Role of RopB in Growth Phase Expression of the SpeB Cysteine Protease of *Streptococcus pyogenes*

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The Rgg family of transcription regulators is widely distributed among gram-positive bacteria; however, how the members of this family control transcription is poorly understood. In the pathogen *Streptococcus pyogenes*, the Rgg family member RopB is required for transcription of the gene that encodes the secreted SpeB cysteine protease. Expression of the protease follows distinct kinetics that involves control of transcription in response to the growth phase. In this study, the contribution of RopB to growth phase control was examined. The gene encoding the protease (*speB*) and *ropB* are transcribed divergently from a 940-bp intergenic region. Primer extension analyses, in conjunction with reporter fusion studies, revealed that the major region controlling the transcription of both *speB* and *ropB* is adjacent to *ropB* and that the promoters for the two genes likely overlap. Furthermore, it was found that RopB is a DNA-binding protein that specifically binds to sequences in this control region. The interrelationship between *ropB* and *speB* expression was further reflected in the observation that transcription of *ropB* itself is subject to growth phase control. However, while expression of *ropB* from a promoter expressed during the early logarithmic phase of growth could complement a *ropB* deletion mutant, ectopic expression of *ropB* did not uncouple the expression of *speB* from its growth phase signal. These data implicate other factors in growth phase control and suggest that regulation of *ropB* expression itself is not the central mechanism of control.

The pathogenesis of a bacterial infection is a multistage process characterized by adaptive responses on the part of both the pathogen and the host. For the pathogen, transmission to a new host, specific interaction with tissues at the initial site of infection, and growth in the initial host compartment, often followed by dissemination to other host tissues, all represent stages that require the expression of distinct subsets of the pathogen's genome. The host's adaptive response involves sensing of the pathogen and then modification of the site of infection to produce a harsh environment that is not conducive to microbial growth. Successful pathogens have evolved sensory programs to recognize the host's response in order to mount an appropriate adaptive response. Thus, it is not surprising that the expression of most bacterial virulence genes is highly regulated and that mutations in these genes generally result in an attenuated ability to cause infection.

Virulence regulatory genes have been most thoroughly studied among the gram-negative bacteria, where it has become apparent that many are members of several large conserved families of proteins that modulate transcription. Each of these families is characterized by a common structure and mechanism of action that have been adapted to individual regulatory programs. An understanding of the general paradigm by which a particular family functions often provides crucial insight into how any individual member functions in the regulation of virulence. Much less is known about virulence regulatory networks in gram-positive bacteria. However, the recent influx of genomic information has revealed large conserved families of regulators that are unique to the gram-positive pathogens. One example is the family of regulators that share homology with the prototype member *rgg* of the oral bacterium *Streptococcus gordonii* (38). Members of the *rgg*-like family have been identified in the genomes of *S. pyogenes* (8, 27), *S. pneumoniae* (40), *S. agalactiae* (17), *S. mutans* (32), *S. oralis* (15), *S. sanguis* (42), *S. equi* (M. N. Neely and M. Caparon, unpublished data), and *Listeria monocytogenes* (16). Some genomes, including those of *S. pyogenes* (13, 27) and *S. gordonii* (25, 41), contain multiple family members, and *rgg*-like genes have also been found in nonpathogens as well, including *Lactococcus lactis* (35) and *Lactobacillus sakei* (33). How the members of this extensive family function to regulate gene expression is not understood. For example, while the Rgg protein of *S. gordonii* has been shown to associate with DNA (43), it has not been clearly established that any member of this extensive family of proteins binds specifically to DNA to regulate transcription.

In *S. pyogenes*, one of the major secreted proteins during the transition from the logarithmic to the stationary phase of growth in vitro is the SpeB cysteine protease. This organism is the cause of many different suppurative and nonsuppurative diseases, including pharyngitis (strep throat), cellulitis, necrotizing fasciitis, and rheumatic fever (2). The contribution of SpeB to the pathogenesis of any of these diseases is not well understood (1, 3, 19, 23, 24, 26, 39). However, it is clear that expression of the gene that encodes the protease (*speB*) is regulated by the *rgg* family member *ropB* (also known as *rgg*) (8, 27). Consistent with other *rgg* family members, *ropB* is located adjacent to *speB* in the *S. pyogenes* chromosome (27), transcription of *speB* has an absolute dependence on *ropB*, and *speB* encodes a protein that is secreted from the bacterial cell. Proteomic profiling has revealed that *ropB* regulates the ex-

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pression of several other proteins that are preferentially secreted from cells in the stationary phase of growth, including acting as a repressor of the mitogenic factor DNase (11). Transcription profiling has suggested that *ropB* may have a much greater influence over expression of the proteome via its ability to influence the expression of a number of unrelated virulence regulatory programs (10). The importance of *ropB* is further illustrated by the fact that a *ropB* mutant has a reduced ability to cause disease in an animal model of streptococcal myositis (29).

Similar to other *rgg* family members, little is known about how *ropB* functions to regulate the growth phase pattern of transcription of *speB* or any of its other target genes. In the present study, we characterized the intergenic region between *ropB* and *speB* to identify elements involved in transcriptional control and showed that RopB binds to DNA in the vicinity of the major *speB* promoter. This study also revealed that transcription of *ropB* itself is regulated in a growth phase-dependent manner that resembles that of *speB*. However, ectopic expression of *ropB* during the logarithmic phase of growth does not alter the expression pattern of *speB*. Thus, control of *ropB* transcription does not represent the major determinant for control of the *speB* regulatory program. These data suggest that additional factors must interact with *ropB* in order to control secreted protease production in *S. pyogenes*.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. Streptococcus experiments were done with derivatives of *S. pyogenes* HSC5 (20) cultured as described previously (20). When appropriate, antibiotics were added at the following concentrations: kanamycin at 25 μg/ml for *Escherichia coli* and 500 μg/ml for *S. pyogenes*, erythromycin at 750 μg/ml for *E. coli* and 1 μg/ml for *S. pyogenes*, chloramphenicol at 15 μg/ml for *E. coli* and 3 μg/ml for *S. pyogenes*, and ampicillin at 50 μg/ml for *E. coli*.

Northern blot analysis. RNA samples were prepared from cultures harvested at the onset of stationary phase (approximately 6 h) as described elsewhere (27), and 10-μg samples were subjected to Northern blot analysis as described previously (27). The probe used for the *speB* transcript consisted of a 550-bp DNA fragment that was amplified by PCR from HSC5 chromosomal DNA with primers *speB*1850for and *speB*2400rev (Table 1) as described elsewhere (27). Relative levels of the *speB* transcripts were determined by comparison to a probe that hybridized to the transcript for *recA*, similar to that described previously (27).

Primer extension analysis. The 5′ ends of the *speB* transcript(s) was determined with (i) avian myeloblastosis virus reverse transcriptase (Promega) in accordance with the manufacturer's directions and (ii) RNA samples prepared as described above. The primers used for extension included *speB*-20primerext and *speB*-600primerext, which were end labeled with ^{32}P as previously described (27). Extended products were visualized by autoradiography following electrophoresis through a 6% polyacrylamide-urea sequencing gel, and the 5′ ends were determined by comparison to a sequencing reaction generated with a modified T7 DNA polymerase (Sequenase 2.0; Amersham), the same end-labeled primer and a plasmid template that contained a DNA segment that encompassed the entire *speB*-*ropB* intergenic region.

Transcript analysis by RT-PCR. *S. pyogenes* cultures were grown in C medium; aliquots were harvested at 3, 5, 7, and 24 h; and total RNA was isolated as described above. First-strand cDNA synthesis was performed with SuperScript II reverse transcriptase (Invitrogen) with 5 μg of RNA, 250 ng of random hexamers as primers (Invitrogen), 0.5 mM deoxynucleoside triphosphate mix, 10× reverse transcription (RT) buffer, 5 mM MgCl₂, and 10 mM dithiothreitol in accordance with the manufacturer's directions. With 2 μl of the cDNA reaction mixture, specific transcripts were detected with primer pairs that amplified an internal segment of the target cDNAs, including *ropB* (*ropB*-intergene and *ropB*-stop-*Bam*HI), *speB* (*speB*-3′-fwd and *speB*-3′-rev), and the housekeeping gene *glnQ* (*glnQ*-5′ and *glnQ*-3′). To ensure that no contaminating DNA was present in the RNA samples and that all cDNA synthesis was derived from RNA templates,

TABLE 1. Primers used in this study

Name	Sequence[a]
speB-20primerext	CTGCATTTGCTTTTAACGCTAC
speB-600primerext	GTTTGTCAATGCTTTTATTGAC
ropB intergene	GGAAGACTGTCTGACAGAGCGG
ropB-stop-*Bam*HI	CGC<u>GGATCC</u>CCTCAGGACAGTTTATGTTTAATGG
speB-3′-fwd	GGCGATTTCAGAATTGATGGCTG
speB-3′-rev	GCCACCAGTACCAAGAGCTGAAGGG
glnQ-5′	GGAATTGATGCCAAATTCTACGAAGGTG
glnQ-3′	GATAACACGGTTAGCTACTTGGCGAGC
pSPE-*speB*+180*Eco*RI	ATATCTTCTG<u>GAATTC</u>GTCAACCTGCTTTG
pSPE10-*speB*-1000*Bam*HI	GAATGCCTAAT<u>GGATCC</u>AACGGTTTCACAA
pSPE11-*speB*-800*Bam*HI	GTCATATGT<u>GGATCC</u>TTTCTAATC
pSPE12-*speB*-700*Bam*HI	GCAGCTATGAAG<u>GGATCC</u>ATAAGGTTAAAAGGAAG
pSPE13-*speB*-400*Bam*HI	GGTGTTGT<u>GGATCC</u>TATCACGATC
pSPE14-*speB*-250*Bam*HI	GATGATAAGT<u>GGATCC</u>ACTCATAGCGTC
pSPE15-*speB*-20*Bam*HI	GTACCGTTAAAAG<u>GGATCC</u>GCAGTAG
pSPE17-700*ropB*-*Xho*I	CCTTATGGC<u>CTCGAG</u>ATAGCTGC
pSPE17-inverse-250*speB*-*Xho*I	GATGAGAATA<u>CTCGAG</u>TTGGGTTGTC
pROP-*ropB*+100*Eco*RI	GTTTTTT<u>GAATTC</u>CTAATCTATTCAACGG
pROP10-*speB*360*Bam*HI	CTTCTGGCTTC<u>GGGATCC</u>TGCTTTG
pROP11*ropB*1600*Bam*HI	CGATGTCGC<u>GGATCC</u>CATGAATGGTAATAG
pROP12-*ropB*1400*Bam*HI	GCTAGGCAGAC<u>GGATCC</u>GCAAGATCAGC
pROP13-*ropB*1200*Bam*HI	CCATTAGTTGACTCGTA<u>GGATCC</u>TCCCTGG
pROP14-*ropB*1000*Bam*HI	CCTTTTAACCTTAT<u>GGATCC</u>ATCATAGCTGC
pROP15-*ropB*900*Bam*HI	CATATGACAGT<u>GGATCC</u>AACTATCGCATCTG
5′-*ropB*-IFD	AAAA<u>CTGCAG</u>GGGAGGTCGTGACCAAAAAGGCGGC
3′-*ropB*-IFD	AAAA<u>CTGCAG</u>GGGAGGTCGTGACCAAAAAGGCGGC
ropB-5′-rev-IFD	TCC<u>CCCGGG</u>TTCACCAATTTCCATATGTCAAGC
ropB-3′-fwd-IFD	TCC<u>CCCGGG</u>ATTAAACATAAACTGTCCTGAGGC
rofA-5′-*Bgl*II	GGA<u>AGATCT</u>CATATATGAGCTTACCACG
rofA-3′-*Bam*HI	CGC<u>GGATCC</u>AGTTCCTCACAATAATGG
ropB-5′-RBS-*Bam*HI	CGC<u>GGATCC</u>GACATCAACTAGGAAGGC
ropB-stop-*Pst*I	AAAA<u>CTGCAG</u>CCTCAGGACAGTTTATGTTTAATGG
ropB-5′-*Sma*I	TCC<u>CCCGGG</u>ATGGAAATTGGTGAAACCG
5′-Upstream-pro	CAAGCCTTCCTAGTTGATGTC
3′-Upstream-pro	GTCAATGCTTTTATTGACTTCTC
5′-Downstream-pro	GAGAACGGTCCCATATCATC
*speB*pro-20rev	TCC<u>CCCGGG</u>GCTGCATTTGCTTTTAACGGTAC

[a] Underlined sequences denote restriction site incorporated into primer.

PCR amplification was performed on RNA samples in the absence of the first-strand cDNA synthesis reaction.

Analysis of promoter structure with reporter fusions. Reporter fusions were constructed with a gene encoding an alkaline phosphatase that has been modified for secretion (PhoZF) (18). Regions of interest were amplified from chromosomal DNA templates by PCR and inserted between the *Eco*RI and *Bam*HI sites of the *phoZF*-containing *E. coli*-streptococcal shuttle plasmid pAGB5 (18). All amplifications of the *speB* promoter fragments used primer *speB*+180*Eco*RI and one of the following primers (the 5′ end of the resulting fragment is in parentheses): *speB*-1000*Bam*HI (full length), *speB*-800*Bam*HI (−792), *speB*-700*Bam*HI (−700), *speB*-400*Bam*HI (−397), *speB*-250*Bam*HI (−166), and *speB*-20*Bam*HI (−43). The plasmids containing these fragments were designated pSPE10 to pSPE15, respectively (see Fig. 2). An internal deletion was constructed by inverse PCR with plasmid pSPE10 (see Fig. 2) and primers 700*ropB*-*Xho*I and inverse-250*speB*-*Xho*I. The resulting plasmid (pSPE17) contains a deletion between bp −700 and −166 (see Fig. 2). All amplifications of *ropB*

promoter fragments used primer *ropB*+100*Eco*RI and one of the following primers (the 5′ end of the resulting fragment is in parentheses): *speB*360*Bam*HI (full length), *ropB*1600*Bam*HI (−827), *ropB*1400*Bam*HI (−654), *ropB*1200*Bam*HI (−480), *ropB*1000*Bam*HI (−238), and *ropB*900*Bam*HI (−127). The plasmids containing these fragments were designated pROP10 to pROP15, respectively (see Fig. 3). Transformation of HSC5 with pSPE10 to pSPE17 and pROP10 to pROP15 by electroporation produced strains that were designated SPE100 to SPE107 and ROP100 to ROP105, respectively. For analysis of reporter activity, strains were cultured as described above and secreted alkaline phosphatase activity was determined after 9 h of growth as explained elsewhere (18).

Construction of a *ropB* deletion mutant. An in-frame deletion in *ropB* was constructed with primers 5′-*ropB*-IFD and 3′-*ropB*-IFD to amplify a 1,594-bp fragment containing the entire HSC5 *ropB* open reading frame. The fragment was inserted into a standard vector, and an in-frame deletion of 807 bp was made by inverse PCR with the primers *ropB*-5′-rev-IFD and *ropB*-3′-fwd-IFD. The resulting mutant allele (*ropB*$_{\Delta6-274}$) was inserted between the *Pst*I and *Bam*HI sites of the shuttle vector pJRS233 (31) to create pDLR3, which was used to replace the wild-type *ropB* allele in HSC5 by a previously described method (34). The chromosomal structure of the resulting mutant, MNN100, was confirmed by PCR.

Construction of a *ropB* expression plasmid. A streptococcal expression vector was created with a previously described *S. pyogenes* promoter to express the *ropB* transcript as follows. The *rofA* promoter (18) was amplified by PCR with HSC5 chromosomal DNA and primers *rofA*-5′-*Bgl*II and *rofA*-3′-*Bam*HI, and the resulting fragment was inserted into the *Bam*HI site of pLZ12 (30). The wild-type allele of *ropB* was amplified by PCR with primers *ropB*-5′-RBS-*Bam*HI and *ropB*-stop-*Pst*I, and the restriction sites introduced into the primers were used to insert the resulting fragment between the *Bam*HI and *Pst*I sites downstream of the *rofA* promoter segment, creating pMNN23. The sequence of the inserted fragments was confirmed, and then pMNN23 was used to transform the wild type (HSC5) and the *ropB* mutant (MNN100) for the analyses described later in the text.

Protease activity assays. Analysis of SpeB proteolytic activity was determined by measurement of zones of clearance on skim milk agar medium following growth at 37°C in an anaerobic environment or in cell-free supernatants with the substrate fluorescein isothiocyanate-casein as described previously (21). Uninoculated C medium was used to determine background values, and proteolytic activity is presented as relative to the activity of wild-type strain HSC5 at 20 h. Cell numbers were adjusted to that of HSC5 for each time point tested. For selected samples, inclusion of the cysteine protease inhibitor E64 (Sigma) (27) confirmed that typically >95% of the proteolytic activity was due to SpeB.

Construction of MBP-RopB. A fusion protein joining maltose-binding protein (MBP) and RopB was constructed as follows. The *ropB* open reading frame was amplified by PCR from HSC5 genomic DNA with primers RopB-5′-*Sma*I and *ropB*-stop-*Pst*I. The resulting fragment was digested with *Sma*I and *Pst*I (sites in primers) and inserted between the *Xmn*I and *Pst*I sites of pMAL-c2 (New England Biolabs), which produced an in-frame fusion of the entire *ropB* coding sequence to the 3′ end of the *malE* gene encoded on the vector. The fidelity of the construct was confirmed by DNA sequencing, and the resulting plasmid was designated pMAL-*ropB*. Cell lysis and protein purification by amylose affinity chromatography were performed in accordance with the manufacturer's (New England Biolabs) specifications. Purified protein was concentrated with Centricon YM-10 centrifugal filter units (Millipore), and concentrations were determined by the bicinchoninic acid method with bovine serum albumin as the standard (37). Electrophoresis through sodium dodecyl sulfate-polyacrylamide gels and staining with Coomassie blue confirmed the expected size of MBP-RopB (~74 kDa) and that preparations were typically >95% pure.

DNA-binding studies. Probes for DNA-binding studies were generated by PCR amplification from HSC5 chromosomal DNA and biotinylated with a commercial DNA biotinylation kit (DNADetector; KPL). Probe 1 is 345 bp long and was synthesized with primers 5′-Upstream-pro and 3′-Upstream-pro. Probe 2 is 319 bp long and was synthesized with primers 5′-Downstream-pro and SpeBpro-20rev. The chromosomal regions corresponding to the probes are shown in Fig. 4A. Electrophoretic mobility shift assays were performed under the following conditions. A constant amount of labeled and gel-purified probe (5 ng) was mixed with increasing amounts of purified MBP-RopB protein (6.75 to 13.5 pmol) in reaction mixtures containing 20 mM HEPES (pH 7.2), 1 mM EDTA, 10 mM KCl, 10 mM MgCl$_2$, 1 mM dithiothreitol, 5% glycerol, and 500 ng of poly(dI-dC) in a total volume of 20 µl. A 20-fold excess of unlabeled probe was added to selected reaction mixtures. Following incubation at room temperature for 45 min, samples were run on a nondenaturing Tris-borate-EDTA 10% polyacrylamide gel and then transferred to a nylon membrane (Biodyne B; Pall) by alkaline transfer in accordance with the manufacturer's (KPL) directions.

Chemiluminescent detection of biotinylated DNA on membranes was accomplished with a commercial reagent (DNADetector; KPL), followed by exposure to X-ray film.

RESULTS

Analysis of the *speB* promoter region. The genes encoding SpeB and RopB are located adjacent to one another on the streptococcal chromosome but transcribed in opposite directions (Fig. 1A). An interesting feature of this locus is a long 941-bp intergenic region between *speB* and *ropB* that does not contain any long open reading frame. The region does contain numerous small open reading frames of 56 or fewer codons, and most annotations of the genomic information available for several *S. pyogenes* strains recognize only a single small open reading frame of 56 residues (13) (e.g., Spy2040 of strain SF370). The importance of any feature of the intergenic region is unknown. Previous data demonstrated that two distinct transcripts could be detected by a *speB*-specific probe in a Northern blot analysis of RNA isolated from *S. pyogenes* HSC5 (27). To further define the regions involved in control of *speB* expression, the ends of these two transcripts were identified by primer extension with primers that annealed to various sequences within the intergenic region. Extension products that terminated at two distinct nucleotides were consistently obtained (the products obtained by extension of two representative primers are shown in Fig. 1B). These transcript ends were located 578 bp apart at positions −697 and −137 relative to the first nucleotide of the initiation codon of the *speB* open reading frame. The loci corresponding to the distal and proximal transcript ends were designated P$_1$ and P$_2$, respectively (Fig. 1C). Regions similar to the consensus −10 element of a σ^{70} promoter were located upstream of P$_1$ and P$_2$, although regions similar to a consensus −35 sequence are not present in either promoter (Fig. 1C). The P$_1$ promoter was preceded by two copies of a long nearly identical direct repeat (identical at 33 of 35 positions), and each direct repeat contained an internal 9-bp inverted repeat (Fig. 1C). An inverted repeat identical at 12 of 14 positions lies directly upstream of the P$_2$ promoter (Fig. 1C).

Sequences required for *speB* transcription. The importance of the sequence features that flank each transcript end was analyzed through the construction of fusions between the *speB* promoter regions and a reporter gene. This analysis was done with a gene that encodes a modified alkaline phosphatase (PhoZF) that was previously used for analysis of the divergently transcribed *rofA* and *prtF* promoters of *S. pyogenes* (18). The initial construct resulted from the introduction of the entire intergenic region in a position to transcribe *phoZF* in the same direction as *speB* transcription in the vector pABG5 (18) (pSPE10, Fig. 2). Following introduction into *S. pyogenes* HSC5, the pattern of alkaline phosphatase expression paralleled the pattern of expression of SpeB cysteine protease activity and characteristically initiated late in log phase, just prior to the entry of the culture into the stationary phase of growth (data not shown). Truncations of the intergenic region were then analyzed after 9 h of growth, in comparison to the full-length region, to determine the minimal required sequence for transcription of *speB*. A truncation that terminated 95 bp upstream of P$_1$ retained high levels of activity, although they were
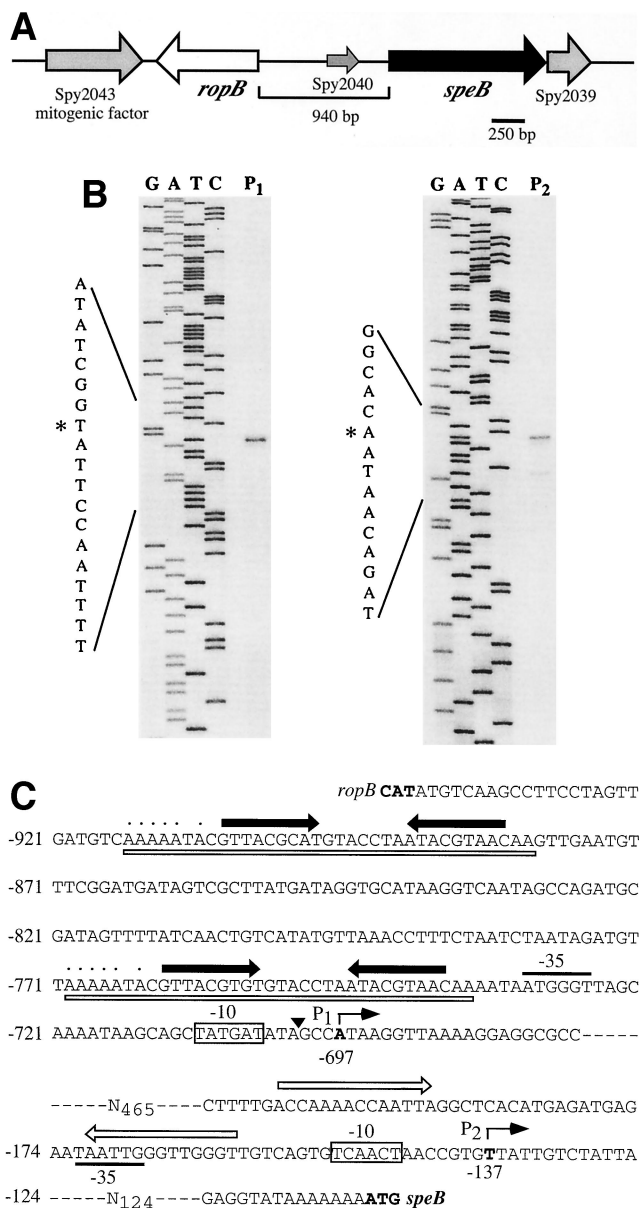
FIG. 1. Identification of the 5′ ends of the *speB* transcripts. (A) Organization of the *ropB-speB* region on the streptococcal chromosome. Gene designations are based on the genome sequence of *S. pyogenes* SF370 (18). (B) Primer extension analyses to identify the 5′ ends of the two transcripts previously determined for *speB*. The primer extension products obtained for the longer (P$_1$) and shorter (P$_2$) transcripts are shown. The sequencing ladder used to determine the terminal nucleotides of each transcript (denoted by an asterisk) are shown at the left of the extension products. (C) Features of the P$_1$ and P$_2$ promoters. The initiation nucleotides of the P$_1$ and P$_2$ promoters are in bold and marked by the bent arrow above the sequence. The numbers below the nucleotides and at the left of the sequence indicate nucleotide positions relative to the first nucleotide of the *speB* start codon. The closed arrows above the sequence show the locations of an inverted repeat present in two copies near P$_1$, and the open boxes beneath the sequence indicate a large direct repeat. Nucleotides that encode a poly(U) tract adjacent to the inverted repeats on the strand opposite to the one shown are indicated by the dotted line above the sequence. The poly(U) tract and repeats have the characteristics of a rho-independent terminator for transcripts encoded on the opposite strand.
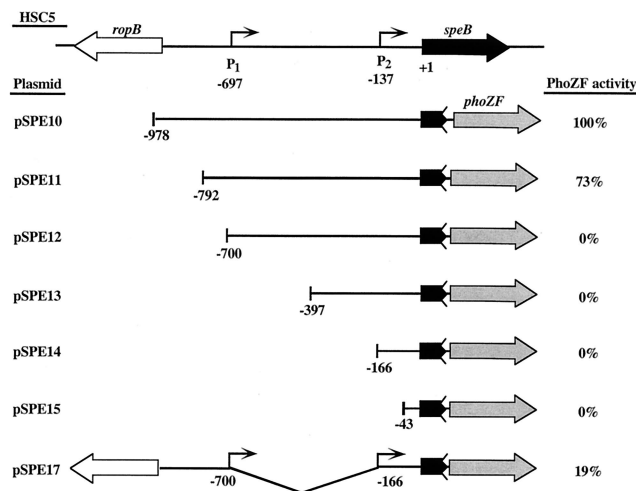

FIG. 2. Analysis of the *speB* promoter. The importance of sequences in the *speB-ropB* region for transcription of the *speB* promoter were evaluated with fusions to the reporter gene *phoZF*. The alkaline phosphatase activities produced by various truncations of the intergenic regions are shown relative to the activity produced from the full-length intergenic region (pSPE10). One hundred percent activity for pSPE10 was 125 U, calculated as enzyme units per unit of optical density at 600 nm as described by Brinkman and Beckwith (6). The number at the left end of each truncation indicates the 5′-terminal nucleotide relative to the first nucleotide of the *speB* start codon. Bent arrows represent 5′ ends of the *speB* transcripts identified by primer extension. The zigzag line after *speB* in each fusion construct indicates that transcription and not translation fusions to *phoZF* were constructed. Construct pSPE17 has an internal deletion that is shown by the connecting line below the line illustrating the intergenic sequence.

reduced about 25% relative to the activity of the full-length construct (compare pSPE11 to pSPE10, Fig. 2). In contrast, a truncation that removed the putative −10 and −35 regions of P$_1$ resulted in complete loss of transcriptional activity, despite the fact that P$_2$ was unaltered (pSPE12, Fig. 2). Additional P$_2$-containing constructs also demonstrated no activity (pSPE13 and pSPE14, Fig. 2), as did a construct that lacked both P$_1$ and P$_2$ (pSPE15, Fig. 2). The observed drop from high to background levels of activity between truncations at −792 and −700 suggested that the intervening 92 bp contain sequences essential for expression of *speB*. An internal deletion of the region between P$_1$ and P$_2$ (−700 to −166 bp) reduced activity even though the region upstream of P$_1$ remained intact (pSPE17, Fig. 2). Taken together, these data suggest (i) that the entire extensive intergenic region is important in *cis* for transcription of *speB*; (ii) that P$_1$ represents the principal promoter of *speB* and is controlled by elements, which may include the direct repeats, in sequences upstream of the −35 region; (iii) that P2 is not an autonomous promoter; and (iv) that important *cis*-acting control regions for *speB* transcription are located adjacent to the *ropB* open reading frame.

Identification of the regions required for *ropB* transcription. The proximity of *speB* control regions to *ropB* suggests that the *speB* and *ropB* promoters are oriented such that they oppose each other. However, despite the use of numerous primers, all attempts to obtain an extension product to define the end of the *ropB* transcript were unsuccessful, possibly because of secondary structure generated by interactions between the exten-
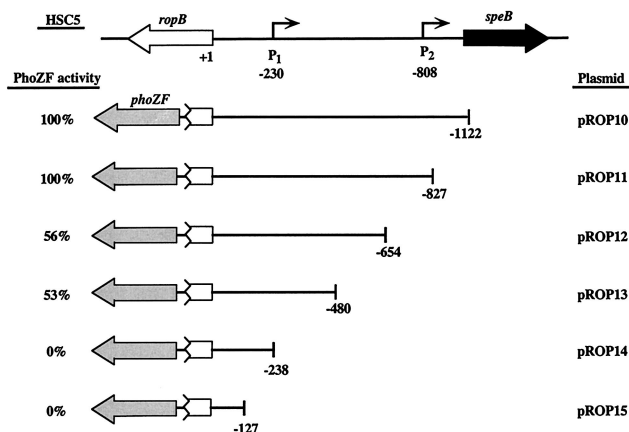
FIG. 3. Analysis of the *ropB* promoter. The importance of sequences in the *speB-ropB* region for transcription of the *ropB* promoter was evaluated as described in the legend to Fig. 2. The number at the right end of each truncation indicates the 5′-terminal nucleotide relative to the first nucleotide of the start codon for *ropB*. Bent arrows represent 5′ ends of the *speB* transcripts and are numbered relative to the start codon of *ropB*. Alkaline phosphatase activity is shown as a percentage of that produced by the full-length intergenic region (pROP10). One hundred percent activity for pROP10 was 32 U, calculated as enzyme units per unit of optical density at 600 nm as described by Brinkman and Beckwith (6). All other designations are the same as those described for Fig. 2.

sive repetitive sequences in this region. Thus, to define the region that controls transcription of *ropB* and to determine the relative distance between the *ropB* and *speB* promoters, the region upstream of *ropB* was analyzed with fusions to the *phoZF* reporter (Fig. 3). When introduced so that the promoter for *ropB* would direct transcription of *phoZF*, the entire intergenic region and a truncation to approximately the location of P_2 produced equivalent activities (pROP10 and pROP11, Fig. 3). However, truncation to −654 bp relative to the start of *ropB* reduced activity to about half of maximum (pROP12, Fig. 3) and this level was unaffected by the removal of an additional 174 bp (pROP13, Fig. 3). All further truncations reduced activity to background values (pROP14 and pROP15, Fig. 3). These data indicate (i) that, similar to that of *speB*, transcriptional control of *ropB* requires extensive sequences in *cis* that extend up to 800 bp upstream of *ropB*; (ii) that elements essential for control of *ropB* reside in the vicinity of P_1 between 238 and 480 bp upstream of *ropB*; and (iii) that it is highly likely that the *ropB* and *speB* P_1 promoters oppose each other.

RopB binds specific sequences in the *ropB-speB* intergenic region. Numerous members of the Rgg family of regulators have been shown to be essential activators of transcription of their target genes. However, these regulators lack homology to any other known class of transcription activator, and how they function to regulate transcription is unknown. For example, it has not been shown whether these regulatory proteins have DNA binding activity. In order to determine if RopB can bind DNA, the protein was overexpressed and purified from *E. coli* as a fusion to MBP (MBP-RopB; see Materials and Methods). The ability of MBP-RopB to bind to the *speB-ropB* intergenic region was examined with an electrophoretic mobility shift assay. Reactions between a 345-bp probe that included the

sequences flanking P_1 that were shown above to be critical for expression of *speB* (probe 1, Fig. 4A) and MBP-RopB resulted in decreased mobility of the probe compared to the mobility of the probe alone, and the amount of DNA in the shifted band was dependent on the concentration of MBP-RopB in the reaction mixture (compare lane 1 with lanes 2 and 3, Fig. 4B). In contrast, a second probe derived from sequences that included P_2 (probe 2, Fig. 4A) showed little to no decreased mobility when MBP-RopB was added to the reaction mixture (compare lane 5 to lanes 6 and 7, Fig. 4B). The addition of an
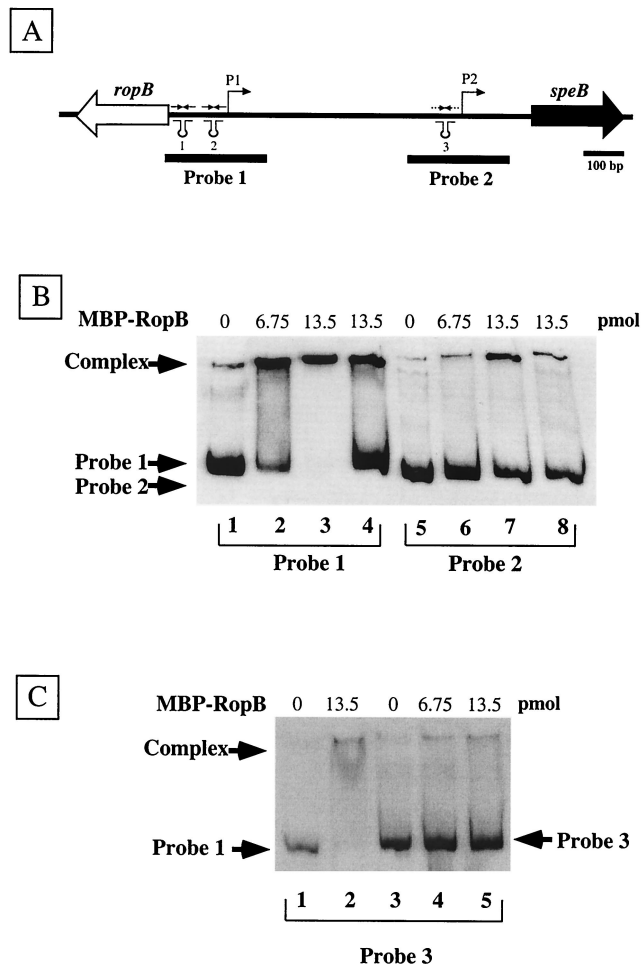

FIG. 4. RopB binds to sequences in the vicinity of the *speB* P_1 promoter. (A) The DNA contained on two labeled probes encompassing sequences in the vicinity of the P_1 and P_2 promoters is shown by the solid bars under the chromosome. (B) The ability of MBP-RopB to bind to the probes was evaluated in an electrophoretic mobility shift assay. Lanes 1 to 4 contain 5 ng of probe 1 incubated with no protein (lane 1) or with 6.75 and 13.5 pmol of MBP-RopB (lanes 2 and 3, respectively). Lanes 5 to 8 contain 5 ng of probe 2 incubated with no protein (lane 5) or with 6.75 and 13.5 pmol of MBP-RopB (lanes 6 and 7, respectively). A 20-fold excess of unlabeled probe 1 or 2 was added to reaction mixtures with 13.5 pmol of MBP-RopB analyzed in lanes 4 and 8, respectively. (C) Ability of MBP-RopB to bind to an unrelated fragment outside of the *speB-ropB* intergenic region. Lanes 1 and 2 contain 5 ng of probe 1 with no protein (lane 1) and 13.5 pmol of MBP-RopB (lane 2). Lanes 3 to 5 contain 5 ng of probe 3 incubated with no protein (lane 3) or with 6.75 and 13.5 pmol of MBP-RopB (lanes 4 and 5, respectively).
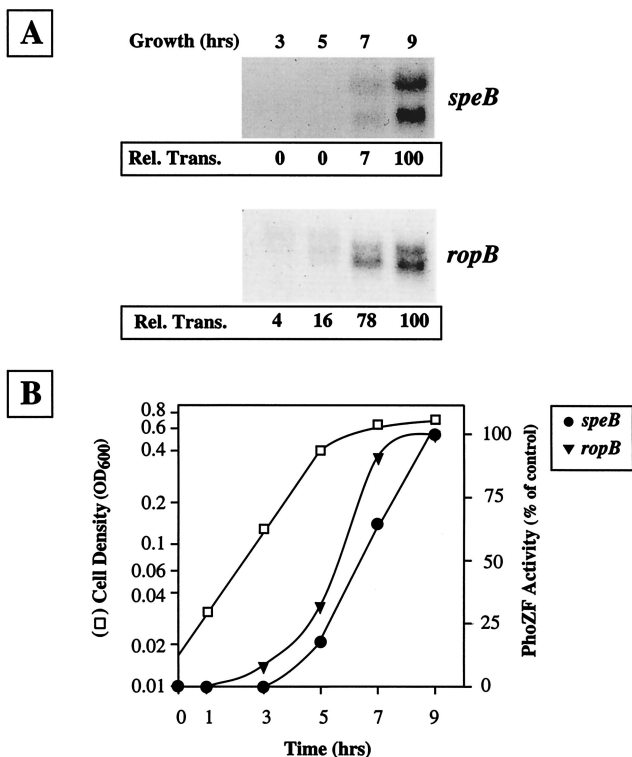
FIG. 5. Kinetics of *ropB* and *speB* expression. (A) Northern blot analysis of total RNA harvested at the indicated time points during growth. Transcripts for the two genes were visualized with either *ropB*-specific or *speB*-specific probes. The value for relative transcription (Rel. Trans.) listed under each lane was determined by comparison to the same RNA sample hybridized with a probe for the *recA* transcript. (B) Expression kinetics evaluated with reporter fusions. Activity of the PhoZF reporter in HSC5 strains containing a fusion of the *speB* and *ropB* promoters contained in the full-length intergenic region (pSPE10 and pROP10, respectively) was determined relative to the cell density of the culture as shown. Activity is presented relative to the maximal activity obtained in early stationary-phase cultures and is normalized for the density of the culture. OD_{600}, optical density at 600 nm.

only detected at later, rather than earlier, time points during culture growth. However, expression of *ropB* appeared to initiate at an earlier time point than that of *speB* (compare *ropB* and *speB* at 7 h, Fig. 5A). The kinetics of *ropB* expression was confirmed with *phoZF* fusions containing the entire intergenic region in *speB*- and *ropB*-specific orientations. Consistent with data from the Northern blot analyses, expression of *ropB* promoter-dependent PhoZF activity initiated in late log phase in a pattern similar to that of *speB* promoter-dependent activity (Fig. 5B).

Role of *ropB* in growth phase control. Since *ropB* also demonstrates growth phase expression kinetics, one model proposes that the signal for expression of *speB* is channeled directly via *ropB*. In this model, the signal acts exclusively to initiate transcription of *ropB*. Once RopB is present, it acts as the sole factor required to trigger expression of *speB*. The model predicts that the major event required for *speB* expression is the time point at which *ropB* is transcribed. To test this model, a vector was established for ectopic expression of *ropB* from a promoter that is expressed in the early, rather than the late, log phase of growth. The promoter for *rofA* has this characteristic and has the additional advantage that it has been shown to be highly active when plasmid encoded under the growth conditions used in these experiments for expression of *speB* (14, 18). A plasmid was constructed that contains *ropB* under the control of the *rofA* promoter (pMNN23) and was introduced into both the wild type (HSC5) and a mutant (MNN100) that contains an in-frame deletion in *ropB*. The deletion resulted in removal of the region encoding 271 of the 281 amino acids in the internal region of the *ropB* polypeptide but left the *ropB*-*speB* intergenic promoter region intact. Consistent with previous data (8, 27), mutant MNN100 made little proteolytic activity on protease indicator plates compared to HSC5 (Δ*ropB* versus wild type, Fig. 6). Introduction of a plas-

excess of unlabeled probe 1 to a reaction mixture containing labeled probe 1 and MBP-RopB resulted in a decreased amount of labeled DNA in the shifted complex, demonstrating that binding of MBP-RopB is specific (compare lanes 3 and 4, Fig. 4B). Additional controls for specificity included that an unrelated MBP fusion protein did not bind probe 1 or 2 (data not shown) and a probe made from a different region of the streptococcal chromosome did not to bind MBP-RopB (probe 3, Fig. 4C).

Expression kinetics of *ropB* and *speB*. As noted above, *speB* is regulated in a striking growth phase pattern and transcriptional expression is dependent on *ropB*. Since the major control region for expression of *speB*, including the P_1 promoter and RopB binding sites, are located adjacent to *ropB* and the control regions for *ropB*, it was of interest to determine whether expression of *ropB* is constitutive or shows a distinct kinetic pattern. When a Northern blot analysis was used to directly compare the kinetics of expression of *speB* and *ropB*, it was found that *ropB* was expressed in a growth phase pattern that resembled that of *speB* (Fig. 5A). Similar to *speB*, a *ropB*-specific probe hybridized to two distinct bands, which were
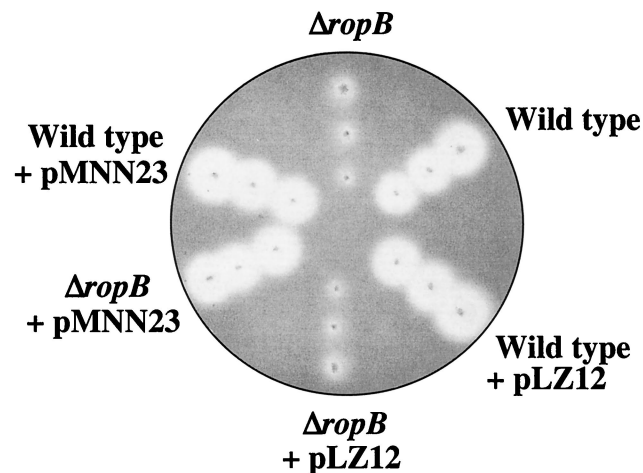

FIG. 6. Complementation of RopB deletion demonstrated by protease expression. Derivatives of *S. pyogenes* strain HSC5 (wild type) were grown on protease indicator plates overnight at 37°C in an anaerobic environment. The strains evaluated included MNN100 (Δ*ropB*), HSC5 with the vector alone (wild type + pLZ12), MNN100 with the vector alone (Δ*rop* + pLZ12), MNN100 with a *ropB*-complementing plasmid (Δ*rop* + pMNN23), and HSC5 with a *ropB*-complementing plasmid (wild type + pMNN23). Clear zones around colonies indicate protease activity.
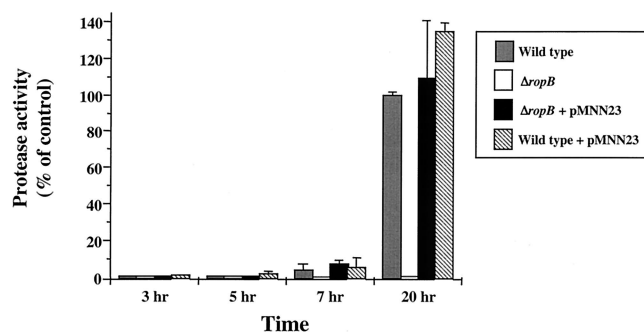
FIG. 7. Ectopic expression of *ropB* does not alter the expression kinetics of *speB*. The chimeric plasmid pMNN23 contains *ropB* under the control of a promoter expressed during the early logarithmic phase of growth. Levels of protease activity produced in various strains at the indicated time points were measured with the substrate fluorescein isothiocyanate-casein and are reported relative to the activity of the wild-type strain at 20 h. The strains analyzed included HSC5 (wild type), MNN100 (Δ*ropB*), MNN100 plus ectopically expressed *ropB* (Δ*ropB* + pMNN23), and HSC5 plus ectopically expressed *ropB* (wild type + pMNN23). The data presented represent the mean and standard error of the mean derived from seven independent experiments.

mid alone had no effect on the protease expression profiles of the mutant or HSC5 (wild type + pLZ12 and Δ*ropB* + pLZ12, Fig. 6). However, when the plasmid contained *ropB* under the control of the *rofA* promoter, the protease activity of the complemented mutant strain was indistinguishable from that of HSC5 (compare Δ*ropB* + pMNN23 to wild type and wild type + pMNN23, Fig. 6). These data show that ectopic expression of *ropB* can complement a chromosomal deletion of *ropB*. However, the protease indicator plates measure the accumulation of protease over time and are not sensitive for kinetics. Analysis of SpeB protease activity in cell-free supernatants at different time points revealed that ectopic expression of *ropB* had no influence on the pattern of expression of *speB*. Both the mutant and wild-type strains complemented with pMNN23 expressed protease activity in a pattern similar to that of the wild-type strain alone (Fig. 7). Since the protease zymogen must be activated following its secretion from the cell, it was possible that protease activity did not accurately measure expression because activating conditions are only generated following the log phase of growth. Analysis of transcript levels by RT-PCR demonstrated that this was not the case. Although not quantitative for absolute message levels, no transcripts for *speB* could be detected in wild-type and complemented wild-type and mutant strains at early time points during growth (3 and 5 h for *speB*, Fig. 8). This was despite the fact that transcripts of *ropB* could be detected at the earliest point analyzed (3 h, Fig. 8). No transcripts for *speB* or *ropB* were detected in the *ropB* mutant at any time point (Fig. 8), and samples subjected to PCR without prior treatment with reverse transcriptase failed to show a band at any time point to indicate that contamination by DNA did not influence the results (data not shown). Finally, RT-PCR with primers to detect the housekeeping gene *glnQ* demonstrated that amplifiable RNA was present in each sample (Fig. 8). Because shifting of the timing of *ropB* expression did not influence the kinetics of *speB* transcription, these data do not support growth phase control of *ropB* as the event required for expression of *speB*.

DISCUSSION

The SpeB cysteine protease of *S. pyogenes* is remarkable for its multiple layers of regulation. Not only are the secretion and processing of the protease polypeptide precursor into the active protease subject to regulation by accessory gene products, the folding and activation of the precursor following its secretion are strongly influenced by environmental factors. The expression of the gene that encodes the protease is also subject to a multilayered regulatory program that includes growth phase, nutritional, and environmental cues. How all of these factors interact to control the expression of proteolytic activity during infection of host tissue is not well understood.

Previous studies have shown that activation of transcription of *speB* has an absolute dependence on the *rgg* family regulator *ropB*. Thus, understanding how *ropB* functions is essential for understanding the biogenesis of proteolytic activity, as well as for understanding how this widely distributed novel family of regulators has been adapted for control of transcription of other genes. Consistent with the complexity of the protease regulatory program, we have found in the present study that very few alterations can be made to the extensive *ropB-speB* intergenic region without affecting protease expression. The reason that this extensive regulatory region is required is not known.

When examining the repeats near the 5′ end of the *ropB* message, both inverted repeats in the RNA are followed by a run of U nucleotides (UAUUUUU, Fig. 1C) resembling the structure of a factor-independent transcription termination signal (5). Inverted repeats may play a key role in the regulation of genes by other *rgg* family members. Both *rgg* itself and *gadR* (35, 38) are located immediately upstream of their target genes. Unlike *ropB*, *rgg* and *gadR* are located in a direct orientation relative to their regulatory targets and have very short intergenic regions. Similar to *ropB*, the intergenic regions contain prominent rho-independent terminator-like structures that overlap the promoter elements of the target genes (35, 38). It has been proposed that Rgg influences the formation of this RNA secondary structure in order to promote the transcription and/or translation of the *gftG* message. The overlap of the *ropB* and *speB* promoters and the location of the termi-
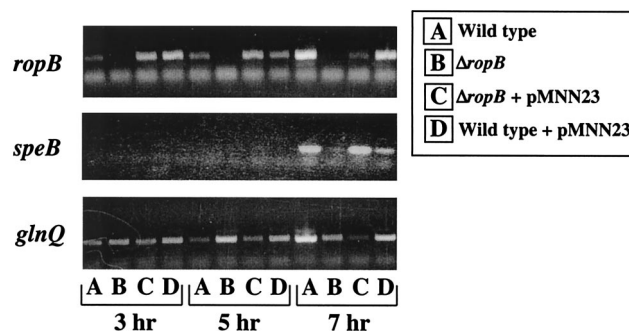

FIG. 8. Confirmation of ectopic expression of *ropB*. The presence of mRNA for *ropB* (top), *speB* (middle), and the housekeeping gene *glnQ* (bottom) was determined for cultures at the time points indicated by nonquantitative RT-PCR. The strains evaluated were identical to those listed in Fig. 7. The results shown are representative of at least three different RT-PCR assays from three separate RNA isolations.

nator structures near both promoters strongly implicate these structures in the program for control of protease expression.

Similar to that of other promoters that are subject to activation, the −10 site of the *speB* promoter is quite close to consensus (TATgAT); however, the −35 site only matches at two of the six consensus nucleotides (aTGggt). In *E. coli*, many transcriptional activators bind to a specific site next to the −35 region of the promoter and interact with polymerase in order to compensate for the poor consensus −35 region and recruit polymerase to the promoter, thereby increasing the rate of transcription of the gene (4). The direct and inverted repeat sequences occurring directly upstream of the −35 site of the *speB* promoter fit into this category of regulatory sequences and resemble the palindromic sequences that are often bound by DNA-binding proteins to activate transcription. Consistent with this was the observation that RopB bound with the highest affinity to a DNA fragment that contained the repetitive region adjacent to P_1.

The closest relative of *ropB*, *lasX*, regulates the expression of an operon for synthesis of the lantibiotic lactocin S in *Lactobacillus sakei* (36). Similar to the *S. pyogenes* cysteine protease, many lantibiotic operons are regulated in a growth phase-dependent pattern. In some of these operons, developmental control involves a bacterial cell density-dependent mechanism that involves direct monitoring of the concentration of the lantibiotic peptide itself by a two-component phosphorelay. However, for other operons, including lactocin S and mutacin of *S. mutans*, regulation is under the control of an *rgg* family regulator (32, 33, 36). Other similarities between *lasX* and *ropB* include the fact that both are located adjacent to their regulatory targets but are oriented in the opposite direction. Furthermore, both have intergenic regions containing several directly repeated sequences, both have promoters that overlap those of their regulatory targets, and lastly, regulation of both of their target genes is influenced by environmental cues such as pH (28). One difference is that, unlike that of *speB* and many other lantibiotics, expression of lactocin S is not subject to growth phase regulation (36). Nevertheless, the fact that *ropB*, *lasX*, and other members of the *rgg* family have many characteristics in common suggests that they use very similar mechanisms to interact with their regulatory programs to process information.

Regulation of *speB* is subject to both environmental and growth phase cues (7, 9, 12, 27). In the present report, we have shown that the program for expression of *speB* is not solely dependent on *ropB*, suggesting that other elements are involved. Prior studies have suggested that developmental control does not function through a mechanism that senses culture density (9) but may involve the two-component regulator *csrRS/covRS* since mutants with changes at this locus typically overexpress *speB* (22). Interestingly, *ropB* had a negative influence on transcription of a number of other virulence regulatory genes, including *csrRS/covRS* (10), suggesting a complex interplay between global regulators for control of *S. pyogenes* virulence. The details of these interactions remain to be elucidated and will be crucial for understanding the role that *speB* and other virulence factors of *S. pyogenes* contribute to interaction with the host.

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