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The *rgg* Gene of *Streptococcus pyogenes* NZ131 Positively Influences Extracellular SPE B Production

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Streptococcus pyogenes **produces several extracellular proteins, including streptococcal erythrogenic toxin B (SPE B), also known as streptococcal pyrogenic exotoxin B and streptococcal proteinase. Several reports suggest that SPE B contributes to the virulence associated with *S. pyogenes*; however, little is known about its regulation. Nucleotide sequence data revealed the presence, upstream of the *speB* gene, of a gene, designated *rgg*, that was predicted to encode a polypeptide similar to previously described positive regulatory factors. The putative Rgg polypeptide of *S. pyogenes* NZ131 consisted of 280 amino acids and had a predicted molecular weight of 33,246. To assess the potential role of Rgg in the production of SPE B, the *rgg* gene was insertionally inactivated in *S. pyogenes* NZ131, which resulted in markedly decreased SPE B production, as determined both by immunoblotting and caseinolytic activity on agar plates. However, the production of other extracellular products, including streptolysin O, streptokinase, and DNase, was not affected. Complementation of the *rgg* mutant with an intact *rgg* gene copy in *S. pyogenes* NZ131 could restore SPE B production and confirmed that the *rgg* gene product is involved in the production of SPE B.**

Streptococcus pyogenes produces a variety of extracellular proteins including streptococcal erythrogenic toxin B (SPE B), also known as streptococcal pyrogenic exotoxin B and streptococcal proteinase (7, 17, 19, 33). The *speB* gene encodes a polypeptide consisting of 371 amino acids with a predicted molecular weight of 40,000 (19). Following secretion, the SPE B zymogen is thought to be activated by proteolysis and reduction to form a 28-kDa sulfhydryl proteinase (15, 16, 27). In contrast to the genes encoding streptococcal erythrogenic toxins A and C, which are bacteriophage encoded, the *speB* gene is present in the chromosome of all strains of *S. pyogenes* (55), although certain strains, including some isolates from invasive infections, do not produce the protein in vitro (8, 14, 15, 22, 32, 38, 42, 43, 47). Although it is not known if those isolates which did not produce SPE B in vitro were capable of SPE B production in vivo, recent results showed that in a murine model of necrotizing fasciitis, *speB* is not required for soft-tissue necrosis (1). Nonetheless, other studies indicate that SPE B does contribute to the virulence of *S. pyogenes* in mice (25, 29).

The proteolytic activity of SPE B has been suggested to enable *S. pyogenes* to alter its interaction with the human host during the course of infection. Specifically, SPE B has been shown to cleave the streptococcal adhesin M1 protein from the bacterial surface (4) and to degrade host cell extracellular matrix factors that also participate in adherence, including fibronectin and vitronectin (2, 24, 51). This strategy could facilitate dissemination of *S. pyogenes* after colonization has been established. In addition, the proteolytic activity of SPE B has been shown to activate a variety of host cell molecules including kininogens, which results in the release of kinins (20), interleukin-1β (23), and a human matrix metalloprotease (5). Such activation may be relevant to the clinical manifestations associated with streptococcal infection.

Despite the potential importance of SPE B in streptococcal pathogenesis, relatively little is known about the molecular mechanisms involved in the regulation of its production. Previous research has identified culture conditions that influence SPE B production, including an acidic pH and the presence of neopeptone (12). In addition, SPE B was detected in sterile culture supernatant fluid primarily during the transition from the exponential to the stationary phase of growth when strain NZ131 was grown in dialyzed Todd-Hewitt (TH) medium (9), which confirmed an earlier report by Lo et al. (28). However, SPE B production in NZ131 did not strictly correlate with the growth phase of the culture, based on the observation that the addition of catabolic compounds during the exponential phase of growth, including glucose and Casamino Acids, inhibited SPE B production even as the culture entered the stationary phase (9). Production of SPE B also did not strictly correlate with nutrient depletion, based on the finding that exponential phase cultures resuspended in conditioned medium did not secrete SPE B (9). Not surprisingly, the molecular mechanisms involved in the regulation of SPE B expression are also proving to be complex. Podbielski et al. (37) reported that SPE B is expressed as part of the multiple gene activator (Mga) regulon, which consists of several coregulated virulence-associated genes (6, 10, 34). In addition, inactivation of components of both the oligopeptide and dipeptide transport systems diminished *speB* mRNA levels, suggesting that a regulatory link exists between peptide transport and the expression of the extracellular proteinase (35, 36). Thus, several previous studies suggest that SPE B production is regulated, at least in part, by the metabolic state of the culture; however, the molecular mechanisms responsible for this regulation are not well understood.

The purpose of this study was to assess the potential role of the streptococcal *rgg* gene, which was identified upstream of the *speB* locus, in the production of SPE B. Inactivation of the *rgg* gene in *S. pyogenes* NZ131 resulted in a marked decrease in SPE B production, as determined by immunoblotting and an abrogation of caseinolytic activity on agar plates. Extracellular

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference[b]
Strains		
S. pyogenes NZ131		D. R. Martin, Porirua, New Zealand
E. coli		
DB11	Erythromycin sensitive	13
JM109	JM107 *recAJ*	
DH10B	*recA1*	BRL
Plasmids		
pCRII	Ap^r; cloning vector	Invitrogen
pUC18::*ermAM*	Em^r	44
pOU118	1,500-bp I-PCR product cloned into pCRII; independently isolated colony	This study
pOU424	1,500-bp I-PCR product cloned into pCRII; independently isolated colony	This study
pVA891-2	Em^r	H. Malke, Jena, Germany 31
pOU500	*Eco*RI fragment from pOU118 consisting of nucleotides 25–599 of the *rgg* gene cloned into pVA891-2; Em^r	This study
pCRII::*rgg*	1.185-kb PCR product (Rgg-F, Rgg-R) cloned into pCRII	This study
pAM401	Shuttle vector; Cm^r	B. Jett, University of Oklahoma HSC, (53)
pAM401::*rgg*	*Eco*RV-*Bam*HI 1.18-kb fragment of pCRII::*rgg* cloned into pAM401	This study

[a] Ap^r, Em^r, and Cm^r, resistance to ampicillin, erythromycin, and chloramphenicol, respectively.

[b] BRL, Gibco-Bethesda Research Laboratories Inc., Gaithersburg, Md.; HSC, Health Sciences Center.

SPE B production could be restored by complementation of the *rgg* mutant with an intact *rgg* gene copy, confirming that the *rgg* gene is involved in the production of SPE B.

MATERIALS AND METHODS

Bacterial strains and media. Bacterial strains and plasmids used in this study are described in Table 1. *S. pyogenes* NZ131 was grown in TH medium (Becton Dickinson, Cockeysville, Md.) containing 0.2% yeast extract (Difco Laboratories, Detroit, Mich.). Dialyzed TH medium was prepared by suspending 30 g of TH medium (Becton Dickinson) in 100 ml of water and dialyzing (in dialysis tubing with an approximate molecular weight cutoff of 3,500; Spectrum Medical Industries, Inc., Los Angeles, Calif.) against 1 liter of water overnight at 4°C. The dialyzed material was filter sterilized by using a 0.2-μm-pore-size filter sterilization unit (Nalge Nunc International, Rochester, N.Y.). When appropriate, erythromycin and chloramphenicol were added to TH medium at final concentrations of 3.0 and 10.0 μg/ml, respectively. *Escherichia coli* was grown in Luria-Bertani (LB) broth or LB agar plates containing, when appropriate, erythromycin (10 μg/ml) or chloramphenicol (80 μg/ml).

PCR. Southern hybridization analysis using a radiolabeled probe specific to the 5′ portion of the *speB* gene indicated the presence of *Cla*I restriction endonucleases sites within and upstream of the *speB* gene that resulted in a fragment of suitable size for inverse PCR (I-PCR). Based on these results, chromosomal DNA was isolated from *S. pyogenes* NZ131 and digested with *Cla*I overnight at 37°C. Following digestion, total DNA was purified by using a Qiaex DNA purification kit (Qiagen, Chatsworth, Calif.). The DNA was eluted in water, and T4 DNA ligase (New England Biolabs Inc., Beverly, Mass.) was used to ligate the DNA under conditions that favored intramolecular ligation. A 1-μl aliquot of the ligation mix was used as template DNA, and the genomic DNA upstream of the *speB* gene was amplified by using oligonucleotide primers purchased from Integrated DNA Technologies, Inc. (Coralville, Iowa). The first primer, designated SpeB-1 (5′-ACGAGCAAAGTTTTGATC-3′), corresponded to bp 99 to 82 of the *speB* gene (19). The second primer, designated SpeB-2 (5′-AAAGTAGGC GGACATGCC-3′), was complementary to nucleotides 1006 to 1023 of the *speB* gene. Thirty cycles of amplification were carried out in a Perkin-Elmer (Branchburg, N.J.) DNA thermocycler with strand denaturation (1 min at 94°C), annealing (1 min at 48°C), and elongation (3 min at 72°C). The total volume of the PCR mixture was 50 μl and consisted of 1 μl of template DNA, 0.4 μM each primer, 20 μM deoxynucleoside triphosphates, 4 mM Mg_2^+, and 2.5 U of *Taq* polymerase (Promega Corp., Madison, Wis.). Following amplification, the amplicon was cloned into the TA cloning vector pCRII (Invitrogen, San Diego, Calif.) as described by the manufacturer. Two independently isolated clones were selected for further analysis and designated pOU118 and pOU424.

Southern hybridization. Streptococcal chromosomal DNA was isolated as previously described (7). DNA was digested with various restriction endonucleases obtained from New England Biolabs, and the fragments were separated by agarose gel electrophoresis. Following electrophoresis, DNA was transferred to Nytran (Schleicher & Schuell, Keene, N.H.) by the method of Southern (46) as described elsewhere (40). Probes were labeled with [α-^{32}P]dATP (New England Nuclear, Dupont Corp.) using a random-primer DNA labeling kit (U.S. Biochemical Corp. Cleveland, Ohio) as instructed by the manufacturer. The *rgg*-specific probe consisted of nucleotides 25 to 559 of the *rgg* gene and was isolated from an agarose gel by using a Qiaex purification kit (Qiagen) following digestion of pOU118 with *Eco*RI. The *ermAM*-specific probe was prepared by digesting pUC18::*ermAM* (obtained from D. Simon) (44) with *Eco*RI and *Bam*HI. The approximately 1.1-kb *ermAM* fragment was then purified following agarose electrophoresis using a Qiaex purification kit (Qiagen). Hybridization was done under stringent conditions (65°C, 0.5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate [SDS]) essentially as previously described (40).

Cloning of the *rgg* gene from NZ131. Using the nucleotide sequence data obtained from sequencing of pOU118 and pOU424 and data from the unannotated streptococcal genome sequencing project (39), oligonucleotides were designed to amplify a 1.185-kb fragment containing the complete *rgg* gene from NZ131. The oligonucleotides, Rgg-F (5′-TTATGGCTATATCATAGCTGC-3′) and Rgg-R (5′-ATCGCCCTGGAGCTGTTGAG-3′), were synthesized by Genemed Biotechnologies, Inc. (San Francisco, Calif.). Rgg-F corresponded to the nucleotide sequence beginning 247 bases upstream of the predicted translational start codon of *rgg*, and thus the amplicon may have included a functional promoter. Thirty cycles were carried out in a Perkin-Elmer 9600 DNA thermocycler with strand denaturation (15 s at 94°C), annealing (30 s at 47°C), and elongation (1.5 min at 70°C). The total volume of the PCR mixture was 50 μl and consisted of 0.1 μg of genomic DNA isolated from strain NZ131, 0.4 μM each primer, 20 μM deoxynucleoside triphosphates, 1.5 mM Mg_2^+, and 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer). Following amplification, the amplicon was cloned into the TA cloning vector pCRII (Invitrogen) as described by the manufacturer. Restriction enzyme fragment analysis and PCR confirmed that *rgg* had been cloned into pCRII. The recombinant plasmid was designated pCRII::*rgg*.

Nucleotide sequence determination. Plasmid DNA was isolated from pOU118 and pOU424 by using a Wizard MiniPrep plasmid isolation kit (Promega) according to the manufacturer's instructions. The nucleotide sequences of both strands of the insert DNA were determined by using Sequenase as described by the manufacturer (U.S. Biochemical Corp.) and custom-designed oligonucleotides purchased from Integrated DNA Technologies. The complete *rgg* gene of NZ131 was amplified from purified genomic DNA in a Perkin-Elmer 9600 thermocycler with High Fidelity polymerase (Boehringer Mannheim), using primers Rgg-F and Rgg-R. The approximately 1.1-kb PCR product was purified by using a Centricon-100 column (Amicon Inc., Beverly, Mass). Subsequently, the nucleotide sequences of both strands were determined by using custom-designed oligonucleotide primers (Genemed Synthesis) and a Dye Terminator Cycle Sequencing Ready Reaction kit (PE Applied Biosystems, Foster City, Calif.). The sequencing reactions were analyzed on an Applied Biosystems 373 DNA sequencer (PE Applied Biosystems). The sequencing data were analyzed with Sequencer version 3.0 software (Gene Codes Corp., Ann Arbor, Mich.).

Inactivation of the *rgg* gene in *S. pyogenes* NZ131 and complementation of the mutant with an intact gene. The chromosomal *rgg* gene of NZ131 was insertionally inactivated following electrotransformation (45) of *S. pyogenes* NZ131 with the suicide plasmid pOU500. Erythromycin-resistant (Em^r) transformants were selected on agar plates containing 3.0 μg of erythromycin per ml. Three Em^r transformants were selected for further study.

To complement the *rgg* mutant, the recombinant plasmid pAM401::*rgg* was constructed by subcloning the complete *rgg* gene from pCRII::*rgg* into pAM401 and transforming *E. coli* DH10B as previously described (11). The purified plasmid was then used to electrotransform the *rgg* mutant, and transformants were selected on agar plates containing chloramphenicol. Cell lysates from selected transformants were prepared by boiling bacterial cell suspension in an alkaline solution as previously described (18). PCR was performed with cell lysates as template DNA and oligonucleotide primers specific to the *rgg* gene and to the gene conferring chloramphenicol resistance. The results from the PCR analysis confirmed that transformants possessed the recombinant plasmid pAM401::*rgg*.

Caseinolytic assay. The proteolytic activity of SPE B was assessed by the casein agar plate assay as described by Hynes and Tagg (22). Briefly, the strain to be tested was stab inoculated into TH agar plates containing 10% skim milk (Difco).

The plates were then incubated anaerobically at 37°C for approximately 18 h in an anaerobic GasPak (Becton Dickinson). Caseinolytic activity resulted in a zone of translucence surrounding the stab site.

Streptokinase and DNase assays. A plate assay was used to assess streptokinase activity in broth culture supernatant fluid as previously described (21). DNase activity was assessed on agar plates containing methyl green (Becton Dickinson).

Supernatant fluid preparation, SDS-PAGE, and immunoblot analysis. Culture supernatant fluid was prepared following growth in dialyzed TH medium at 37°C. The A_{600} of each culture was adjusted to 0.5 by dilution with sterile medium. Typically 1 ml of the suspension was centrifuged to pellet the cells, and the supernatant fluid was removed and passed through a 0.2-μm-pore-size filter unit (Millipore Corp., Bedford, Mass.). Between 2 and 5 μl of culture supernatant fluid was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) using a 12.5% acrylamide resolving gel (26). Following electrophoresis, proteins were transferred to Immobilon-NC nitrocellulose membranes (Millipore Corp.), using a Bio-Rad transfer apparatus and Towbin's buffer (50). After transfer, the nitrocellulose membrane was blocked with 5% skim milk in phosphate-buffered saline containing 0.02% Tween 20 (PBST) for 45 min. The membrane was washed with PBST, rabbit antiserum raised against purified SPE B (diluted 1:1,000 in PBST) was added, and the blot was incubated for 1 h. Next, protein A-horseradish peroxidase conjugate (Amersham) was added at a 1:2,000 dilution in PBST for 1 h. The antibody-antigen complexes were visualized by using the Amersham enhanced chemiluminescence Western blotting detection system as described by the manufacturer.

Nucleotide sequence accession number. Nucleotide sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession no. AF091252.

RESULTS

I-PCR and nucleotide sequence analysis of *rgg*. To identify potential regulatory elements involved in the production of SPE B, the genomic region upstream of the *speB* gene was amplified by I-PCR. The PCR product was cloned, and the nucleotide sequences of both strands of the amplicon were determined from two independently isolated clones (designated pOU118 and pOU424). One terminus of the I-PCR product included 99 bp that were identical to the first 99 bp of the *speB* structural gene, including the sequence used to design SpeB-2, one of the I-PCR oligonucleotide primers. Adjacent to the structural gene were 160 bp that corresponded to the region upstream of the *speB* translational start site. The other terminus of the amplicon contained 30 bp that corresponded to nucleotides 1006 to 1036 of the *speB* structural gene (19) and included the sequence used to design SpeB-1, the second I-PCR primer. Thus, the nucleotide sequence at the termini of the I-PCR product indicated that the intervening 1,500 bp of sequenced DNA represented the region of the streptococcal chromosome that is upstream of *speB* in strain NZ131.

A single incomplete open reading frame (ORF) was identified upstream of the *speB* gene. The translational start site of the ORF is 941 bp upstream of the translational start site of *speB*. The ORF is encoded on the strand opposite that encoding SPE B. A putative ribosome-binding site (GGAAGG) was identified 8 bp upstream of the predicted ATG translational start site. The ORF did not include a termination codon within the I-PCR-amplified region, and thus the entire gene was not amplified. Using nucleotide sequence data from the streptococcal genome sequencing project, we amplified the complete ORF from NZ131 genomic DNA by PCR and determined the nucleotide sequence of both strands. Subsequently, the nucleotide sequence of the region upstream of *speB* in strain CS101 was submitted to GenBank (54). The nucleotide sequence from strain NZ131 had five single base pair differences in the noncoding region of NZ131 compared to the nucleotide sequence determined from strain CS101; both NZ131 and CS101 are M49 serotypes. In addition, there were several single base pair differences in the noncoding region compared to strain SF370, the strain used in the *S. pyogenes* genome sequencing project (data not shown). The complete ORF identified in strain NZ131 was predicted to encode a polypeptide composed of 280 amino acids that is 22% identical and 34% similar to the Rgg polypeptide of *S. gordonii* (49). The polypeptide is also 22% identical and 35% similar to the GadR polypeptide of *Lactobacillus lactis* (41). An alignment of these polypeptides is shown in Fig. 1. Based on the similarity of ORF to Rgg of *S. gordonii*, we have designated the gene *rgg* and the corresponding polypeptide Rgg.

Inactivation of the *rgg* gene in *S. pyogenes* NZ131 and complementation of the mutant with an intact gene copy. To assess the potential role of the *rgg* gene in SPE B production, isogenic strains of NZ131 that differed with respect to the presence of an intact *rgg* gene were created by directed mutagenesis. To inactivate *rgg*, we constructed a recombinant plasmid, designated pOU500, that contained 536 bp of the *rgg* gene to facilitate homologous recombination with the genomic *rgg* locus. The plasmid also contained a marker that conferred erythromycin resistance but not an origin of replication that is functional in *S. pyogenes*. Following electrotransformation of NZ131 with pOU500, transformants were selected on agar plates containing erythromycin. To confirm that Em^r transformants resulted following integration of pOU500 into the genomic *rgg* locus, Southern hybridization was performed with chromosomal DNA isolated from the parental *S. pyogenes* NZ131 strain (NZ131 wild type [wt]) and from three independently isolated Em^r transformants. A schematic representation of the Southern analysis is shown in Fig. 2A. As expected, *Cla*I restriction of chromosomal DNA isolated from NZ131 wt resulted in a single fragment of approximately 2.5 kb that hybridized with the *rgg*-specific probe (Fig. 2B). In contrast, *Cla*I restriction of chromosomal DNA isolated from three Em^r transformants resulted in two fragments that hybridized to the *rgg*-specific probe; the results obtained with a representative transformant are shown in Fig. 2B. Only chromosomal DNA isolated from the Em^r transformants hybridized to the erythromycin-specific probe (Fig. 2B). These results were confirmed by Southern hybridization following *Pst*I restriction of chromosomal DNA isolated from the wild-type NZ131 strain and from the three Em^r transformants (results not shown). Together, these results demonstrated that pOU500 had integrated into the *rgg* locus of the streptococcal chromosome.

To control for potential polar affects associated with the insertion of pOU500 into the streptococcal chromosome, the complete *rgg* gene was cloned into the shuttle plasmid pAM401, and the recombinant plasmid was designated pAM401::*rgg*. Plasmid pAM401 replicates episomally in *S. pyogenes* (10) and confers chloramphenicol resistance (3). The recombinant plasmid (pAM401::*rgg*) was used to electrotransform the *rgg* mutant, and the resulting transformants (designated NZ131 *rgg*/pAM401::rgg^+) were selected on agar plates containing chloramphenicol. The presence of the intact *rgg* gene and the gene which conferred chloramphenicol resistance was confirmed by PCR (data not shown). As a control, NZ131 wt was similarly electrotransformed with pAM401::*rgg*; however, no transformants were obtained. In contrast, both NZ131 wt and the *rgg* mutant were readily transformed with the vector (pAM401) alone.

SPE B caseinolytic activity in NZ131 requires an intact *rgg* gene. The casein agar assay was used as an initial step to determine if inactivation of the *rgg* gene affected the caseinolytic activity previously associated with SPE B production (7). The caseinolytic activity associated with SPE B creates a zone of translucence surrounding either a colony or stab site following growth on opaque, casein-containing TH agar medium due to the proteolytic activity of SPE B (Fig. 3) (22). As shown in Fig. 3, a zone of translucence formed around the wild-type stab site, indicative of SPE B production. In contrast, an NZ131

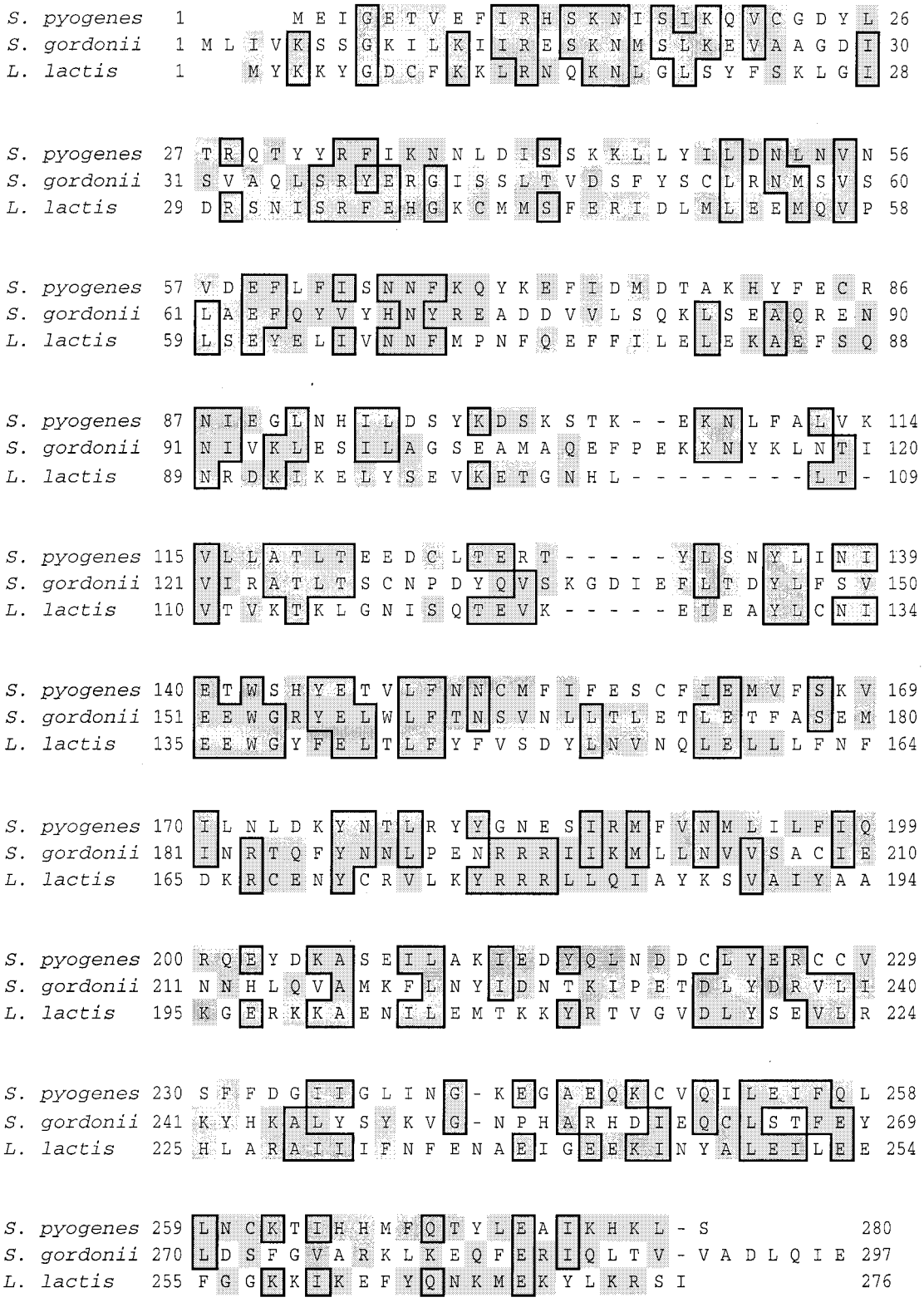

FIG. 1. Alignment of the deduced amino acid sequence of the Rgg polypeptide from *S. pyogenes* NZ131 with amino acid sequences of the Rgg polypeptide from *S. gordonii* (49) and the GadR polypeptide of *L. lactis* (41). Boxed and shaded regions indicate identical and similar amino acids, respectively.

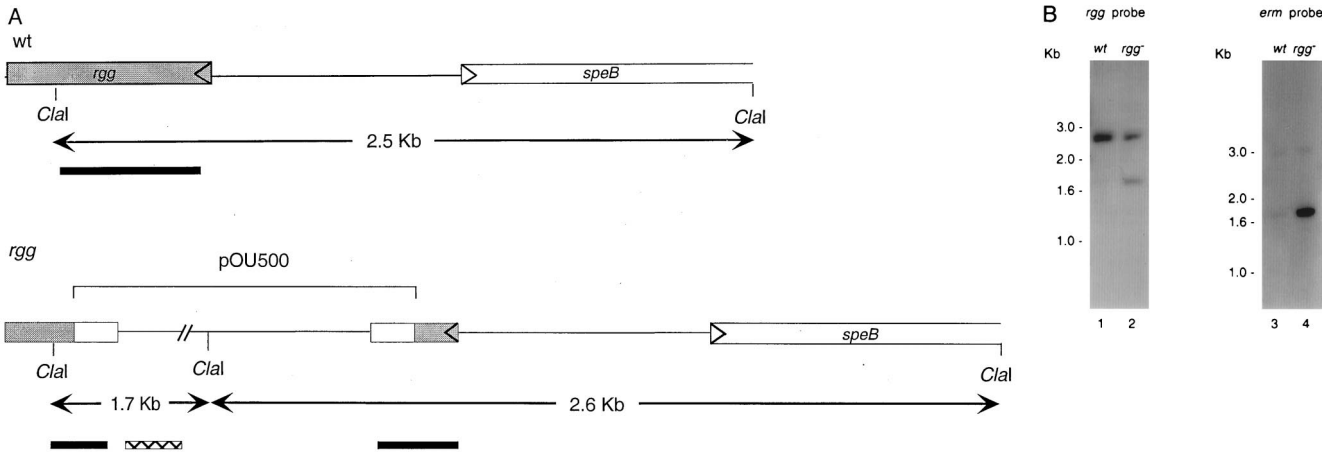

FIG. 2. Southern blot analysis of chromosomal DNA isolated from NZ131 wt and an NZ131 *rgg* mutant. DNA isolated from NZ131 wt and an *rgg* mutant strain was digested with *Cla*I and analyzed by Southern blot hybridization. (A) Schematic presentation of the *rgg* locus showing relative positions of the *Cla*I restriction sites, approximate sizes of the *Cla*I fragments, and locations of the *rgg* (solid bar) and *ermAM* (hatched bar) probes. (B) DNA from NZ131 wt (lane 1) and the NZ131 *rgg* mutant (lane 2) was hybridized with an *rgg*-specific probe. DNA from NZ131 wt (3) and the NZ131 *rgg* mutant (4) was probed with a probe specific to *ermAM*.

speB mutant, created by insertional duplication mutagenesis, showed markedly reduced caseinolytic activity; however, as previously reported (7), a slight zone of clearing was still evident surrounding the stab site (Fig. 3). Inactivation of the *rgg* gene ablated caseinolytic activity (Fig. 3). Two phenotypes were observed on skim milk-containing plates among transformants obtained following electroporation of the *rgg* mutant with pAM401::*rgg*. The majority of the chloramphenicol-resistant (Cm^r) colonies showed a low level of caseinolytic activity, reminiscent of that observed with a *speB* mutant (NZ131 *rgg*/pAM401::rgg^+-2) (Fig. 3). The second phenotype (designated NZ131 *rgg*/pAM401::rgg^+-1) was observed in 1 of 20 Cm^r transformants and showed a significantly greater level of caseinolytic activity compared to the parental strain (Fig. 3).

To confirm that inactivation of the *rgg* gene diminished SPE B production, culture supernatant fluid was prepared from cultures with an equal A_{600} (following approximately 18 h of growth in dialyzed TH broth medium) from strains NZ131, NZ131 *speB*, NZ131 *rgg*, NZ131 *rgg*/pAM401::rgg^+-1, and NZ131 *rgg*/pAM401. Sterile culture supernatant fluid was prepared and analyzed by immunoblotting using antiserum prepared against purified SPE B. As expected, culture supernatant fluid prepared from the parental NZ131 strain (Fig. 4, lane 1) contained a significant amount of protein that migrated similarly to purified SPE B (lane 6) and reacted with antiserum to SPE B. Also detected were several faster-migrating species (lane 1) which likely represent modified forms of the SPE B zymogen. As a control, supernatant fluid was similarly prepared from the *speB* mutant, and relatively little protein that reacted with antiserum to SPE B was present (lane 2). Similarly, the amount of anti-SPE B-reactive protein in supernatant fluid prepared from both the *rgg* mutant (lane 3) and the *rgg* mutant transformed with pAM401 (lane 5) was less than for the wild-type strain. In supernatant fluid prepared from the

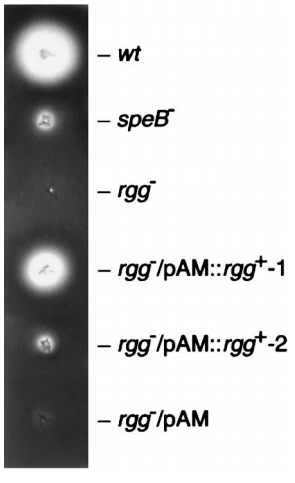

FIG. 3. Casein agar assay for SPE B proteolytic activity. Genetic derivatives of NZ131 were stab inoculated into agar plates containing skim milk and incubated for 18 h anaerobically. Proteinase activity is manifest as a zone of translucence surrounding the stab sites of NZ131 wt and NZ131 *rgg*/pAM::rgg^+-1.

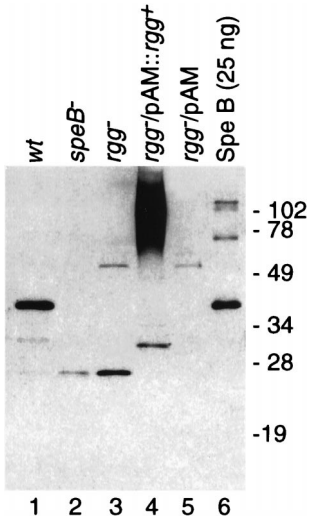

FIG. 4. Immunoblot analysis of culture supernatant fluid from isogenic derivatives of strain NZ131. Sterile supernatant fluid was prepared from derivatives of strain NZ131. Proteins in the supernatant fluid were separated by SDS-PAGE and transferred to a nitrocellulose membrane. SPE B was then detected by using rabbit antiserum raised against purified SPE B. Lanes 1 to 5, 2 μl of supernatant fluid prepared from the strains indicated at the top; lane 6, 25 ng of purified SPE B protein. Sizes are indicated in kilodaltons.

complemented *rgg* mutant (NZ131 *rgg*/pAM401::*rgg*$^+$-1), anti-SPE B-reactive protein that migrated as a high-molecular-weight smear was detected (lane 4). The smear did not resolve into distinct bands following either serial dilution of the sample or treatment with various reducing agents (results not shown). In contrast, no protein was detected with antiserum to SPE B in supernatant fluids prepared from the complemented *rgg* mutants (NZ131 *rgg*/pAM401::*rgg*$^+$-2) that showed only a minor level of caseinolytic activity (not shown). Thus, the results obtained from immunoblots of culture supernatant fluid and the casein agar assay showed that the *rgg* gene positively influences the production of SPE B in *S. pyogenes* NZ131.

Inactivation of the *rgg* gene does not affect extracellular hemolytic, streptokinase, or DNase activity. Despite the similarity of Rgg to previously described transcriptional regulatory factors, it remained possible that inactivation of the *rgg* gene had a general negative effect on the secretion apparatus of NZ131. In this regard, NZ131 *rgg* mutants were beta-hemolytic on blood agar plates, indicating that the production of the extracellular streptolysin S and O was not affected by inactivation of the *rgg* gene (data not shown). In addition, the *rgg* mutant showed normal production of both extracellular streptokinase and DNase activities (data not shown). Taken together, these results suggest that the lack of SPE B production in *rgg* mutants is unlikely to be related to secretion defects. In addition, these results showed that *rgg* is not required for the production of all extracellular products.

DISCUSSION

The *rgg* gene product of *S. pyogenes* is similar in amino acid sequence to the *rgg* gene product of *S. gordonii* (49). In *S. gordonii*, the *rgg* gene is present upstream and in the same transcriptional orientation as the *gtfG* gene, which encodes an extracellular glucosyltransferase (52). Sulavik and Clewell recently showed that Rgg is a positive transcriptional regulator of *gtfG* expression (48). In addition, the *rgg* genes of *S. gordonii* and *S. pyogenes* are also similar to the *gadR* gene of *L. lactis*, which encodes an activator of the *gadCB* operon (41). The location of the *rgg* gene upstream of *speB* and the similarity of the protein it encodes to other positive regulatory proteins prompted this investigation to determine whether the Rgg protein is involved in the regulation of extracellular proteins in *S. pyogenes*. Toward this end, inactivation of the *rgg* gene in strain NZ131 resulted in an ablation of caseinolytic activity and a decrease in the secretion of SPE B, as determined by immunoblots of sterile culture supernatant fluid. However, the *rgg* mutant did possess hemolytic, streptokinase, and DNase activity, indicating that production of the exoproteins responsible for those activities was not affected by *rgg* inactivation. Complementation of the *rgg* mutant with an intact *rgg* gene could restore SPE B production, confirming that the gene is involved in the production of SPE B in strain NZ131.

As previously reported (7), a *speB* mutant constructed in NZ131 by insertional duplication mutagenesis had markedly reduced caseinolytic activity when grown on skim milk-containing agar plates compared to the parental strain. Nonetheless, some caseinolytic activity remained (Fig. 3). Interestingly, the *rgg* mutant showed no detectable caseinolytic activity under these conditions (Fig. 3). The results from immunoblotting of sterile culture supernatant fluid prepared from both the *speB* and *rgg* mutant showed a similar, albeit minor, amount of an approximately 28-kDa protein that reacted with SPE B antiserum. Therefore, the amount of SPE B detected in supernatant fluid from these mutants did not correlate with the level of caseinolytic activity observed following growth on solid medium. This result suggested that the difference in caseinolytic activity observed between *speB* and *rgg* mutants was not due to low level SPE B production. The disparity in the levels of caseinolytic activity between the mutants on solid medium may be related to the relative amount of acid produced by these strains. *S. pyogenes* generates ATP by fermentation and produces primarily lactic acid as the fermentation end product, which could potentially hydrolyze casein to result in a small zone of translucence on casein-containing medium. However, no difference in the pH of the medium during or following growth of the mutants in broth medium was apparent (6a). Nonetheless, it is unclear if the production of lactic acid or the buffering capacity of the medium was different following growth in broth medium compared to growth on solid medium containing skim milk. An additional explanation is that *rgg* inactivation may have affected the production of a previously uncharacterized protein (i.e., a protein other than SPE B) with caseinolytic activity. The *rgg* mutant was hemolytic on blood agar plates and produced levels of streptokinase and DNase activity similar to those produced by the wild-type strain, which indicated that the *rgg* mutation did not hinder exoprotein transport in general, nor was it required for the production of all extracellular products. Further experiments are necessary to determine if the production of other proteins, in addition to SPE B, is influenced by *rgg*.

Complementation of the *rgg* mutant with an intact gene resulted in partial restoration of caseinolytic activity on skim milk-containing agar plates compared to both the mutant and the mutant transformed with the plasmid only (Fig. 3). This result confirmed that the *rgg* gene positively influenced SPE B production. In addition, these results indicated that the *rgg* gene product can function in *trans* to influence production. The majority of the anti-SPE B-reactive material in the supernatant fluid prepared from the complemented strain (NZ131 *rgg*/pAM401::*rgg*$^+$-1) migrated in the gel as a high-molecular-weight smear. To complement the mutant, an intact *rgg* gene was introduced into the mutant on a multicopy plasmid. Thus, it seems likely, based on the Western blotting results (Fig. 4), that SPE B was overproduced due to the presence of multiple copies of the *rgg* gene. Overproduction of SPE B may have resulted in the formation of aggregates of SPE B with other polypeptides. Thus, the smear likely represents a heterologous aggregate of SPE B-associated proteins, since it did not resolve into distinct bands upon dilution of the sample as would be expected if the smear was composed entirely of SPE B. In this regard, we noted that purified SPE B formed homoaggregates that migrated as distinct bands (Fig. 4, lane 6).

Two phenotypes were observed following growth of complemented *rgg* mutants on skim milk-containing agar. The majority of the Cm^r colonies showed a low level of caseinolytic activity, reminiscent of that observed with the *speB* mutant (Fig. 3). However, approximately 1 of 20 Cm^r transformants showed significantly greater caseinolytic activity (approximately 70% of that detected in the parental strain [Fig. 3]). Results from PCR analysis of colonies displaying both phenotypes showed the presence of a full-length *rgg* gene; however, we cannot rule out the possibility that point mutations were present in the plasmid-borne *rgg* gene. This seems unlikely, however, since the caseinolytic activities of all Cm^r colonies were significantly greater than those of both the mutant and the mutant transformed with the vector alone, both of which did not produce any detectable caseinolytic activity (Fig. 3). Thus, all of the Cm^r colonies analyzed appeared to be at least partially complemented for caseinolytic activity. The recombinant plasmid was unstable and NZ131 *rgg*/pAM401::*rgg*$^+$-1 failed to grow after several passages on selective medium,

suggesting that there was a toxic effect associated with the presence of *rgg* on a multicopy plasmid. Consistent with this interpretation was the lack of transformants when wild-type NZ131 was electrotransformed with pAM401::*rgg*. Thus, it seems reasonable to speculate that the colonies that showed a low level of caseinolytic activity following complementation may have had additional genotypic or phenotypic changes that were selected for during growth on media containing chloramphenicol. However, the molecular basis for the different phenotypes remains unknown. Thus, in general the results following complementation of the *rgg* mutant indicated that an intact *rgg* gene could restore SPE B production and caseinolytic activity to an *rgg* mutant, but production was not restored to the levels observed in the parental strain.

The similarity of the *rgg* gene product to previously described positive transcriptional regulators suggested that the basis for the lack of SPE B production in an *rgg* mutant was likely to be at the level of transcription. Recently Lyon et al. (30) showed that inactivation of the *rgg* gene (*ropB*) in strain HSC5 was associated with an absence of *speB* transcription. Together, the data indicate that Rgg is a positive transcriptional activator required for the expression of *speB*; however, it remains to be determined if Rgg acts directly or indirectly to regulate *speB* expression.

The *rgg* gene product is homologous to the *gadR* gene product of *L. lactis* throughout the primary structure of the polypeptide. This information, coupled with results of the present study, suggests that the regulation of SPE B production may be related to pH homeostasis in *S. pyogenes*. In *L. lactis*, GadR positively regulates the expression of *gadC*, which encodes a putative glutamate–γ-aminobutyrate (GABA) antiporter, and *gadB*, which encodes a glutamate decarboxylase. Together, these proteins contribute to pH homeostasis by facilitating the exchange of an acidic extracellular glutamate residue for a less acidic intracellular derivative (GABA). In addition, an intracellular proton is consumed during the decarboxylation of glutamate to form GABA. The expression of *gadCB* begins in the late exponential phase of growth and is maximal during the stationary phase of growth (41). Thus, one possible function of SPE B may be to degrade host cell proteins, such as fibronectin and vitronectin, to form peptides and amino acids that could potentially be important in pH homeostasis. To date, however, homologues of GadC and GadB have not been identified in *S. pyogenes*. In addition, peptides formed by the proteolytic activity of SPE B could potentially serve as a nitrogen source, and we note that the expression of *speB* has been previously shown to be linked to both the oligopeptide (36) and dipeptide transport (35) systems of *S. pyogenes*, as well as nutrient starvation (7). Clearly the regulation of SPE B production is complex, and additional information is required to understand how the various regulons involved in SPE B production interact. Understanding the conditions of SPE B production coupled with the knowledge of the molecular basis for SPE B regulation should enhance our understanding of how SPE B potentially affects the interaction between *S. pyogenes* and its human host.

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