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ORIGINAL ARTICLE

Deletion of gene encoding methyltransferase (*gidB*) confers high-level antimicrobial resistance in *Salmonella*

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The glucose-inhibited division gene (*gid*)*B*, which resides in the *gid* operon, was thought to have a role in the modulation of genes similar to that of *gidA*. Recent studies have indicated that GidB is a methyltransferase enzyme that is involved in the methylation of the 16S ribosomal RNA (rRNA) in *Escherichia coli*. In this study, we investigated the role of GidB in susceptibility to antibiotics and the overall biology of *Salmonella*. A *gidB* isogenic mutant of *Salmonella* was constructed and subsequently characterized under different conditions. Our data indicated that growth and invasion characteristics of the *gidB* mutant were similar to those of the wild type (WT). The *gidB* mutant was outgrown by the WT in a competitive growth assay, indicating a compromised overall bacterial fitness. Under the stress of nalidixic acid, the *gidB* mutant's motility was significantly reduced. Similarly, the mutant showed a filamentous morphology and smaller colony size compared with the rod-shaped and large colonies of the WT in the presence of nalidixic acid. Most importantly, deletion of *gidB* conferred high-level resistance to the aminoglycoside antibiotics streptomycin and neomycin. A primer extension assay determined the methylation site for the WT to be at G527 of the 16S rRNA. A lack of methylation in the mutant indicated that GidB is required for this methylation. Taken together, these data indicate that the GidB enzyme has a significant role in the alteration of antibiotic susceptibility and the modulation of growth and morphology under stress conditions in *Salmonella*.
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Keywords: antibiotic resistance; *gidB* gene; methyltransferase enzyme; RNA methylation; *Salmonella* Typhimurium; stress conditions

INTRODUCTION

Salmonella is one of the most common food-borne pathogens in the United States.[1] *Salmonella* is transmitted by the fecal-oral route through the consumption of contaminated food or water.[2,3] In the United States, the number of salmonellosis cases has been estimated to be 1.39 million per year, with 415 deaths, costing about $2.65 billion annually due to medical costs and loss of productivity.[4]

The glucose-inhibited division (*gid)AB* operon, which includes the *gidA* and *gidB* genes, was originally related to the glucose-mediated inhibition of cell division in *Escherichia coli* (*E. coli*).[5] *GidA* is a highly conserved gene, first described in *E. coli* and was thought to have a role in chromosomal division and replication due to its location near the origin of replication.[6,7] The role of *gidA* as a global virulence gene regulator has been established in several bacterial pathogens including *Pseudomonas syringae*, *Aeromonas hydrophila*, *Pseudomonas aeruginosa* and *Streptococcus pyogenes.*[8–11] Most recently, a study from our laboratory has shown that *gidA* affects *Salmonella* virulence.[12] Deletion of *gidA* resulted in attenuation of *Salmonella in vitro* and in a murine model of infection, indicating a role in modulation of virulence factors.[12] Studies in *E. coli* indicate that GidA uses a posttranscriptional-based mechanism to modify transfer RNA.[8–11,13–16] On the other hand, the *gidB* gene encodes for a methyltransferase enzyme[17,18] and therefore, proposed to be redesignated to *rsmG* (ribosomal small subunit methyltransferase G).[17] The *gidB* gene is highly conserved in both Gram-negative and Gram-positive bacteria, and binds the 30S subunit of the ribosome and methylates the 16S ribosomal (rRNA).[17–19] Mutation in the *gidB* homolog (*rsmG*) in *E. coli, Mycobacterium tuberculosis*, *Thermus thermophilus* and *Bacillus subtilis* showed a low level of streptomycin resistance due to lack of methylation of the 16S rRNA.[17–20] Additionally, the *gidB* mutant of *T. thermophilus* was outgrown by the wild type (WT) in a competitive growth assay, indicating a physiological cost of the methylation deficiency.[19] These data indicate that GidB affects antimicrobial susceptibility in bacteria by a mechanism that involves a posttranscriptional modification.

Antibiotics are one of the most effective methods of treatment for infectious diseases in modern medicine. However, antibiotic-resistant bacterial strains are increasingly emerging, causing a significant public health threat.[21] Antimicrobial resistance in non-typhoidal *Salmonella* serotypes has become a serious problem worldwide.[22–28] For example, *Salmonella enterica* serovar Typhimurium DT104 is resistant to multiple antimicrobial drugs, including ampicillin, chlor-

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amphenicol, streptomycin, sulfonamide and tetracycline.[29] During the early 1990s, there was a very significant increase in the *Salmonella* Typhimurium multidrug-resistant isolates, making up about one-third of all human isolates in 1996.[30]

The aminoglycoside family of antibiotics includes several medically important drugs, such as streptomycin, neomycin and kanamycin.[31] They are highly active against Gram-negative bacteria and, to some extent, Gram-positive bacteria.[32] Aminoglycosides act by interfering with protein synthesis by binding to the 30S subunit of the bacterial ribosomes and inducing codon misreading, as well as inhibiting translocation.[31,33] Resistance mechanisms to aminoglycosides are mainly by alteration of ribosomal binding sites, decreased antibiotic uptake and accumulation, and production of aminoglycoside-modifying enzymes.[31] Enzymatic modification is the most common type of aminoglycoside resistance. Several enzymes have been identified and classified according to the type of modification, such as acetyltransferases, adenyltransferases, and phosphotransferases.[34,35] The genes encoding for aminoglycoside-modifying enzymes are usually found on plasmids and transposons, which have an important role in their dissemination. Streptomycin binds to the 30S ribosomal subunit and interacts with the C526 and G527 residues of the 16S rRNA, resulting in inhibition of protein synthesis.[36,37] On the other hand, neomycin targets the conserved sequences in the A-site of 16S rRNA.[38]

In this work, we investigated the role of GidB on *Salmonella* phenotype and biology. Deletion of *gidB* conferred a high-level of resistance to aminoglycoside antibiotics in *Salmonella*. We further determined the precise location of the methylation target of *Salmonella* GidB. Additionally, analysis of the mutant indicated that deletion of *gidB* showed defects in morphology, motility and competitive growth characteristics of the bacteria under stress conditions, suggesting a significant role in stress response for ribosomal RNA modification in *Salmonella*.

MATERIALS AND METHODS

Bacterial strains, plasmids and cell lines

The *Salmonella enterica* serovar Typhimurium 14028 strain was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and a spontaneous nalidixic acid-resistant strain was created in our laboratory and used as the WT.[12] All *Salmonella* strains were grown either in lysogeny broth (LB) medium or on *Salmonella–Shigella* plates. Nalidixic acid (150 mg ml^{-1}), kanamycin (50 mg ml^{-1}), tetracycline (15 mg ml^{-1}) or ampicillin (100 mg ml^{-1}) was added to the media as necessary. T84 human intestinal epithelial cells were obtained from ATCC and maintained in Dulbecco's modified Eagle's medium/nutrient mixture F12 medium supplemented with 10% fetal bovine serum, and incubated at 37 °C with 5% CO_2.

Construction of *gidB* mutant

The *gidB* mutant was generated using the Lambda Red system following the described methods.[39] Briefly, WT *Salmonella* Typhimurium 14028 was transformed with the plasmid pKD46, which carries the Lambda red recombinase genes.[39] Arabinose-induced WT *Salmonella* carrying pKD46 (WT-pKD46) was cultured and used to generate electro-competent cells. The kanamycin resistance (Km) gene was PCR amplified from the pKD4[39] plasmid using F4/R4 primers set (5′-TTTAACACAAGCCGCGTCGTTTTTTTTCTATTTTTTTTGA TAAAAATGTGTAGGCTGGAGCTGCTTCG-3′/5′-GCCCGATAAGCGAAAGC GCATCGGGCATTTGATTATTAACAGGTACATATGAATATCCTCCTTAG-3′; primers relative locations are shown in Figure 1). These primers are homologous to the flanking sequences of the *Km*^{r} gene on pKD4. The 5′-end of the F4 primer carries an extra 47 bases homologous to the upstream sequence of *Salmonella gidB* gene, whereas the 5′-end of the R4 primer carries an extra 45 bases homologous to *gidB* downstream sequence. The PCR product was electroporated into the WT-pKD46 electro-competent cells. The transformation reaction was incubated for 5 h at 37 °C, and plated on LB agar supplemented with kanamycin. Colonies were picked and *gidB* deletion was confirmed by sequencing, and PCR amplification using primer sets F1/R1 (5′-CTGTGCTCGACGTTGTCACT-3′/5′-GCTCTTCGTCCAGATCATCC-3′) was used to amplify the km^r cassette, the F3/R1 (5′-GCGTGCACCAGATCA CACA-3′/R1) primers were used to amplify the upstream *gidB* sequence along with the km^r cassette, and the F1/R3 (F1/5′-TGCTGCGTCGTAGCGCTTG-3′) was used to amplify the downstream *gidB* sequence along with the km^r cassette. The F2/R2 primer set (5′-AGGCATAAAAACGCCCTTTC-3′/5′-GGCTATTG TTCTCCCTGACG-3′) was used to amplify the *gidB* gene.

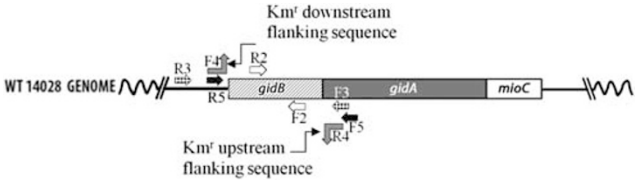

Figure 1 Schematic diagram showing the relative locations of primer sets used in PCR amplification for confirmation of the *gidB* deletion.

Complementation of the *gidB* mutant

The *gidB* gene insert was PCR amplified from WT chromosomal DNA using the F5/R5 primers (5′-AGAAGCGACGTTGTCATGTT-3′/5′-AAGGTATGCT GCGTCGTA-3′). The PCR product was blunt-ended using a PCR polishing kit (Stratagene, Santa Clara, CA, USA), ligated to a blunt-ended pBR322 vector (lab stock) and digested with *Sca*I restriction enzyme. The recombinant vector was transformed into the *gidB* mutant strain by electroporation. To confirm the complementation, recombinant pBR322 was isolated from the *gidB*-complemented strain and digested with *Bam*HI restriction enzyme. The product was separated by gel electrophoresis to analyze bands sizes. Additionally, the recombinant plasmid was PCR amplified, using primers F5/R5 flanking the *gidB* upstream and downstream sequences, and analyzed by gel electrophoresis.

Morphology

To observe colony morphology, LB agar plates with and without nalidixic acid were plated with the WT, *gidB* mutant and *gidB* complement. Bacterial colony morphology was examined following overnight incubation at 37 °C.

Bacterial cell morphology for *gidB*, WT and complemented strains was examined by growing bacteria in LB broth medium with and without nalidixic acid. Bacterial cells were Gram-stained and examined under AxioCam HRm light microscopy and analyzed by Zeiss AxioVision Rel. 4.8.1 software (Carl Zeiss Microscopy, Thornwood, NY, USA).

Growth assays

The growth characteristic of the *gidB* mutant was determined using a conventional growth assay. Overnight cultures of various *Salmonella* strains were quantified and standardized to the same OD_{600}. An equal number of cells were inoculated into a fresh LB broth media with and without nalidixic acid and incubated at 37 °C for 8 h. Culture samples were taken every hour to determine the OD_{600}.

A competitive growth assay was used to determine the ability of the *gidB* mutant to compete with the WT under stressful growth conditions. The assay was performed following the described methods.[40] In brief, overnight cultures of the WT and the *gidB* mutant were sub-cultured in a fresh LB broth media. At an OD_{600} of 0.6, both cultures were mixed together in the same culture flask in a ratio of 1:1; then, the mixture was inoculated in 100 ml of fresh LB broth media in a dilution of 1:100 and incubated at 37 °C shaking at 160 r.p.m. for 2 weeks. A sample of culture was taken every second day, serially diluted and plated on LB agar plates with the appropriate antibiotics. The CFU was determined for each strain, and a growth curve was plotted.

Antibiotic susceptibility

The *gidB* mutant's susceptibility to antibiotics was tested by determining the MIC using conventional tube method and an automated Sensititre Susceptibility System (Trek Diagnostic Systems, Cleveland, OH, USA). Cultures

of the WT, *gidB* mutant and the *gidB* complement were streaked on LB agar plates containing streptomycin, incubated overnight at 37 °C, and growth was examined. For the conventional MIC test, streptomycin, gentamicin and chloramphenicol were serially diluted two-fold in LB medium ranging from 700 to 1.37 μg ml^{-1} for streptomycin, and from 20 to 0.625 μg ml^{-1} for gentamicin and chloramphenicol. An equal number of cells from overnight cultures of the WT, *gidB* mutant and the complemented strain were inoculated into each antibiotic dilution and incubated overnight at 37 °C with shaking. The next day, growth versus no growth was scored. The automated Sensititre Susceptibility test was performed at the Wisconsin Veterinary Diagnostic Laboratory to determine the MIC of a wide range of the most commonly used antibiotics in veterinary medicine (in the treatment of bovine and avian species, as well as in companion animals) for *gidB* mutant and the WT. Briefly, colonies of the *gidB* mutant and the WT were suspended in demineralized water to 0.5 McFarland standard (1×10^5 CFU ml^{-1}). A volume of 10 μl of the standardized cells were transferred to 11 ml of Mueller Hinton Broth, and the Sensititre plates (microtiter plates containing increasing doses of lyophilized antimicrobials) were inoculated with 50 μl of the cell suspension. The plates were then incubated on the Sensititre machine at 37 °C overnight.

Motility assay

Cultures of various *Salmonella* strains used in this study were grown in LB broth. An equal number of bacteria (1×10^6) of each strain was stabbed into a 0.35% agar (semi-solid) plates, with or without nalidixic acid, and incubated overnight at 37 °C. The motility was determined by measuring the distance migrated from the center of inoculation to the periphery of the plate.

Primer extension

This assay was performed to detect the presence or absence of methylation at G527 in the 16S rRNA of the *gidB* mutant and the WT of *Salmonella*, and therefore, determine the antibiotic resistance mechanism. Total RNA was isolated from the *gidB* mutant and WT using the Bacterial RiboPure RNA extraction kit (Ambion, Austin, TX, USA). N^7-methylguanosines in the RNAs were cleaved by reduction with $NaBH_4$, followed by β-elimination with acetic acid–aniline.[41,42] A yeast transfer RNA was used as an RNA carrier and was hypermodified at N^7 of guanosine by dimethyl sulfate treatment, which enhanced cleavage at the N^7-methylated guanosines in the rRNA.[43] The rRNAs were scanned by reverse transcription using the radiolabeled primers R6 (reverse internal primer for 16S rRNA, 5′-CCGGGGATTTCACATCCGACTTGACAGAC-3′) and R7 (reverse internal primer for 16S rRNA, 5′-TTCGAATGCAGTTCCCAGGTTGAGCC-3′). The primers were ^{32}P-labeled by mixing 12 μl of 25 ng μl^{-1} of the stock primers with 5 μl of [γ- ^{32}P]ATP (Perkin-Elmer, Waltham, MA, USA), 2 μl of 10× polynucleotide kinase buffer and 1 μl of T4 polynucleotide kinase (NEB, New England Biolabs, Ipswich, MA, USA). The mixture was incubated for 30 min at 37 °C and the free nucleotides were removed by QIAquick Nucleotide Removal Kit (Qiagen, Valencia, CA, USA). For the primer extension reaction, 19 μl (approximately 1 μM) of the treated RNA were hybridized with 1 μl of ^{32}P-labeled oligo in the presence of 5 μl 5× first strand buffer for 10 min at 80 °C, followed by 1 min incubation on ice. The reverse-transcription reaction was carried out by addition of 1 μl 5× first strand buffer, 1 μl 0.1 M dithiothreitol, 2.5 μl 5 mM dNTPs and 0.5 μl of SuperScript III reverse transcriptase (Promega, Madison, WI, USA) enzyme to the RNA/oligo mix. The reaction was incubated for 30 min at 48–50 °C. To stop the reaction, one volume of formamide stop solution (95% formamide, 20 mM EDTA pH8.0, 0.05% bromophenol blue and 0.05% xylene cyanol) was added. Samples were heated to 90 °C for 1 min, then loaded on to a 9% polyacrylamide urea gel with a sequencing ladder made with the Thermo Sequenase Cycle Sequencing Kit (USB, Cleveland, OH, USA) using the pN01301 plasmid, which carries the entire *rrnB* rRNA operon from *E. coli*.[44] The gel was dried, exposed to a phosphorimager screen, scanned using the Typhoon imager and analyzed using the ImageQuant software package (GE Healthcare, Piscataway, NJ, USA).

Statistical analysis

Wherever appropriate, data were analyzed using Microsoft Office Excel and a Student's *t*-test. *P*-values ≤ 0.05 were considered to be significant. Experiments were repeated at least three times, and data were expressed as arithmetic means with s.d.

RESULTS

Analysis of the *gidB* mutant and complement

The *gidB* mutant was generated using the Lambda Red system and confirmed by DNA sequencing and PCR analysis. The PCR amplification using chromosomal DNA from the *gidB* mutant revealed expected bands for the F1/R1, F3/R1 and F1/R3 primers, indicating insertion of the Km^r cassette. The amplification gave no bands with the F2/R2 primers, indicating deletion of the *gidB* gene. Likewise, PCR analysis using the DNA from the WT strain confirmed the presence of *gidB* gene and the absence of km^r cassette.

Restriction digestion analysis of the recombinant pBR322 isolated from the *gidB*-complemented strain showed the expected sizes of 1509 (including *gidB* insert) and 3733 base fragments, indicating correct orientation of the insertion. PCR amplification of the recombinant plasmid using F5/R5 primers showed the expected size (881 bp) for the *gidB* gene with its upstream- and downstream-flanking sequences.

GidB mutant is defective in morphology under stress condition

Examination of colony morphology indicated no significant difference in the size of colonies for the *gidB* mutant, compared with the WT and complemented strains (Figure 2a) when grown on LB agar without nalidixic acid. However, the *gidB* mutant colonies were significantly smaller than the WT and complemented strain colonies when they were grown on a nalidixic acid-containing LB agar plate (Figure 2b). Microscopic examination of Gram-stained bacterial cells indicated no significant difference in the cellular morphology of the *gidB* mutant compared with the WT and the complemented strains (Figure 2c) when grown in LB broth without nalidixic acid. In contrast, bacterial cultures grown in nalidixic acid-containing media showed *gidB* with filamentous morphology compared with the rod shape of the WT and the complemented strains (Figure 2d). These data indicated that GidB may have an important role in response of *Salmonella* to stress conditions such as nalidixic acid.

Deletion of *gidB* affects growth and survival under stress condition

Growth curves indicated no significant difference in the growth pattern of the *gidB* mutant compared with the WT and the complemented strains grown with or without nalidixic acid (Figure 3a), indicating that deletion of *gidB* did not affect *Salmonella* growth. However, the competitive growth assay showed that during the first 7 days of the assay, both *gidB* mutant and WT strains showed similar growth rates and equally competed for the available nutrients. However, after day 8, the mutant strain showed progressive decrease in growth compared with the WT, indicating decreased overall fitness and lower survival rate (Figure 3b).

Deletion of *gidB* confers antibiotic resistance

Although the *gidB* mutant was able to grow on LB plates containing 250 μg ml^{-1} of streptomycin, the WT and the complemented strains were not (data not shown). Table 1 shows the antibiotic resistance pattern of the *gidB* mutant compared with the WT for streptomycin, gentamicin and chloramphenicol. Although the data showed no significant difference in the gentamicin and chloramphenicol MICs between the WT and the *gidB* mutant, the streptomycin MIC was 700 μg ml^{-1} for the *gidB* mutant, 87.5 μg ml^{-1} for the WT and 175 μg ml^{-1} for the complemented strain. These data indicated that deletion of *gidB* conferred streptomycin resistance to *Salmonella*.

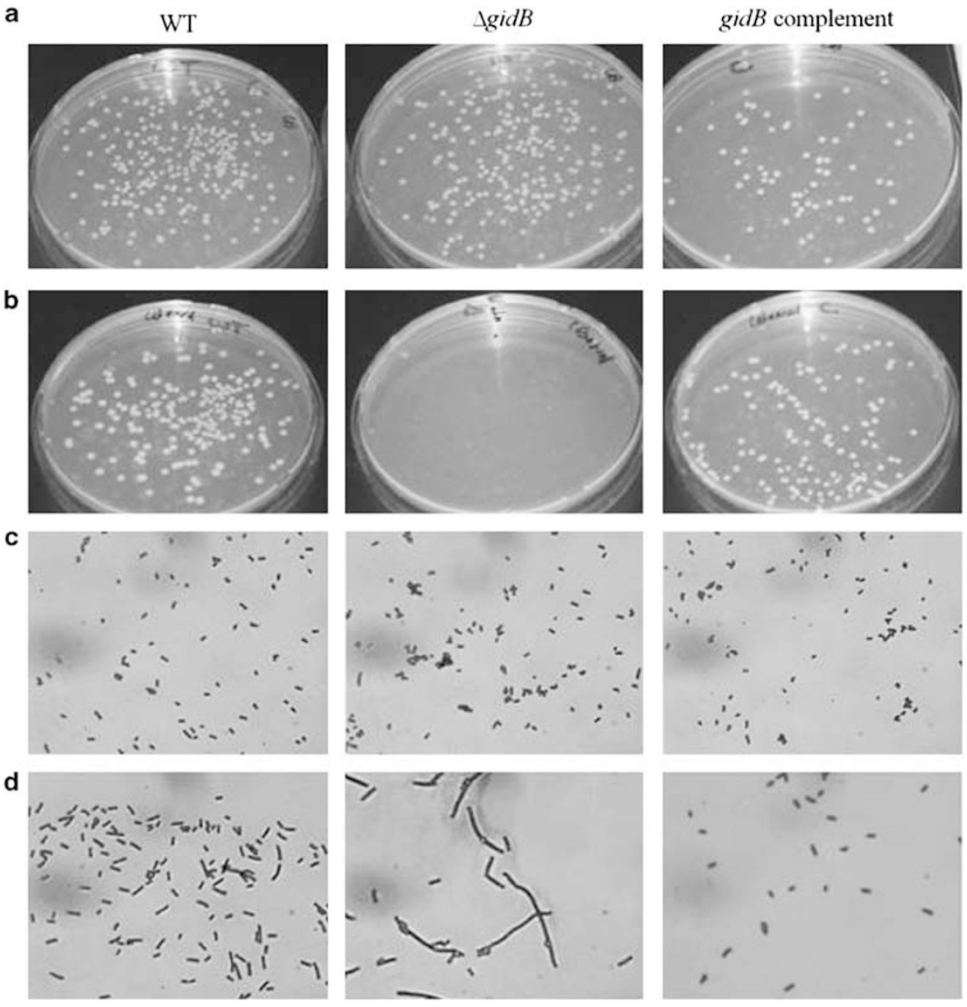

Figure 2 Colony and cellular morphology of the WT, *gidB* mutant and complemented strains. In panel (**a**), cells were grown in LB agar without nalidixic acid, and displayed no difference in colony morphology in the *Salmonella* strains. Panel (**b**) showed the *gidB* mutant with tiny colonies compared with the WT and complemented strains when grown on nalidixic acid-containing medium. Panel (**c**) showed Gram-stains for WT, *gidB* mutant and complemented strains having the normal rod-shaped cells when grown in LB broth without nalidixic acid. Panel (**d**) indicated filamentous morphology in the *gidB* mutant when grown in nalidixic acid-containing media compared with the normal rod-shaped WT and complemented strains. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

The automated Sensititre Susceptibility test showed little difference in the MIC pattern for florfenicol (WT: intermediate, *gidB* mutant: sensitive) and spectinomycin (WT: resistant, *gidB* mutant: intermediate) between the WT and the *gidB* mutant. However, there was a significant difference for neomycin (WT: sensitive ($\leqslant 4\,\mu g\,ml^{-1}$), *gidB* mutant: resistant ($\geqslant 32\,\mu g\,ml^{-1}$)) and streptomycin (WT: sensitive ($16\,\mu g\,ml^{-1}$), *gidB*: resistant ($128\,\mu g\,ml^{-1}$)) MICs in the *gidB* mutant compared with the WT, indicating a significant role for GidB in altering antibiotic sensitivity in *Salmonella*. Interestingly, streptomycin and neomycin are from the same antibiotics family, aminoglycosides, which work by binding to the small ribosomal subunit and interfering with protein synthesis.

GidB mutant is defective in bacterial motility under stress condition

Motility assay indicated that the distance migrated from the point of inoculation to the periphery was 45.3 mm for the *gidB* mutant and 43.33 mm for the WT when inoculated in media with no nalidixic acid (Figure 4a). However, motility plates supplemented with nalidixic acid showed that *gidB* mutant migrated 30.5 mm from the point of inoculation to the periphery, whereas the WT and the complemented strains migrated 63.5 mm and 51.5 mm, respectively. These data indicate that the presence of nalidixic acid in the medium significantly reduced *gidB* mutant motility (Figure 4b) and not the WT.

Lack of 16S rRNA methylation in *gidB* mutant

Autoradiogram analysis showed that the *gidB* mutant and the WT termination patterns were different in the reverse transcription at nucleotide C526, which lies immediately upstream of G527 on the 16S rRNA templates (Figure 5). The band formed in the WT at C526, and not in the *gidB* mutant, indicated the presence of the N^7-methylated guanosine in the WT, which resulted in its cleavage, and that the lack of such methylation in the *gidB* mutant was due to the loss of GidB activity. This data indicated that GidB is responsible for N^7 methylation of G527 of the 16S rRNA in *Salmonella*.

DISCUSSION

To investigate the potential effects of the *gidB* deletion on *Salmonella* growth and overall fitness, we performed conventional and competitive growth assays. This assay has been used widely to measure the

overall fitness and determine ability of bacterial mutants to survive and compete with the WT under stressful conditions.[19,40,45] Consistent with what has been shown in *T. thermophilus*, deletion of *gidB* significantly decreased the overall ability of *Salmonella* to survive and compete with the WT under stressful growth conditions. This could be due to the physiological cost of the methylation (m^7G527) deficiency in the *gidB* mutant,[19] or alterations in biosynthetic pathways due to *gidB* deletion, which needs further investigation.

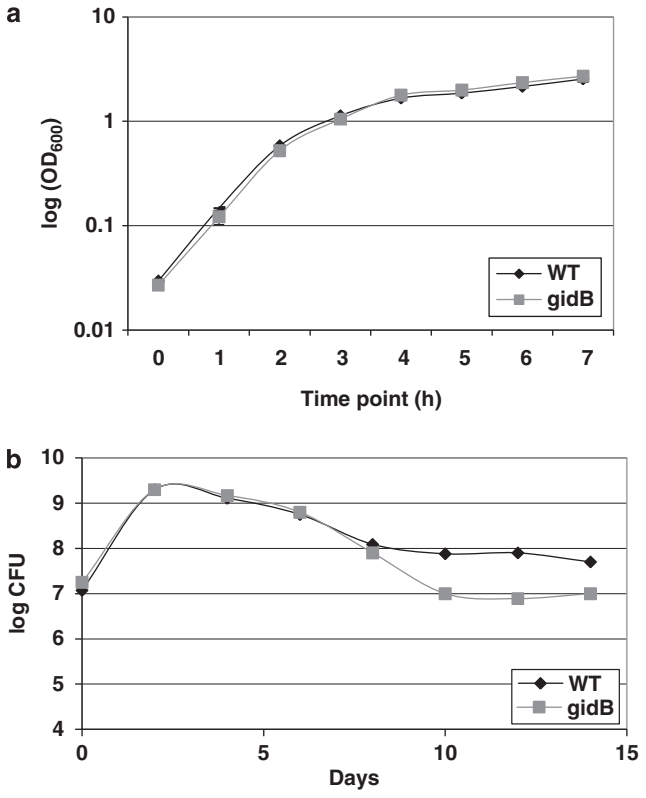

Figure 3 Growth curves for *gidB* mutant and WT strains. No significant difference in the growth pattern was observed for the two strains when grown with or without nalidixic acid. Panel (**a**) showed the *gidB* mutant and WT strains grown in LB broth without nalidixic acid (growth curve in LB broth with nalidixic acid is not shown). Panel (**b**) showed the competitive growth assay for *gidB* mutant and WT strains. The *gidB* mutant was unable to compete for limited nutrient sources. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

The *Salmonella gidB* mutant showed filamentous cells in the presence of nalidixic acid. Filamentation is the continued cell growth without cell division, mostly associated with metabolic changes, DNA damage and mutations in cell division machinery.[46] It has been shown that filamentation is a common strategy for survival in many bacteria under stress conditions, such as nutrient limitations, host effectors and antimicrobial therapies.[46,47] For example, magnesium deficiency induces filamentation in *Clostridium welchii* by a mechanism involving inhibition of cell division.[48,49] In *E. coli*, filamentation was found to serve as a survival strategy to evade neutrophil phagocytosis.[50] The β-lactam antibiotics have also been found to induce filamentation in bacteria.[51–53] *Burkholderia pseudomallei* showed filamentous cells when exposed to ceftazidime.[54] In *Salmonella*, a filamentous phenotype was found to be associated with the *in vitro* survival within the macrophages. It was suggested that nitric oxide radicals inhibited cell division and that filamentation was mediated by the DNA-damage response (SOS).[55] Nalidixic acid has also been implicated in the filamentation of *E. coli* cells.[56,57] In the present study, nalidixic acid did not cause significant filamentation in the WT *Salmonella.* Therefore, it is most likely that *gidB* has a potential role in responses to stress conditions and induction of a filamentous morphology in *Salmonella*.

Alterations in bacterial colony morphology could be either in size or shape of the colony. Changes in colony shape have been related to the bacterial response to different stress stimuli, such as starvation, high temperature, presence of antibiotics in a sub-IC and genetic mutations.[58,59,60] Several genes were shown to be involved in colony morphological change in bacteria.[61,62] In *E. coli*, the *flu* (coding for the O-antigen) and *fim* (coding for type-1 fimbriae) genes significantly influenced the colony morphology.[62] In *Salmonella*, smaller size colonies have been detected in an *lpd* (coding for dihydrolipoamide dehydrogenase) mutant, *hemL* (coding for glutamate-1-semyaldehyde aminotransferase) mutant, and *aroD* (coding for 3-dehydroquinate dehydratase) mutant. Interestingly, these *Salmonella* mutants were also less susceptible to aminoglycoside antibiotics.[61] Small colonies were also observed in *Staphylococcus aureus* when exposed to gentamicin.[63–72] Smaller colonies have shown a slower rate of growth in many bacteria, including *S. aureus*.[73] In this study, the *gidB* mutant formed small colonies in the presence of nalidixic acid without showing an effect on the growth rate.

Reduced motility in the *gidB* mutant under the stress of nalidixic acid might be related to its filamentation. It was shown that filamentous *Burkholderia pseudomallei* exhibited a significant decrease in motility. This reduction in motility has been suggested to be due to suppression of energy metabolism in these filamentous cells, which is

Table 1 Conventional MIC test results

Streptomycin (μg ml^{-1})	700	350	175	87.5	43.75	21.88	10.94	5.47	2.73	1.37
WT	–	–	–	–	+	+	+	+	+	+
Δ*gidB*	–	+	+	+	+	+	+	+	+	+
gidB complement	–	–	–	+	+	+	+	+	+	+
Gentamicin (μg ml^{-1})	20	10	5	2.5	1.25	0.625	0.3125	0.156	0.078	0.039
WT	–	–	–	+	+	+	+	+	+	+
Δ*gidB*	–	–	+	+	+	+	+	+	+	+
Chloramphenicol (μg ml^{-1})	20	10	5	2.5	1.25	0.625	0.3125	0.156	0.078	0.039
WT	–	–	–	+	+	+	+	+	+	+
Δ*gidB*	–	–	–	–	+	+	+	+	+	+

Abbreviations: *gidB*, glucose-inhibited division gene; WT, wild type.

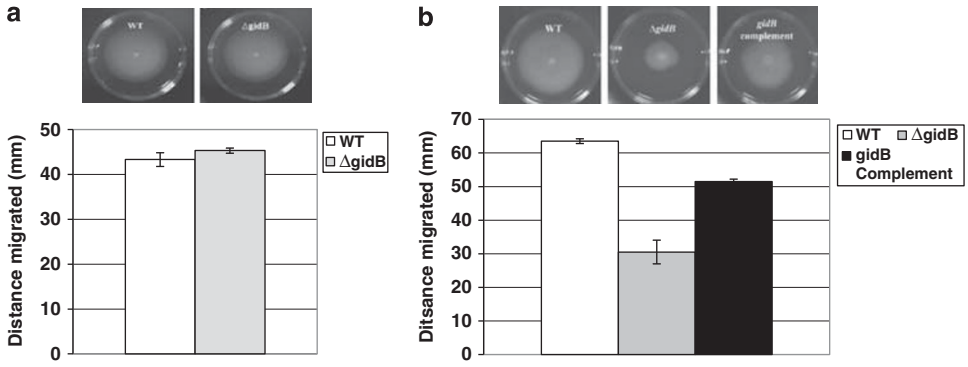

Figure 4 Motility assay for various *Salmonella* strains. Panel (**a**) showed the *gidB* mutant and WT strains grown in LB motility plates (without supplementation with nalidixic acid), with no significant difference in their migration distance ($P>0.05$). Panel (**b**) showed a significant reduction in *gidB* mutant motility compared with the WT and complemented strains when grown in nalidixic acid-containing LB motility plates ($P<0.005$).

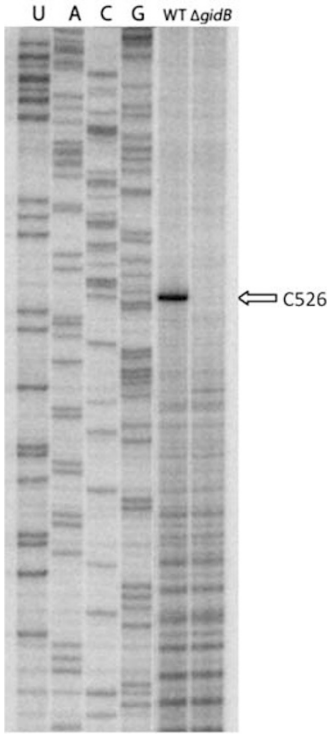

Figure 5 Identification of GidB methylation site. Gel autoradiogram shows the primer extension products of the $NaBH_4$ and acetic acid–aniline-treated *gidB* mutant and WT RNAs. Only WT RNA was cut in the phosphodiester backbone at m^7G527, giving a band corresponding to C526. This band was not detected in the *gidB* mutant due to the lack of methylation at G527.

essential for flagella movement.[54] Such a proposed mechanism may have also resulted in motility defect in the *Salmonella gidB* mutant in the presence of nalidixic acid.

In agreement with many other findings in *E. coli*, *T. thermophilus*, *Bacillus subtilis* and *M. tuberculosis*, *gidB* mutation conferred streptomycin resistance to *Salmonella*.[17–19] Although it conferred a low level of streptomycin resistance in other bacteria, our mutant displayed a high level of resistance. Resistance to antimicrobial agents was independently confirmed by a commercially available automated MIC test. This test showed that the *gidB* mutant had, in addition to streptomycin, a high resistance to neomycin. Interestingly, streptomycin and neomycin both belong to the aminoglycoside antibiotic family. Both antibiotics act by inhibiting bacterial protein synthesis by binding to the 16S rRNA of the 30S subunit of the ribosome.[33,36,74–77] During this binding, streptomycin makes contact with the 16S rRNA residues C526 and G527, resulting in the genetic code misreading in the translation process.[63,78] It has been reported that the loss of methylation at G527 (*E. coli* numbering) as a result of a *gidB* mutation caused streptomycin resistance in *B. subtilis*, *M. tuberculosis* and other bacteria.[17,18] Our data showed a lack of methylation in the 16S rRNA of the *gidB* mutant, indicating a similar mechanism of resistance in *Salmonella*. On the other hand, neomycin binds to the 16S rRNA at a different site from that of streptomycin. The method by which GidB confers neomycin resistance in *Salmonella* is not clear. It is possible that lack of methylation in the *gidB* mutant may have affected the binding site of neomycin or by another mechanism yet to be determined.

In conclusion, we developed a *Salmonella* strain from which *gidB* was deleted from the chromosome. Subsequently, the *gidB* mutant was characterized using biological and virulence assays. Although GidB had no effect on the growth of *Salmonella*, a competitive growth assay indicated that deletion of *gidB* significantly affected the overall fitness of the bacteria, specifically under limited stressful growth conditions. In the presence of nalidixic acid, *gidB* mutant showed smaller sized colonies, filamentous cellular morphology and reduction in bacterial motility compared with the WT. Most importantly, the *gidB* mutant gained a high level of streptomycin resistance compared with the WT. These effects of GidB could be restored to the WT phenotype by complementation of the *gidB* mutant. The streptomycin resistance was associated with the loss of methylation at the G527 of the 16S rRNA of the *gidB* mutant, as demonstrated by the primer extension assay. The MIC data indicated that the *gidB* mutant was also resistant to another aminoglycoside antibiotic neomycin. Taken together, these data strongly suggest that *gidB* has an important role in response to stress conditions in *Salmonella*. It will be crucial to examine the effect of GidB on the ribosomal structure and function in *Salmonella*. Further investigation is needed to determine the role of GidB in stress response and in modulation of metabolic pathways. Such studies are now underway in our laboratory.

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