

Dynamics of *speB* mRNA Transcripts in *Streptococcus pyogenes*

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***Streptococcus pyogenes* (group A streptococcus [GAS]) is a human-specific pathogen that causes a variety of diseases ranging from superficial infections to life-threatening diseases. SpeB, a potent extracellular cysteine proteinase, plays an important role in the pathogenesis of GAS infections. Previous studies show that SpeB expression and activity are controlled at the transcriptional and posttranslational levels, though it had been unclear whether *speB* was also regulated at the posttranscriptional level. In this study, we examined the growth phase-dependent *speB* mRNA level and decay using quantitative reverse transcription-PCR (qRT-PCR) and Northern blot analyses. We observed that *speB* mRNA accumulated rapidly during exponential growth, which occurred concomitantly with an increase in *speB* mRNA stability. A closer observation revealed that the increased *speB* mRNA stability was mainly due to progressive acidification. Inactivation of RNase Y, a recently identified endoribonuclease, revealed a role in processing and degradation of *speB* mRNA. We conclude that the increased *speB* mRNA stability contributes to the rapid accumulation of *speB* transcript during growth.**

Streptococcus pyogenes (group A streptococcus [GAS]) is a Gram-positive pathogen that causes a variety of human diseases. GAS infections range from mild superficial infections, such as pharyngitis and impetigo, to life-threatening systemic diseases, such as toxic shock syndrome and necrotizing fasciitis (15). GAS also plays a significant role in the development of poststreptococcal infection sequelae, including acute rheumatic fever, acute glomerulonephritis, and reactive arthritis (15). The pathogenesis of GAS infection involves a complex host-pathogen interaction in which the streptococcal proteinase SpeB (streptococcal pyrogenic exotoxin B) plays a crucial role (47).

SpeB is a secreted cysteine proteinase with a broad spectrum of activities. SpeB cleaves human extracellular matrix proteins, such as fibrin, fibronectin, vitronectin, and matrix proteoglycans (16, 21, 41), and degrades human immunoglobulins (13, 14) and inflammatory mediators, such as complement factor C3b (46) and cathelicidin LL-37 (19). In addition, SpeB cleaves *S. pyogenes* surface proteins, releasing C5a peptidase and M protein (8). These observations indicate that SpeB can facilitate bacterial dissemination and survival and induce inflammation and tissue damage in the host. Clinical observations and animal experiments have clearly demonstrated the importance of SpeB in the pathogenesis of GAS infection. Accordingly, it has been observed that SpeB was abundantly present in necrotic human tissue (19) and that a decreased SpeB proteinase activity led to reduced tissue damage in a primate model for necrotizing fasciitis (36).

Since SpeB is an important virulence factor in GAS infection, it is not surprising that SpeB production is tightly regulated. Under laboratory conditions, the SpeB proteinase is usually not detected during early and mid-exponential growth phases, but it becomes highly abundant when the culture reaches late exponential and stationary phases (11, 38). SpeB production is strongly affected by culture pH and nutrient availability; for example, the optimal pH for SpeB synthesis ranges from pH 6.0 to pH 6.5 (11, 12, 28, 35), and supplementation of glucose or peptides in the growth medium usually inhibits production of the proteinase (11, 12, 38). Molecular biological studies show that SpeB is controlled at both the transcriptional and posttranslational levels. The transcription of SpeB is repressed by CovR/S (17) and Srv (39) and is activated by Rgg, which is also referred to as RopB (30), CcpA (22), and Mga

(40). Among these regulators, RopB is essential for *speB* transcription by binding to the promoter region and facilitating transcription initiation (4, 10, 29, 33). It has yet to be determined whether *speB* is regulated at the posttranscriptional level.

The steady-state level of mRNA is determined by both transcript synthesis and degradation. Traditionally, the regulation of mRNA decay has been considered insignificant in prokaryotes, though this opinion has slowly changed, as a growing body of literature suggested that the regulation of mRNA turnover is widely distributed in many bacterial species (5). Barnett et al. (7) reported that the expression of certain “growth phase-dependent” genes, such as *sagA* and *sda* (encoding streptolysin S and streptodornase, respectively), were primarily regulated at the mRNA decay level, indicating an important role of posttranscriptional regulation on *S. pyogenes* virulence. These transcripts were more abundant in stationary phase than in exponential phase, mainly because their stability increased dramatically in the stationary phase (7). It was later found that ribonucleases J1 and J2 were involved in the decay process of these genes (9). Additionally, the mRNAs of prominent genes (*mga*, *covR*, and *ska*) have been shown to exhibit widely differing half-lives (45).

The aim of this study was to determine how *S. pyogenes* regulates SpeB at the posttranscriptional level leading to the rapid accumulation of *speB* transcripts during growth. By combining Northern blot analysis and quantitative reverse transcription-PCR (qRT-PCR), we observed that *speB* mRNA stability increased gradually during exponential growth and that the mRNA degradation process was pH dependent. RNase Y (encoded by *cvfA*), a recently identified endoribonuclease of *S. pyogenes* (20), is involved in *speB* mRNA processing and degradation, but other yet unidentified nucleases are also required. We conclude that the

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TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
Bacterial strains		
<i>S. pyogenes</i>		
NZ131	Wild type	32
Δ speB mutant	speB mutant strain	This study
Δ pnpA mutant	pnpA mutant strain	This study
Δ acpA mutant	acpA mutant strain	This study
Δ cvfA mutant	cvfA mutant strain	This study
Δ cvfA + pDL278::cvfA	Δ cvfA mutant strain carrying pDL278::cvfA	This study
<i>E. coli</i> DH5 α	Cloning strain	New England Biolabs
Plasmids		
pDL278	Shuttle vector	24
pDL278::cvfA	pDL278 carrying a gene encoding cvfA	This study

increased *speB* mRNA stability contributes to the rapid accumulation of *speB* transcript during growth.

MATERIALS AND METHODS

Bacterial strains and growth condition. Bacterial strains used in this study are listed in Table 1. *S. pyogenes* NZ131 (serotype M49) was routinely grown in C medium (0.5% proteose peptone 3, 1.5% yeast extract, 10 mM K₂HPO₄, 0.4 mM MgSO₄, 17 mM NaCl) (30) at 37°C without aeration. Erythromycin and spectinomycin, when required, were added at a final concentration of 2 μ g/ml and 100 μ g/ml, respectively.

Total RNA extraction, cDNA synthesis, and real-time PCR analysis. Overnight cultures of *S. pyogenes* were diluted 1:40 in fresh C medium and grown at 37°C to the desired growth phase. Streptococcal cells were harvested by centrifugation (5,000 \times g, 5 min, 4°C). Cell pellets were resuspended in TRIzol (Invitrogen) and stored at -80°C. To isolate RNA, cells were disrupted three times for 30 s each time using lysing matrix B (MP Biomedicals, Solon, OH) in a FastPrep FP210 homogenizer (Thermo Scientific) (speed setting of 6.5). Total RNA isolation was carried out according to the manufacturer's instructions (isolation of total RNA using TRIzol, Invitrogen). RNA samples were treated with Turbo DNase (Ambion) to remove traces of chromosomal DNA. RNeasy MiniElute cleanup kit (Qiagen) was used to purify RNA samples after DNase treatment. cDNA was synthesized from 1 μ g of total RNA by using the iScript cDNA synthesis kit (Bio-Rad). Real-time PCR was performed as described previously (2, 31). Briefly, the relative amounts of gene-specific cDNA were quantified by the comparative threshold cycle (C_T) method using the Bio-Rad (Hercules, CA) MyiQ real-time PCR detection system with fluorescein-spiked SYBR green as the fluorophore. The primers were designed to have a melting temperature between 59.5°C and 60.5°C and to amplify 110- to 115-bp fragments. The amplification efficiency was between 90% and 110%, which was determined by analysis of the standard curve of real-time PCR with template dilution over 5 orders of magnitude. The C_T value of each gene of interest (C_{T_goi}) was normalized against the C_T value of the 16S rRNA (C_{T_16S}) ($\Delta C_T = C_{T_goi} - C_{T_16S}$). The 16S rRNA was used as the endogenous reference, because its abundance was consistent from early exponential phase to early stationary phase (data not shown). The relative abundance (RA) of each gene compared to that of 16S rRNA was calculated using the equation $RA = 2^{-\Delta C_T}$.

Northern blot analysis. Total RNA without DNase treatment was used for Northern blot analysis. RNA samples were frozen and thawed no more than once to minimize degradation. One to five micrograms of total RNA was separated on a 1% agarose-0.66 M formaldehyde gel in a buffer consisting of 40 mM 3-[N-morpholino]propanesulfonic acid (MOPS)

TABLE 2 Primers used in this study

Primer	Sequence
Primers for qRT-PCR	
speB_1l	GTGGAGTCTCTGACGGCTTC
speB_1r	TGCCTACAACAGCACTTTGG
ropB_1l	TGCCTTGGTCAAGGTGTT
ropB_1r	GCACAGTCTCATAGTGACTCCA
16S rRNA_1l	AAGCAACGCGAAGAACCCTTA
16S rRNA_1r	GTCTCGCTAGAGTGCCCAAC
Primers for construction of mutants	
Erm-L	CCGGGCCCCAAAATTTGTTTGTAT
Erm-R	AGTCGGCAGCGACTCATAGAAT
Erm-F(ptls)	GAAGGAGTGATTACATGAACAAAA
cvfA_P1	GGACGCTCAAAAGGTTCTCA
cvfA_P2(erm)	CAAATCAAACAAAATTTGGGCCCGG
	CCAATGAGGGCAGAAAACAAT
cvfA_P3(erm)	ATAATTCTATGAGTCGCTGCCGACT
	GAGCGGTTGATTATGCCAAG
cvfA_P4	AGCTGAAGGATCTGGGTGAA
speB_P1	GGTCAATAGCCAGATGCGGATA
speB_P2(erm)	CAAATCAAACAAAATTTGGGCCCGG
	TGATCGGCAAAATACCTGGTTA
speB_P3(erm)	ATAATTCTATGAGTCGCTGCCGACT
	CGCACTAAACCCTTCAGCTC
speB_P4	TCGAGACGAGTTTGGTGTG
pnpA_P1	CAGGTTTGGTCACAGGCTTT
pnpA_P2(erm_ptls)	TTTTTGTTCATGTAATCACTCCTTC
	GGTTTCCCTGCAAATGTTGT
pnpA_P3(erm)	ATAATTCTATGAGTCGCTGCCGACT
	CCCACAAAACCAGAGAAAA
pnpA_P4	TGAAGACTCCAGGAGCGATT
acpA_P1	AGCCATGACGCTATTGATCC
acpA_P2(erm_ptls)	TTTTTGTTCATGTAATCACTCCTTC
	GGAGACGATGCTCGTTAGC
acpA_P3(erm)	ATAATTCTATGAGTCGCTGCCGAC
	TCCCTTGCCCAACTTAGTGAG
acpA_P4	TCTCTGCTTCTTGCCACTT
cvfA_L(BamHI)	AAAAAAGGATCCCATCATGGACGAC
	TTGCTACA
cvfA_R(HindIII)	AAAAAAAAGCTTGAACATTTTCAAA
	GGCAAGTCA
Primers for DIG-labeled probe synthesis	
speB_L (probe)	CTATCAAAGCAGGTGCACGA
speB_R (probe)	TAATTTGAGCAGTTGCAGTAGCA
ropB_L (probe)	GGAAATGGGTGAAACCGTTG
ropB_R (probe)	AAACATATGATGGATCGTTTTCG

(pH 7.0), 10 mM sodium acetate, and 0.2 mM EDTA. RNA was then transferred to a Hybond-N membrane (Amersham) in a Trans-Blot SD semidry transfer cell (Bio-Rad) and immobilized to the membrane by UV cross-linking (Stratagene). Gene-specific digoxigenin (DIG) probes were PCR generated by using a PCR DIG probe synthesis kit (Roche Diagnostic). Primers used for DIG probe synthesis are listed in Table 2. Hybridization of the DIG-labeled DNA probes to RNA on the membrane and visualization of the hybrid with CDP-*Star* were carried out according to the manufacturer's instructions (DIG application manual, Roche Diagnostic). The abundance of gene transcript, represented by the averaged pixel intensity of a band with defined size, was quantified with ImageJ software (1). The amount of 23S rRNA, which was visualized by ethidium

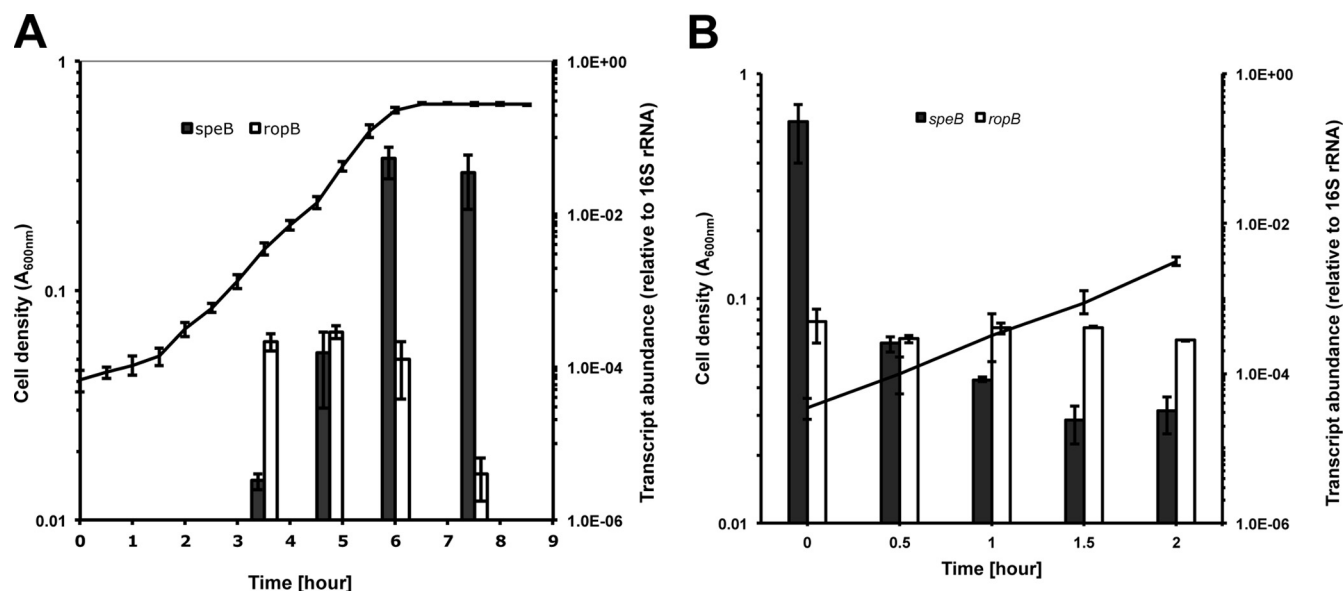


FIG 1 Changes in the levels of *speB* and *ropB* transcripts during growth. (A) *S. pyogenes* cells were continuously grown in C medium from early exponential phase to early stationary phase. (B) An aliquot of cell culture from the late exponential phase was diluted 1:20 in fresh C medium and incubated for 2 h. qRT-PCR analysis was used to determine the levels of *speB* and *ropB* transcripts at different time points. Data are presented as means \pm standard deviations (error bars) from three (A) or two (B) independent experiments.

bromide staining on an agarose gel prior to transfer, served as a loading control.

mRNA decay assay. Streptococcal cells were grown in C medium to the desired cell density. Rifampin was added to the culture to a final concentration of 1 mg/ml. Five-milliliter aliquots of the culture were withdrawn at different time points after the addition of rifampin, rapidly chilled to 0°C by mixing with 10 ml crushed ice, and harvested by centrifugation (5,000 \times g, 5 min, 4°C). The transcript abundance of a particular gene at each time point was determined by qRT-PCR or Northern blot analyses. Excel software (Microsoft) was used to perform regression analysis and to calculate mRNA decay rates.

SpeB and RNase mutant construction. Overlap extension PCR technique (44) was used to construct the *S. pyogenes* mutants shown in Table 1. Primer sequences for the construction of mutants are given in Table 2. For *speB* mutant (Δ *speB*) construction, the upstream and downstream regions of the *speB* gene were PCR amplified with primer pairs *speB*_P1/*speB*_P2(*erm*) and *speB*_P3(*erm*)/*speB*_P4, respectively. An erythromycin resistance cassette (*erm*) was PCR amplified from a shuttle vector pHS17 (18) with primer pair *Erm*-L/*Erm*-R (L stands for left, and R stands for right). The *speB*_P2(*erm*) and *speB*_P3(*erm*) primers were oligonucleotide hybrids with 5'-end sequences complementary to the *erm* cassette and 3'-end sequences complementary to the flanking regions of the *speB* gene. PCR-generated fragments of the region upstream of *speB*, the *erm* cassette, and the region downstream of *speB* were mixed at 1:1:1 molar ratio and amplified with primer pair *speB*_P1/*speB*_P4. This amplification led to the "ligation" of the three fragments in the order of 5'-*speB*_upstream-*erm*-*speB*_downstream-3'. The resulting PCR product was used to transform *S. pyogenes* NZ131 to generate the *speB* mutant. The same strategy was applied for construction of other mutants. For *pnpA* and *acpA* mutant construction, a promoterless *erm* fragment [generated by a primer pair *Erm*-F(*ptls*)/*Erm*-R (*ptls* stands for promoterless)] was used to replace the *pnpA* or *acpA* gene so that expression of the gene downstream was not affected.

Δ *cvfA* mutant complementation. A shuttle vector, pDL278 (24), was used to introduce the *cvfA* gene into the Δ *cvfA* mutant. The *cvfA* gene was PCR amplified with primer pairs *cvfA*_L(BamHI)/*cvfA*_R(HindIII). The PCR product was digested with restriction enzymes BamHI and HindIII

(Promega) and ligated to the pDL278 vector that was similarly digested. The resulting plasmid, pDL278-*cvfA*, was introduced into *S. pyogenes* Δ *cvfA* mutant via electroporation to generate the Δ *cvfA* complemented strain (Δ *cvfA* mutant carrying plasmid pDL278::*cvfA* [Δ *cvfA*+pDL278::*cvfA*]).

SpeB proteinase activity assay. The overnight cultures of wild-type *S. pyogenes* and its derivatives were stab inoculated on a C-medium-based agar plate containing 1.5% skim milk. The plate was incubated at 37°C for 18 h in a candle jar. Caseinolytic activity results in a translucent zone around the stab site (10).

RESULTS

Increased *speB* mRNA abundance during exponential growth.

Previous studies demonstrated growth-dependent production of SpeB in *S. pyogenes*, with the highest proteinase activity detected in late exponential and stationary phases (11, 28). Furthermore, a novel peptide-mediated regulatory system has recently been shown to be involved in expression control of *speB* in late exponential and early stationary phase (43). In this study, we examined the level of *speB* transcript during bacterial growth with qRT-PCR. An overnight culture of *S. pyogenes* was diluted 1:100 in C medium and incubated at 37°C, and bacterial samples were withdrawn at different growth phases (Fig. 1A). The *speB* transcript level was extremely low in early exponential phase ($A_{600} = 0.15$). It increased by 50-fold from early to mid-exponential phase ($A_{600} = 0.3$) and increased by 340-fold from mid- to late exponential phase ($A_{600} = 0.6$). The overall *speB* transcript abundance increased by over 10,000-fold from early to late exponential phase. After the cells entered the stationary phase, the *speB* transcript abundance slightly decreased but was still considerably higher than in the early and mid-exponential phases. We also examined the transcription profile of the *ropB* gene, which encodes a positive transcriptional regulator of *speB* (33). The *ropB* gene was expressed at a constant level during exponential growth and dimin-

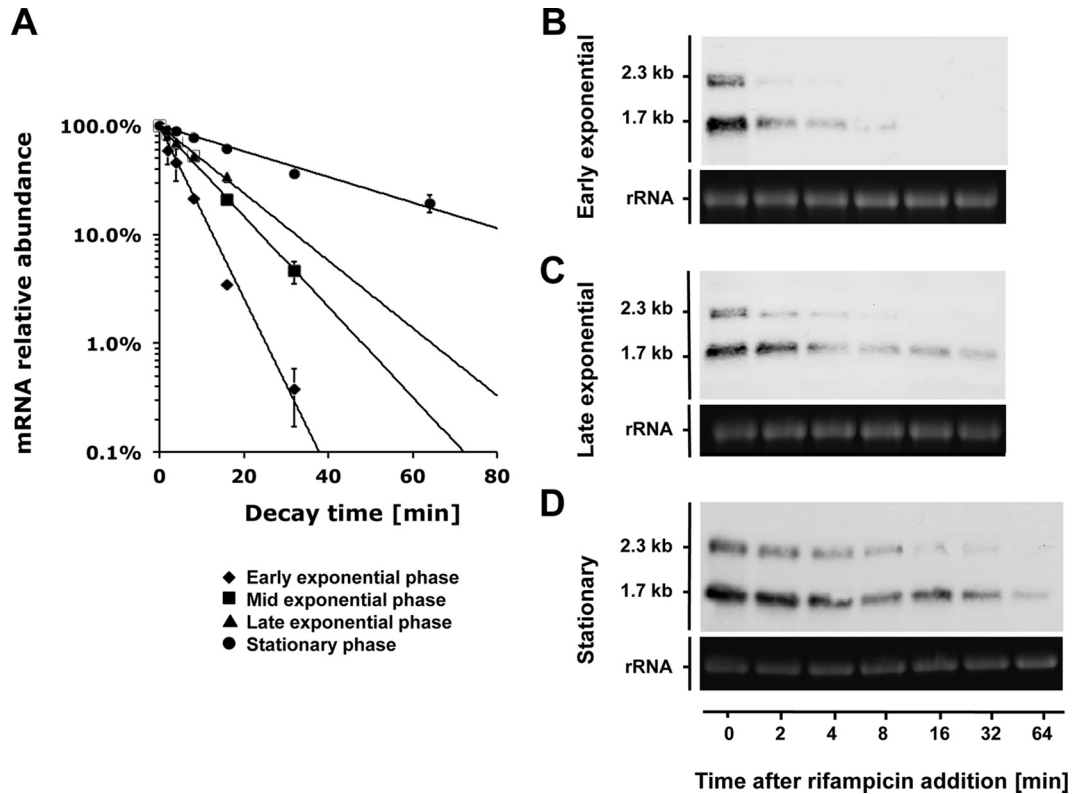


FIG 2 Growth phase-dependent *speB* mRNA decay. Decay rates of *speB* mRNA in different growth phases were determined by qRT-PCR (A) and Northern blot analyses (B, C, and D). Note that to analyze *speB* mRNA decay in early exponential phase, we used stationary culture that was diluted in fresh medium to mimic the condition (see Results for details). qRT-PCR data are presented as means \pm standard deviations (error bars) from two independent experiments. For Northern blot analysis, 23S rRNA was used as a loading control. One microgram of total RNA was loaded per lane.

ished quickly upon entering stationary phase (Fig. 1A). These observations suggested that *speB* and *ropB* transcripts were regulated by distinct mechanisms.

Our data showed a rapid increase of *speB* transcript abundance when cells grew from early to late exponential phase. Correspondingly, one would expect a rapid decrease of *speB* transcript abundance when cells are diluted to simulate a low-cell-density environment. To confirm this hypothesis, we grew *S. pyogenes* cells until late exponential phase and diluted the culture 1:20 in fresh prewarmed medium so that the cells reentered the early exponential phase. qRT-PCR analysis detected a rapid decrease of the *speB* transcript level immediately after dilution (Fig. 1B). The most significant change occurred within the first 30 min, when the total amount of *speB* transcripts decreased by almost 1,000-fold. After that, the *speB* transcript level continued to decrease and became 10,000-fold less than that of the inoculum after 1.5 h. In contrast, the *ropB* transcript remained at a constant level throughout the incubation. These data clearly show that *S. pyogenes* can accumulate or specifically destroy a large amount of *speB* transcripts over a short period of time and suggest that *speB* and *ropB* mRNA degradation is regulated by distinct mechanisms.

Increased *speB* mRNA stability during growth. We initially hypothesized that the *speB* mRNA stability might increase during exponential growth, which might contribute to the rapid accumulation of *speB* mRNA from early to late exponential phase. To test this hypothesis, *speB* mRNA stability was measured in different growth phases with Northern blot analyses and qRT-PCR.

Northern blot analysis is a standard method for mRNA stability assays (3). It allows detection of differently sized transcripts from the same gene. Since each *speB* and *ropB* mRNA species has two transcripts of different sizes (33), Northern blot analysis makes it possible to measure the decay rate of each individual transcript. However, signal detection and quantification become difficult when the target mRNA level is very low. To circumvent the problem of low mRNA abundance, qRT-PCR detection was employed because of its high sensitivity, broad dynamic range, and direct quantitative measurement and because several studies have confirmed the validity of qRT-PCR in mRNA decay analysis (25, 34, 42). In this study, qRT-PCR primers were designed to target the protein-encoding regions of *speB* and *ropB* genes so that the results reflected the overall mRNA decay rate of each gene (Table 2). To verify qRT-PCR results for mRNA decay measurements, Northern blot analysis was performed to determine the decay rate of each transcript when target mRNA was sufficiently abundant for reliable detection.

The *speB* mRNA decay rates appeared to follow first-order kinetics with corresponding exponential regression coefficients (R^2) greater than 0.95 under all prevailing growth conditions. qRT-PCR analysis revealed an increasing trend of *speB* mRNA stability along the growth curve (Fig. 2A and Table 3). We could not directly measure the *speB* mRNA stability in the early exponential phase ($A_{600} = 0.15$), because the transcript abundance was extremely low in that condition (Fig. 1). An alternative strategy was used instead. Fifty milliliters of *S. pyogenes* culture was grown until early stationary phase when *speB* was highly expressed, and the

TABLE 3 Estimation of *speB* mRNA half-lives in different growth phases

Growth phase	Estimated <i>speB</i> mRNA half-life (min)		
	qRT-PCR ^a	Northern blotting ^b	
		Short transcript	Long transcript
Early exponential	3.5 ± 0.3	2.0	0.8
Mid-exponential	7.1 ± 0.6	ND	ND
Late exponential	10.6 ± 1.3	16.3	1.4
Stationary	26.6 ± 2.1	18.1	8.2

^a Half-life calculated from qRT-PCR analysis in Fig. 2A. The values are means ± standard deviations from two independent experiments.

^b Half-life calculated from Northern blot analysis in Fig. 2B, C, and D. ND, not determined.

cells were collected by centrifugation. The cell pellet was immediately suspended in 250 ml prewarmed C medium (1:5 dilution) to simulate the early exponential phase. qRT-PCR results showed that *speB* mRNA degraded rapidly under this condition (Fig. 2A). The stability of *speB* mRNA gradually increased as the cells grew into mid- and late exponential phases, and *speB* mRNA became very stable at early stationary phase (Fig. 2A). These findings were confirmed by Northern blot analysis (Fig. 2B to D). *speB* mRNA has two transcript sizes, 2.3 kb and 1.7 kb, with the short transcript (1.7 kb) being the dominant form. The short transcript was also more stable than the long transcript (2.3 kb) in a given growth phase. Nevertheless, both transcripts displayed an increasing trend of stability when cells grew from early exponential to stationary phase.

In contrast to *speB*, the stability of *ropB* mRNA remained unchanged during exponential growth and increased dramatically when cells entered the stationary phase (Fig. 3 and Table 4). *ropB* mRNA also has two sizes of transcripts, 1.1 kb and 1.0 kb. The two *ropB* transcripts were similar in abundance at the beginning of the decay assay ($t = 0$ min), though the long transcript (1.1 kb) di-

TABLE 4 Estimation of *ropB* mRNA half-lives in different growth phases

Growth phase	Estimated <i>ropB</i> mRNA half-life (min)		
	qRT-PCR ^a	Northern blotting ^b	
		Short transcript	Long transcript
Early exponential	2.2 ± 0.4	ND	ND
Mid-exponential	1.9 ± 0.2	1.2	0.5
Late exponential	1.9 ± 0.1	1.0	0.7
Stationary	17.7 ± 2.6	ND	ND

^a Half-life calculated from qRT-PCR analysis in Fig. 3A.

^b Half-life calculated from Northern blot analysis in Fig. 3B and C. ND, not determined.

minished faster than the short transcript (1.0 kb), indicating that the short transcript was more stable.

Effect of environmental pH on *speB* mRNA stability. Two environmental factors, pH and nutrient availability, change continuously when bacteria actively grow in a batch culture. Because C medium has a weak buffering capacity, the culture pH decreased from pH 7.4 to pH 6.2 as *S. pyogenes* cells grew from early to late exponential phase and remained constant at pH 6.2 after the cells entered stationary phase (data not shown). C medium is used in this study because it supports a high level of expression of SpeB (30). The medium is rich in peptides and poor in carbohydrate (30). We assumed that the availability of an energy source was the limiting factor of bacterial growth, the depletion of which led to entry into the stationary phase. This assumption was supported by the fact that the bacterial culture in early stationary phase resumed growth immediately after the addition of exogenous glucose (data not shown). We hypothesized that either environmental pH or energy source availability or both affected *speB* mRNA stability.

To test this hypothesis, *S. pyogenes* cells were grown until late exponential phase, when *speB* mRNA was highly abundant. The culture was then divided into four aliquots. Each aliquot was adjusted with 2 M Bis-Tris buffer (the final concentration of Bis-Tris

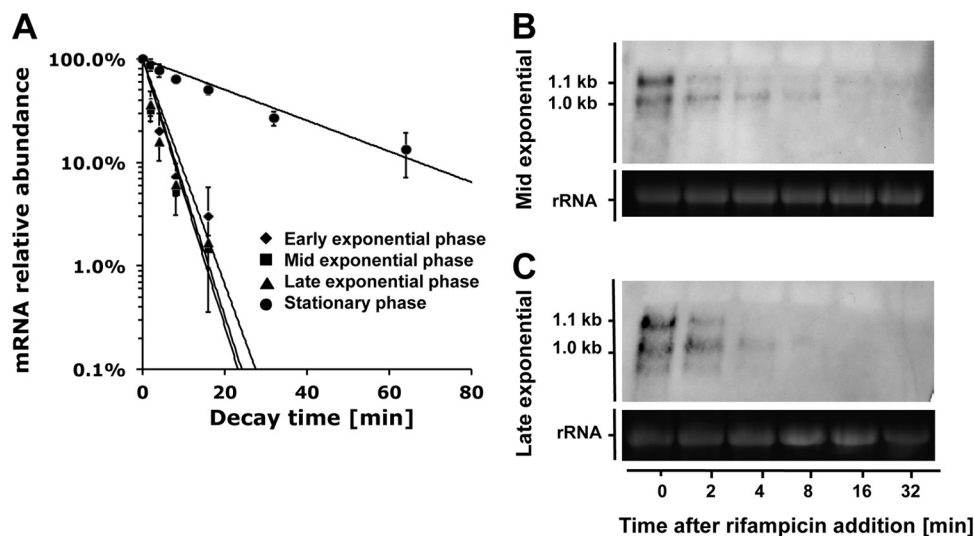


FIG 3 Growth phase-dependent *ropB* mRNA decay. Decay rates of *ropB* mRNA in different growth phases were determined by qRT-PCR (A) and Northern blot analyses (B and C). qRT-PCR data are presented as means ± standard deviations from two independent experiments. For Northern blot analysis, 23S rRNA was used as a loading control. Five micrograms of total RNA was loaded per lane.

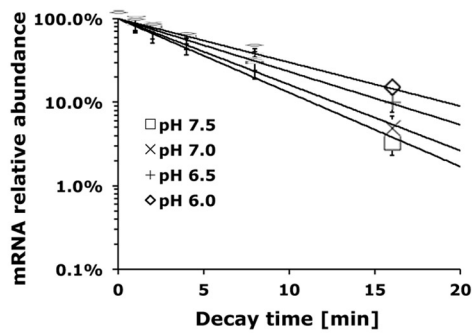
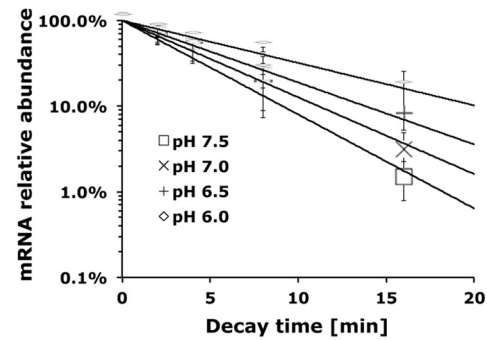
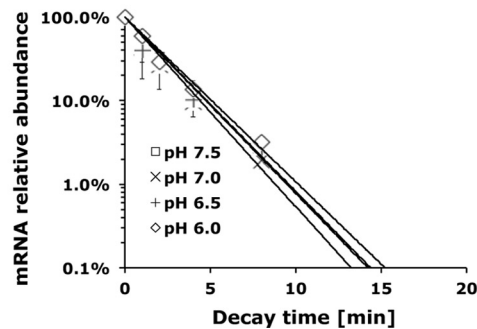
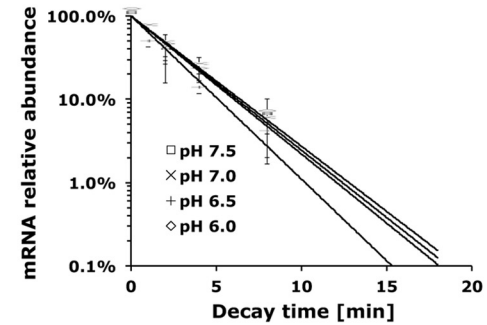
A *speB*, no glucose**B** *speB*, with glucose**C** *ropB*, no glucose**D** *ropB*, with glucose

FIG 4 *speB* and *ropB* mRNA decay under different environmental conditions. *S. pyogenes* NZ131 was grown to late exponential phase ($A_{600} = 0.55$) and divided into four aliquots. The culture pH was artificially adjusted from pH 7.5 to pH 6.0 with Bis-Tris buffer (final concentration of 0.1 M). Glucose (1% [wt/vol]) was added to the culture as indicated. The mRNA decay assay was conducted immediately after the medium was modified. qRT-PCR was used to determine the decay rates of *speB* mRNA (A and B) and *ropB* mRNA (C and D). Data are presented as the averages \pm standard deviations from two independent experiments.

buffer is 0.1 M) to pH 7.5, pH 7.0, pH 6.5, or pH 6.0 and was supplemented with 1% glucose or not supplemented with glucose. Rifampin was added simultaneously with Bis-Tris buffer and glucose to inhibit new mRNA synthesis. qRT-PCR results showed that the adjusted culture pH had an instant effect on *speB* mRNA stability (Fig. 4A and B and Table 5). The *speB* mRNA degraded rapidly at neutral pH and became more stable when the culture medium was gradually acidified. This pattern was observed in cultures supplemented with glucose or cultures not supplemented with glucose. At a given environmental pH, the supplementation of glucose did not obviously change *speB* mRNA stability. We conclude that environmental pH, rather than energy source availability, played a major role in regulating *speB* mRNA stability. This finding can at least partially explain the increased SpeB activity

TABLE 5 Estimation of *speB* and *ropB* mRNA half-lives under different pH conditions based on qRT-PCR analysis in Fig. 4

pH	Estimated half-life (min) of:			
	<i>speB</i> mRNA		<i>ropB</i> mRNA	
	Without glucose	With glucose	Without glucose	With glucose
7.5	3.3 \pm 0.2	2.8 \pm 0.5	1.5 \pm 0.1	2.0 \pm 0.5
7.0	3.7 \pm 0.5	3.6 \pm 0.8	1.5 \pm 0.0	1.8 \pm 0.0
6.5	5.1 \pm 0.5	5.2 \pm 0.1	1.6 \pm 0.1	1.7 \pm 0.3
6.0	6.1 \pm 0.2	8.4 \pm 2.0	1.6 \pm 0.1	2.0 \pm 0.1

measured under low pH as shown before (28). We also measured *ropB* mRNA stability in different environmental conditions. qRT-PCR results showed that *ropB* mRNA degraded at a constant rate under all pH conditions. The presence of glucose led to a slight increase of *ropB* mRNA stability, though the change was too small to be considered biologically significant (Fig. 4C and D and Table 5). These findings indicated that in contrast to *speB* mRNA variations in stability, *ropB* mRNA stability was not affected by environmental pH or energy source availability.

Search for RNase(s) involved in *speB* mRNA degradation. The degradation of RNA molecules is a tightly controlled process involving different types of RNases. The genome of *S. pyogenes* NZ131 contains genes that encode at least 11 RNases (32), 4 exoribonucleases, and 9 endoribonucleases (RNases J1 and J2 have both exo- and endoribonuclease activities). In this study, RNases polynucleotide phosphorylase (PNPase) (encoded by *pnpA*), RNase III (encoded by *acpA*), and RNase Y (encoded by *cvfA*) were chosen and investigated for their possible roles in *speB* mRNA degradation. RNases J1 and J2 are essential and cannot be inactivated (9).

PNPase is an exoribonuclease that catalyzes the 3'-5' phosphorolytic degradation of RNA (6). PNPase in *S. pyogenes* is involved in the decay of at least two gene transcripts, *sagA* and *sda* (7). RNase III is an endoribonuclease that specifically cleaves double-stranded RNAs (37). Its function in *S. pyogenes* remains to be determined. RNase Y from *Bacillus subtilis* is an endoribonuclease that is the functional equivalent of RNase E from *Escherichia coli*

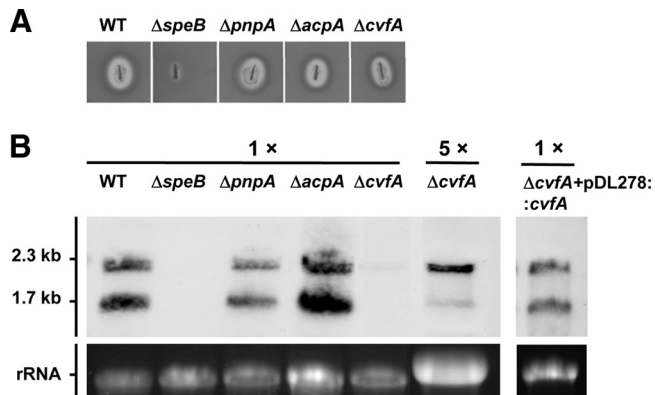


FIG 5 SpeB expression in RNase mutants and $\Delta cvfA$ complementation strain. (A) SpeB proteinase activities in RNase mutants were visualized on a 1.5% skim milk agar plate. The wild type (WT) and $\Delta speB$ mutant were included as positive and negative controls, respectively. (B) The level of *speB* mRNA transcript in each strain at early stationary phase was determined by Northern blot analysis. The samples were used as found (1 \times) or concentrated fivefold (5 \times). One microgram of total RNA was loaded per lane for each strain except for the $\Delta cvfA$ mutant. For the $\Delta cvfA$ mutant, 5 μ g of total RNA was used so that the hybridization signal was comparable to the other strains.

(26, 27). Its ortholog in *S. pyogenes* (*CvfA*) is involved in the expression of multiple virulence factors, including SpeB (20).

We constructed isogenic null mutants of PNase ($\Delta pnpA$), RNase III ($\Delta acpA$), and RNase Y ($\Delta cvfA$) by allelic exchange. All RNase mutants were viable, suggesting that these RNases were dispensable for the organism. All three mutants formed a translucent zone on a skim milk plate (Fig. 5A), indicating that functionally active SpeB proteinase was produced and secreted in these strains. This observation is at variance with a previous study that the proteinase activity was undetected in the *cvfA* mutant (20). We attribute this variance to the different serotypes of the strains used in the two studies (strain NZ131 [serotype M49] versus strain HSC5 [serotype M5]). Northern blot analysis was then performed to determine the level of *speB* mRNA in these strains at early stationary phase. No hybridization signal was detected in the $\Delta speB$ mutant, confirming that the *speB* probe was highly specific (Fig. 5B). The wild type and PNase ($\Delta pnpA$) and RNase III ($\Delta acpA$) mutants produced similar amounts of *speB* mRNA, with the short transcript being more abundant than the long transcript (Fig. 5B). These two RNase mutants were not further studied. In contrast, the RNase Y mutant ($\Delta cvfA$) produced considerably less *speB* mRNA than the other strains. The hybridization signal was detected only when the sample was concentrated (Fig. 5B). One striking feature was that the relative levels of two *speB* transcripts were inverted in the $\Delta cvfA$ mutant, with the long transcript being much more abundant than the short transcript. To rule out a possible polar effect or unexpected mutation that may occur during mutant construction, the *cvfA* gene was reintroduced into the $\Delta cvfA$ mutant on a shuttle vector, pDL278. Northern blot analysis showed that the *speB* transcript pattern was restored to the wild-type level after the *cvfA* gene complementation (Fig. 5B), which confirmed that the altered *speB* transcript pattern was due to the *cvfA* gene deletion.

Role of RNase Y in *speB* mRNA degradation. The *speB* mRNA abundance in $\Delta cvfA$ strain was further examined. qRT-PCR results showed that, unlike the wild-type strain, the level of *speB*

mRNA in the $\Delta cvfA$ mutant was very low during exponential growth and increased rapidly only immediately before the cells entered stationary phase (data not shown). This expression pattern made it technically difficult to collect $\Delta cvfA$ samples for an mRNA decay assay, because the assay required samples expressing high levels of *speB* mRNA but were still exponentially growing. To solve this problem, 1% glucose was added to bacterial cultures in the early stationary phase (ES plus 1% glucose) so that the cells “reentered” the exponential phase. A similar *speB* mRNA decay rate under this condition (ES plus 1% glucose) and in late exponential phase (compare Fig. 6A and Fig. 2C) in the wild-type strain was observed. Cells treated this way (ES plus 1% glucose) should behave similarly to those in late exponential phase.

Subsequently, *speB* mRNA decay rates in the wild type and $\Delta cvfA$ mutant were compared. Cells were grown until early exponential phase (30 min after reaching the maximal A_{600}) and supplemented with 1% glucose. The culture was either unbuffered (pH 6.2), or buffered to pH 7.5 or 6.0 with Bis-Tris buffer (final concentration of 0.1 M), and mRNA decay was determined immediately after modification. The $\Delta cvfA$ mutant showed a fivefold-reduced abundance of *speB* mRNA compared to the wild type. To compensate for the low abundance of *speB* mRNA, the total RNA amount was therefore increased 5-fold and used in the Northern blot assay. Again, we observed an altered *speB* transcript pattern in the $\Delta cvfA$ strain, with the long transcript being the most dominant form under all three conditions (Fig. 6). We also observed the following. (i) In a given strain (wild type or $\Delta cvfA$) under a given environmental condition (unbuffered, pH 7.5, or pH 6.0), the *speB* short transcript was always more stable than the long transcript was. (ii) In a given strain, both short and long transcripts were more stable at low pH (unbuffered, pH 6.0) than at high pH (pH 7.5). (iii) Under a given environmental condition, RNase Y contributes to the stability of the *speB* short transcript, but not long transcript (Fig. 6 and Table 6). On the basis of these observations, we conclude that RNase Y is not responsible for pH-dependent regulation of *speB* mRNA degradation, although it apparently plays a general role in determining the stability of the *speB* short transcript.

DISCUSSION

The degradation of mRNA appears to play an important regulatory role in the actual availability of several virulence factors in GAS (9). Furthermore, it has been suggested that growth phase-dependent regulation of GAS gene expression is predominantly regulated at the level of mRNA stability (9, 20). In the present study, we used the important GAS virulence gene *speB* to further dissect posttranscriptional regulation in *S. pyogenes*. Using Northern blot analysis and qRT-PCR to detect the level of *speB* mRNA, we measured *speB* mRNA abundance and decay under several environmental conditions. The main results of mRNA abundance and decay measurements suggest that the apparent increase in *speB* mRNA abundance is the result of increased *speB* mRNA stability in the late logarithmic and early stationary phase. The smooth accumulation processes of *speB* mRNA abundance suggest that *speB* gene expression is controlled by the same regulatory mechanism from early to late exponential phase.

The rapid accelerating degradation rate of *speB* mRNA implies the existence of a strong mRNA degradation mechanism in *S. pyogenes*. A search for putative RNases in the genome of *S. pyogenes* NZ131 revealed 11 candidates (32). Our previous microar-

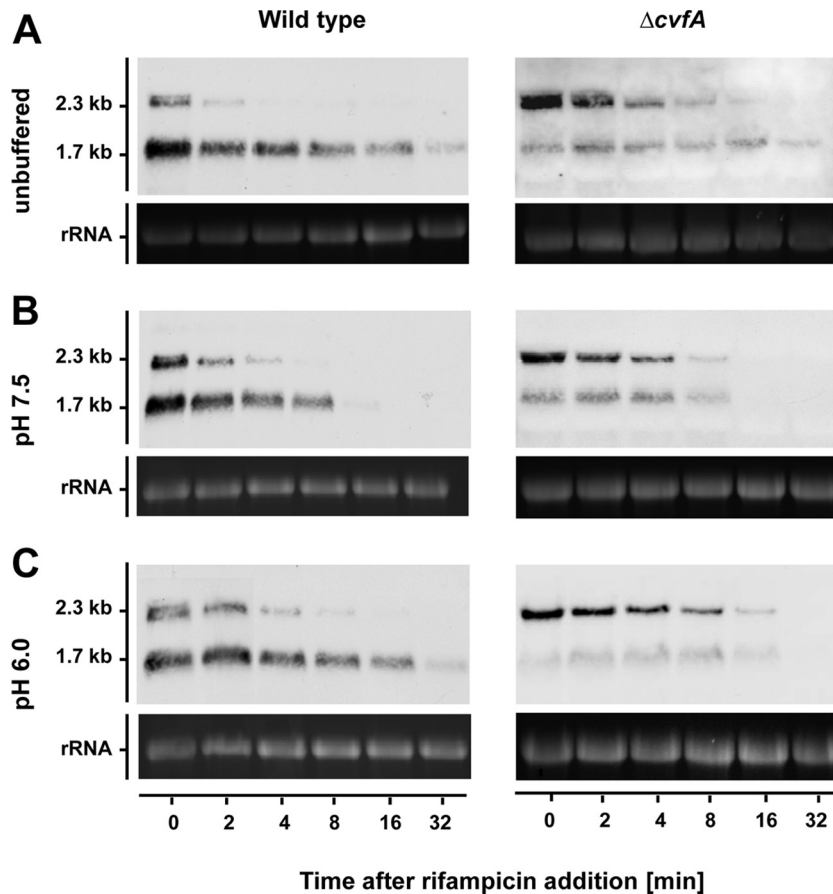


FIG 6 *speB* mRNA decay in wild type and $\Delta cvfA$ strains under different pH conditions. Bacterial strains were grown until early stationary phase, and 1% glucose was added to the culture so that the cells resumed growth. The culture pH was either unbuffered (pH 6.2) (A) or buffered to pH 7.5 (B) or pH 6.0 (C). Rifampin was added immediately after the pH was modified. For Northern blot analysis, 23S rRNA was used as a loading control. One microgram (wild type) or 5 μ g ($\Delta cvfA$) of total RNA was loaded per lane.

ray data showed that most RNase-encoding genes were actively transcribed in both exponential and early stationary phases (data not shown) (23). On the basis of these findings, we propose that the mRNA degradation mechanism is highly active throughout exponential growth. The accumulation of *speB* mRNA is because more transcripts are synthesized than can be serviced by the degradosome. A highly active degradation mechanism would predict that disruption of *speB* mRNA synthesis would result in a quick degradation of the accumulated mRNA. This was observed in our dilution experiment and during the challenge experiment with different pHs. We observed an immediate increase in *speB* mRNA degradation during the shift to pH 7.5, demonstrating the

presence of an active degradosome and excluding new synthesis since rifampin was administered simultaneously. If *speB* mRNA was simply stabilized by a stopped degradation after entry into stationary phase, it is unlikely that the message would be so rapidly degraded upon the dilution of stationary-phase cells. Our data also suggest a pH-dependent regulation of the *speB*-specific degradosome.

What could be the evolutionary rationale for not simply stopping mRNA degradation entirely to accumulate *speB* transcripts during exponential growth? A highly active degradation process at any given time means that the cell can eliminate the *speB* transcript and therefore SpeB production by preventing its translation. Because SpeB is a potent extracellular proteinase, control of *speB* mRNA abundance by its synthesis and degradation rates may enable *S. pyogenes* to respond to environmental changes in a more time-efficient manner. One example shown in this study is the dilution experiment, in which the *speB* mRNA abundance decreases by 1,000-fold within 30 min after the cells from late exponential phase are diluted in fresh medium (Fig. 1B). This drastic change can occur only when (i) the synthesis of new *speB* mRNA is completely stopped and (ii) the existing mRNA molecules are rapidly degraded.

One major environmental signal influencing *speB* mRNA stability and SpeB abundance is the culture pH (Fig. 4) (28). The *speB*

TABLE 6 Estimation of *speB* transcript half-lives based on Northern blot analysis in Fig. 6

pH	Estimated <i>speB</i> mRNA half-life (min)			
	WT		$\Delta cvfA$ mutant	
	Short transcript	Long transcript	Short transcript	Long transcript
6.2 (unbuffered)	16.9	3.5	27.0	3.7
7.5	3.3	1.7	5.4	1.6
6.0	10.0	2.8	14.5	3.4

transcript is more stable at a low culture pH than neutral pH. This explains why the *speB* mRNA stability increased during exponential growth, when the culture medium was gradually acidified by metabolic products (lactic acid, etc.). In contrast to *speB*, *ropB* mRNA stability is not affected by environmental pH. This observation further supports the notion that the pH-dependent regulation of mRNA stability is not a universal phenomenon but seems to be gene specific, since *ropB* mRNA abundance remains constant during exponential growth with a short half-life. The constitutive expression of the *ropB* gene suggests a balanced synthesis and degradation of *ropB* mRNA. This observation is at variance with a previous study (33), in which the *ropB* gene was expressed in the late stage of exponential growth. We consider that the variance may be due to the different serotypes of the strains used in the two studies (serotypes M14 and M49). However, in the same study (33), the authors also demonstrated that ectopic expression of *ropB* (i.e., *ropB* was expressed in the early stage of exponential growth) had no influence on the pattern of *speB* gene expression. This result is consistent with the results of our study, since we did not detect any correlation between *speB* mRNA abundance and *ropB* expression.

RNase Y (also known as CvfA) is a recently identified endoribonuclease that controls SpeB abundance in *S. pyogenes* (20). RNase Y seems to be part of the RNA degradosome in Gram-positive bacteria, which also includes the metabolic enzyme enolase and phosphofructokinase. We found in the present study that the *speB* mRNA abundance was obviously reduced in the RNase Y mutant, confirming previous findings (20). We also report for the first time that the mutation of RNase Y led to an altered *speB* transcript pattern. The long transcript, but not the short transcript, was the dominant form of *speB* mRNA species. The exact mechanism of how *S. pyogenes* produces two sizes of *speB* transcripts is still unclear. Our findings favor the hypothesis that the short transcript might be a processed product from the primary transcript through endonucleolytic cleavage and that RNase Y might be a major enzyme responsible for the cleavage. Since the *speB* short transcript is significantly more stable than the long transcript, the conversion of *speB* mRNA from long to short transcript may increase the overall stability of *speB* mRNA, which in turn promotes the production of protein. We are currently testing this hypothesis.

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REFERENCES

- Abramoff MD, Magelhaes PJ, Ram SJ. 2004. Image processing with ImageJ. *Biophotonics Int.* 11:36–42.
- Ajdic D, Pham VT. 2007. Global transcriptional analysis of *Streptococcus mutans* sugar transporters using microarrays. *J. Bacteriol.* 189:5049–5059.
- Alwine JC, Kemp DJ, Stark GR. 1977. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. *Proc. Natl. Acad. Sci. U. S. A.* 74:5350–5354.
- Anbalagan S, McShan WM, Dunman PM, Chaussee MS. 2011. Identification of Rgg binding sites in the *Streptococcus pyogenes* chromosome. *J. Bacteriol.* 193:4933–4942.
- Anderson KL, Dunman PM. 2009. Messenger RNA turnover processes in *Escherichia coli*, *Bacillus subtilis*, and emerging studies in *Staphylococcus aureus*. *Int. J. Microbiol.* 2009:525491.
- Andrade JM, Pobre V, Silva IJ, Domingues S, Arraiano CM. 2009. The role of 3'-5' exoribonucleases in RNA degradation. *Prog. Mol. Biol. Transl. Sci.* 85:187–229.
- Barnett TC, Bugrysheva JV, Scott JR. 2007. Role of mRNA stability in growth phase regulation of gene expression in the group A streptococcus. *J. Bacteriol.* 189:1866–1873.
- Berge A, Bjorck L. 1995. Streptococcal cysteine proteinase releases biologically active fragments of streptococcal surface proteins. *J. Biol. Chem.* 270:9862–9867.
- Bugrysheva JV, Scott JR. 2010. The ribonucleases J1 and J2 are essential for growth and have independent roles in mRNA decay in *Streptococcus pyogenes*. *Mol. Microbiol.* 75:731–743.
- Chaussee MS, Ajdic D, Ferretti JJ. 1999. The *rgg* gene of *Streptococcus pyogenes* NZ131 positively influences extracellular SpeB production. *Infect. Immun.* 67:1715–1722.
- Chaussee MS, Phillips ER, Ferretti JJ. 1997. Temporal production of streptococcal erythrogenic toxin B (streptococcal cysteine proteinase) in response to nutrient depletion. *Infect. Immun.* 65:1956–1959.
- Cohen JO. 1969. Effect of culture medium composition and pH on the production of M protein and proteinase by group A streptococci. *J. Bacteriol.* 99:737–744.
- Collin M, Olsen A. 2001. Effect of SpeB and EndoS from *Streptococcus pyogenes* on human immunoglobulins. *Infect. Immun.* 69:7187–7189.
- Collin M, Olsen A. 2001. EndoS, a novel secreted protein from *Streptococcus pyogenes* with endoglycosidase activity on human IgG. *EMBO J.* 20:3046–3055.
- Cunningham MW. 2000. Pathogenesis of group A streptococcal infections. *Clin. Microbiol. Rev.* 13:470–511.
- Elliott SD. 1945. A proteolytic enzyme produced by group A streptococci with special reference to its effect on the type-specific M antigen. *J. Exp. Med.* 81:573–592.
- Graham MR, et al. 2002. Virulence control in group A *Streptococcus* by a two-component gene regulatory system: global expression profiling and in vivo infection modeling. *Proc. Natl. Acad. Sci. U. S. A.* 99:13855–13860.
- Haake SK, Yoder SC, Attarian G, Podkaminer K. 2000. Native plasmids of *Fusobacterium nucleatum*: characterization and use in development of genetic systems. *J. Bacteriol.* 182:1176–1180.
- Johansson L, et al. 2008. Cathelicidin LL-37 in severe *Streptococcus pyogenes* soft tissue infections in humans. *Infect. Immun.* 76:3399–3404.
- Kang SO, Caparon MG, Cho KH. 2010. Virulence gene regulation by CvfA, a putative RNase: the CvfA-enolase complex in *Streptococcus pyogenes* links nutritional stress, growth-phase control, and virulence gene expression. *Infect. Immun.* 78:2754–2767.
- Kapur V, et al. 1993. A conserved *Streptococcus pyogenes* extracellular cysteine protease cleaves human fibronectin and degrades vitronectin. *Microb. Pathog.* 15:327–346.
- Kietzman CC, Caparon MG. 2010. CcpA and LacD.1 affect temporal regulation of *Streptococcus pyogenes* virulence genes. *Infect. Immun.* 78:241–252.
- Kreth J, Chen Z, Ferretti J, Malke H. 2011. Counteractive balancing of transcriptome expression involving CodY and CovRS in *Streptococcus pyogenes*. *J. Bacteriol.* 193:4153–4165.
- LeBlanc DJ, Lee LN, Abu-Al-Jaibat A. 1992. Molecular, genetic, and functional analysis of the basic replicon of pVA380-1, a plasmid of oral streptococcal origin. *Plasmid* 28:130–145.
- Leclerc GJ, Leclerc GM, Barredo JC. 2002. Real-time RT-PCR analysis of mRNA decay: half-life of beta-actin mRNA in human leukemia CCRF-CEM and Nalm-6 cell lines. *Cancer Cell Int.* 2:1.
- Lehnik-Habrink M, et al. 2011. RNase Y in *Bacillus subtilis*: a natively disordered protein that is the functional equivalent to RNase E from *Escherichia coli*. *J. Bacteriol.* 193:5431–5441.
- Lehnik-Habrink M, et al. 2011. RNA processing in *Bacillus subtilis*: identification of targets of the essential RNase Y. *Mol. Microbiol.* 81:1459–1473.
- Loughman JA, Caparon M. 2006. Regulation of SpeB in *Streptococcus pyogenes* by pH and NaCl: a model for in vivo gene expression. *J. Bacteriol.* 188:399–408.
- Lyon WR, Caparon MG. 2003. Trigger factor-mediated prolyl isomerization influences maturation of the *Streptococcus pyogenes* cysteine protease. *J. Bacteriol.* 185:3661–3667.
- Lyon WR, Gibson CM, Caparon MG. 1998. A role for trigger factor and an rgg-like regulator in the transcription, secretion and processing of the cysteine proteinase of *Streptococcus pyogenes*. *EMBO J.* 17:6263–6275.

31. Malke H, Steiner K, McShan WM, Ferretti JJ. 2006. Linking the nutritional status of *Streptococcus pyogenes* to alteration of transcriptional gene expression: the action of CodY and RelA. *Int. J. Med. Microbiol.* **296**:259–275.
32. McShan WM, et al. 2008. Genome sequence of a nephritogenic and highly transformable M49 strain of *Streptococcus pyogenes*. *J. Bacteriol.* **190**:7773–7785.
33. Neely MN, Lyon WR, Runft DL, Caparon M. 2003. Role of RopB in growth phase expression of the SpeB cysteine protease of *Streptococcus pyogenes*. *J. Bacteriol.* **185**:5166–5174.
34. O'Driscoll L, Daly C, Saleh M, Clynes M. 1993. The use of reverse transcriptase-polymerase chain reaction (RT-PCR) to investigate specific gene expression in multidrug-resistant cells. *Cytotechnology* **12**:289–314.
35. Ogburn CA, Harris TN, Harris S. 1958. Extracellular antigens in steady-state cultures of the hemolytic *Streptococcus*: production of proteinase at low pH. *J. Bacteriol.* **76**:142–151.
36. Olsen RJ, Musser JM. 2010. Molecular pathogenesis of necrotizing fasciitis. *Annu. Rev. Pathol.* **5**:1–31.
37. Panganiban AT, Whiteley HR. 1983. Purification and properties of a new *Bacillus subtilis* RNA processing enzyme. Cleavage of phage SP82 mRNA and *Bacillus subtilis* precursor rRNA. *J. Biol. Chem.* **258**:12487–12493.
38. Podbielski A, Woischnik M, Kreikemeyer B, Bettenbrock K, Buttaro BA. 1999. Cysteine protease SpeB expression in group A streptococci is influenced by the nutritional environment but SpeB does not contribute to obtaining essential nutrients. *Med. Microbiol. Immunol.* **188**:99–109.
39. Reid SD, et al. 2006. Inactivation of the group A *Streptococcus* regulator *srv* results in chromosome wide reduction of transcript levels, and changes in extracellular levels of Sic and SpeB. *FEMS Immunol. Med. Microbiol.* **48**:283–292.
40. Ribardo DA, McIver KS. 2006. Defining the Mga regulon: comparative transcriptome analysis reveals both direct and indirect regulation by Mga in the group A streptococcus. *Mol. Microbiol.* **62**:491–508.
41. Schmidtchen A, Frick IM, Bjorck L. 2001. Dermatan sulphate is released by proteinases of common pathogenic bacteria and inactivates antibacterial alpha-defensin. *Mol. Microbiol.* **39**:708–713.
42. Schmittgen TD, et al. 2000. Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: comparison of endpoint and real-time methods. *Anal. Biochem.* **285**:194–204.
43. Shelburne III, et al. 2011. An amino-terminal signal peptide of Vfr protein negatively influences RopB-dependent SpeB expression and attenuates virulence in *Streptococcus pyogenes*. *Mol. Microbiol.* **82**:1481–1495.
44. Song JH, et al. 2005. Identification of essential genes in *Streptococcus pneumoniae* by allelic replacement mutagenesis. *Mol. Cells* **19**:365–374.
45. Steiner K, Malke H. 2002. Dual control of streptokinase and streptolysin S production by the *covRS* and *fasCAX* two-component regulators in *Streptococcus dysgalactiae* subsp. *equisimilis*. *Infect. Immun.* **70**:3627–3636.
46. Terao Y, et al. 2008. Group A streptococcal cysteine protease degrades C3 (C3b) and contributes to evasion of innate immunity. *J. Biol. Chem.* **283**:6253–6260.
47. von Pawel-Rammingen U, Bjorck L. 2003. IdeS and SpeB: immunoglobulin-degrading cysteine proteinases of *Streptococcus pyogenes*. *Curr. Opin. Microbiol.* **6**:50–55.