Inactivation of the Streptococcal Erythrogenic Toxin B Gene (*speB*) in *Streptococcus pyogenes*

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Streptococcal proteinase precursor (SPP) is a zymogen secreted by *Streptococcus pyogenes* that becomes activated to a cysteine proteinase. SPP has been shown to be immunologically identical to streptococcal erythrogenic toxin B (SPE B), and sequence comparison has shown a high degree of homology between the two proteins. In this study, we have constructed a *speB* mutant strain of *S. pyogenes* by insertional inactivation. An internal fragment of the cloned *speB* gene in plasmid pCR1000 was replaced with an erythromycin resistance determinant, and the recombinant plasmid was introduced into strain NZ131 by electrotransformation. Following the selection of erythromycin-resistant clones, Southern hybridization experiments confirmed the presence of the recombinant plasmid containing the *erm* gene in the chromosome of the resistant strains. Analysis of extracellular proteins produced by the wild-type and *speB* mutant strains by Ouchterlony immunodiffusion and isoelectric focusing revealed the presence of SPE B in the wild-type strain but not the *speB* mutant. Additionally, SPP, which has an isoelectric focusing pattern similar to that of SPE B and reacts with SPE B antiserum, was not detected among the extracellular proteins of the *speB* mutant strain. Proteinase activity as assayed by two different methods was present in the extracellular proteins produced by the wild-type strain, but the *speB* mutant strain had no extracellular proteinase activity. The mutant strain had a growth rate similar to that of the wild-type strain and produced normal levels of other extracellular products, suggesting that proteinase was not essential for viability as previously suggested. Our data are consistent with the view that a single gene (*speB*) produces a single protein that has been identified and/or assayed as either SPE B or SPP.

Streptococcus pyogenes produces a variety of extracellular products, including erythrogenic toxin B (SPE B) and the streptococcal proteinase precursor (SPP). SPE B and SPP share several properties and have been proposed to be either identical proteins (9) or variants of the same protein (11). The nucleotide sequence of the *speB* gene from *S. pyogenes* 86-858 was determined recently (11), and the inferred amino acid sequence of SPE B predicts a protein with a molecular weight of 40,314 that is 84% identical in amino acid sequence to SPP. The amino acid sequence of the SPP protein isolated from *S. pyogenes* was determined (23, 24), and the protein had a calculated molecular weight of 36,708. SPP and SPE B also have similar isoelectric focusing patterns; SPP has isoelectric points of 8.2, 8.4, 8.6, and 9.0 (10), and SPE B has isoelectric points of 8.0, 8.4, and 9.0 (1). In addition, Gerlach et al. (9), using Ouchterlony immunodiffusion, have demonstrated that SPP is immunologically identical to SPE B. SPP is secreted as a zymogen and is activated by reduction and proteolysis to form a sulfhydryl protease (6, 7, 15). In contrast, the SPE B protein isolated from *S. pyogenes* 86-858 and the protein expressed from the cloned *speB* gene in *E. coli* did not have proteolytic activity (11). These results suggested that SPP and SPE B were variants of the same protein that are similar in structure, with only SPP having proteolytic activity.

Extracellular products such as SPP are generally considered to be accessory proteins that are not essential for the viability of the organism. However, a recent report showed that a synthetic peptide derivative of cystatin C (a human cysteine protease inhibitor) was capable of inhibiting the growth of *S. pyogenes* and further identified a cysteine

proteinase known to be extracellular in nature as the specific enzyme that was inhibited by the synthetic peptide (2). To test the validity of this possibility and as an additional step in determining the relationship between SPP and SPE B, we constructed an *speB* mutant by insertional inactivation of the *speB* gene.

MATERIALS AND METHODS

Bacterial strains and media. *S. pyogenes* NZ131, which does not contain phage-specific genes encoding erythrogenic toxin A or erythrogenic toxin C (provided by D. R. Martin, New Zealand Communicable Disease Center, Porirua, New Zealand), was grown in Todd-Hewitt medium containing 0.2% yeast extract and, when appropriate, erythromycin at a final concentration of 3.0 µg/ml. *Escherichia coli* DB11, an erythromycin-sensitive strain (5), and *E. coli* LE392 (3) were grown with LB broth or LB agar plates containing, when appropriate, erythromycin at a final concentration of 10 µg/ml and kanamycin at a final concentration of 100 µg/ml. Restriction enzymes, the Klenow fragment of DNA polymerase I, and T4 DNA ligase were purchased from Bethesda Research Laboratories, Gaithersburg, Md., and used as instructed by the manufacturer. *Taq* polymerase was purchased from Promega, Madison, Wis.

Cloning of *speB*. The *speB* gene was amplified from *S. pyogenes* NZ131 by the polymerase chain reaction (PCR) (17), using oligonucleotides synthesized by the Molecular Biology Resource Facility, Oklahoma Center for Molecular Medicine. The first primer (CCA TTC ATG GTA TCA GCG) corresponded to –124 to –107 of the *speB* gene (11), and the second primer (CAT GGT TGA GGT CCA GG) was complementary to *speB* nucleotides 1606 to 1623. Thirty cycles were carried out in a Perkin Elmer DNA thermocycler with

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strand denaturation (1 min at 94°C), annealing (1 min at 42°C), and elongation (3 min at 72°C). The total volume of the PCR mixture was 100 µl and consisted of 1 ng of genomic DNA (25), 4.0 µM each primer, 200 µM deoxynucleoside triphosphates (dNTPs), 0.5 mM Mg²⁺, and 5 U of *Taq* polymerase. Following amplification, the 1.6-kb product was cloned into the TA cloning vector pCR1000 (Invitrogen, San Diego, Calif.) and sequenced by the dideoxy-chain termination method (19).

In vitro mutagenesis of *speB*. An internal portion of the cloned *speB* gene was removed by digestion of pCR1000::*speB* with *AccI*. *AccI* restriction sites were present at nucleotides 816 and 989 of the cloned *speB* gene. The 4.4-kb plasmid was separated from the smaller fragment by electrophoresis. The ends of the plasmid were made blunt by using the Klenow fragment, and a 1.1-kb, blunt-ended fragment containing an *erm* gene was ligated to the plasmid with T4 DNA ligase. The *erm* gene was obtained from pIL253, a derivative of pAMβ1 (20). The ligation mixture was used to transform *E. coli* DB11 as described previously (4). Transformants were selected on agar plates containing erythromycin. The construction of the recombinant plasmid was confirmed by restriction enzyme analysis and Southern hybridization, using a radiolabeled 1.0-kb *erm* fragment as a probe.

Transformation of NZ131. The recombinant plasmid was introduced into *S. pyogenes* NZ131 by electrotransformation (21). Erythromycin-resistant (*Em*^r) *S. pyogenes* strains were screened for proteolytic activity by inoculating agar plates containing casein (13). Proteolytic activity was detected as a circle of translucence surrounding the stab due to proteolytic digestion of casein in the medium. Two protease-deficient colonies were selected for further study and designated SPP⁻¹ and SPP⁻².

Colony hybridization. *Em*^r strains were analyzed by colony hybridization as previously described (26). A radiolabeled 1.0-kb *erm* gene was used to probe the blots. The probe was labeled with [α -³²P]dATP (New England Nuclear, Dupont Corp.), using a random-primer DNA labeling kit (U.S. Biochemical Corp., Cleveland, Ohio) as instructed by the manufacturer.

Southern hybridization. Chromosomal DNA from wild-type *S. pyogenes*, SPP⁻¹, and SPP⁻² was isolated as previously described (25). Purified DNA was digested with *HindIII* and *ClaI*, and the DNA fragments were separated by 0.7% agarose electrophoresis. The DNA was transferred to a Nytran membrane (Schleicher & Schuell, Keene, N.H.) by the method of Southern (22) as described elsewhere (18). Probes were labeled with [α -³²P]dATP (New England Nuclear, Dupont Corp.), using a random-primer DNA labeling kit (U.S. Biochemical Corp.) as instructed by the manufacturer. Probes were removed by using formamide as described previously (18).

PCR analysis. Two oligonucleotide primers corresponding to nucleotides 328 to 344 and 1606 to 1623 of the *speB* gene were used to amplify the *speB* gene from the wild-type, SPP⁻¹, and SPP⁻² strains. Template DNA was prepared as described previously (12). Thirty cycles of amplification were carried out in a Perkin Elmer DNA thermocycler with strand denaturation (1 min at 94°C), annealing (1 min at 55°C), and elongation (3 min at 72°C). The total volume of the PCR mixture was 50 µl and consisted of 2 µl of cell lysate, 1.7 µM each primer, 25 µM dNTPs, 0.5 mM Mg²⁺, and 1 U of *Taq* polymerase. Following amplification, a 10-µl aliquot was analyzed by agarose gel electrophoresis. The amplified products were then further characterized by

Southern hybridization, using the cloned 1.6-kb *speB* gene and the 1.0-kb *erm* gene as probes, and by restriction enzyme analysis following excision of the amplicon from the gel and purification with GeneClean (Bio 101, La Jolla, Calif.).

Analysis of culture supernatant. The SPP⁻¹, SPP⁻², and wild-type strains were grown under identical conditions in I nutrient broth (Merck, Darmstadt, Germany), which consisted of the following per liter: 15 g of peptone, 3 g of yeast extract, 6 g of NaCl, and 1 g of D-(+)-glucose. The bacteria were grown for 24 h at pH 6.0 in a 100-ml fermenter with automatic feeding of glucose to 1 g/100 ml and pH regulation with 5 M NaOH, based on the method of Fuvesy et al. (8). Following centrifugation, the culture supernatant (100 ml) was concentrated by ammonium sulfate precipitation (75% saturation). The precipitate was dissolved in 2 ml of water and dialyzed against 0.01 M Tris-HCl buffer (pH 8.1). Isoelectric focusing of the culture supernatants was performed with ampholine polyacrylamide gels (pH 3.5 to 9.5) (Pharmacia, Piscataway, N.J.) as instructed by the manufacturer. Rabbit serum developed against SPE B isolated from *S. pyogenes* T19 was used in Ouchterlony immunodiffusion (16) experiments.

Purification of SPE B from NZ131. *S. pyogenes* NZ131 was grown in tryptic soy broth (pH 6.0) at 30°C for 30 h. The sterile supernatant (850 ml) was precipitated with 325 g of ammonium sulfate (75% saturation). The precipitate was resuspended in 1 ml of water and dialyzed against 40% saturated ammonium sulfate (pH 5.2) at 4°C. The precipitate was discarded, and the supernatant was dialyzed against 75% ammonium sulfate (pH 8.0) to precipitate SPE B. The precipitate was solubilized in water and dialyzed against buffer A (0.02 M acetate buffer [pH 5.1]), and 14 ml was applied to an S-Sepharose column (2 by 18 cm) equilibrated with buffer A. After washing with buffer A, the proteins were eluted with a linear gradient of 200 ml of buffer A and 200 ml of buffer A plus 0.4 M NaCl. SPE B was detected with antiserum in fractions 35 to 50, and fractions 41 to 43 were collected. The collected fractions contained 18 ml of solution and had an *A*₂₇₉ of 0.145. The solution was concentrated by ammonium sulfate precipitation, reduced with 0.05 M β-mercaptoethanol (pH 8.0) for 1 h at 25°C, and dialyzed against 0.02 M phosphate buffer containing 10⁻⁵ M dithiothreitol and 2 mM EDTA (pH 7.5). Proteolytic activity was determined by the method of Kortt and Liu (14). Briefly, 3 ml of 0.2 M sodium acetate buffer (pH 5.5) was mixed with 100 µl of Z-glycine-4-nitrophenolate (0.92 mg/ml in acetonitrile), and 50 or 100 µl of activated enzyme was added. The hydrolysis of Z-glycine-4-nitrophenolate was measured by recording the change in *A*₃₁₇.

RESULTS AND DISCUSSION

The *speB* gene from *S. pyogenes* NZ131 was obtained by PCR amplification of genomic DNA, using primers complementary to regions upstream and downstream of the *speB* gene. The amplified 1.6-kb fragment was cloned into pCR1000 and sequenced to confirm that it was the *speB* gene. The nucleotide sequence of the cloned gene contained seven base pair substitutions compared with the published sequence of *speB* from *S. pyogenes* 86-858, which resulted in three amino acid substitutions, i.e., residues 111 (Val for Ala), 317 (Ser for Ala), and 385 (Asp for Gly). Thus, the *speB* gene cloned from strain NZ131 is 99% identical to the published sequence of *speB* from strain 86-858. We do not know whether these nucleotide changes are due to errors by

FIG. 1. Autoradiograph following Southern hybridization of *Hind*III-*Cla*I-cut genomic DNA isolated from wild-type *S. pyogenes* NZ131 (lanes 1 and 3) and the *speB* mutant (SPP⁻¹) (lanes 2 and 4). Lanes 1 and 2 show hybridization with a *Hind*III-*Cla*I *speB* radiolabeled probe. Following autoradiography, the *speB* probe was removed and the blot was hybridized with a radiolabeled probe specific for the *erm* gene (lanes 3 and 4). Sizes are indicated in kilobases.

Taq polymerase during amplification or represent true polymorphisms at this locus.

To inactivate the *speB* gene in pCR1000, an internal 173-bp region of the cloned *speB* gene was replaced with a gene encoding resistance to erythromycin (*erm*) and known to express in streptococci. This recombinant plasmid was purified by electrophoresis and introduced by electroporation into *S. pyogenes* NZ131. EM^r transformants were selected on agar plates containing erythromycin. The average frequency of transformation was two transformants per microgram of DNA and varied proportionally with the DNA concentration. Colony hybridization of 58 EM^r strains with a probe specific to the *erm* gene showed that 57 of the strains had the *erm* gene inserted in the chromosome. EM^r transformants were screened for decreased proteolytic activity on agar plates containing casein, and all of the strains that had the *erm* gene in the chromosome (57 strains) produced minimal zones of translucence (<3 mm in diameter) compared with the wild-type strain, which produced zones 12 to 15 mm in diameter, after 18 h of growth. Two EM^r, proteolytically deficient strains were selected for further characterization and designated SPP⁻¹ and SPP⁻².

The genotype of strains SPP⁻¹ and SPP⁻² was analyzed by PCR and Southern hybridization. Two oligonucleotide primers, complementary to nucleotides 328 to 344 and 1606 to 1623 of the *speB* gene, were used to amplify the gene from SPP⁻¹, SPP⁻², and the wild-type strain of *S. pyogenes*. A single product of 1.3 kb, the size predicted from the *speB* nucleotide sequence, was amplified from the wild-type cell lysate. However, two products were amplified from the SPP⁻¹ and SPP⁻² cell lysates. One product was approximately 2.3 kb and hybridized with probes specific to both the *erm* and *speB* genes, indicating that the *erm* gene was flanked by 5' and 3' fragments of the *speB* gene. This product was identical to the mutated form of *speB* constructed in vitro, as judged from restriction enzyme analysis of the amplified products purified from the agarose gel by using GeneClean. The other product was 1.3 kb in size and hybridized only with a probe to the *speB* gene. This DNA was also purified from the gel and had a restriction enzyme profile identical to that of the wild-type *speB* gene. Southern hybridization analysis (Fig. 1) was used to confirm the PCR results. Genomic DNA isolated from the wild-type and mutant strains was cleaved with *Hind*III and *Cla*I, which

FIG. 2. Isoelectric focusing of culture supernatants from the wild-type strain of *S. pyogenes* (lane C) and SPP⁻¹ and SPP⁻² strains (lanes B and D), using ampholine polyacrylamide gels (pH 3.5 to 9.5). Lane A contained pI marker proteins.

resulted in a 0.84-kb fragment from the wild-type strain that hybridized with an *speB*-specific probe (Fig. 1, lane 1), as predicted from the nucleotide sequence. In the mutant strain, the *speB* probe hybridized to both a 0.84-kb and an approximately 1.7-kb *Hind*III-*Cla*I fragment (Fig. 1, lane 2). The 1.7-kb fragment of the mutant strain also hybridized with a probe specific to the *erm* gene (Fig. 1, lane 4), while the *erm* gene was not detected in the wild-type strain (Fig. 1, lane 3). These results indicated that the mutant strains had two copies of the *speB* gene; one copy was identical to the mutated allele, and the other copy was identical to the wild-type allele.

Southern hybridization analysis of the wild-type strain confirmed that only a single copy of the *speB* gene is present in the chromosome of *S. pyogenes*. Using a probe specific for pCR1000, it was also shown that the recombinant plasmid (pCR1000::*speB*Δ*erm*) used to transform NZ131 had inserted into the chromosome of transformed cells (data not shown). The insertion of the recombinant plasmid into the chromosome by a single homologous crossover event is consistent with the presence of two copies of the *speB* gene in transformed cells, one copy the mutated form of the gene containing the *erm* gene and the second copy a hybrid *speB* gene.

Analysis of culture supernatants from wild-type and *speB* mutant strains by isoelectric focusing showed protein bands of the expected isoelectric points only in the wild-type supernatant (Fig. 2). It was noted that inactivation of *speB* results in the absence of both SPE B and SPP, which have similar isoelectric focusing patterns. Additionally, Ouchterlony immunodiffusion of concentrated culture supernatants using antibody to SPE B, which has been shown to react identically with SPP, showed a reaction only with the supernatant from the wild-type strain (Fig. 3). The absence of SPE B and SPP in the mutant strains is dependent upon inactivation of the structural gene and not a result of *erm* expression, since insertion of the erythromycin resistance determinant into the streptokinase gene of NZ131 did not affect proteolytic activity (21).

The SPE B protein was isolated from *S. pyogenes* NZ131 and had a molecular weight of approximately 28,000. When assayed for protease activity, the purified SPE B protein was found to have a specific activity of 12.8 U/mg, which is only slightly higher than the specific activity of 10.5 U/mg re-

FIG. 3. Ouchterlony immunodiffusion of culture supernatants from wild-type *S. pyogenes* and two *speB* mutants. The wells contained sera from a rabbit immunized with SPE B isolated from *S. pyogenes* T19 (a), purified SPE B used to develop the antibody (b), concentrated culture supernatant from the wild-type strain of *S. pyogenes* (c), and concentrated culture supernatant from the SPP⁻¹ (d) and SPP⁻² (e) strains.

ported by Kortt and Liu (14). The loss of both SPP and SPE B in the *speB* mutant strains and the purification of a proteolytically active SPE B protein from *S. pyogenes* NZ131 support the previous conclusion that the two proteins are identical (9). Our evidence is consistent with the view that a single gene (*speB*) specifies a single protein that has been identified in two different ways, as either SPE B or SPP. We suggest that this gene continue to be referred to as *speB*, recognizing that it produces the protein referred to as SPE B or SPP, and discourage the use of *spp*.

Björck et al. (2) showed that a synthetic peptide derivative of a human cysteine protease inhibitor was capable of inhibiting the growth of group A streptococci and identified a cysteine proteinase known to be extracellular as the specific enzyme inhibited by the synthetic peptide. This observation suggested that SPP may be essential for the growth of *S. pyogenes*. The results of the present study indicate that inactivation of the *speB* gene has no effect on the in vitro viability of this organism. The synthetic peptide derivative inhibited the growth of NZ131 and the *speB* mutant equally under the conditions described by Björck et al. (2). Moreover, the *speB* mutant strain grew at a rate comparable to that of the wild-type strain and produced apparently normal levels of other extracellular proteins (streptolysins S and O, streptokinase, and DNases). It remains possible that *speB* is being expressed at a level which is below the level of detection by the methods used in this study or that another enzyme with a more critical function is inhibited by the peptide derivative described by Björck et al. (2). In this regard, it is interesting to note that small zones of clearing could be detected around SPP⁻¹ and SPP⁻² colonies on casein-containing Todd-Hewitt agar plates.

The contribution of the extracellular proteinase to cellular metabolism is not known, and there has been speculation that it may be involved in the virulence associated with group A streptococci. In this regard, the availability of an *speB* mutant will be important for future studies aimed at identifying the role of SPE B in streptococcal infections and their sequelae.

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