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Identification of Rgg-Regulated Exoproteins of *Streptococcus pyogenes*

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Streptococcus pyogenes **secretes many proteins that influence host-pathogen interactions. Despite their importance, relatively little is known about the regulation of these proteins. The *rgg* gene (also known as *ropB*) is required for the expression of streptococcal erythrogenic toxin B (SPE B), an extracellular cysteine protease that contributes to virulence. Proteomics was used to determine if *rgg* regulates the expression of additional exoproteins. Exponential- and stationary-phase culture supernatant proteins made by *S. pyogenes* NZ131 *rgg* and NZ131 *speB* were separated by two-dimensional electrophoresis. Differences were identified in supernatant proteins from both exponential- and stationary-phase cultures, although considerably more differences were detected among stationary-phase supernatant proteins. Forty-two proteins were identified by peptide fingerprinting with matrix-assisted laser desorption mass spectrometry. Mitogenic factor, DNA entry nuclease (open reading frame [ORF 226]), and ORF 953, which has no known function, were more abundant in the culture supernatants of the *rgg* mutant compared to the *speB* mutant. ClpB, lysozyme, and autolysin were detected in the culture supernatant of the *speB* mutant but not the *rgg* mutant. To determine if Rgg affected protein expression at the transcriptional level, real-time (TaqMan) reverse transcription (RT)-PCR was used to quantitate Rgg-regulated transcripts from NZ131 wild-type and *speB* and *rgg* mutant strains. The results obtained with RT-PCR correlated with the proteomic data. We conclude that Rgg regulates the transcription of several genes expressed primarily during the stationary phase of growth.**

Infection with *Streptococcus pyogenes* (group A streptococcus) typically results in pharyngitis and is self-limiting. Rarely, severe infections such as necrotizing fasciitis and toxic shock syndrome occur. Although the molecular mechanisms of severe streptococcal infections are poorly understood, *S. pyogenes* secretes to the extracellular environment many proteins that may contribute to disease. For example, the extracellular cysteine protease streptococcal erythrogenic toxin B (SPE B) degrades human extracellular matrix proteins (18) and activates human enzymes involved in host tissue remodeling (4). In this manner, SPE B may contribute to the massive tissue destruction and concomitant dissemination of infection that is characteristic of necrotizing fasciitis and myositis. In addition, extracellular superantigens, in conjunction with cell-associated components, undoubtedly contribute to the systemic effects that characterize streptococcal toxic shock syndrome (10). Genetic studies support the idea that extracellular proteins (ECPs) contribute to severe streptococcal disease. Inactivation of *speB* (28, 30) and the *sic* gene (29), encoding the extracellular streptococcal inhibitor of complement, reduced virulence compared to the isogenic wild-type strains in animal models of infection. In addition, the extracellular plasminogen activator streptokinase A (SKA) has been linked to the development of acute post-streptococcal glomerulonephritis in animal models (33, 34). Thus, ECPs are important determinants of host-pathogen interactions and are potential targets for chemotherapeutic intervention designed to prevent or treat severe disease.

Several loci that influence the expression of streptococcal ECPs have been identified. Mga (multiple gene activator) regulates the expression of several cell-associated proteins including M protein, M-like proteins (Mrp, Enn, and FcR), and C5a peptidase, in addition to the ECPs SIC and serum opacity factor (10). Mga-regulated genes are expressed primarily during the exponential phase of growth (32). A two-component regulatory system designated *csrRS* (2, 14, 25), also known as *covRS* (11), regulates the expression of several proteins, including ECPs. Specifically, nonpolar inactivation of *csrR* enhanced expression of the *has* operon responsible for capsule formation and of the ECPs SKA, SagA (streptolysin S-associated gene A), SPE B, and mitogenic factor (MF) (11, 14). Inactivation of the pleiotropic effect locus (*pel*) altered the expression of genes encoding SPE B, SKA, and streptolysin S (26). Despite recent progress, much remains to be learned about the regulation of ECP expression and how expression is coordinated with additional regulatory networks.

The expression of *speB* is dependent on the *rgg* gene (6), also known as *ropB* (31), which is located proximal to *speB* in the chromosome. Rgg is homologous to the transcriptional regulatory factor Rgg of *Streptococcus gordonii* (41, 42), which is required for the transcription of the gene encoding glucosyltransferase G (*gtfG*). GtfG is a secreted enzyme responsible for the polymerization of glucose to form water-soluble and insoluble glucans important in bacterial adherence to tooth enamel (27). Rgg of *S. pyogenes* is also similar to GadR of *Lactococcus lactis*, which is required for expression of the GadABCD regulon (37). GadC is an antiporter that transports glutamate into the cytoplasm and exports glutamate-γ-aminobutyrate, formed by GadA and GadB-mediated decarboxylation of glutamate.

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The GadABCD regulon is required for glutamate-dependent acid resistance and is maximally expressed in the stationary phase of growth (37). In addition, Rgg of *S. pyogenes* is similar to MutR of *Streptococcus mutans* (36), which is required for the expression of an operon encoding the lantibiotic mutacin II (MutA), the modifying enzyme MutM, a transport protein (MutT), and three polypeptides required for immunity to the lantibiotic (MutF, MutE, and MutG) (36). Mutacin activity is maximal in the stationary phase of growth, although the level of *mutA* transcripts does not vary between the exponential and stationary phases of growth (36). Although *rgg* and *speB* are physically linked, it is unclear if *rgg* acts directly to influence *speB* expression. Moreover, the influence of *rgg* on the expression of additional ECPs of *S. pyogenes* is unknown.

The determination of the complete nucleotide sequence of an *S. pyogenes* serotype M1 genome (B. Roe, S. P. Linn, L. Song, X. Yuan, S. Clifton, R. E. McLaughlin, M. McShan, and J. J. Ferretti, streptococcal genome sequencing project, online [http://www.genome.ou.edu/strep.html]) facilitates the use of functional genomic methods to study global changes in gene expression. Proteomics involves separating and identifying proteins composing a defined proteome, such as the ECPs of *S. pyogenes*. High-resolution separation of complex protein mixtures is typically done by two-dimensional electrophoresis (2-DE). Identification of proteins of interest can be accomplished by determining the masses of peptides after in-gel trypsinization. The masses represent a fingerprint of the protein and are used to identify the corresponding gene in a genomic database. Criteria used to describe the quality of protein identifications include the number of tryptic peptides detected, the coverage of the identified protein with detected peptides, and the accuracy of peptide mass determinations. As few as three peptides are sufficient to identify a protein; however, confidence in the identification increases with the detection of additional peptides (15). Although coverage values greater than 35% are typical of unambiguous identifications, posttranslational modifications such as proteolytic removal of the signal peptide or proteolytic modification of proproteins or zymogens are not considered when calculating the coverage because the precise modification is typically not defined. Finally, differences in the calculated mass and observed mass of a peptide that are less than 10 ppm approach the technical limitations of many mass spectrometers. Protein identification by mass spectrometry (MS) offers a relatively rapid method to identify proteins of interest, such as those whose expression is altered by inactivation of a regulatory gene.

The objective of this study was to determine if the *rgg* gene influences expression of ECPs other than SPE B. To achieve this objective, proteomics was used to identify differences in ECP expression between NZ131 *speB* and *rgg* mutants. Real-time (TaqMan) reverse transcription-PCR (RT-PCR) showed that the differences in protein expression were due to changes in the level or stability of the corresponding transcripts. The results indicate that Rgg regulates the expression of several genes in the stationary phase of growth.

MATERIALS AND METHODS

Strains and media. *S. pyogenes* NZ131 (serotype M49) and isogenic mutant derivatives NZ131 *speB* and NZ131 *rgg* have been previously described (6, 8). Strains were grown on Trypticase soy agar containing 5% sheep blood (Becton Dickinson, Cockeysville, Md.) overnight at 37°C in 5% CO_2. Todd-Hewitt broth containing 0.2% (wt/vol) yeast extract (THY; Difco Laboratories, Detroit, Mich.) was passed through a 10,000-molecular-weight cutoff (MWCO) filter using a Millipore ProFlux M12 tangential flow filtration system to prepare protein-free THY.

Preparation of extracellular proteins. Plate-grown bacteria were used to inoculate 10 ml of protein-free THY in 15-ml polypropylene tubes (Corning, New York, N.Y.), and the cultures were incubated for 8 h at 37°C in 5% CO_2. Each 10-ml culture was added to 40 ml of prewarmed protein-free THY and incubated for approximately 14 h prior to inoculation into 1-liter bottles containing 950 ml of protein-free THY equilibrated overnight in a 37°C incubator containing 5% CO_2. The cultures were incubated at 37°C with 5% CO_2 with no agitation. Exponential-phase cultures had an A_{600} of 0.2 to 0.3 and corresponded to 2 to 3 h of growth. Stationary-phase cultures had an A_{600} of 0.5 to 0.6 and were grown for approximately 18 h. Following growth in protein-free THY broth, bacteria were centrifuged for 15 min at 13,679 × *g* at 4°C, and the supernatant fluids were sterilized with a 0.2-μm-pore-size filter (NalgeNunc, Rochester, N.Y.). Culture supernatant proteins were concentrated approximately 10-fold with a Millipore ProFlux M12 tangential flow concentrator fitted with an Amicon S3Y10 spiral cartridge with a 10,000-MWCO filter. Culture supernatant proteins were precipitated by adding 85% (wt/vol) ammonium sulfate (Sigma Chemical Co., St. Louis, Mo.). Proteins were resuspended in 3 ml of water and dialyzed extensively with a Slide-A-Lyzer 10,000-MWCO cartridge (Pierce Chemical Co., Rockford, Ill.). When necessary, protein preparations were further concentrated by lyophilization with a Savant (Hicksville, N.Y.) SpeedVac concentrator. Total protein concentration was determined with an ESL protein determination kit, as described by the manufacturer (Boehringer Mannheim, Mannheim, Germany).

2-DE. First-dimension isoelectric focusing was done with an IPGphor isoelectric focusing system as described by the manufacturer (Amersham Pharmacia Biotech, Piscataway, N.J.). Immobiline dry strips (18 cm) with a 3 to 10 linear separation range were rehydrated with 350 μl of protein sample in rehydration buffer consisting of 8 M urea (Amersham Pharmacia Biotech), 2% (wt/vol) 3-[3-(cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate (CHAPS; Amersham Pharmacia Biotech), 0.5% (vol/vol) IPG buffer (Amersham Pharmacia Biotech), and 2.8 mg of dithiothreitol (Amersham Pharmacia Biotech) per ml at 20°C for 14 h. Isoelectric focusing was done with 500 V for 500 V·h, 1,000 V for 1,000 V·h, and 8,000 V for 32,000 V·h. The strips were then incubated in sodium dodecyl sulfate (SDS) equilibration buffer (50 mM Tris-Cl [pH 8.8], 6 M urea, 30% [vol/vol] glycerol, 2% SDS, bromophenol blue) for 10 min. SDS-polyacrylamide gel electrophoresis separation was done with a DALT electrophoresis system (Amersham Pharmacia Biotech) and a 12% acrylamide resolving gel (1.5 by 23.4 by 19.5 mm; Bio-Rad, Hercules, Calif.) containing 1% SDS for approximately 18 h at 115 V. Following staining with Coomassie colloidal blue (Bio-Rad), the gels were scanned with a calibrated UMAX transmission scanner (Amersham Pharmacia Biotech). Spot volume was determined with ImageMaster 2D Elite software (Amersham Pharmacia Biotech) and is defined as the sum of the pixel values comprising the protein spot minus the sum of background pixel values.

In-gel tryptic digestion of proteins. Proteins of interest were excised from the SDS-polyacrylamide gels and washed three times for 15 min in 400 μl of 25 mM NH_4HCO_3–50% acetonitrile (ACN; Aldrich, Milwaukee, Wis.). The proteins were incubated in 100% ACN for 5 min and lyophilized in a SpeedVac for 30 min. The dried gel plugs were rehydrated with 25 mM NH_4HCO_3 containing 10 μg of sequencing-grade trypsin (Sigma) per ml. Following incubation at 37°C for approximately 16 h, the trypsin solution was aspirated to a microcentrifuge tube, and additional peptides were recovered from the gel plugs by extraction twice with 50% ACN–5% trifluoroacetic acid (TFA; Applied Biosystems, Foster City, Calif.) for 1 h. The extracted peptides were lyophilized in a SpeedVac, resuspended in 10 μl of 0.1% TFA, and purified with ZipTip Microcolumns (Millipore, Bedford, Mass.). The peptides were recovered from the ZipTip columns by elution with 30, 50, and 80% ACN in 0.1% TFA, lyophilized, and resuspended in 3 μl of 50% ACN–0.1% TFA.

MALDI-TOF MS. The mass of each extracted peptide was determined with a Voyager STR MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) mass spectrometer (PE Biosystems, Framingham, Mass.). Peptides were crystallized on a stainless steel MALDI plate by using a dry-drop method with an equal volume of 10.0 mg/ml α-cyano-4-hydroxycinnamic acid matrix (Aldrich) in 50% ACN–0.1% TFA. The masses of the peptides were determined in positive reflector mode with internal calibrants obtained from PE Biosystems (des-Arg1-bradykinin [M_r 904.56] and adrenocorticotropin [clip 18–39; M_r 2,465.20]).

Database searches and protein identification. Protein Prospector (University of California, San Francisco, Mass Spectrometry Facility) was used to search a genomic database of *S. pyogenes* containing 2,241 open reading frames (ORFs).

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TABLE 1. Oligonucleotide primers and fluorescent probes used to quantitate cDNA

Gene	Forward primer (5′ - 3′)	Reverse primer (5′ - 3′)	Fluorescent probe[a](5′ - 3′)
mf	CCCAAAATGTAGGAGGTCGTG	CATTCTTGAGCTCTTTGTTCGGT	CCAAAAAGGCGGCATGCGCT
orf226	TCCTCCTGGCTGGCATAATT	CTAAATGGCCACGGTCCATT	AAATTGACTGACGCTAATGGAAAAACAACTTGG
orf953	TTGGTTAGGAATGGTATCAGTCTTTTT	AATTTGCGAGCTAGAGTTATTATGATTG	CGATTCTCCTTTTTTTAACTGCAGCATCGA
orf204	GGCTGTCGCAGCAGTAGCA	GAACCTATTGGACGTTTACCATCATC	TGCTATTCGACGAAATCGGGCAGGTT
orf1669	TTAAGGGTGCCTATAACGGTTCTT	TGATCAATCGTATAGGTATTGCCATTA	TGTGACGATGTCAACTTGGGAAGATGATG
orf1324	TTCAAATGGCAATGCTTACGAT	GTCTGCGTAGCCATCCCAA	TTGATGGATCGCTTGGTGCGCAAT
gyrA	CGACTTGTCTGAACGCCAAA	TTATCACGTTCCAAACCAGTCAA	CGACGCAAACGCATATCCAAAATAGCTTG

[a] Covalently linked at the 5′ end to 5-carboxyfluorescein and at the 3′ end to *N,N,N*-tetramethyl-6-carboxyrhodamine.

The database included ORFs identified by WIT2 analysis (www.genome.ou.edu) of *S. pyogenes* strain SF370 (serotype M1), by genome sequencing projects in the Laboratory of Human Bacterial Pathogenesis, and by contigs of genomic sequences of *S. pyogenes* Manfredo strain (serotype M5) assembled by the Sanger Institute (www.sanger.ac.uk).

RNA isolation. *S. pyogenes* was grown in 10 ml of protein-free THY broth in 15-ml tubes (Corning) for approximately 10 h (A_{600} of 0.5 to 0.6). Cultures were centrifuged; the bacteria were resuspended in 200 μl of diethyl pyrocarbonate (DEPC; Sigma)-treated water and frozen in liquid nitrogen. Bacterial pellets (200 μl) were thawed on ice and added to 2-ml FastPrep Blue tubes containing ceramic matrices, 500 μl of acid phenol, and 500 μl of CPRS-Blue, as described by the manufacturer (Bio 101, La Jolla, Calif.). The bacteria were lysed with a FastPrep instrument (Bio 101) at setting 6 for 11 s and immediately placed on ice for 1 min. Samples were incubated at 65°C for 10 min and centrifuged at 10,000 × *g* for 5 min at 4°C. The upper aqueous phase was aspirated to a 2-ml phase-lock microcentrifuge tube (Eppendorf Scientific, Westbury, N.Y.) and extracted with an equal volume of acid-phenol heated to 65°C. The phases were separated by centrifugation at 10,000 × *g* for 4 min at 4°C, and the extraction was repeated with acid-phenol:chloroform (1:1, vol/vol) and chloroform:isoamyl alcohol (24:1, vol/vol). The aqueous phase was treated with 100 U of DNase I (Roche Molecular Biochemicals, Mannheim, Germany) for 2 h at 37°C and then extracted three times with acid-phenol and chloroform; the nucleic acid was precipitated by adding an equal volume of isopropanol. Samples were centrifuged at 10,000 × *g* for 15 min at 4°C, and the pellets were washed with 75% DEPC-treated ethanol. RNA was suspended in 50 μl of DEPC-treated water and stored at −80°C. The quality of the RNA was assessed by agarose gel electrophoresis and spectrophotometry.

Real-time RT-PCR. Oligonucleotide primers and probes (Table 1) were designed with Primer Express 1.0 software (ABI Prism; PE Biosystems) and purchased from MegaBases Inc. (Evanston, Ill.). The probes consisted of an oligonucleotide labeled at the 5′ end with the reporter dye 5-carboxyfluorescein and at the 3′ end with the quencher *N,N,N′N′*-tetramethyl-6-carboxyrhodamine. RT-PCR was done with the TaqMan One-Step RT-PCR Master Mix Reagents kit (PE Applied Biosystems) as described by the manufacturer. The RT-PCR mixture (25 μl) contained 6.25 U of Multiscribe reverse transcriptase, 10.0 U of RNase inhibitor, 500 nM each gene-specific primer, 100 nM each probe, and 25 ng of total RNA template. Amplification and detection of specific products was performed with the ABI Prism 7700 sequence detection system (PE Applied Biosystems) with the following cycle profile: 1 cycle at 48°C for 30 min, 1 cycle at 95°C for 10 min, 40 cycles at 95°C for 15 s, and 60°C for 1 min. The critical threshold cycle (Ct) is defined as the cycle at which the fluorescence becomes detectable above background and is inversely proportional to the logarithm of the initial number of template molecules. A standard curve was plotted for each primer-probe set with Ct values obtained from amplification of known quantities of genomic DNA isolated from strain NZ131. The standard curves were used to transform Ct values to the relative number of DNA molecules. The amount of contaminating chromosomal DNA in each sample was determined with control reactions that did not contain reverse transcriptase. The amount of contaminating DNA was subtracted from each experimental value to give the quantity of cDNA. The quantity of cDNA for each experimental gene was normalized to the quantity of *gyrA* cDNA in each sample.

RESULTS

Comparison of supernatant proteins of NZ131 *speB* and NZ131 *rgg* grown to the mid-exponential phase. The role of *rgg* in the regulation of ECP expression was assessed by comparing 2-DE patterns of ECPs from NZ131 *speB* and NZ131 *rgg*. NZ131 *speB* was used rather than the wild-type strain because SPE B accounts for nearly 95% of culture supernatant protein when strain NZ131 is grown in protein-free THY broth (7). The abundance of SPE B would interfere with the detection of other ECPs potentially influenced by Rgg. In addition, SPE B, which is not detected in strain NZ131 *rgg* (6), degrades a variety of human and streptococcal proteins (1, 7, 18).

NZ131 *speB* and NZ131 *rgg* were grown in protein-free THY broth medium to mid-exponential phase. The concentrated ECPs were separated by 2-DE and stained with Coomassie colloidal blue. Representative gels from two independent protein isolations are shown in Fig. 1. The majority of ECPs from each strain had an isoelectric point (pI) between 4 and 6. The protein spots were designated on the basis of the source strain (NZ131 *rgg* or NZ131 *speB*) and arbitrarily numbered. Differences were identified in the protein composition of exponential-phase culture supernatant fluids from NZ131 *speB* and NZ131 *rgg*. For example, proteins (designated Rgg-15 to Rgg-17) present in supernatant fluid from NZ131 *rgg* were not detected in the analogous region of the 2-DE gels of the *speB* mutant (Fig. 1). Protein Rgg-16 was identified as DNA K and migrated similarly to the inferred molecular weight and pI (Table 2). Proteins designated Rgg-32, Rgg-33, and Rgg-34 were detected in the supernatant fluid of NZ131 *rgg* but not NZ131 *speB* (SpeB-32, SpeB-33, and SpeB-34 [Fig. 1]). Finally, Rgg-39 and Rgg-40 were detected in the ECP obtained from the *rgg* mutant but not the *speB* mutant.

Comparison of supernatant fluid proteins of NZ131 *speB* and NZ131 *rgg* grown to stationary phase. The Rgg-regulated exoprotein SPE B is expressed by strain NZ131 primarily in the stationary phase of growth (9). Thus, it was of interest to characterize stationary-phase supernatant proteins from NZ131 *speB* and NZ131 *rgg*. NZ131 *speB* and NZ131 *rgg* were grown in protein-free THY broth medium for approximately 18 h, and the concentrated culture supernatant proteins were analyzed by 2-DE (Fig. 2).

Considerable differences were detected in the protein composition of stationary-phase supernatant fluids between the two strains (Fig. 2). The proteins identified by peptide mass fingerprinting in stationary-phase supernatant fluids are summarized in Table 3. Approximately 4.8-fold more MF was detected in the supernatant of NZ131 *rgg* cultures than in NZ131 *speB* culture supernatant (total spot volumes of 2,606 and 540, respectively) (Fig. 2; Table 4). Five positional variants of MF were detected in the NZ131 *rgg* supernatant fluid (Rgg-39, Rgg-40, Rgg-42, Rgg-43, and Rgg-45), whereas only one

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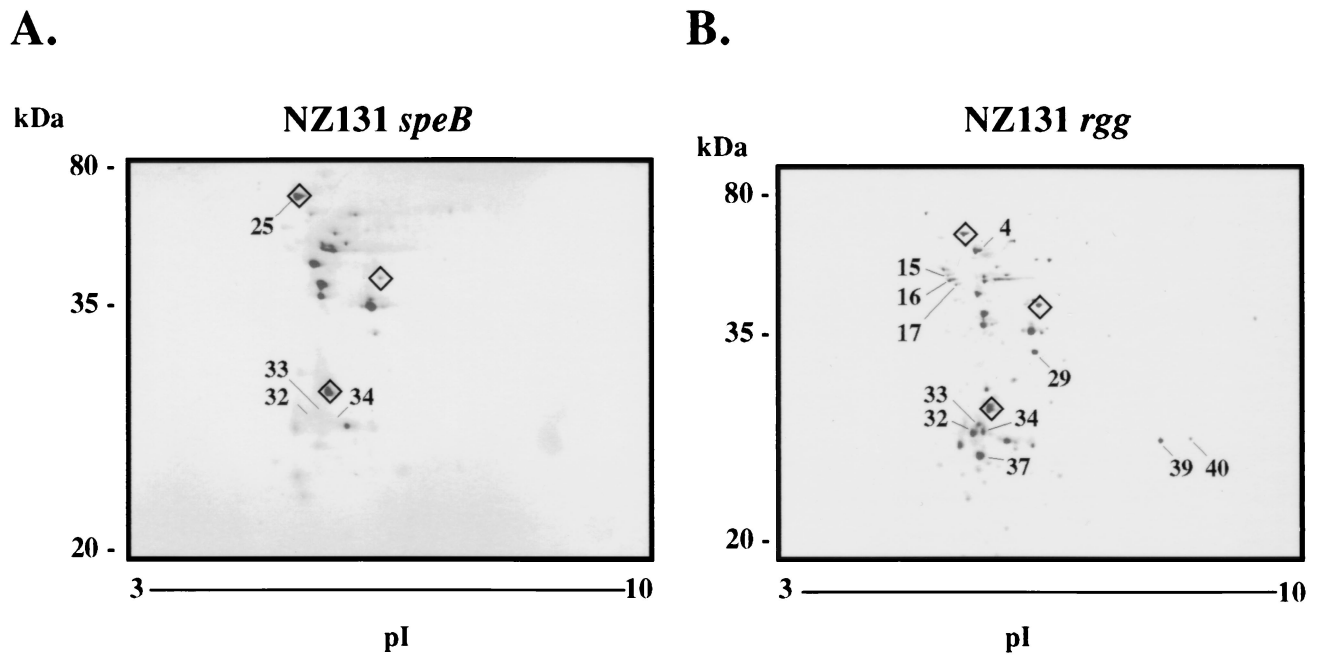

FIG. 1. Coomassie colloidal blue-stained 2-DE gels of supernatant proteins from exponential-phase cultures of NZ131 *speB* (A) and NZ131 *rgg* (B). Proteins identified by peptide mass fingerprinting are summarized in Table 3. Diamonds around selected proteins are used to orient the gels to each other. The migration of molecular mass standards is indicated. The gels (oriented with acidic proteins to the left) are representative of two independent experiments.

was identified in the supernatant fluid from NZ131 *speB* (SpeB-43). In addition, proteins designated Rgg-39 and Rgg-40 in the exponential phase 2-DE map (Fig. 1) migrated similarly to protein spots identified as MF in the stationary phase (Fig. 2, Rgg-42 and Rgg-43), which suggested that NZ131 *rgg* produced detectable MF in the exponential phase of growth whereas the *speB* mutant did not (cf. Fig. 1 and 2). In addition, DNA entry nuclease (ORF 226) was highly expressed by the *rgg* mutant compared to the *speB* mutant (Table 4). Proteins Rgg-17, Rgg-19, Rgg-36, Rgg-44, and Rgg-47 were identified as DNA entry nuclease in NZ131 *rgg* supernatant fluid (Table 3); however, only SpeB-26 was identified as DNA entry nuclease in ECPs from the *speB* mutant. Protein Rgg-20, uniquely expressed by the *rgg* mutant, was identified as ORF 953 and has no known function (Fig. 2; Table 3).

Several proteins were detected in the supernatant from NZ131 *speB* cultures but not in the supernatant of the *rgg* mutant (Fig. 2). Peptide mass fingerprinting indicated that SpeB-28 had peptides that corresponded to ClpB (ORF 204); SpeB-38 and SpeB-40 were identified as autolysin (ORF 1669) and lysozyme (ORF 1324), respectively (Table 3). The results indicate that the *rgg* mutant did not synthesize detectable levels of autolysin, lysozyme, and ClpB.

Proteins whose expression was not significantly altered by *rgg* inactivation were also identified. For example, proteins identified as CAMP factor included SpeB-27, SpeB-33, SpeB-34, and SpeB-35 from NZ131 *speB* supernatant and protein Rgg-23 from the supernatant of NZ131 *rgg*. Proteins identified as streptokinase included SpeB-12, SpeB-22, and SpeB-25 from NZ131 *speB* supernatant and Rgg-5, Rgg-8, Rgg-21, Rgg-32, and Rgg-37 from NZ131 *rgg* supernatant.

Real-time quantitative RT-PCR. The results of the proteomic analysis of ECPs from NZ131 *speB* and NZ131 *rgg* indicated that the quantity of several proteins in culture supernatants was affected by *rgg* inactivation. Inasmuch as Rgg is similar to known transcriptional regulatory proteins, the results

TABLE 2. Exponential-phase supernatant proteins identified by peptide mass fingerprinting

Designation	Name	Accession no.[a]	M_r/pI[b]	No. of peptides (coverage)[c]	Δppm[d]
SpeB-25	Heat shock protein	U72721	64,920/4.62	10 (18)	2.9 ± 3.2
Rgg-4	Heat shock protein	X89236, RST0771	50,497/4.68	14 (37)	4.0 ± 1.8
Rgg-16	DNA K	RST00473	58,144/4.58	6 (16)	9.9 ± 5.4
Rgg-29	Enolase	RST00465	37,330/4.83	8 (34)	12.2 ± 2.3
Rgg-37	Enolase	RST00465	37,330/4.83	7 (24)	0.5 ± 2.3

[a] NCBI accession number or WIT2 RST designation.
[b] Inferred from the nucleotide sequence.
[c] Number of tryptic peptides of the identified protein detected by MALDI-TOF MS (percentage of the protein covered with detected peptides).
[d] Mean difference ± standard error between masses detected by MALDI-TOF MS and calculated masses of the tryptic peptides.

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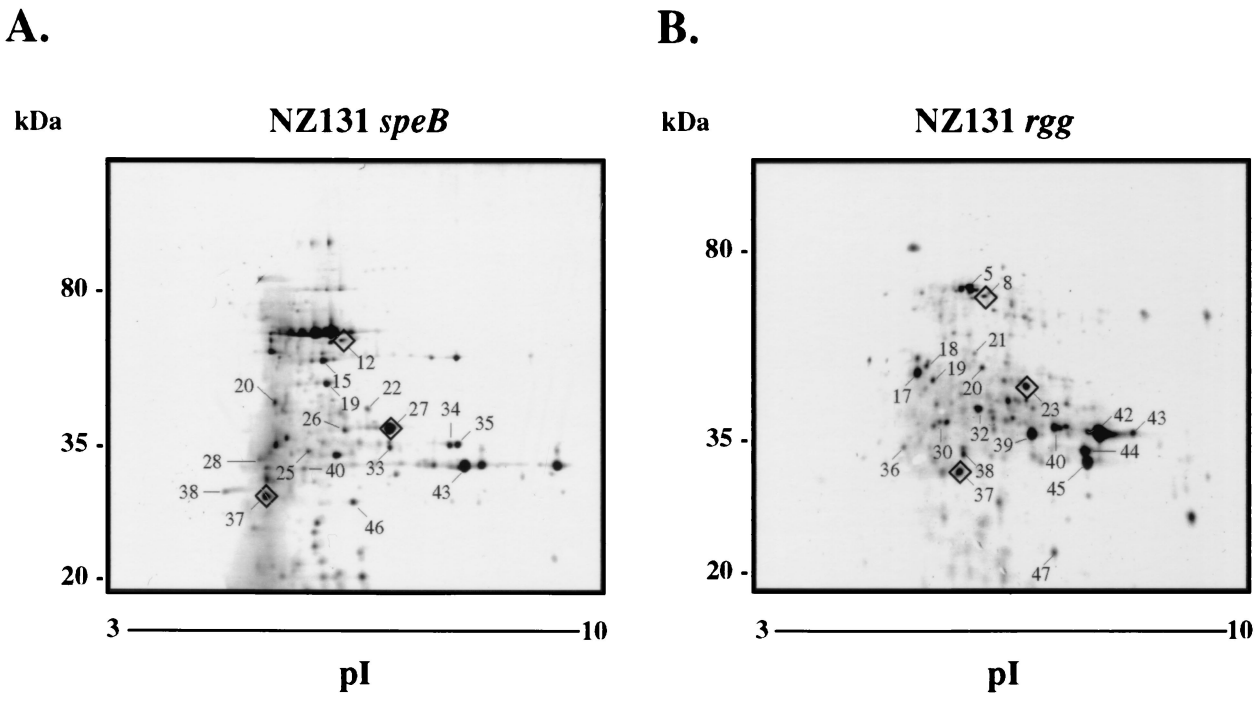

FIG. 2. Coomassie colloidal blue-stained 2-DE gels of supernatant proteins from stationary-phase cultures of NZ131 *speB* (A) and NZ131 *rgg* (B). Proteins identified by peptide mass fingerprinting are summarized in Table 2. Diamonds around selected proteins are used to orient the gels to each other. The migration of molecular mass standards is indicated. The gels (oriented with acidic proteins to the left) are representative of two independent experiments.

suggested that Rgg influenced ECP expression at the level of transcription. To determine if this was the case, the following six genes were analyzed by real-time RT-PCR: *mf*, *orf 226* (encoding DNA entry nuclease), *orf953*, *orf204 (clpB)*, *orf1669* (encoding autolysin), and *orf1324* (encoding lysozyme). Standard curves were generated for each gene with genomic DNA isolated from the wild-type strain of NZ131 to determine the relative quantity of amplified cDNA. The quantity of cDNA for each gene was then normalized to the quantity of *gyrA* cDNA present in each RNA preparation. To confirm that *gyrA* was constitutively expressed, the amount of *gyrA* mRNA was measured in three separate RT-PCR experiments with two independent RNA samples isolated from both stationary- and exponential-phase cultures of NZ131. The results confirmed that *gyrA* is constitutively transcribed in NZ131 wild-type, *speB*, and *rgg* strains in both growth phases (data not shown).

Equal amounts of total RNA from stationary-phase (10-h) cultures of wild-type, *speB*, and *rgg* strains were used to quantify the transcript levels of the Rgg-regulated exoproteins. Representative results, confirmed by analysis of two or three independently isolated sets of RNA, are shown in Fig. 3. Importantly, the RT-PCR and proteomic data were qualitatively cognate. The *mf, orf226*, and *orf953* transcript levels were higher in the *rgg* mutant than in the wild-type strain and the *speB* mutant (Fig. 3). Levels of autolysin and *clpB* transcripts were lower in the *rgg* mutant than in the wild-type strain and the *speB* mutant (Fig. 3). Primers and probes based on an M1 nucleotide sequence (Roe et al., online) did not amplify lysozyme cDNA or the lysozyme structural gene when purified genomic DNA from NZ131 was used a control. However, the primers did amplify the lysozyme gene when genomic DNA from an M1 serotype (SF370) was used (data not shown).

To determine if *rgg* transcription correlated with the influence of Rgg primarily on stationary-phase gene expression, total RNA was isolated from NZ131 wild-type and *speB* cultures grown to exponential (2 h) and stationary (10 h) phases. The *rgg* transcript level was higher in the stationary phase than in the exponential phase for both strains (Fig. 4). Interestingly, the *rgg* transcript was more abundant in the *speB* mutant than in the wild-type strain (Fig. 4).

DISCUSSION

Several ECPs of *S. pyogenes* have been extensively characterized, but the function and contribution to virulence of many others are not well understood. Insight into protein function is often gained by determining the conditions under which a gene is expressed. Rgg is required for the expression of SPE B (6, 31), an extracellular cysteine protease that contributes to virulence (28, 30). In this study, we used proteomics and real time RT-PCR to identify additional Rgg-regulated exproteins. Comparative analysis of the extracellular proteomes of NZ131 *speB* and NZ131 *rgg* showed that the *rgg* mutant expressed less lysozyme (ORF 1324), autolysin (ORF 1669), and ClpB (ORF 204) in the stationary phase of growth. In addition, the *rgg* mutant expressed considerably more MF (ORF 1835), DNA entry nuclease (ORF 226), and ORF 953. The results show that Rgg regulates the expression of several genes expressed primarily in the stationary phase of growth.

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TABLE 3. Stationary-phase supernatant proteins identified by peptide mass fingerprinting

Designation	Name	Accession no.[a]	M_r/pI[b]	No. of peptides (coverage)[c]	Δppm[d]
SpeB-12	Streptokinase	M19347	50,084/5.54	8 (13)	2.8 ± 6.0
SpeB-15	Glyceraldehyde-3-phosphate dehydrogenase/plasmin receptor	M95569	35,958/5.34	8 (30)	2.9 ± 4.5
SpeB-19	6-Phosphofructokinase	RST00803	35,748/5.33	7 (23)	0.8 ± 2.6
SpeB-20	Immunogenic secreted protein	RST00809, U31811	53,292/5.5	5 (10)	8.2 ± 7.2
SpeB-22	Streptokinase	M19347	50,084/5.54	5 (14)	1.3 ± 3.6
SpeB-25	Streptokinase	M19347	50,084/5.54	5 (18)	3.3 ± 9.6
SpeB-26	DNA entry nuclease	RST00226	30,398/4.93	4 (16)	0.3 ± 5.8
SpeB-27	CAMP factor	RST0342, AF079502	28,480/6.25	4 (15)	6.9 ± 1.8
SpeB-28	ClpB	RST00204	63,349/5.41	5 (9)	10.4 ± 17.8
SpeB-33	CAMP factor	RST0342, AF079502	28,480/6.25	5 (26)	2.8 ± 2.7
SpeB-34	CAMP factor	RST0342, AF079502	28,480/6.25	6 (29)	0.1 ± 4.9
SpeB-35	CAMP factor	RST0342, AF079502	28,480/6.25	6 (29)	0.1 ± 4.9
SpeB-37	Enolase	RST00465	37,330/4.83	6 (23)	7.1 ± 1.2
SpeB-38	Autolysin	RST01669	23,020/4.66	4 (15)	11.7 ± 2.7
SpeB-40	Lysozyme	RST01324	49,729/5.04	5 (15)	1.5 ± 16.5
SpeB-43	Mitogenic factor	D13428	30,061/9.24	7 (37)	7.6 ± 1.8
SpeB-46	Ribosome recycling factor	RST00322	20,572/5.68	8 (45)	11.7 ± 4.7
Rgg-5	Streptokinase	M19347	50,084/5.54	8 (23)	1.7 ± 3.2
Rgg-8	Streptokinase	M19347	50,084/5.54	7 (17)	5.4 ± 6.7
Rgg-17	DNA entry nuclease	RST00226	30,398/4.93	8 (23)	4.2 ± 3.5
Rgg-18	Immunogenic secreted protein	RST00809, U31811	53,292/5.50	5 (37)	4.7 ± 5.5
Rgg-19	DNA entry nuclease	RST00226	30,398/4.93	6 (27)	6.2 ± 3.1
Rgg-20	Unknown	RST00953	32,053/5.79	4 (22)	2.6 ± 6.2
Rgg-21	Streptokinase	M19347	50,084/5.54	9 (20)	1.7 ± 1.7
Rgg-23	CAMP factor	RST0342, AF079502	28,480/6.25	10 (38)	6.0 ± 2.6
Rgg-30	Immunogenic secreted protein	RST00809, U31811	53,292/5.50	4 (11)	15.2 ± 13.4
Rgg-32	Streptokinase	M19347	50,084/5.54	7 (18)	18.7 ± 6.1
Rgg-36	DNA entry nuclease	RST00226	30,398/4.93	5 (24)	1.0 ± 4.1
Rgg-37	Streptokinase	M19347	50,084/5.54	8 (13)	1.7 ± 7.3
Rgg-38	Phosphoglycerate kinase	RST00513	42,130/4.82	5 (17)	4.9 ± 11.8
Rgg-39	Mitogenic factor	D13428	30,061/9.24	5 (28)	18.3 ± 9.1
Rgg-40	Mitogenic factor	D13428	30,061/9.24	8 (37)	9.1 ± 5.0
Rgg-42	Mitogenic factor	D13428	30,061/9.24	9 (45)	3.1 ± 15.9
Rgg-43	Mitogenic factor	D13428	30,061/9.24	4 (19)	11.4 ± 10.6
Rgg-44	DNA entry nuclease	RST00226	30,398/4.93	6 (27)	11.2 ± 1.9
Rgg-45	Mitogenic factor	D13428	30,061/9.24	5 (26)	18.0 ± 7.6
Rgg-47	DNA entry nuclease	RST00226	30,398/4.93	6 (14)	11.2 ± 1.9

[a] NCBI accession number or WIT2 RST designation.
[b] Inferred from the nucleotide sequence.
[c] Number of tryptic peptides of the identified protein detected by MALDI-TOF MS (percentage of the protein covered with detected peptides).
[d] Mean difference ± standard error between masses detected by MALDI-TOF MS and calculated masses of the tryptic peptides.

Transcriptional regulation by Rgg. Real time RT-PCR analysis showed that Rgg influenced the expression of MF, DNA entry nuclease, ORF 953, autolysin, and ClpB by altering the quantity or stability of the corresponding transcripts (Fig. 3). This finding is consistent with transcriptional regulation of *speB* by Rgg, as previously described (31). The results are also consistent with the observation that the amino acid sequence of Rgg is similar to those of several other gram-positive transcriptional regulatory proteins, including Rgg of *S. gordonii* (41, 42), GadR of *L. lactis* (37), and MutR of *S. mutans* (36). The *gadR* gene expressed primarily in the stationary phase of growth (37). Mutacin II activity, which is regulated by MutR, is also maximally expressed in the stationary phase (36). Similarly, the majority of *S. pyogenes* Rgg-regulated exoproteins were detected in the stationary phase of growth, which correlated with increased *rgg* expression (Fig. 4). No information is available regarding growth phase-dependent expression of *rgg* in *S. gordonii*. Several potential regulatory elements were identified in the promoter regions of *S. pyogenes* Rgg-regulated genes, but a common motif was not apparent.

Interestingly, more *rgg* transcript was detected in NZ131 *speB* than in the wild-type strain (Fig. 4), suggesting that SPE B represses the expression of *rgg*. The possibility that *speB* inactivation influenced the expression of *orf204* (*clpB*), *orf1669* (encoding autolysin), *orf953, mf*, and *orf226* (encoding DNA entry nuclease) was excluded by RT-PCR, which showed that the transcript levels for each gene were similar between the wild-type strain and the isogenic *speB* mutant (Fig. 3). Additional experiments are required to determine if the *speB* transcript, SPE B protease, or SPE B degradation products are responsible for the inhibition of *rgg* expression.

Proteomics was used to identify several Rgg-regulated genes. It remains possible that additional exoproteins, not detected in this study, are regulated by Rgg. Although variation was periodically observed upon repeated analysis of culture supernatant proteins, MF, ORF 226, ORF 953, ClpB, autoly-

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TABLE 4. Quantitation of Rgg-regulated exoproteins

Name	NZ131 *speB*		NZ131 *rgg*	
	Designation	Vol[a]	Designation	Vol
Mitogenic factor	SpeB-43	540.2	Rgg-39	430.4
			Rgg-43	200.3
			Rgg-45	465.5
			Rgg-42	1,171.5
			Rgg-40	338.3
DNA entry nuclease (ORF 226)	SpeB-26	102.1	Rgg-17	318.8
			Rgg-19	91.2
			Rgg-36	50.6
			Rgg-44	334.7
			Rgg-47	149.9
ORF 953	ND[b]		Rgg-20	90.3
Lysozyme (RST1324)	SpeB-40	46.4	ND	
Autolysin (RST1669)	SpeB-38	89.8	ND	
ClpB (RST204)	SpeB-28	55.4	ND	

[a] Sum of pixel values comprising the protein spot minus background pixel values.
[b] ND, not detected.

sin, and lysozyme were selected for further study because their expression consistently differed between NZ131 *speB* and NZ131 *rgg*. Several exoproteins, including four that were detected in NZ131 *speB* supernatants but not in supernatants from NZ131 *rgg*, were not identified by peptide fingerprinting. The genomic database used to identify proteins by peptide fingerprinting was constructed primarily with nucleotide sequences from a serotype M1 genome sequencing project prior to its completion (Roe et al., online). Thus, the database did not contain all M1 ORFs. Moreover, NZ131 (serotype M49) may possess exoproteins not present in the M1 genome used to construct the database.

MF was more abundant in the stationary-phase supernatant of the *rgg* mutant compared to the *speB* mutant (Fig. 2). The *mf* gene is located proximal to *rgg* in the streptococcal chromosome. The intergenic region between *mf* and *rgg* is 240 bp in strain MGAS 8232 (serotype M18) (J. C. Smoot, unpublished data) and 241 bp in strain SF370 (serotype M1) (R. Overbeek, G. D. Pusch, M. Dsouza, N. Larsen, and E. Selkov, Functional overview of *Streptococcus pyogenes*, online [http://129.15.12.51

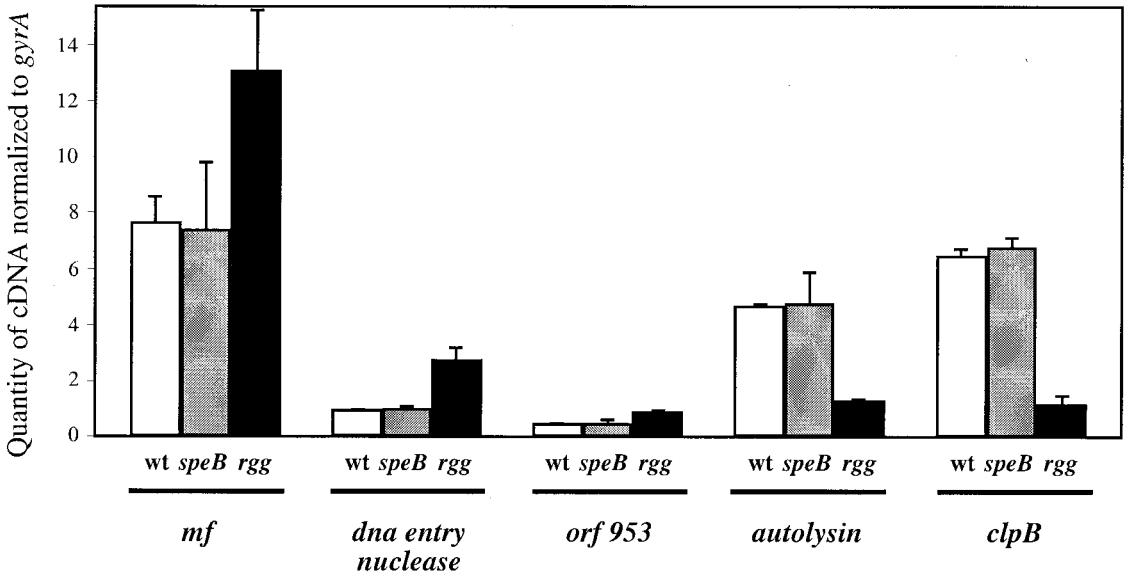

FIG. 3. Relative quantities of Rgg-regulated gene transcripts assessed by TaqMan assays. cDNA detected from stationary-phase cultures of NZ131 wild-type (wt), *speB*, and *rgg* strains was quantified for *mf*, the DNA entry nuclease gene (*orf226*), *orf953*, the autolysin gene (*orf1669*), and *clpB* (*orf204*). The cDNA values were normalized to the quantity of *gyrA* cDNA in each sample. The experiments were repeated using at least two independently isolated RNA preparations, and representative results are shown.

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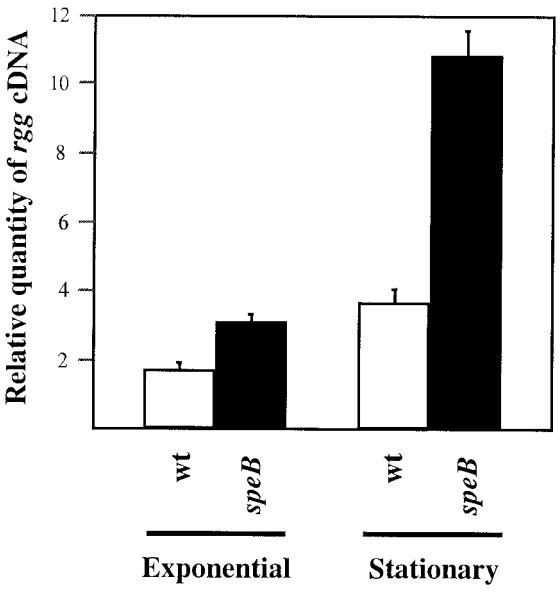

FIG. 4. Relative quantities of *rgg* cDNA in the exponential and stationary phases of growth assessed by TaqMan assays. The amount of *rgg* cDNA was determined and normalized to the amount of *gyrA* cDNA following reverse transcription of total RNA isolated from NZ131 wild-type (wt) and *speB* strains. The results shown are representative of those obtained with two independently isolated RNA preparations.

:8080/WIT2/CGI/index.cgi?user=]); in both strains, the genes are divergently oriented. Promoter activity potentially contained within the heterologous DNA used to insertionally inactivate *rgg* is unlikely to have enhanced *mf* expression, because the insertion is downstream of the *mf* gene. Nonetheless, we cannot formally exclude the possibility that *mf* expression was altered by insertion of heterologous DNA into the *rgg* locus.

Function of Rgg-regulated proteins. Four immunologically and electrophoretically distinct nucleases, designated DNases A, B, C, and D, have been previously identified in *S. pyogenes* supernatant fluids (45, 46). DNase D is encoded by the *sdaD* gene (35), and MF is thought to be identical to the protein previously described as DNase B (16). It is unclear if DNA entry nuclease (ORF 226) is identical to enzymes previously designated DNase A or C. ORF 226 is approximately 30% identical to several nucleases, including streptodornase (accession number X84793), EndA of *Streptococcus pneumoniae* (23), and MF (accession number D13428). In addition to ORF 226, RST00413 and RST0049 were also designated as DNA entry nucleases in the WIT2 analysis of an M1 streptococcal genome (Overbeek et al., online). However, in contrast to ORF 226, neither RST00413 nor RST00491 has an apparent signal sequence. Rgg thus regulates the expression of at least two of four extracellular nucleases described, suggesting that control of extracellular nuclease activity is an important component of the Rgg regulon.

Many gram-positive and gram-negative bacteria secrete nucleases. Although their function is unclear, it has been hypothesized that extracellular nucleases (i) provide phosphate, nitrogen, and carbon for catabolism following the transport of oligonucleotides and nucleotides to the cytoplasm, (ii) protect the bacterial cell against potentially mutagenic heterologous DNA, and (iii) contribute to host-pathogen interactions. Secreted nuclease activity among streptococci is primarily associated with *S. pyogenes* (44). This observation suggests that this enzyme activity is not an important component of streptococcal metabolism, since the activity is not conserved among related groups of streptococci that share many metabolic features. In addition, in the absence of evidence for natural DNA transformation, it seems unlikely that secreted nucleases are necessary to protect *S. pyogenes* from heterologous DNA, since the cell wall is an efficient barrier against DNA entry. Host mucus may contain significant amounts of DNA that can inhibit the adherence of microorganisms to epithelial cells. DNA is also present in pus, and secreted DNase may decrease the viscosity of pus and facilitate bacterial dissemination. As noted by Wannamaker (44), all strains of *S. pyogenes* have extracellular nuclease activity and produce significantly more activity compared to other groups of streptococci, suggesting that the activity may contribute to virulence. In this regard, the toxicity of the cytolethal distending toxin of *Campylobacter jejuni* was found to be dependent on its DNase activity (24). Nonetheless, the function and potential contribution to virulence of streptococcal exonucleases remain to be determined.

Autolysin and lysozyme were detected in the supernatant from stationary-phase cultures of NZ131 *speB* but not NZ131 *rgg* (Fig. 2). Bacterial peptidoglycan hydrolases are typically involved in cell wall turnover, cell separation, competence, and sporulation. Interestingly, their activity is often posttranslationally regulated by proteases. For example, in *Enterococcus hirae*, muramidase activity is activated by an extracellular protease (19). Similarly, an extracellular protease activates the ATL peptidoglycan hydrolase of *Staphylococcus aureus* (20). Alternatively, proteases may down regulate autolysin activity, as described for *Bacillus subtilis* (17) and exemplified by the degradation of the autolysin AcmA by the serine protease PrtP of *L. lactis* (3). The coordinate regulation of the extracellular protease SPE B and peptidoglycan hydrolases (autolysin and lysozyme) suggests a functional relationship.

Function of the Rgg regulon. Exoproteins are often referred to as accessory gene products that are not essential for growth in nutrient-rich conditions. Typically expressed under conditions of stress, exoproteins are likely to be critical for survival in hostile environments, such as those encountered during infection. The comparison of ECPs in exponential- and stationary-phase culture supernatant fluids of NZ131 *speB* and NZ131 *rgg* showed that significantly more ECPs were produced in the stationary phase of growth (cf. Fig. 1 and 2). In addition, NZ131 produces SPE B in the stationary phase of growth in response to nutrient starvation (9), consistent with the general theme that many secreted proteins comprise a bacterial response to nutritional stress.

The expression of extracellular nuclease by *Serratia marcescens* is enhanced by induction of the SOS stress response (13). In addition, the extracellular thermonuclease of *S. aureus* is secreted at a higher level in a *sigB* mutant (22). SigB is a stationary-phase sigma factor involved in the cellular response to stress (5, 21). Similarly, we observed increased expression of MF (DNase B) and the putative DNase ORF 226 in the *rgg* mutant in the stationary phase of growth. Inactivation of *rgg*

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also resulted in decreased expression of autolysin, lysozyme, and ClpB. The ClpB heat shock proteins of *Saccharomyces cerevisiae* and *Escherichia coli* are necessary for survival at elevated temperatures (38, 39, 43). Peptidoglycan hydrolases such as autolysin and lysozyme are required for sporulation in *B. subtilis* (12), a developmental response induced, at least in part, by nutritional stress (40). Thus, Rgg regulates a variety of genes whose products potentially comprise a response to stress. It remains to be determined if the *rgg* mutant is deficient in responding to stress or in the detection and signaling of stressful conditions.

In conclusion, *rgg* was initially described as being required for *speB* expression (6, 31). The results described in the present study show that inactivation of the gene affects the expression of several extracellular proteins, some of which are likely to influence host-pathogen interactions. It remains unclear if Rgg acts directly or indirectly on each gene to alter expression. In addition, the influence of Rgg on the expression of a variety of secreted proteins may be due, at least in part, to the interaction of the Rgg regulon with additional regulatory networks. This hypothesis is currently being tested by DNA microarray analysis.

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