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tRNA Modification by GidA/MnmE Is Necessary for *Streptococcus pyogenes* Virulence: a New Strategy To Make Live Attenuated Strains▽†

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Studies directed at vaccine development and mucosal immunity against *Streptococcus pyogenes* would benefit from the availability of live attenuated strains. Our approach for production of candidate live attenuated strains was to identify mutations that did not alter growth in vitro and did not alter the overall complement of virulence factors produced but did result in reduced levels of expression of multiple secreted virulence factors. A global reduction but not elimination of expression would likely lead to attenuation while maximizing the number of antigenic targets available for stimulation of immunity. Adaptation of Tn*5*-based transposome mutagenesis to *S. pyogenes* with initial screening for reduced expression of the SpeB protease resulted in identification of mutations in *gidA*, which encodes an enzyme involved in tRNA modification. Reduced SpeB expression was due to delayed onset of *speB* transcription resulting from reduced translation efficiency of the message for RopB, a transcriptional activator. Overall, GidA$^-$ mutants had a nearly normal global transcription profile but expressed significantly reduced levels of multiple virulence factors due to impaired translation efficiencies. A translation defect was supported by the observation that mutants lacking MnmE, which functions in the same tRNA modification pathway as GidA, phenocopied GidA deficiency. The mutants stimulated a cytokine response in cultured macrophages identical to that in the wild type, with the exception of reduced levels of tumor necrosis factor alpha and interleukin-23. Significantly, GidA$^-$ mutants were highly attenuated in the murine ulcer model of soft tissue infection. These characteristics suggest that GidA pathway tRNA modification mutants are attractive candidates for further evaluation as live attenuated strains.

Despite the exquisite sensitivity of *Streptococcus pyogenes* (group A streptococcus) to penicillin, diseases caused by this organism remain a major public health concern in both developed and developing countries (6). This pathogenic gram-positive bacterium is the causative agent of myriad diseases, ranging from severe invasive diseases (cellulitis, necrotizing faciitis), toxigenic diseases (scarlet fever, toxic shock syndrome), and sequlae (rheumatic heart disease, glomerulonephritis) to more self-limiting diseases, including pyoderma and pharyngitis. A recent analysis of the prevalence of group A streptococcal diseases indicated that the minimum global burden is upwards of 18 million cases of severe disease, with 1.78 million new cases arising each year, resulting in at least 517,000 deaths (6). This degree of mortality places group A streptococci among the major human pathogens, exceeded only by human immunodeficiency virus, *Mycobacterium tuberculosis*, *Plasmodium falciparum*, and *Streptococcus pneumoniae* (6). While children in developing countries bear the greatest burden of severe disease (40), the impact on the health care system of the United States is also considerable, with cases of acute pharyngitis accounting for as many as 11 million office visits per year (3). The burden imposed by group A streptococci on the global population has stimulated considerable interest in the development of new therapies for prevention of these diseases.

A vaccination approach offers many advantages. However, development of a safe and effective group A streptococcal vaccine has met with several significant challenges. Among these challenges are the fact that the pathogenesis of most group A streptococcal diseases is not well understood and may involve an autoimmune component in certain diseases, such as rheumatic fever (11). Similarly, both the contribution of the large number of secreted enzymes, toxins, and surface-associated factors produced by *S. pyogenes* to the pathogenesis of specific diseases and the importance of the immune response against these components to protective immunity are also not well understood (11). Early studies established that serum immunoglobulin can be opsonic and can promote the neutrophil-mediated killing of *S. pyogenes* in human blood (25). The antibodies function by neutralizing the antiphagocytic property of a streptococcal surface protein known as the M protein (for a review, see reference 15), and the generation of these antibodies has provided the rationale for several M protein-based vaccine formulations (for a review, see reference 3). However, several issues have complicated the development of vaccines based on the M protein, including (i) the fact that there are over 150 distinct serotypes of M protein and (ii) the fact that M proteins can elicit the production of antibodies that are cross-reactive with human tissues (10).

The use of peptides representing epitopes common to multiple serotypes, the use of multivalent peptides, and the use of peptides that have been engineered to lack prominent cross-reactive epitopes have all been used as strategies to increase the safety and efficacy of M protein vaccines (3). However, continuing concerns have stimulated investigation into other

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potential formulations, including the use of conserved surface proteins (3), and have spurred several large-scale interrogations of the surface proteome in the search for additional vaccine candidates (34, 47, 51). A combination of these studies with a thorough understanding of the immune response against *S. pyogenes* would offer a more rationale approach to vaccine design. Of particular utility would be a vaccine capable of preventing *S. pyogenes* from colonizing the mucosal surfaces of the nasopharynx, which is the primary site of most streptococcus-host interactions (11). However, the factors that contribute to immunity against *S. pyogenes* at a mucosal surface are not well understood.

Effective immunity at a mucosal surface generally requires engagement of the mucosal immune system, which is responsible for the production of defense molecules, like secretory immunoglobulin A, that are well adapted for defense in the mucosal environment (31). Most mucosal vaccines in current use accomplish this through the use of live attenuated strains that can be administered by a route that mimics the natural route of infection. The attenuated strains typically can undergo the initial stages of pathogenesis but have mutations that restrict them to producing a self-limiting infection with minimal symptomatology (12, 29). Unfortunately, there has been relatively little work directed at the development of live attenuated strains for analyses of immunity to *S. pyogenes*.

Since *S. pyogenes* pathogenesis is dependent upon secretion of a large array of surface proteins, extracellular enzymes, and toxins (11), our approach to construction of a candidate for a live attenuated strain was to conduct a screen for a mutant with the following characteristics: (i) it should have no obvious defect in growth characteristics in vitro; (ii) it should have no obvious defect in the number of different virulence factors that it expresses; and (iii) it should have a measurable defect that causes it to produce a large subset of its secreted virulence factors at significantly reduced levels. We reasoned that a global reduction in, but not elimination of, virulence factor expression would render the mutant attenuated, while maximizing the repertory of antigens that could be presented to the immune system. In this report, we describe mutants defective in a tRNA modification pathway as candidates for such a live attenuated strain. The pathway involves the proteins GidA and MnmE, which is responsible for the addition of a carboxymethylaminomethyl group to uridine 34 of a subset of tRNAs that is crucial for the appropriate decoding of many mRNAs (48). While these mutants have no obvious in vitro growth defect and a nearly normal global transcription profile, the level of expression of multiple secreted virulence factors by them is reduced but expression is not eliminated. In a subset of the mutants, reduced expression results from a defect at the level of translation of at least one key transcription regulator. The mutants induce a nearly normal pattern of cytokine expression in macrophages in vitro and are highly attenuated in a murine ulcer model of soft tissue infection. The fact that this pathway is highly conserved suggests that mutation of the genes encoding the factors is a strategy that can be applied to the construction of live attenuated strains of other species of pathogenic streptococci.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains and plasmids used in this study are listed in Table 1. Molecular cloning experiments were performed with *Escherichia coli* DH5α which was cultured in Luria-Bertani broth (38) at 37°C. For routine culture of *S. pyogenes*, Todd-Hewitt medium (BBL) supplemented with 0.2% yeast extract (Difco) (THY medium) was employed. Unless otherwise indicated, for growth of *S. pyogenes* for SpeB activity assays, Western blot analyses, and preparation of RNA for real-time reverse transcription (RT)-PCR C medium was used (37). For growth in liquid media, *S. pyogenes* was cultured at 37°C in sealed tubes without agitation. To produce solid media, Bacto agar (Difco) was added to a final concentration of 1.4% (wt/vol). When appropriate, antibiotics were added to the media at the following concentrations: spectinomycin, 100 μg/ml for *E. coli* and *S. pyogenes*; chloramphenicol, 15 μg/ml for *E. coli* and 3 μg/ml for *S. pyogenes*; kanamycin, 50 μg/ml for *E. coli* and 500 μg/ml for *S. pyogenes*; and erythromycin, 750 μg/ml for *E. coli* and 1 μg/ml for *S. pyogenes*.

Manipulation and computational analyses of DNA. Plasmid DNA was isolated via standard techniques and used to transform *S. pyogenes* as described previously (5). Restriction endonucleases, ligases, and polymerases were used according to the manufacturer's recommendations. The fidelity of all constructs derived by PCR was confirmed by DNA sequencing analyses. All references to genomic loci and predictions of protein function are based on the genome of *S. pyogenes* strain SF370 (14) and were supported by interrogation of the nonrepetitive sequence database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) using a gapped BLAST algorithm (2).

Construction of EZ-Tn*5*<ΩErm>. For application of Tn*5*-based "transposome" mutagenesis (22) to *S. pyogenes*, the BamHI site of pMOD-2<MCS> (Epicenter Biotechnologies) was used to insert a BamHI fragment that contains the ΩErm element of pJDM-STM (30). The resulting plasmid was designated pMOD-2::Tn*5*<ΩErm>, and the resulting transposable element was designated EZ-Tn*5*<ΩErm> (Fig. 1A). The erythromycin resistance gene of the ΩErm element can be used for selection in both *E. coli* and *S. pyogenes* (30).

Transposome mutagenesis. A gel-purified PvuI fragment of pMOD-2::Tn*5*<ΩErm> that included Tn*5*<ΩErm> (Fig. 1A) was mixed in vitro with the EZ-Tn*5* transposase according to the directions of the manufacturer (Epicenter Biotechnologies). Electroporation was then used to introduce the transposome complex into competent cells of *S. pyogenes* that had been prepared by a standard method (5) and then washed once at room temperature in a 15% (vol/vol) glycerol solution containing 1 mM EDTA to remove Mg^{2+}. This additional step was required for stabilization of the transposon-transposase complex prior to its introduction into the streptococcal cell (22). Transformants with insertions of Tn*5*<ΩErm> were then recovered following overnight incubation at 37°C on THY medium plates supplemented with erythromycin. For mutants of interest, the transposon insertion site was identified by direct sequencing of chromosomal DNA using a transposon-specific primer (SqTM-r) (see Table S1 in the supplemental material) as described previously (18).

Directed mutagenesis and complementation. Insertional inactivation of specific genes and complementation analyses were conducted using a standard recombinational strategy (8). For mutagenesis, an approximately 0.7-kbp fragment of the gene of interest that lacked both its 5′ and 3′ ends was amplified by PCR, inserted into pSPC18 (37), and then introduced into *E. coli*. The plasmid was purified and then used to transform *S. pyogenes*. Since pSPC18 cannot replicate in *S. pyogenes*, most transformants resistant to spectinomycin have undergone a single homologous recombination event that results in disruption of the targeted gene (8). For complementation of a GidA$^-$ mutant, an intact copy of *gidA* was introduced into the chromosome of the mutant as follows. A chromosomal segment containing the entire predicted *gidA* open reading frame and putative promoter region was amplified by PCR and inserted into the integrational plasmid pCIV2 (34). Transformation followed by recombination of the resulting plasmid into the mutant's chromosome resulted in introduction of an intact copy of *gidA*. Details for each integrating plasmid are shown in Table 1, and the sequences of primers used for amplification of gene segments are shown in Table S1 in the supplemental material. The chromosomal structure of each mutant was confirmed by PCR using primers for the appropriate sequences. To ensure that mutant phenotypes were specifically due to gene disruption and not due to a nonspecific effect of integration of pSCP18 into the chromosome, a strain with pSPC18 inserted into the intergenic region downstream of *recF* (SPy_2204) (9), which is referred to here as HSC5spc (Table 1), was included in selected analyses.

Analysis of protein expression. Analyses of cell growth and use of protease indicator medium, Western blot analysis of SpeB secretion, and quantitative measurement of SpeB activation and SpeB cysteine protease activity were all

TABLE 1. Bacterial strains and plasmids

Strain(s) or plasmid	Relevant characteristics	Reference or source
E. coli DH5a	*recA1 endA1 hsdR17*	38
S. pyogenes strains		
HSC5	Wild type	
TM-GidA1,TM-GidA2, and TM-GidA3	Transposon-generated *gidA* mutants[a]	This study
HSC5Spc	Chromosomal insertion of pSPC18 downstream of *recF* in the HSC5 chromosome without disruption of any gene or operon; previously referred to as "Control"	9
HSC5::pKHC100	HSC5 mutant with *gidA* disruption created with pKHC100[b]	This study
HSC5::pKHC101	HSC5 mutant created with pKHC101; pSPC18 is inserted between *gidA* and SPy_2184[b]	This study
HSC5::pKHC102	HSC5 mutant with SPy_2184 disruption created with pKHC102[b]	This study
HSC5::pKHC100::pKHC103	Insertion of pKHC103 into HSC5::pKHC100; contains an intact *gidA* gene	This study
HSC5::pKHC104	HSC5 mutant with *mnmE* disruption created with pKHC104	This study
HSC5::pKHC105	HSC5 mutant with SPy_1173 disruption created with pKHC105	This study
HSC5Spc(pRopB-HA)	HSC5Spc transformed with pRopB-HA; produces RopB-HA	This study
HSC5::pKHC100(pRopB-HA)	HSC5::pKHC100 transformed with pRopB-HA; produces RopB-HA	This study
HSC5::pKHC104(pRopB-HA)	HSC5::pKHC104 transformed with pRopB-HA; produces RopB-HA	This study
HSC5Spc(pSPN-HA)	HSC5Spc transformed with pSPN-HA; produces SPN-HA	This study
HSC5::pKHC100(pSPN-HA)	HSC5::pKHC100 transformed with pSPN-HA; produces SPN-HA	This study
HSC5::pKHC104(pSPN-HA)	HSC5::pKHC104 transformed with pSPN-HA; produces SPN-HA	This study
HSC5Spc(pMF-HA)	HSC5spc transformed with pMF-HA; produces MF-HA	This study
HSC5::pKHC100(pMF-HA)	HSC5::pKHC100 transformed with pMF-HA; produces MF-HA	This study
HSC5::pKHC104(pMF-HA)	HSC5::pKHC104 transformed with pMF-HA; produces MF-HA	This study
Plasmids		
pMOD-2<MCS>	pUC-based vector containing EZ-Tn*5*<MCS>	Epicenter Inc.
pMOD-2::Tn*5*<ΩErm>	pUC-based vector containing EZ-Tn*5*<ΩErm>; derived from pMOD-2<MCS>[c]	This study
pSPC18	pUC18-based streptococcal integration vector; contains *aad9* conferring spectinomycin resistance	28
pCIV2	pUC18-based streptococcal integration vector; contains *aphA3* conferring kanamycin resistance	34
pSPC18::*'recF*	pSPC18 containing a 0.70-kbp C-terminal segment of *recF*	9
pKHC100	pSPC18 containing a 0.70-kbp internal fragment of *gidA* generated by PCR with primers GidA-f and GidA-r	This study
pKHC101	pSPC18 containing a 0.62-kbp fragment spanning the *gidA*-SPy_2184 intergenic region generated by PCR with primers Separate-f and Separate-r	This study
pKHC102	pSPC18 containing a 0.73-kbp internal fragment of SPy_2184 generated by PCR with primers SPy2184-f and SPy2184-r	This study
pKHC103	pCIV2 containing a 2.43-kbp DNA fragment including *gidA* generated by PCR with primers GidACisComp-f and GidACisComp-r	This study
pKHC104	pSPC18 containing 0.52-kbp internal fragment of *mnmE* generated by PCR with primers MnmE-f and MnmE-r	This study
pKHC105	pSPC18 containing 0.58-kbp internal fragment of SPy_1173 generated by PCR with primers SPy1173-f and SPy1173-r	This study
pRopB-HA	pABG5 derivative containing HA-tagged *ropB*	26
pSPN-HA	pABG5 derivative containing HA-tagged *spn*; same as pJOY7	17
pMF-HA	pABG5 derivative containing HA-tagged *mf*; same as pJOY2	17

[a] For detailed information, see Fig. 1.
[b] See Fig. 2.
[c] See Fig. 1.

performed as described previously (37). Sample preparation and experiments with antisera for Western blot analyses to quantitate expression of streptolysin O (SLO), NAD glycohydrolase (SPN), mitogenic factor (MF), and RopA-hemagglutinin (RopA-HA) were conducted as described elsewhere (22, 35). Cells were fractionated as described previously (36), and M protein in cell wall fractions was analyzed by Western blotting using an antiserum developed against a peptide (QELAKKEEQNKISDASRKG) that was shown to be involved in streptococcal cell aggregation (16). The level of expression of a specific protein in various mutants was expressed relative to its expression in the wild type by comparison of band intensities in digital images of Western blots analyzed using IMAGEJ software (1).

Analysis of transcription. RNA from the various streptococcal strains was isolated and analyzed using real-time RT-PCR as described previously (27), employing the primers listed in Table S1 in the supplemental material. Transcript abundance was normalized to the results for *recA*, and the data presented below are the means and standard deviations derived from at least two independent experiments that were performed on different days, with each individual sample analyzed in triplicate. Comparison of global transcriptional profiles using DNA microarrays was conducted as described in detail elsewhere (6, 13, 36).

Infection of macrophages. Murine bone marrow-derived macrophages were obtained by a standard procedure (7) and were cultured in RPMI 1640 containing L-glutamine (Cellgro Mediatech, Inc.) with 20% (vol/vol) heat-inactivated fetal bovine serum (BioWhittaker, Inc.), 30% (vol/vol) L-cell conditioned medium (a source of macrophage colony-stimulating factor), penicillin G (50 IU ml^{-1}), and streptomycin (50 $\mu g\ ml^{-1}$). Macrophages were cultured for 8 days, harvested with ice-cold phosphate-buffered saline, resuspended in macrophage medium (Dulbecco's minimal essential medium [BioWhittaker, Inc.] containing 50 mM HEPES, 10% fetal bovine serum, and 8 mM L-glutamine), and then plated at a density of 2.5×10^6 cells per well in a six-well plate (catalog no. 3506; Corning Inc.). For infection, overnight cultures of *S. pyogenes* in THY medium

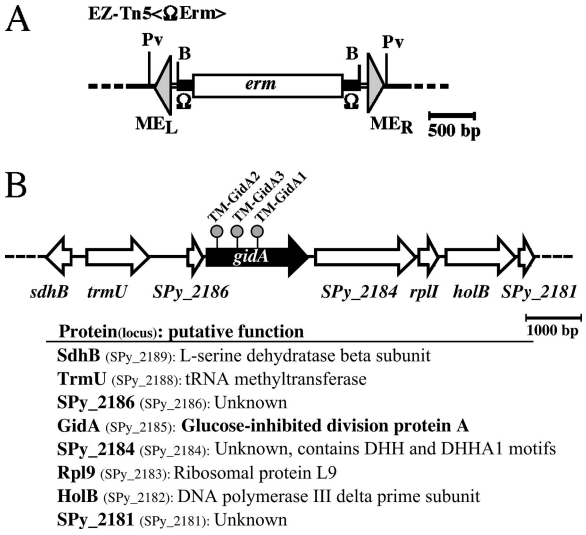

FIG. 1. (A) Transposable element EZ-Tn*5*<ΩErm>. The structure of the EZ-Tn*5*<ΩErm> transposon developed for transposome mutagenesis (22) is shown. The ΩErm element contains an erythromycin resistance determinant (*erm*) that is flanked by strong transcription and translation terminators (indicated by "Ω") (30). The ΩErm element is flanked by the left and right mosaic ends of Tn*5* (arrowheads ME_L and ME_R). For clarity, only a limited region of plasmid pMOD-2::Tn*5*<ΩErm> (dashed line), a derivative of pMOD-2 <MCS> (Epicenter Biotechnologies), is shown. Restriction sites: Pv, PvuI; B, BamHI. (B) Reduced SpeB expression as a result of transposon insertion in *gidA*. The organization of the chromosomal region containing *gidA* is shown at the top. The arrows indicate individual open reading frames and their orientations and are labeled based on the annotation of the *S. pyogenes* SF370 genome (14). The predicted proteins encoded by these open reading frames and their putative functions are shown at the bottom. The markers above *gidA* indicate the sites of EZ-Tn*5*<ΩErm> insertion in three mutants (TM-GidA1 to TM-GidA3) that resulted in reduced expression of the SpeB cysteine protease. The specific sites of transposon insertion are as follows: TM-GidA1, within a lysine codon (L368); TM-GidA2, following an alanine codon (A16); and TM-GidA3, within a serine codon (S179).

were harvested, washed twice with phosphate-buffered saline, and then resuspended in macrophage medium to an optical density at 600 nm of 0.76. Portions of this suspension ranging from 50 to 100 μl were then added directly to wells containing macrophages. Final multiplicities of infection were determined by plating the streptococcal suspensions on THY agar following brief sonication to disrupt the streptococcal chains. In selected experiments, streptococci were inactivated by exposure to UV light (254 nm, 500 mJ/cm^2) prior to incubation with macrophages. This dose exceeds the lethal dose for *S. pyogenes* (44), which was routinely confirmed by plating on THY agar.

Cytokine assays. Following 4 h of infection of macrophage cultures, the supernatant fluids were harvested and then sterilized by filtration (Millex-GP; 0.22 μm; Millipore Corp). Cytokine levels in these fluids were determined using a commercial antibody-based multiplex bead assay (Bio-Plex, Mouse Cytokine 23-Plex; catalog no. 171-F11241; Bio-Rad Laboratories) to measure the 23 different murine cytokines that are listed in Fig. 8 and Fig. S2 in the supplemental material. For this analysis the directions of the manufacturer (Bio-Rad Laboratories) were followed, and the analysis was conducted by the High Speed Cell Sorter Core Lab at the Siteman Cancer Center of Washington University. The data presented are the means and standard errors derived from three different infections, which were conducted on different days, and each experiment was analyzed in duplicate. Any differences in the mean values for specific cytokines induced in response to mutants compared with the wild type were tested for significance by the unpaired *t* test (19), and the null hypothesis was rejected when the *P* value was <0.05.

Murine subcutaneous infection model. The abilities of selected mutants to cause disease in soft tissue were evaluated by subcutaneous infection of 6- to 8-week-old SKH1 hairless mice (Charles River Labs) as described previously (4). Various strains were analyzed using groups containing five mice, and each experiment was repeated at least twice on different days. The area of the draining ulcers that formed was documented every 24 h by digital photography, and the precise area for each ulcer was calculated from the digital record using MetaMorph image analysis software (version 4.6; Universal Imaging Corp.). Any differences in the areas of ulcers between experimental groups was tested for significance by the Mann-Whitney U test (19), and the null hypothesis was rejected when the *P* value was <0.05.

Microarray data accession number. The microarray data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo) under GEO Series accession number GSE9678.

RESULTS

Transposome mutagenesis identified the *gidA* gene. We adapted a Tn*5*-based transposome mutagenesis strategy to screen for a mutant with reduced virulence factor expression (see Materials and Methods). This method has the advantages that it is simple, target site selection is random, and, because the inserted element itself does not encode transposase, insertions are stable in the absence of continued antibiotic selection (22). Introduction of a transposome complex generated with EZ-Tn*5*<ΩErm> (Fig. 1A) into *S. pyogenes* HSC5 produced transformants at an efficiency between 10^3 and10^4 CFU/μg DNA. An insertion library was then examined for mutants that displayed reduced activity of the secreted SpeB cysteine protease following overnight culture on a protease indicator medium (see Materials and Methods). Approximately 4,000 colonies were examined, and nine mutants with reduced activity were identified that were subsequently found to express cysteine protease activity at levels between 5 and 20% of the wild-type level when they were examined using a quantitative proteolysis assay with a casein substrate (see Materials and Methods) following growth in two different media (THY and C media). Identification of the transposon insertion loci (see Materials and Methods) revealed that six mutants had insertions in the gene encoding RopB, which is a positive transcriptional regulator of SpeB, and three mutants had insertions at different sites within the *gidA* open reading frame (SPy_2185) (Fig. 1B), whose orthologous gene products have been implicated in the flavin adenine dinucleotide (FAD)-dependent modification of tRNAs decoding two-family box triplet codons (47). Mutation of *gidA* in *E. coli* causes cell elongation and slow growth in rich media supplemented with glucose (45). However, the *S. pyogenes* mutants did not exhibit any measurable defect in either the growth rate or cell morphology upon culture in several rich media (THY and C media) in the absence or presence of additional glucose (0.5%, wt/vol) (data not shown). Also, like the genomes of many bacterial species (46), the *S. pyogenes* genome encodes a protein that is highly homologous to, although somewhat smaller than, GidA (SPy_1173) (20.1% identical and 55.1% similar). Insertional inactivation of SPy_1173 did not lead to any observable defect in SpeB biogenesis (data not shown).

Characterization of the *gidA* chromosomal region. Examination of the genomic data available for multiple *S. pyogenes* strains revealed that the structure of the *gidA* chromosomal region is strongly conserved and that this region includes genes whose products are implicated in the function of ribosomes or DNA polymerase or have an unknown function (Fig. 1B), all of which may contribute to the biogenesis of SpeB and other

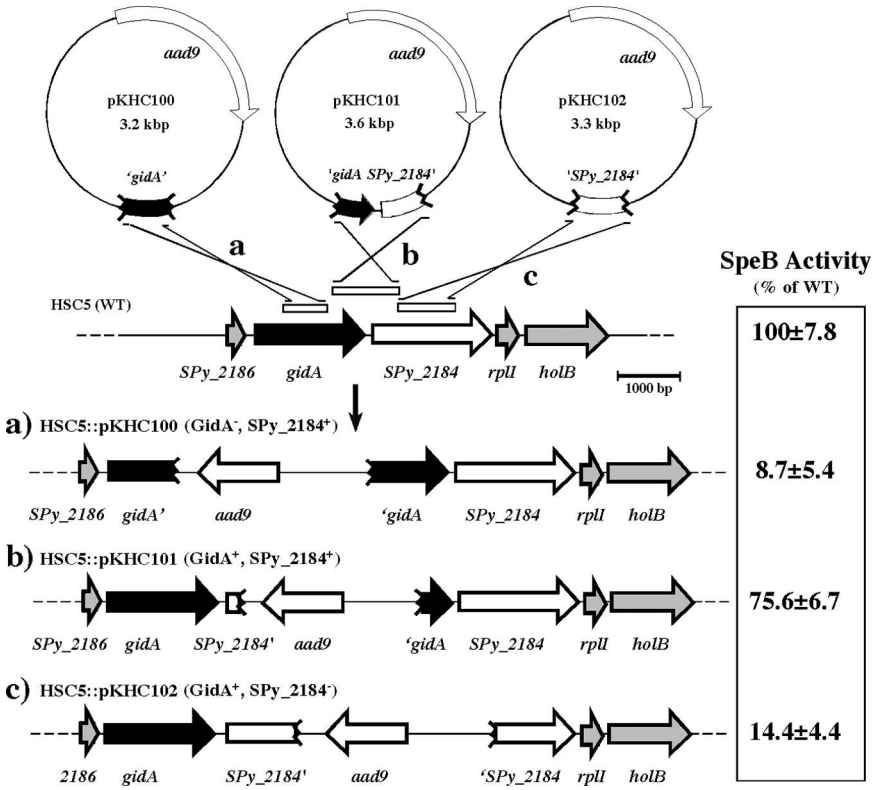

FIG. 2. Confirmation of the contribution of *gidA* to SpeB expression. The integrational plasmids shown at the top were used to disrupt various regions of the *gidA* locus in *S. pyogenes* HSC5. The broken ends of the plasmid-encoded boxes indicate that a gene segment lacks the 5′ or 3′ end of the corresponding chromosomal open reading frame, and these regions are indicated by the open boxes above the open reading frames. Chromosomal structures resulting from the single recombination events (labeled a, b, and c) are indicated below the solid arrow, and the designations and genotypes of the resulting strains are shown on lines a, b, and c. The open reading frame labeled *aad9* encodes a spectinomycin resistance determinant. The SpeB cysteine protease activities produced by these strains were determined relative to the activity produced by the wild-type strain (HSC5) (WT) and are indicated in the box on the right. The data are the means and standard errors of the means derived from at least three independent experiments.

virulence factors. Since the EZ::Tn*5*<ΩErm> element includes strong transcription and translation terminators to ensure generation of strongly polar insertions (Fig. 1A), it was necessary to examine the contribution of the adjacent genes to the GidA$^-$ phenotype. Insertion duplication mutations constructed to inactivate *gidA* and the open reading frame immediately downstream (SPy_2184) (Fig. 2) produced two mutants that both had reduced expression of SpeB (HSC5::pKHC100 and HSC5::pKHC102) (Fig. 2). However, when the amounts of SpeB protein that were secreted into the supernatant were compared using a Western blot analysis, the two mutants had distinct phenotypes. When tested after 10 h of culture, the GidA$^-$ mutant secreted a reduced amount of SpeB protein, like the original transposon insertion mutants. In contrast, the SPy_2184 mutant secreted wild-type levels of the protease zymogen at this time point but failed to process the inactive precursor to the active protease (data not shown). A comparison of the transcript levels of the genes downstream of *gidA* (SPy_2184 and *rplI*) (Fig. 1B) between the wild type and a transposon insertion mutant (TM-GidA1) (Fig. 1B) revealed no significant differences when a real-time RT-PCR was used (GidA$^-$ mutant/wild-type strain expression ratio for SPy_2184, 0.92 ± 0.17; GidA$^-$ mutant/wild-type strain expression ratio for *rplI*, 0.91 ± 0.30), showing that insertions into *gidA* are not polar for expression of downstream genes. Taken together, these data suggested that both GidA and SPy_2184 are required for SpeB biogenesis but contribute to different steps. A requirement for both genes was confirmed by construction of an additional insertion duplication mutation that placed the integrated plasmid between the intact *gidA* and SPy_2184 open reading frames. The resulting strain (HSC5::pKHC101) (Fig. 2) expressed nearly wild-type levels of SpeB proteolytic activity. Finally, reintroduction of an intact copy of *gidA* into the chromosome of a GidA$^-$ mutant (see Materials and Methods) resulted in a complemented strain whose protease activity more closely resembled that of the wild type than that of the GidA$^-$ mutant on protease indicator plates (Fig. 3A) and which produced SpeB activity in culture supernatants at nearly wild-type levels (Fig. 3B). Based on these data, we concluded that the reduced-expression phenotype observed in the transposon insertion mutants was due to the loss of *gidA*.

Transcription of *speB* is delayed in GidA$^-$ mutants. Expression of *speB* is known to be highly regulated at the level of transcription, and this gene is expressed in a growth phase-dependent pattern (26). Since GidA$^-$ mutants produced a reduced amount of SpeB protein at the time points described above, it was of interest to determine if the loss of *gidA* had any effect on the levels or timing of *speB* transcription. The growth

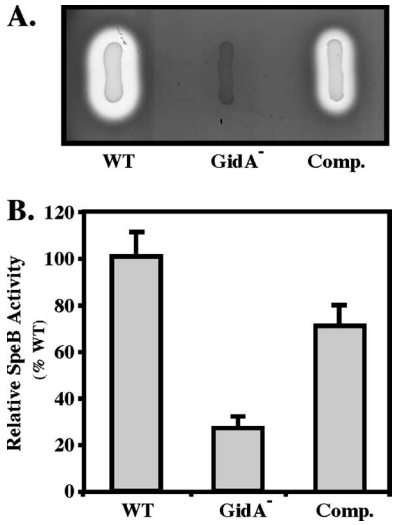

FIG. 3. Reconstitution of disrupted *gidA* restores SpeB expression. An intact copy of *gidA* was inserted into the chromosome of a GidA$^-$ mutant (see Materials and Methods for details). (A) Protease production on indicator medium. The indicated strains were patched on protease indicator medium and examined following overnight incubation. A zone of clearing resulted from protease activity. (B) Quantitative assessment of protease activity. The SpeB cysteine protease activities in supernatants of 12-h cultures are shown relative to the SpeB activity of the wild-type strain. The data are the means and standard errors of the means derived from at least three independent experiments. The following strains were used: wild type (WT), HSC5; GidA$^-$ mutant (GidA$^-$), HSC5::pKHC100; and GidA$^-$ complemented (Comp.), HSC5::pKHC100::pKHC103.

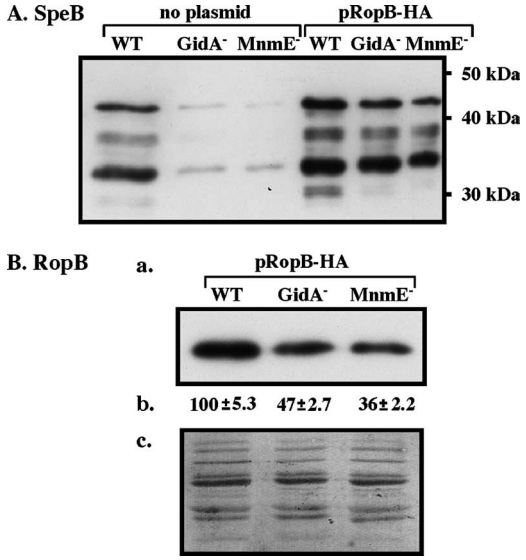

FIG. 5. Overexpression of RopB restores SpeB expression in GidA pathway mutants. (A) Western blot analysis to detect SpeB in strains containing a plasmid that overexpresses RopB (pRopB-HA) or in the absence of a plasmid (no plasmid). Note that multiple proteolytic cleavages are required for activation of the 46-kDa SpeB zymogen to the 28-kDa active protease. (B) Western blot analysis to determine the amount of RopB-HA present in the indicated strains (panel a), with protein levels quantitated by densitometry and expressed relative to the wild-type level (line b). Panel c shows the relevant region of the membrane stained with Coomassie blue for comparison of the amounts of total protein present in each lane. The following strains were used: wild type (WT), HSC5; GidA$^-$ mutant (GidA$^-$), HSC5::pKHC100; and MnmE$^-$ mutant (MnmE$^-$), HSC5::pKHC104.

patterns of the strains (the wild type, the GidA$^-$ mutant, and the SPy_2184 mutant) were almost identical (Fig. 4; see Fig. S1 in the supplemental material), so the levels of *speB* transcript abundance of the strains at different time points were examined by real-time RT-PCR. The levels of the *speB* message in the wild type exhibited the expected growth phase pattern, with initiation at the onset of stationary phase and an increase over the next several hours, followed by a gradual decline (Fig. 4). The SPy_2184 mutant, which had reduced SpeB activity but not a reduced level of secreted SpeB protein, exhibited a pattern identical to that of the wild type (Fig. 4). In contrast, the GidA$^-$ mutant displayed an aberrant pattern of expression. At the onset of stationary phase, the abundance of the *speB* transcript was over 60-fold less than that of the wild type (Fig. 4). The transcript became more abundant over time, until it reached a level that was not significantly different from the wild-type level when it was measured in 10-h cultures (Fig. 4).

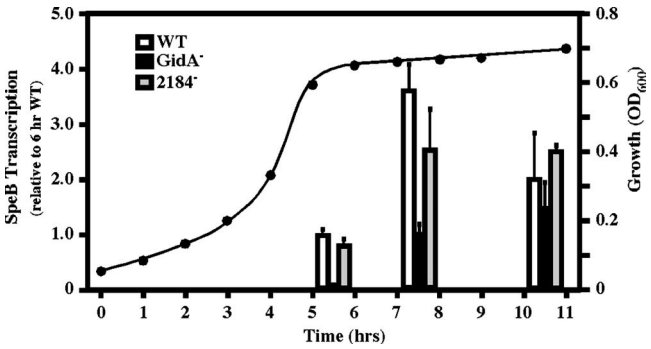

FIG. 4. Disruption of *gidA* results in altered pattern of *speB* transcription. The relative abundance of the *speB* message was determined over the course of growth in several strains using real-time RT-PCR. For each strain, transcript abundance is shown relative to the abundance of the *speB* message in the wild-type strain at 6 h. For clarity, only the growth pattern of the wild-type strain is shown, as the growth characteristics of all strains were indistinguishable under these conditions (see Fig. S1 in the supplemental material). The transcription data are the means and standard deviations of three independent experiments. The following strains were used: wild type (WT), HSC5; GidA$^-$ mutant (GidA$^-$), HSC5::pKHC100; and SPy_2184 mutant (2184$^-$), HSC5::pKHC102. OD_{600}, optical density at 600 nm.

Disruption of *mnmE* showed the same phenotype as mutation of *gidA*. In addition to GidA, modification of U34 in tRNAs decoding two-family box triplet codons requires additional enzymes, including MnmE (4, 8, 14, 47). This relationship is supported by the observation that GidA and MnmE form a heterotetrameric complex in *E. coli* (47). To examine whether the SpeB expression defect of GidA$^-$ mutants was specific to *gidA* or was a consequence of the loss of the ability to modify tRNA, a strain lacking *mnmE* was constructed (see Materials and Methods). A Western blot analysis revealed that the MnmE$^-$ mutant had a SpeB expression defect identical to that of the GidA$^-$ mutant (Fig. 5A), indicating that the entire pathway for U34 modification may be important for SpeB biogenesis.

GidA influences SpeB expression via the transcription regulator RopB. In *Shigella flexneri*, mutation of several genes

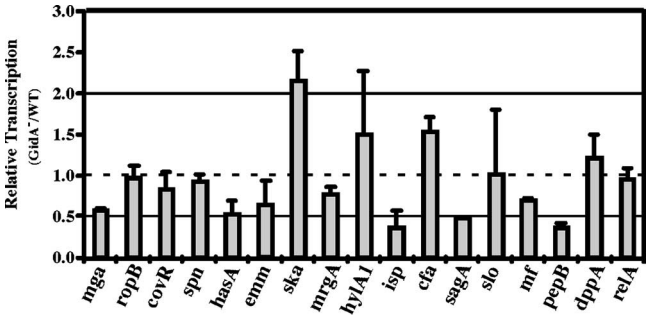

FIG. 6. Nearly normal transcription of multiple virulence factors in a GidA$^-$ mutant. The relative levels of abundance of the messages for the indicated virulence factors in a GidA$^-$ mutant were determined by real-time RT-PCR following 6 h of culture in C medium and are expressed as changes relative to the wild-type strain. Lines indicate expression that was not different from the wild-type expression (dashed line) and expression that was twofold different from the wild-type expression (solid lines). The chromosomal locus and the primers used for analysis of each gene are listed in Table S1 in the supplemental material. The transcription data are the means and standard errors of the means derived from at least two independent experiments. The following strains were used: wild type, HSC5Spc; and GidA$^-$ mutant, HSC5::pKHC100.

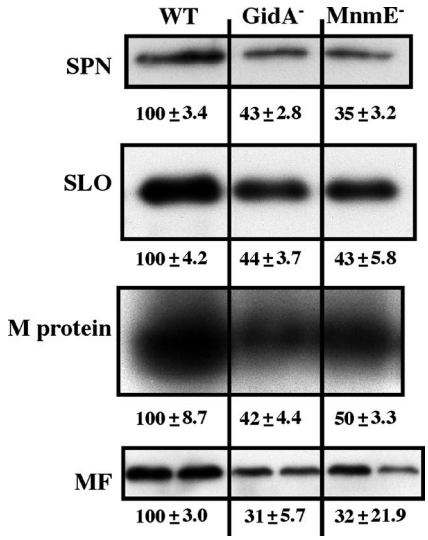

FIG. 7. GidA pathway mutants have reduced expression of several virulence proteins. The results of Western blot analyses to detect the levels of expression of the indicated virulence proteins in several GidA pathway mutants are shown. Strains were cultured in THY medium and harvested at the point of maximal expression of each factor in the wild-type strain (6 h for SPN, SLO, and M protein; 16 h for MF). A cysteine protease inhibitor (10 μM E64) was added to culture media to inhibit possible degradation of virulence proteins by SpeB. Expression levels were determined by densitometry and are expressed relative to expression in the wild-type strain (indicated below each panel). The data are the means and standard deviations of two independent experiments. The following strains were used: wild type (WT), HSC5Spc; GidA$^-$ mutant (GidA$^-$), HSC5::pKHC100; and MnmE$^-$ mutant (MnmE$^-$), HSC5::pKHC104.

involved in tRNA modification results in a reduction in expression of selected virulence genes as a consequence of poor translation efficiency of the transcription regulator VirF (13). Expression of such virulence factors can be rescued in *Shigella* mutants by overexpression of VirF (13). In *S. pyogenes*, transcription of *speB* is under control of the transcription activator RopB (26). In the GidA$^-$ mutant, the abundance of the *ropB* transcript was unchanged from the abundance in the wild type (Fig. 6), suggesting that the tRNA processing mutants may not efficiently translate this transcription regulator. In this case, overexpression of RopB should rescue *speB* expression, similar to overexpression of VirF in *Shigella* tRNA modification mutants. To test this, the GidA$^-$ and MnmE$^-$ mutants were transformed with a plasmid that expresses high levels of a RopB protein that has been modified to include an influenza HA tag at its carboxy terminus. This construct produces a functional RopB, as demonstrated by its ability to efficiently complement a *ropB* deletion (26). When these transformed strains were analyzed, it was found that overexpression of RopB restored the amount of SpeB protein secreted by the mutants to wild-type levels (Fig. 5A, compare "no plasmid" to "pRopB-HA"). Even though the amount of RopB that the mutant strains produced was sufficient to rescue SpeB expression, these strains produced less than 50% of the amount of RopB-HA made by the wild-type strain transformed by the overexpression plasmid (Fig. 5B). These data are consistent with the idea that the loss of GidA- and MnmE-dependent tRNA modification alters the pattern of *speB* transcription via an effect on the translation efficiency of the *ropB* message.

Reduced production of multiple secreted virulence proteins. A sodium dodecyl sulfate-polyacrylamide gel electrophoresis-based comparison of cytosolic, cell membrane, and cell wall fractions of GidA$^-$ and MnmE$^-$ mutants and the wild type did not reveal any obvious differences in protein profiles (data not shown), consistent with the lack of any overall growth defect in the mutants. Transcription of *speB* occurs during the early stationary phase of growth; however, many virulence factors are expressed in growing cells. Examination of transcription profiles in growing cells using a microarray analysis indicated that the loss of GidA had a minimal impact on the global transcriptome, with only about 2% of the transcripts examined altered at a level of twofold or greater. Of these, only 0.5% (seven transcripts) were altered at levels greater than threefold and none were altered at levels greater than fivefold (see Tables S2, S3, and S4 in the supplemental material). A lack of an effect on transcription of virulence-associated genes was confirmed by a real-time RT-PCR analysis of a subset of messages, including several messages that encode transcription regulators implicated in virulence (*mga*, *ropB*, and *covR*) and a panel of additional factors representing both intracellular and secreted proteins. This analysis revealed that transcription of this panel of genes varied only about twofold or less from the wild-type transcription at 6 h postinoculation (Fig. 6). In contrast, examination of the levels of several secreted virulence proteins, including SPN, SLO, the surface M protein, and MF, demonstrated that both the GidA$^-$ and MnmE$^-$ mutants expressed each of these proteins at levels that were less than 50% of the wild-type levels in the exponential phase (Fig. 7). Taken together, these data suggest that a reduction in expression of multiple virulence proteins, such as SPN, SLO, M protein, and MF, in the exponential phase is influenced at the level of translation by GidA and MnmE and that there may be a greater impact on virulence factor production than on the

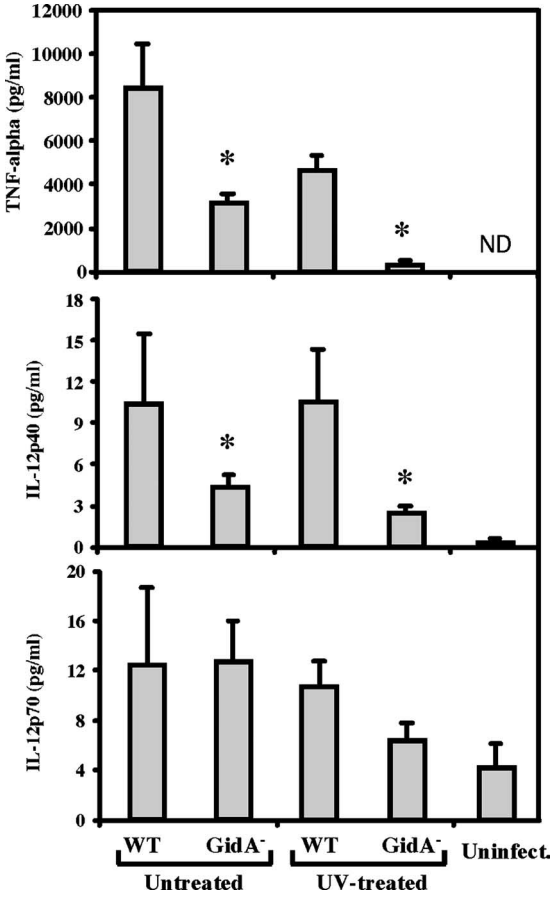

FIG. 8. Cultured macrophages produce reduced levels of TNF-α and IL-12(p40) in response to GidA$^-$ mutants. The levels of production of the indicated cytokines following 4 h of infection of cultured murine bone marrow-derived macrophages are shown. Cytokine levels were measured using an antibody-based multiplex bead assay (Bio-Plex; Bio-Rad Laboratories). Macrophage cultures were infected at a multiplicity of 10 streptococci per macrophage or were not infected (Uninfect.). Streptococcal strains were used immediately following culture (Untreated) or were inactivated by treatment with UV radiation (UV-treated) immediately prior to infection. An asterisk indicates that expression was significantly decreased compared to the wild type ($P < 0.05$), and ND indicates that the level of expression was below the level of detection of the assay. In addition to the expression of IL-12(p70) shown, there was no difference in expression between the wild type and the mutant for the 21 other cytokines analyzed in this experiment (see Fig. S2 in the supplemental material). The data are the means and standard errors of the means from three independent experiments. The following strains were used: wild type (WT), HSC5Spc; and GidA$^-$ mutant (GidA$^-$), HSC5::pKHC100.

production of housekeeping proteins that are involved in cell growth.

Comparison of cytokine profiles from infected macrophages. It has been shown that *S. pyogenes* induces a unique cytokine activation program in macrophages (21) and that macrophages have an important role in controlling *S. pyogenes* infection in a murine model of disease (20). Since macrophages are also a key cell element of the adaptive immune response, the influence of GidA on streptococcus-macrophage interaction was assessed. Murine bone marrow-derived macrophages were infected with either the wild type or the GidA$^-$ mutant or with these strains following UV treatment to block growth. After 4 h of infection, supernatant fluids were monitored for the presence of a panel of 23 different cytokines and chemokines (see Materials and Methods). Overall, the wild type and the GidA$^-$ mutant, whether they were UV treated or not treated, induced identical patterns of expression of 21 cytokines at levels of twofold or greater (Fig. 8; see Fig. S2 in the supplemental material). The cytokines not stimulated by both the mutant and the wild type included interleukin 1α (IL-1α) and eotaxin (see Fig. S2 in the supplemental material). For the induced cytokines, the magnitudes of induction were also similar for the wild type and the GidA$^-$ mutant (see Fig. S2 in the supplemental material), with two exceptions. For both untreated and UV-inactivated cultures, the GidA$^-$ mutant induced tumor necrosis factor alpha (TNF-α) and IL-12(p40) at levels that were less than 50% of the levels induced by the wild type (Fig. 8). Since IL-12(p40) is a subunit of both IL-12 and IL-23 and since the IL-12(p70) subunit of IL-12 was not differentially expressed (Fig. 8), it was likely that IL-23, rather IL-12, was differentially induced. This was confirmed by real-time RT-PCR analysis of the IL-23(p19) subunit, which showed that this transcript was present in macrophages infected by the GidA$^-$ mutant at a level that was only 22% ± 15% of the level induced in macrophages infected with the wild type.

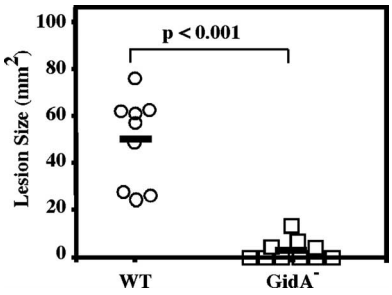

FIG. 9. GidA$^-$ mutants are highly attenuated. The ability of a GidA$^-$ mutant to cause disease in the murine subcutaneous ulcer model is shown. Virulence was evaluated on the basis of the area of the ulcer produced at the time when ulcer formation was maximal in the wild-type strain (3 days postinfection). The circles and squares represent ulcer areas in individual mice for the wild type and GidA$^-$ mutant, respectively. The solid bars indicate the mean values for the strains, and *P* value as determined by a Mann-Whitney U test statistic is indicated above the bracket. The data shown are pooled data from two independent experiments. The following strains were used: wild type (WT), HSC5Spc; and GidA$^-$ mutant (GidA$^-$), HSC5::pKHC100.

GidA$^-$ mutants are highly attenuated in a murine model of soft tissue infection. The ability of the GidA$^-$ mutants to cause disease in soft tissue was evaluated using the murine subcutaneous model. For the strain used in this study (HSC5), injection into the subcutaneous tissue of an SKH1 hairless mouse results in a local draining ulcer, whose area reaches the maximum value 3 days postinfection and which then goes on to heal over the next 10 to 14 days (4). In a comparison of the abilities of a GidA$^-$ mutant (TM-GidA1) and the wild type to cause disease in this model, it was found that the latter produced lesions of the expected size by day 3 postinfection (Fig. 9). In contrast, the GidA$^-$ mutant was significantly attenuated in the ability to cause lesions. While a few infected mice ex-

hibited small lesions, the majority of mice infected with the mutant did not exhibit any visible lesions at day 3 postinfection or at any subsequent time point. Identical results were obtained with a second GidA$^-$ mutant (HSC5::pKHC100) (data not shown). These data indicate that the reduction in virulence factor expression observed in vitro in the GidA$^-$ mutants translates into a severely attenuated ability to cause disease in soft tissue.

DISCUSSION

This analysis showed that *S. pyogenes* GidA/MnmE pathway mutants have a number of characteristics that make them attractive candidates for further evaluation as live vaccine strains. These characteristics include the fact that they have no obvious defects in growth, the fact that they have minimal alterations to the complement of virulence factors that they produce, the fact that they produce a large subset of these factors at significantly reduced levels, and the fact that they are among the most attenuated mutants that have been analyzed to date using the murine subcutaneous model of soft tissue infection. Their avirulence appears to be the result of a global defect in translation efficiency manifested directly on messages encoding several secreted virulence factors or on messages for essential regulators of virulence gene transcription. Taken together, these observations suggest that tRNA modification mutant strains defective in the GidA pathway may be valuable for probing the mucosal immune response to *S. pyogenes*.

While the GidA gene was originally isolated as a gene responsible for a glucose-dependent cell division phenotype in *E. coli* (45), more recent studies have established that GidA is a flavin adenine dinucleotide-dependent enzyme that is required for addition of the carboxymethylaminomethyl group at position 5 of U34 of tRNAs that read mixed codon boxes, including those for Glu, Gln, Lys, Leu, and Arg (4, 29, 32). Also required for this modification is the GTPase MnmE, as both GidA$^-$ and MnmE$^-$ mutants of *E. coli* accumulate the same level of an undermodified form of U34 (48). This modification restricts base pairing at the wobble position with C and U to allow pairing at only G and A residues (63, 64). This prevents both misincorporation and frameshifting, as GidA$^-$ and MnmE$^-$ mutants have an elevated frequency of a +2 frameshift (4, 7, 29). Prevention of the latter error is likely to have a greater impact on protein synthesis because while many missense errors can be tolerated in polypeptides, a frameshift generally produces a truncated nonfunctional protein. Hypomodification of U34 increases the rate of frameshifting between two- and fivefold compared with the basal rate in *E. coli*, depending on the specific codon (43). If these rates are similar in *S. pyogenes*, then this level of frameshifting is well tolerated, as GidA$^-$ and MnmE$^-$ mutants grow at normal rates. However, the observation that virulence factor expression decreased to levels that adversely affected pathogenesis suggests that there is more selective sensitivity of virulence factors than of housekeeping proteins involved in cell growth.

It is possible that secreted proteins are particularly vulnerable to U34 hypomodification. The signal sequence required for protein export includes a positively charged N region and a hydrophobic H region, which in bacteria are dominated by Lys and Leu residues, respectively (32), whose codons both require U34 modifications for efficient decoding. Since many critical virulence factors of *S. pyogenes* are secreted proteins, a more selective defect in expression of these proteins could result in attenuation of virulence. Alternatively, since many transcriptional regulators have clusters of positively charged Lys and Arg residues in the domains that they use to bind DNA, they may be highly sensitive to the loss of GidA and MnmE, since the codons for these residues require decoding by tRNAs with U34 modifications. Even a modest decrease in the levels of a transcription regulator could have a significant pleiotropic effect on virulence depending on the number target genes that are dependent on the regulator for their transcription. The observation that a reduction in the translation efficiency of the *ropB* message is at least one factor contributing to the disregulation of expression of the SpeB protease illustrates the vulnerability of transcription regulators to alterations in the level of modified tRNAs. The *ska* gene was slightly overexpressed in the transcript analysis (Fig. 6), and this might indicate that less of a repressor of *ska* is produced translationally in the GidA$^-$ mutant. Similarly, it has been shown that mutations that alter levels of U34 and A37 tRNA modification attenuate the virulence of *S. flexneri* exclusively via a decrease in the translation efficiency of the gene encoding the VirF transcription activator (13). It has been proposed that tRNA modification can function as a regulatory mechanism to adjust gene expression in response to nutrient deprivation via alterations in pools of cofactors required for the tRNA modification reactions (35). Whether the levels of VirF in *S. flexneri* or RopB is regulated by this mechanism remains to be determined.

In addition to mutants of *S. pyogenes* and *S. flexneri*, tRNA modification mutants of other pathogens have been shown to have robust defects in virulence in the absence of any remarkable defects in growth. For example, defects in virulence and/or virulence factor production associated with defects in tRNA modification have been reported for *Agrobacterium tumefaciens* (23), *Pseudomonas syringae* (24), and *Aeromonas hydrophila* (39). Prominent phenotypes for tRNA modification mutants have also been reported both for nonpathogenic bacteria and for eukaryotic pathogens, including *Myxococcus xanthus* (46) and the phytopathogenic fungus *Colletotrichum lagenarium* (42). The observation that tRNA mutants are frequently attenuated, combined with the fact that tRNA modification is a universal feature of all living cells, suggests that mutation of tRNA modification genes may prove to be a general strategy for construction of live attenuated vaccine strains. Since analysis of mucosal immunity against several other pathogenic streptococcal species, including *Streptococcus agalactiae* and *Streptococcus pneumoniae*, could benefit from the availability of live attenuated strains, it will be of interest to evaluate virulence factor expression and pathogenesis of GidA$^-$ and MnmE$^-$ mutants of these species.

Another characteristic that should make the *S. pyogenes* tRNA modification mutants useful as live attenuated strains is the fact that they do not have a major defect in the number of different virulence factors that they can express relative to the wild type. This suggests that they can engage the adaptive immune system in a manner similar to the manner of the wild type. This idea is supported by the observation that macrophages respond to the mutant and the wild type in similar ways, as revealed by the nearly identical cytokine profiles observed.

The mutants did stimulate several cytokines to a lesser extent than the wild type, most notably TNF-α. Macrophage-derived TNF-α is an important early mediator of the inflammatory cascade, and it is known that *S. pyogenes* infection stimulates production of TNF-α in murine (26, 59) and baboon (41) models of infection and in human patients with severe invasive disease (33). However, in both humans and baboons, high levels of TNF-α were correlated with more serious disease (44, 54), suggesting that overproduction of TNF-α contributes to pathogenesis. Consistent with this, treatment of septic baboons with an anti-TNF-α monoclonal antibody markedly improved both symptoms and survival (41). Thus, the reduced ability of tRNA modification mutants to stimulate TNF-α may prove to be a useful property for a live attenuated strain.

It is less clear whether the attenuated strains will be useful for vaccination of human populations. Possible complications include the fact that the attenuated strains may also express epitopes that contribute to autoimmune sequlae and the fact that they express only a single serotype of the highly variant M protein molecule. It may be possible to engineer the attenuated strains to produce altered M protein variants lacking known cross-reactive epitopes and to express M protein domains common to multiple serotypes. Regardless, the availability of live attenuated strains, like the tRNA mutants described in this work, should be a valuable resource for further vaccine development.

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