).

Forward genetic screens using transposon-based mutagenesis systems can be a powerful method to identify novel bacterial virulence genes. We successfully employed this system in our identification of the clpX gene, encoding part of the intracellular protease ClpXP, as necessary for B. anthracis virulence (8). One challenge of this approach is designing a productive screen. Due to ethical and logistical constraints, it is not possible to perform large-scale virulence screens in vertebrate models. Instead, surrogate in vitro phenotypes that directly or indirectly correlate with virulence are often examined, for example, loss of hemolysis in identification of clpX (8).

A more amenable, whole-animal model to screen for virulence factors emerged with the finding that B. anthracis can infect and kill the nematode Caenorhabditis elegans (11). C. elegans has gained attention as a whole-animal infection model in recent years (12) because it possesses an evolutionarily conserved innate immune system with host defenses such as antimicrobial peptides (AMPs) (13) and reactive oxygen species (ROS) (14), as well as amenable traits, including small size, rapid generation time, and production of large numbers of genetically identical offspring. C. elegans has served as a host model system for several bacterial pathogens, including Pseudomonas aeruginosa and Staphylococcus aureus, with mutants subsequently confirmed to have decreased virulence in murine challenge experiments (12).

In the present study, we employ a transposon mutant library that we developed previously (8) to screen for B. anthracis Sterne mutants unable to infect C. elegans. Our most highly attenuated mutant had a transposon insertion in the second-to-last gene of a six-gene cluster of bacterial tellurite resistance genes. We find that these last two genes are important not only for tellurite resistance but also for resistance to specific reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂), the cathelicidin family of antimicrobial peptides, and human whole-blood killing. This increased susceptibility correlates with reduced virulence of our transposon mutant in C. elegans and murine models of infection.

MATERIALS AND METHODS

Bacterial strains and culture conditions. B. anthracis Sterne (pXO1+ pXO2−) was grown in brain heart infusion (BHI) medium (Hardy Diagnostics) at 37°C with continuous shaking. We used antibiotic selection at the following concentrations: 50 μg/ml kanamycin (Kan) and 5 μg/ml of

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erythromycin (Em). Construction of the transposon mutant library was described previously (8).

Construction of targeted insertional mutant and complementation plasmid. Approximately 600 bp of yceG sequence was amplified using the forward primer 5'-ATGCAAGTCTATGGCACTTCGGCTG-3' and the reverse primer 5'-GTCAGAATTCCGACGATCTGGAAATGGC T-3', which contained 5' extensions with restriction sites for HindIII and EcoRI, respectively. This amplicon was then cloned into the temperature-sensitive plasmid pHY304 (15), a derivative of pVE6007 (16), which contained 5' EcoRI, respectively. This amplicon was then cloned into the temperature-sensitive plasmid pHY304 and the reverse primer 5'-GTCAGAATTCCGACGATCTGGAAATGGC T-3', which contained 5' extensions with restriction sites for HindIII and EcoRI, respectively. Integration was confirmed by PCR using the pHY304-specific primer 5'-AGCAGGTTACCAGCCATTTG-3' and the primer 5'-CCTTCTGTAATCGTTATGGCC-3', which is located downstream of the original ampiclon.

The yceG and yceH genes were amplified as one amplicon using the forward primer 5'-ATGGTACGCCGCAAGCTAAAGAAGATTTGCA CTG-3' and the reverse primer 5'-ATGACCTGACAGCGATATTTGC-3' and cloned into the pDCerm expression vector (17) at the KpnI and SacI restriction sites. This plasmid was designated pGPH and transferred into the TNT1 strain.

C. elegans infection assay. The C. elegans glp-4 (4-hm2) strain, which is sterile at 25°C, was maintained using standard techniques (18). The C. elegans infection assay previously described (11) was modified to accommodate a high-throughput 96-well assay. Two days prior to the infection assay, a synchronous population of L1 worms was seeded onto nematode growth plates and fed Escherichia coli strain OP50. The worms were incubated at 25°C for 48 h to sterilize them and allow them to reach the young adult stage. The worms were then collected from plates, washed twice, and resuspended in 1 ml of S medium (19). Five microliters of this medium (containing approximately 5 to 10 worms) was then transferred to 190 μl of S medium containing Kan and 10 μg/ml Cry5B. Five microliters of an overnight B. anthracis culture grown in 96-well plates was then added for a final volume of 200 μl. The plates were wrapped in Parafilm and incubated at 28°C. Wells were assessed at 48 and 72 h. Nematodes were counted as infected only if they were filled with bacteria, which can be detected as worms being dark, rigid, and immobile. Wells with more than 60% of worms surviving were noted for further confirmation in a larger volume (24-well plates) in an independent assay.

Identification of transposon insertions. Transposon insertions were identified using two different methods. DNA sequencing the transposon insertion site was identified by single-primer PCR as described previously (8). Alternatively, the insertion site was identified using a Y-linker method (20). The transposon insertion site was confirmed using the transposon-specific primer 5'-TATGCAATTATCTATCTGGCAG-3' and one of the following primers located downstream of the insertion site: TN1, 5'-CAAGAAATGCTCGTTTGGCTATC-3'; TN11, 5'-CAAGAAATGCTCGTTTGGCTATC-3'; TN10, 5'-ATAATGGC-3; TN8, 5'-CGTCCACAAT ATGACCTGACG-3'; yceG Rev, 5'-GAGCTACAGGTGTATAGGAAC G-3'; yceH Fwd, 5'-TGCCGATATGCTCGTTTGGCTATC-3'; yceH Rev, 5'-CAGCTGGTGGTGCTG-3'.

Growth curves. Growth curves were started using equal amounts of early-log-phase parental and mutant bacterial strains, and bacteria were grown at 37°C with shaking in BHI with the indicated amount of potassium tellurite (Sigma). Absorbance was recorded at 600 nm. Growth curves were repeated at least three times; a representative graph is shown.

Disk diffusion assays. Parental or mutant B. anthracis stationary-phase cultures (16 h) were swabbed onto BHI agar plates. A 6-mm sterile paper disk (Becton, Dickinson) containing a specific antimicrobial was placed on the agar, the plate was incubated overnight at 37°C, the zone of inhibition was measured, and the area was calculated. Tellurite disks were prepared by soaking the disk in 10 mg/ml of potassium tellurite. Antibiotic disks containing 30 μg chloramphenicol, 30 μg tetracycline, and 10 μg penicillin were obtained from Becton, Dickinson. For antibiotic disk diffusion performed with tellurite, the agar plates contained 30 μg/ml of potassium tellurite.

Oxidant assays. For the growth assays, B. anthracis parental and mutant strains were grown to early log phase; diluted 1:2 (H2O2, 1:10 (hypochlorite), or 1:100 (methyl viologen)); and incubated with the indicated amounts of each specific oxidant (Sigma). The final optical density (OD) after overnight incubation (~16 h) was used as the measure of growth. A MIC assay was performed with 0, 10, and 40 mM methyl viologen (Sigma). For the H2O2 killing assay, bacteria were grown to an optical density of 0.4 at 600 nm and concentrated 10× in phosphate-buffered saline (PBS) and the concentrated bacteria were then diluted 1:2 into 0.1% H2O2 in PBS as a final concentration of 0.05% H2O2. Bacteria were incubated at 37°C. At the indicated time, an aliquot was removed and diluted 1:10 in PBS containing 3,000 U catalase (Sigma) to quench residual H2O2 and surviving bacteria were enumerated by serial dilution plating.

Antimicrobial peptide assays. B. anthracis parental and mutant strains were grown to early log phase, diluted 1:10 in RPMI plus 5% Luria-Bertani broth, and incubated with 1.6 μM LL-37 (Anaspec) overnight at 37°C under static conditions. MIC assays were also performed using RPMI plus 5% Luria-Bertani broth and log-phase bacteria diluted to 1:20 with either 0.125, and 250 μg/ml nisin; 0, 250, 500, and 1,000 μg/ml bacitracin; or 0, 25, 50, and 100 μg/ml polymyxin (all from Sigma) or log-phase bacteria diluted to 1:100 and 0.4, and 0.8 μM HNP-2 (Anaspec).

Whole-blood assay. Blood collected from three different healthy donors (use and procedures approved by the University of California, San Diego, Human Research Protections Program) was incubated with 105 CFU B. anthracis in a total volume of 500 μl and rotated at 37°C. After 15
RESULTS

Identification of an attenuated B. anthracis transposon mutant. We have recently demonstrated that under the proper assay conditions, including supplementation with an exogenous pore-forming toxin, B. anthracis Sterne can infect C. elegans (11). Distinguishing between B. anthracis-infected and uninfected worms is based on visual appearance. Infected nematodes are rigid and curvilinear, displaying typical discernible internal structures. We screened approximately 5,000 B. anthracis transposon mutants created previously (8) for their ability to establish an infection in C. elegans using a high-throughput 96-well screening format. This is a non-saturating screen; however, in our previous use of this library, we found that 5,000 mutants is a sufficient number to identify promising targets (8). Under our infection conditions, the mortality rate of C. elegans with wild-type (WT) B. anthracis Sterne was approximately 75%. We identified 11 transposon mutants that yielded a C. elegans mortality rate of 40% or less (Fig. 1) and determined the site of disruption (Table 1).

The most consistently attenuated mutant, designated TN1, induced a C. elegans mortality rate of less than 20% and contains a disruption in a putative tellurite resistance gene (BAS0389). Tellurite (TeO$_3^{2-}$) is a water-soluble form of tellurium and is highly toxic to bacteria. Tellurite resistance genes are found in a number of bacterial species, although they have not been linked to pathogenesis (22). TN2 has a disruption in a gene containing BNR repeats (also known as ASP-box motifs) that are commonly found in glycosyl hydrolases such as sialidases, which have been linked to bacterial pathogenesis (22). TN3, TN4, and TN5 are all inserted in conserved hypothetical proteins with little information on potential function. TN3 and TN7 also disrupt conserved hypothetical proteins of unknown function. These conserved hypothetical proteins were annotated as hypothetical proteins, but their specific functions are unknown. Other mutants identified in this study are likely to contain disruptions in hypothetical genes.

In vivo infection. Bacteria were grown to an optical density (OD) of 0.4 (600 nm), washed in PBS, and resuspended at a 1:30 dilution in PBS (approximately 7 × 10$^6$ CFU/ml). Parental (wild-type [WT]) and mutant (TN1) B. anthracis Sterne strains were mixed at a 1:1 ratio, and 0.1 ml was injected intravenously via the lateral tail vein into 8- to 10-week-old female CD1 mice. After 2 days, animals were euthanized and the kidneys were isolated and weighed. Kidneys were homogenized (twice for 1 min each at 6,000 rpm) using 1-mm zirconia-silica beads (BioSpec Products) in 2-ml screw-cap tubes containing sterile PBS using a MagNA lyser (Roche). Surviving bacteria were enumerated by serial dilution plating on BHI (total CFU) and BHI Kan50 plates (TN1 CFU). WT CFU was determined by subtracting the Kan-resistant CFU from the total CFU, and bacterial counts were calculated as CFU/g kidney.

Biometrics and statistics. Protein sequences were aligned using the ClustalW program found at http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=NPSA/npsa_clustalw.html. The following sequences were used: the B. anthracis yciD/FGH operon gene symbol BAS0385 through BAS0390, the R478 Ter operon with GenBank accession number U592392, and the Listeria monocytogenes TeA gene symbol lmo1676. Statistical analysis was performed using GraphPad Prism software.

FIG 1 Transposon mutants have attenuated virulence in a C. elegans model of infection. C. elegans mortality rate after 72 h of infection with WT B. anthracis Sterne or transposon mutants (TN1 to -11). Data from at least three independent experiments are presented and compared as means ± standard errors of the means. *, P < 0.05; **, P < 0.01; ***, P < 0.001, from WT by one-way analysis of variance followed by Tukey's post hoc analysis.

### TABLE 1 Summary of attenuated mutants identified in C. elegans infection screen

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Disrupted gene*</th>
<th>Gene symbol</th>
<th>Sequence surrounding insertion site*</th>
</tr>
</thead>
<tbody>
<tr>
<td>TN1</td>
<td>Putative tellurite resistance gene</td>
<td>BAS0389</td>
<td>ACGTATCATTTGTGATGTGATGGCTATATCTATTTTTTGGATTGATGCTT</td>
</tr>
</tbody>
</table>
| TN2 | BNR repeat domain protein | BAS2814 | ACGCTTTGAAAAGATTTT 
| TN3 | Conserved hypothetical protein | BAS0642 | TGGATTTTGTACCTTACATTTCACATGGACAAATTTCCTTACATCAGTTTT|
| TN4 | Protease production regulatory protein | BAS0976 | TTCATTTTGCATTTTGATATTCATTTTTT |
| TN5 | Hypothetical protein | BAS0432 | ATGGATTTTGTACCTTACATTTCACATGGACAAATTTCCTTACATCAGTTTT |
| TN6 | Spore germination protein | BAS3369 | TGGATTTTGCATTTTGATATTCATTTTTT |
| TN7 | Conserved hypothetical protein | BAS0645 | TGGATTTTGCATTTTGATATTCATTTTTT |
| TN8 | Sensor histidine kinase | BAS0869 | TGGATTTTGCATTTTGATATTCATTTTTT |
| TN9 | PTS, IIB component | BAS2290 | TGGATTTTGCATTTTGATATTCATTTTTT |
| TN10 | Acetyl-CoA hydrolase | BAS1726 | TGGATTTTGCATTTTGATATTCATTTTTT |
| TN11 | Oxidoreductase | BAS0712 | TGGATTTTGCATTTTGATATTCATTTTTT |

* Abbreviations: PTS, phosphotransferase system; CoA, coenzyme A.

* Insertion site is shown in bold.
Tellurite resistance genes in B. anthracis. The B. anthracis genome contains a six-gene cluster of putative tellurite resistance genes (BAS3085 to BAS3090) in which the second-to-last gene is disrupted by the transposon insertion in TN1 (Fig. 2A, arrow). Leftward arrows indicate primers used for reverse transcription (RT 1, RT 2, or RT 3). Arrowheads show location of PCR pairs A to G. The bent arrow indicates the predicted promoter region. (B) PCR using cDNA from indicated RT primers. Letters correlate with primer combinations shown in panel A. (C) Semiquantitative RT-PCR expression of the yceC, yceG, and yceH genes from cDNA prepared from WT, TN1, IM1, or TN1 complemented with an expression plasmid containing yceG and yceH (pGH).

**TABLE 2** Homology of B. anthracis Yce proteins to other tellurite resistance proteins

<table>
<thead>
<tr>
<th>B. anthracis</th>
<th>B. subtilis protein(s)</th>
<th>% homology</th>
<th>Ter/Tel protein(s)</th>
<th>% homology</th>
</tr>
</thead>
<tbody>
<tr>
<td>YceC</td>
<td>YceC (62.3)</td>
<td>TerE (40.1), TerD (37.0), TerZ (37.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YceD</td>
<td>YceD (68.6), YceE (67.0)</td>
<td>TerD (54.2), TerE (53.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YceE</td>
<td>YceE (71.1), YceD (63.6)</td>
<td>TerD (55.9), TerE (52.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YceF</td>
<td>YceF (64.3)</td>
<td>TerC (22.9)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>YceG</td>
<td>YceG (41.4)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>YceH</td>
<td>YceH (58.1)</td>
<td>TelA (24.1)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
nants have been identified (21). The *B. anthracis* YceCDEF and YceGH proteins have homology with two of them, TerZABCDEF and TelA (Table 2). The *terZABCDEF* operon was originally identified on the R478 plasmid, and homologues have been found in the chromosomes of numerous species, including *Yersinia pestis*, *E. coli*, *Bacillus subtilis*, and *Proteus mirabilis* (21, 24, 26, 27). The *telA* gene has been recently characterized in the Gram-positive pathogen *L. monocytogenes* (28). At the amino acid level, *B. anthracis* YceCDEF has homology to several Ter proteins (Table 2). YceG, the site of the transposon insertion, has little identity to any other tellurite resistance proteins, while YceH has 24.1% identity to the TelA protein found in *L. monocytogenes*.

To confirm the involvement of the *yceG* gene in the observed phenotype of the transposon mutant, we constructed an independent mutation (IM1) in approximately the same location using targeted insertional plasmid mutagenesis. RT-PCR confirmed that *yceG* gene expression was absent in both the TN1 and IM1 mutants relative to the wild type (Fig. 2C), whereas the first gene, *yceC*, is expressed in all three strains as expected. The last gene, *yceH*, is absent or substantially reduced in TN1 but present in IM1 (Fig. 2C). This differential regulation of *yceH* by TN1 and IM1 was also confirmed by quantitative PCR (qPCR) (data not shown). Therefore, IM1 functions as a genetic knockout of *yceG* whereas the transposon mutant functions as a double knockout of *yceG* and *yceH*. For complementation analysis, *yceG* and *yceH* were cloned on an expression plasmid (designated pGH) and transformed into the TN1 strain. Although transcription of the *yceG* and *yceH* genes was confirmed by RT-PCR in our complemented strain (Fig. 2C, lanes marked pGH), there was no change in phenotype from TN1 lacking the complementation plasmid in any subsequent assays (data not shown). This lack of complementation may result from improper stoichiometry of the proteins involved. No difference in expression of an unrelated control gene, *fusA*, was seen in any strains (data not shown).

Since *yceG* and *yceH* are homologous to putative tellurite resistance genes, we first tested susceptibility to potassium tellurite. We observed that both WT and mutant strains of *B. anthracis* Sterne form the characteristic black precipitate caused by reduction of tellurite (TeO₃⁻) to tellurium (Te⁰) (Fig. 3A). However, there is a small but statistically significant increase in the zone of growth inhibition surrounding a potassium tellurite-impregnated disk in the TN1 and IM1 strains in comparison to WT (Fig. 3A). Exposure to potassium tellurite in liquid culture also results in slower growth for both TN1 and IM1 relative to the WT bacteria (Fig. 3B), although no difference in growth is seen in BHI alone (data not shown). Although other genes likely contribute, *yceGH* appears to play a minor role in tellurite resistance in *B. anthracis* Sterne.

**yceG** and **yceH** genes contribute to innate immune defense mechanisms. Because increased susceptibility to tellurite toxicity cannot by itself explain the attenuated virulence of TN1 in *C. elegans*, we next investigated whether *yceG* could play a role in defense against aspects of the host innate immune system. A critical innate immune defense of *C. elegans* is the production of ROS in response to pathogens (14). In mammalian systems, professional phagocytes such as macrophages and neutrophils generate ROS (29). Tellurite is also a strong oxidant (30) and increases intracellular ROS production in exposed bacteria (31, 32). Therefore, we determined whether *yceG* and *yceH* contribute to ROS resistance. Early-log-phase bacteria were incubated overnight in BHI medium containing 0% and 0.02% H₂O₂. Although no difference in growth as measured by the final optical density was observed in BHI alone, addition of H₂O₂ significantly attenuated growth for both the TN1 and IM1 mutants (Fig. 4A). This killing occurred relatively rapidly, as exposure to 0.05% H₂O₂ killed all TN1 mutants by 45 min and reduced survival of IM1 by 100,000-fold in comparison to WT (Fig. 4B). While loss of *yceG* alone (IM1) appears sufficient to increase ROS sensitivity, *yceH* also likely contributes, since the phenotype seen in the IM1 mutant (loss of both *yceG* and *yceH*) is often stronger (compare survivals of TN1 and IM1 mutants in Fig. 4B). Additionally, we tested susceptibility for two additional ROS, sodium hypochlorite, the active ingredient in bleach, and superoxide generated by methyl viologen. As with H₂O₂, growth of TN1 and IM1 was significantly decreased compared with WT when bacteria were exposed to hypochlorite (Fig. 4C). However, no difference in survival was seen with superoxide (data not shown).

Antimicrobial peptides (AMPs) are a second innate immune defense mechanism employed by *C. elegans* (13). In *L. monocytogenes*, the tellurite resistance gene telA was identified in a transposon-based screen for susceptibility to the AMP nisin (28). Since *yceH* has homology to telA and is absent in our TN1 mutant, we tested whether TN1 and IM1 were more susceptible to several antimicrobial peptides, including the human cathelicidin LL-37, the α-defensin HNP-2, nisin, bacitracin, and polymyxin. A significan-

![FIG 3 Loss of yceG leads to decreased tellurite resistance. Growth inhibition of WT B. anthracis Sterne (black bars), transposon mutant (TN1, white bars), or insertion mutant (IM1, gray bars) spread on plates and exposed to a tellurite-impregnated disk. The area of growth inhibition was calculated and presented as mean ± standard deviation (right). *, \( P < 0.05 \), or **, \( P < 0.01 \), from WT by one-way analysis of variance followed by Tukey’s post hoc analysis. The experiment was repeated at least three times, and representative pictures are shown (left). (B) Representative graph from a 24-hour growth curve in liquid BHI containing 30 μg/ml of potassium tellurite.

**yceG** and **yceH** genes contribute to innate immune defense mechanisms.
icant difference in survival was seen between WT and TN1 in LL-37 susceptibility (Fig. 4D); however, IM1 exhibited an intermediate phenotype (Fig. 4D). This is likely due to the fact that the TN1 insertion disrupts both yceG and yceH whereas IM1 disrupts only yceG. No difference in growth between WT *B. anthracis* Sterne, TN1, and IM1 exposed to HNP-2, nisin, bacitracin, or polymyxin was observed (data not shown). Thus, it appears that both yceG and yceH contribute to resistance to human cathelicidin; however, this did not represent a global change in membrane integrity as no difference in susceptibility was seen with any other AMP tested.

Tellurite enhances susceptibility to antibiotics independently of yceG and yceH. It has recently been reported that exposure to tellurite enhances susceptibility of *E. coli* to a broad range of antibiotics, including chloramphenicol, tetracycline, and ampicillin (33). Similarly, we observed a significantly increased zone of inhibition for *B. anthracis* Sterne, TN1, and IM1 exposed to HNP-2, nisin, bacitracin, or polymyxin was observed (data not shown). Thus, it appears that both yceG and yceH contribute to resistance to human cathelicidin; however, this did not represent a global change in membrane integrity as no difference in susceptibility was seen with any other AMP tested.

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the human cathelicidin LL-37. TN1 has a disruption in yceG, the second-to-last gene in a six-gene cluster of tellurite resistance genes, that results in loss of expression of both yceG and yceH. Although originally identified as a putative operon (24), our results indicate independent regulation of yceCDEF and yceGH. There are at least five unique bacterial TeD determinants that have been identified, including the Ter proteins (similar to YceCDEF) and TelA (similar to YceH) (21). YceG remains poorly characterized with little sequence homology to other known tellurite resistance proteins. YceG has strong hydrophobic regions that may allow for interaction with the membrane, and across bacterial species, it is often found in association with TelA, indicating that these two proteins might be functionally linked (34). Despite their being widespread, surprisingly little is known regarding the actual function of any of the TeD genes.

As their name implies, the TeD genes are important for tellurite resistance in a number of bacterial species, including E. coli, P. mirabilis, and Y. pestis (21, 26, 27). While highly toxic to bacteria, tellurite is not necessarily commonly encountered, which raises the question of why these genes exist in such a wide range of species. It has been argued that the TeD genes are part of a general stress response system rather than solely tellurite resistance (21, 30, 34). In support of this, the Ter genes in E. coli are also implicated in resistance to bacteriophage and colicin (35). In Y. pestis, TerE and TerD proteins are upregulated along with other general stress response proteins during intracellular replication in macrophages (26).

Our data show that loss of yceG and yceH in B. anthracis results in a small growth impairment in the presence of tellurite. However, the more pronounced phenotype is the increased susceptibility to host defenses such as ROS. Both our transposon and insertional mutants demonstrated increased sensitivity to H2O2 and hypochlorite, the active ingredient in bleach. In a separate study in B. anthracis, yceCDEF were identified in an analysis of genes upregulated in response to H2O2 (36). The Ter operon of Proteus mirabilis is also induced by H2O2 and superoxide (27). Tellurite is also a strong oxidant (30), and tellurite toxicity has been linked to ROS production (31, 32). A recent comparative genomics study has found that the Ter genes are functionally linked to many enzymes involved in DNA processing and repair (34). It is therefore possible that these genes may play a role in repairing the DNA damage that would occur after exposure to oxidative stressors such as ROS or tellurite.

B. anthracis yceG and/or yceH is also important for resistance to the human cathelicidin LL-37. YceH has homology to TelA, which has been linked to resistance to nisin and some cell wall-targeting antibiotics in L. monocytogenes (28). Surprisingly, no difference in susceptibility was observed in the TN1 or IM1 mutants with any other AMP tested, including nisin, HNP-2, bacitracin, or polymyxin. This could be due to differences in mechanisms of action of the AMPs and/or in the responses of YceG/H. Although most AMPs form transmembrane pores, peptide insertion into the membrane can differ and some AMPs may have additional bactericidal mechanisms that could alter the bacterial response (37).
L. monocytogenes, loss of TelA increases susceptibility to some cell wall-acting antibiotics (the cephalosporins, cefotaxime, and cefuroxime) but not all (no difference was seen with metillicillin or oxacillin) (28). We also saw no contribution of the yceG or yceH gene to resistance to several common antibiotics, even when antibiotic susceptibility was magnified by coinfection with tellurite. Therefore, although yceGH may function as part of a general stress response system, there is still specificity in their mechanism of action.

A potential role of Te genes in host defense has long been speculated (21), but to our knowledge, this is the first report of decreased survival of B. anthracis in vivo in competition assay, both of which expose the bacteria to a synergistic array of host defenses. Taken together, our results and those of others support the argument that tellurite resistance genes are likely part of a more general chemical stress response system. Loss of these genes could render bacteria more susceptible to a variety of potential environmental stresses, including but not limited to, exposure to strong oxidants. This would help explain their widespread and ubiquitous nature in a wide variety of bacterial species, including several human pathogens. Further study will clarify the roles and mechanism of action of this intriguing and mysterious family of genes.

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