INFECTION AND IMMUNITY, May 1997, p. 1956–1959 Vol. 65, No. 5
0019-9567/97/$04.00+0

Temporal Production of Streptococcal Erythrogenic Toxin B (Streptococcal Cysteine Proteinase) in Response to Nutrient Depletion

MICHAEL S. CHAUSSEE,* ERNEST R. PHILLIPS, AND JOSEPH J. FERRETTI

Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma 73190

Received 19 November 1996/Returned for modification 3 January 1997/Accepted 17 February 1997

The effects of various growth conditions on the production of streptococcal erythrogenic toxin B (streptococcal pyrogenic exotoxin B [SPE B]) by *Streptococcus pyogenes* were analyzed. SPE B was detected in broth culture supernatant fluid only during the stationary phase of growth when glucose and other potential carbon sources were depleted from the medium. Additionally, SPE B production was inhibited when the concentration of glucose in the medium was maintained. These results suggest that SPE B is secreted under conditions of starvation and may be involved in nutrient acquisition.

Erythrogenic toxin B, also known as streptococcal pyrogenic exotoxin B (SPE B), is one of several extracellular products produced by the group A streptococcus, *Streptococcus pyogenes*. In 1983, Gerlach et al. recognized that SPE B was immunologically identical to another extracellular product referred to as streptococcal proteinase (6). Subsequent studies have confirmed that the proteins previously referred to as SPE B and streptococcal proteinase are identical (1, 7, 16, 17). In contrast to the bacteriophage-associated *speA* and *speC* genes, SPE B is chromosomally encoded and all strains of *S. pyogenes* have the gene (22). The nucleotide sequence of *speB* has been determined and predicts a 371-amino-acid protein with a calculated molecular weight of 40,314 (7). The protein is secreted as a zymogen and is activated by proteolysis and reduction to form a sulfhydryl protease with a predicted molecular weight of 27,588 (5, 7, 15).

SPE B is the major extracellular product produced by *S. pyogenes* NZ131 in vitro, yet little is known about the regulation of its production. In this study, the production of SPE B under various growth conditions was examined. The results suggest that SPE B production is part of an adaptive response to nutrient-poor environments and thus may be important in the survival of the organism during periods of nutritional stress.

Bacterial strains, growth conditions, and assays. *S. pyogenes* NZ131 was obtained from D. R. Martin, New Zealand Communicable Disease Center, Porirua, New Zealand. An isogenic derivative (NZ131 *speB* mutant) in which the *speB* gene was specifically inactivated has been previously described (1). *S. pyogenes* was grown at 37°C in dialyzed Todd-Hewitt broth medium with or without 0.2% yeast extract (THY-D and TH-D, respectively). The broth was dialyzed to reduce the amount of protein and other macromolecules in the medium which could compete with SPE B for adsorption to microtiter plates (8). Two methods which differed primarily in the size of the culture were used to grow *S. pyogenes*. (i) Overnight broth cultures of *S. pyogenes* were diluted (1:50 [vol/vol]) in 100 ml of fresh broth and grown at 37°C in a stirred fermentation vessel. When appropriate, 5 ml of one of the following solutions was added: (a) 1.11 M glucose (Sigma Chemical Corp., St. Louis, Mo.), (b) 1.11 M galactose (Sigma); (c) 1% (wt/vol) Casamino Acids (Difco Laboratories, Detroit, Mich.)–1% (wt/vol) neopeptone (Difco), and (d) 0.55 M NaCl (Sigma). (ii) An overnight culture of *S. pyogenes* NZ131 was diluted (1:50 [vol/vol]) into 5 ml of THY-D or TH-D broth medium contained in 10-ml test tubes, and the cultures were incubated at 37°C with shaking to keep the cells suspended. When appropriate, either 200 μl of 1.11 M glucose (Sigma) or 200 μl of 0.55 M NaCl was added. Spent broth was prepared by growing *S. pyogenes* NZ131 *speB* mutant in THY-D or TH-D overnight at 37°C. Following centrifugation to pellet cells, the supernatant fluid was filter sterilized. SPE B was not detected in the spent broth by immunoblotting. An enzyme-linked immunosorbent assay was used to quantitate extracellular SPE B in broth culture supernatant fluid as described previously (2). The concentration of glucose in broth medium was determined by use of a glucose oxidase kit (Sigma). A plate assay was used to detect streptokinase (SKA) activity in broth culture supernatant fluid as described previously (9).

S. pyogenes NZ131 was grown in 5 ml of THY-D broth at 37°C with moderate shaking. At 1-h intervals, the cell density was estimated by determining the A_{600} and aliquots were removed to determine the concentration of SPE B and to detect SKA activity in the broth culture supernatant fluid. SKA has been reported to be constitutively expressed and was used as a control. After an initial lag period, SKA activity was detected throughout the growth of the culture (Fig. 1). In contrast, SPE B was detected only after the culture entered the stationary phase of growth (Fig. 1). Similar results were obtained with a stirred fermentation vessel or with TH-D broth medium.

To determine if the production of SPE B during the stationary phase correlated with the depletion of nutrients, the relationship between the concentration of glucose in the broth medium and SPE B production was analyzed. Two stirred fermentation vessels containing 100 ml of TH-D were used to grow *S. pyogenes* NZ131 at 37°C. In one vessel, the concentration of glucose in the broth medium was maintained at approximately 50 mg/dl by the addition of 1.11 M glucose; as a control, a second vessel was similarly treated with 0.55 M NaCl. The 0.55 M NaCl solution was approximately iso-osmotic compared to the 1.11 M glucose solution and was used to deter-

* Corresponding author. Present address: Laboratory of Microbial Structure and Function, Rocky Mountain Laboratories, NIAID, NIH, Hamilton, MT 59840. Phone: (406) 363-9306. Fax: (406) 363-9204. E-mail: mchaussee@nih.gov.

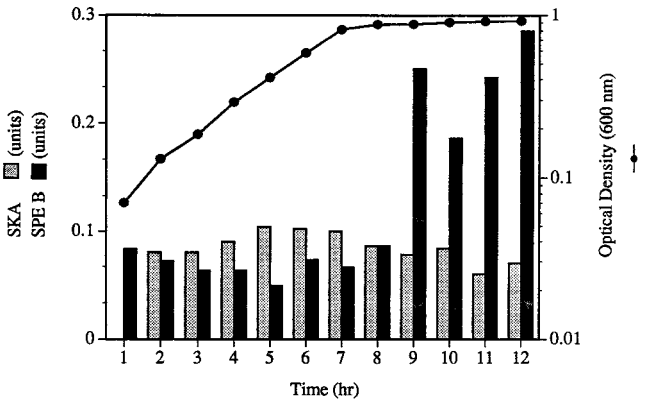

FIG. 1. Production of SKA and SPE B during growth of *S. pyogenes* NZ131. The cell density of *S. pyogenes* NZ131 grown in THY-D was estimated by determining the A_{600}. At regular intervals, samples were removed to detect SKA activity and to determine the concentration of SPE B in supernatant fluid.

mine if an increase in the osmotic pressure of the broth medium affected SPE B production. Aliquots were removed throughout the growth of the cultures, and the concentrations of glucose and SPE B in culture supernatant fluid were determined. The growth of the two cultures was similar (Fig. 2A). In the control culture, SPE B was detected shortly after the culture entered the stationary phase and production coincided with the depletion of glucose from the medium (Fig. 2B). In contrast, when the concentration of glucose in the medium was maintained, SPE B production was inhibited during the stationary phase (Fig. 2C). Similar results were obtained with THY-D broth medium.

To determine if the inhibition of SPE B production was specific to the availability of glucose, the effect of adding alternative carbon sources on SPE B production was examined. Broth cultures of *S. pyogenes* NZ131 were grown in stirred fermentation vessels. After approximately 5 h of incubation (during the exponential phase), 5 ml of one of the following solutions was added to the broth cultures: (a) 1% (wt/vol) Casamino Acids and 1% (wt/vol) neopeptone, (b) 1.11 M glucose, and (c) 0.55 M NaCl. The A_{600} of the cultures at the time of nutrient addition was 0.35 for the NaCl-infused culture, 0.41 for the culture infused with Casamino Acids and neopeptone, and 0.30 for the glucose-infused culture. Following addition of these potential carbon and nitrogen sources, the samples were incubated at 37°C for an additional 8 h. SPE B was detected in the NaCl-infused culture supernatant fluid shortly after the culture entered the stationary phase (Fig. 3). Addition of a solution containing Casamino Acids and neopeptone inhibited the production of SPE B in the stationary phase by approximately 40% compared to the NaCl-infused culture based on a comparison of the average amount of SPE B present in samples removed during the stationary phase of growth (0.95 U of SPE B for the NaCl-infused culture and 0.57 U of SPE B for the Casamino Acids- and neopeptone-infused culture) (Fig. 3). The addition of glucose inhibited SPE B production by approximately 70% (0.29 U of SPE B) compared to the NaCl-infused control culture (0.95 U of SPE B) based on a similar comparison of the average amount of SPE B in samples removed during the stationary phase of growth (Fig. 3). In similar experiments, the addition of 5 ml of 1.11 M galactose also inhibited SPE B production in the stationary phase, similar to the inhibition observed when glucose was added to the culture (data not shown). These results indicate that SPE B production

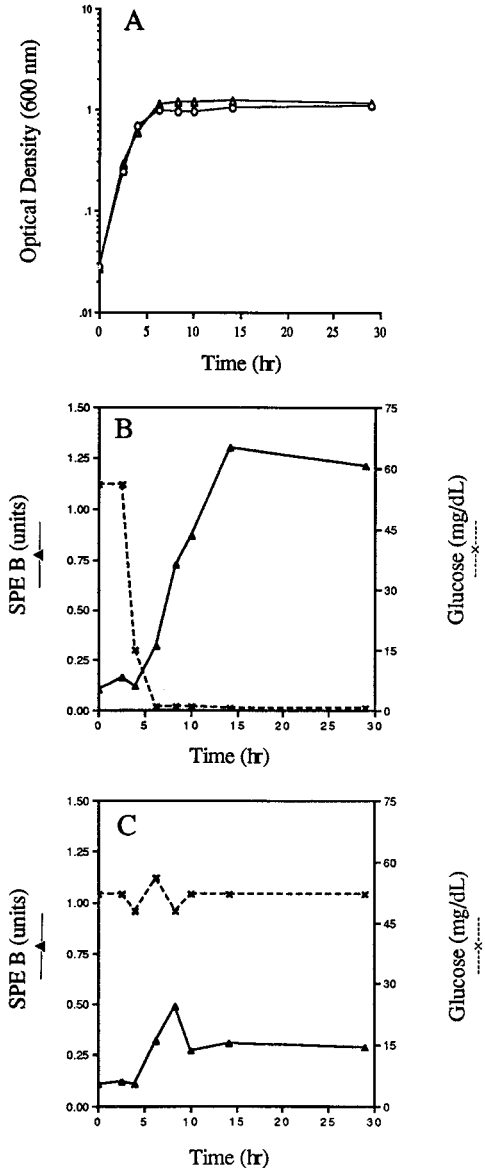

FIG. 2. The effect of glucose availability on SPE B production. The cell density was estimated by A_{600} of both the culture in which the glucose concentration was maintained (△) and the control culture similarly treated with NaCl (○) (A). Throughout the incubation period, aliquots were removed and the concentrations of glucose and SPE B in the supernatant fluid were determined for both the control culture (B) and the culture in which the concentration of glucose was maintained (C).

is inhibited by the availability of various rapidly metabolizable nutrients in the medium including glucose, galactose, and Casamino Acids and neopeptone.

To determine if SPE B was produced solely in response to the depletion of nutrients, the production of SPE B in spent broth was examined. Exponentially growing broth cultures of *S. pyogenes* NZ131 were centrifuged to pellet the cells, and the cells were washed with spent broth and then resuspended in one of the following solutions: THY-D medium, THY-D spent medium, THY-D spent medium containing 0.2% glucose, THY-D spent medium containing 0.02% glucose, and THY-D spent medium containing 0.002% glucose. Glucose was added to spent medium to determine if some form of glucose metab-

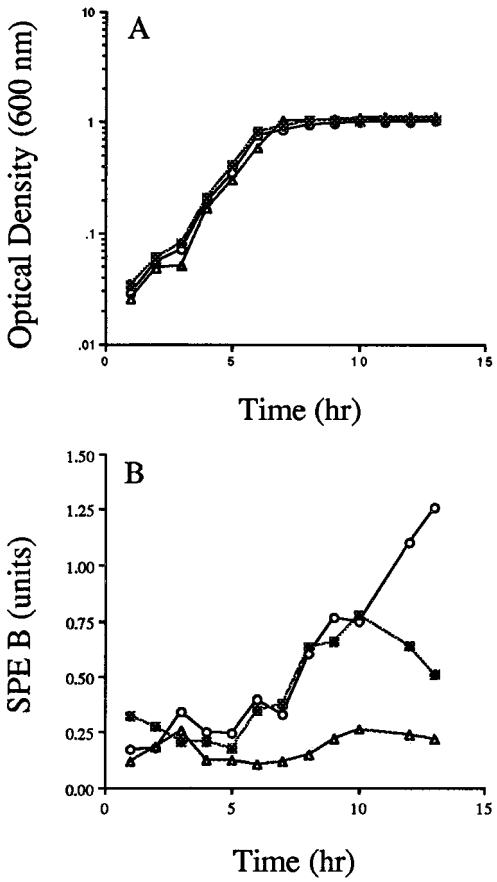

FIG. 3. Inhibition of SPE B production by the addition of rapidly metabolizable nutrients. *S. pyogenes* NZ131 was grown in three fermentation vessels containing TH-D broth medium. (A) Throughout the incubation period, the cell density was estimated by A_{600}. (B) The concentration of SPE B in broth culture supernatant fluid was determined by enzyme-linked immunosorbent assay. During the exponential phase, the vessels were supplemented with glucose (▲), a mixture of Casamino Acids and neopeptone (■), or NaCl (○).

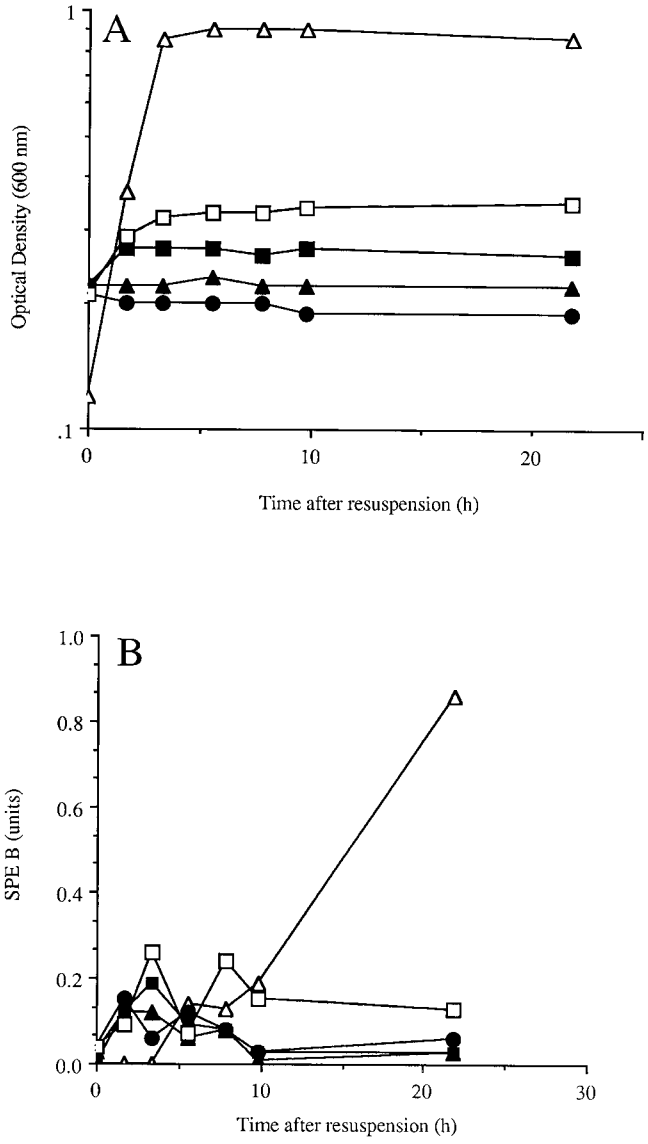

FIG. 4. SPE B production following resuspension of exponential-phase *S. pyogenes* NZ131 in spent medium. A mid-exponential-phase culture of *S. pyogenes* NZ131 was centrifuged, and the cells were washed in spent medium and then resuspended in one of the following solutions: THY-D medium (△), THY-D spent medium (○), THY-D spent medium containing 0.002% glucose (▲), THY-D spent medium containing 0.02% glucose (■), and THY-D spent medium containing 0.2% glucose (□). During the subsequent incubation period, the cell density was estimated by A_{600} (A), and the concentration of SPE B in both culture supernatant fluids was determined (B).

olism was necessary to induce SPE B, since initial results indicated that SPE B was not produced by exponential-phase cells resuspended in spent medium. Following resuspension, the cultures were incubated at 37°C for 22 h, during which time samples were regularly removed to determine the A_{600} and the concentration of SPE B in supernatant fluid. After resuspension in rich THY-D medium, growth resumed and SPE B was produced shortly after the culture entered the stationary phase (Fig. 4). In contrast, exponential-phase cells resuspended in spent medium did not grow, nor was SPE B produced, even after 22 h of incubation (Fig. 4). Similar results were obtained when exponential-phase cells were resuspended in spent broth containing 0.002% glucose (Fig. 4). The addition of 0.02 and 0.2% glucose to spent medium facilitated a small increase in the optical density of the cultures which correlated with the concentration of glucose. SPE B was not detected in cells resuspended in spent medium containing 0.02% glucose, nor was a significant amount of SPE B detected in supernatant fluid from spent medium containing 0.2% glucose. The small amount of SPE B in samples from spent medium containing 0.2% glucose does not appear to be significant given the variation inherent in the assay. In addition, significant levels of SPE B were not detected in similar samples when the experiment was repeated. In general, these results suggest that SPE B production is not induced by the accumulation of metabolites or autoinducers in the medium, although it remains formally possible that such a compound exists but that its activity is labile. Although SPE B production is inhibited by the presence of simple carbon sources such as glucose, galactose, and Casamino Acids and neopeptone, the absence of these nutrients in spent medium was not sufficient to induce SPE B production. Therefore, SPE B production in the stationary phase appears to require an additional, temporal factor which may be formed during growth but does not appear to result solely from glucose metabolism. In this regard, it was noticed that the addition of glucose and other nutrients to the medium late in the exponential phase of growth did not inhibit station-

ary-phase production of SPE B, in contrast to the results obtained when these nutrients were added near the mid-exponential phase of growth. This observation suggests that the temporal factor involved in the regulation of SPE B production may be formed late in the exponential phase of growth.

The results obtained in this study are at variance with those previously reported by Cohen (3). In the latter study, the proteolytic activity was enhanced during culture with a high glucose concentration (1%) and inhibited with a low concentration of glucose (0.25%). A possible explanation for the disparity is that the previous study measured only the enzymatic activity of SPE B, whereas the concentrations of both the SPE B zymogen and the proteolytic form of SPE B were measured in this study. Since proteolytic activation of the SPE B zymogen is most efficient at an acidic pH, the increased acidity produced by growth in 1% glucose compared to growth in 0.25% glucose may have resulted in an increased efficiency of proteolytic activation and not regulation of zymogen production.

Previous studies have shown that the addition of glucose to the culture medium decreases the production of several extracellular proteins produced by *Staphylococcus aureus* including staphylococcal enterotoxins A, B, and C (4, 10, 11). Furthermore, the availability of glucose in the medium decreased the expression of the accessory gene regulator, a positive regulatory factor involved in the production of several extracellular products produced by *S. aureus* (19). In addition to extracellular products, the presence of easily metabolizable compounds including glucose and amino acids in the medium has also been shown to inhibit the expression of the dipeptide transport operon *dpp* of *Bacillus subtilis* (20). The molecular mechanism by which glucose causes repression of these various proteins is not well understood. However, the mechanism appears to differ from that of the relatively well-characterized catabolite repression in *Escherichia coli* which is mediated by cyclic AMP (10, 21). The results from the current study suggest that the regulation of SPE B production in *S. pyogenes* NZ131 may be similar to the regulation of stationary-phase proteins produced by other gram-positive bacteria.

The regulation of SPE B production by both the availability of rapidly metabolizable carbon sources and a temporal factor indicates that SPE B may be important under conditions of nutritional stress. During starvation, the secretion of SPE B may facilitate the acquisition of peptides by *S. pyogenes* following the degradation of host proteins for use as carbon and nitrogen sources. Previous studies have shown that SPE B is capable of cleaving several human host proteins, including interleukin-1β and fibronectin, at specific sites (12, 13). In addition, purified SPE B has been shown to degrade vitronectin, although it remains to be determined if the peptide products are subsequently transported to the cytoplasm of *S. pyogenes* (13). Podbielski et al. have recently identified an oligopeptide permease system (Opps) in *S. pyogenes* CS101 (18). A homologous oligopeptide permease-transport system of *Lactococcus lactis* has been shown to be involved in a number of processes including the transport of peptides produced by the extracellular proteinase PrtP (14). Interestingly, SPE B caseinolytic activity was not detected in OppDF$^-$ mutants of *S. pyogenes* CS101 (18). Thus, starvation of *S. pyogenes* during infection may result in the production of SPE B and subsequent degradation of host proteins for metabolism. In addition, host cell protein degradation may facilitate invasion by *S. pyogenes* of nutrient-rich environments and dissemination of streptococcal infections within its human host.

Editor: V. A. Fischetti

We thank Dieter Gerlach for providing the antiserum to SPE B and purified SPE B.

This work was supported by a PHS award (AI19304) from the National Institutes of Health.

REFERENCES

1. **Chaussee, M. S., D. Gerlach, C.-E. Yu, and J. J. Ferretti.** 1993. Inactivation of the streptococcal erythrogenic toxin B gene (*speB*) in *Streptococcus pyogenes*. Infect. Immun. **61:**3719–3723.
2. **Chaussee, M. S., J. Liu, D. L. Stevens, and J. J. Ferretti.** 1996. Genetic and phenotypic diversity among isolates of *Streptococcus pyogenes* from invasive infections. J. Infect. Dis. **173:**901–908.
3. **Cohen, J. O.** 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.
4. **Duncan, J. L., and G. J. Cho.** 1972. Production of staphylococcal alpha toxin. II. Glucose repression of toxin formation. Infect. Immun. **6:**689–694.
5. **Elliott, S. D., and V. P. Dole.** 1947. An inactive precursor of streptococcal proteinase. J. Exp. Med. **85:**305–320.
6. **Gerlach, D., H. Knöll, W. Köhler, J. H. Ozegowski, and V. Hríbalova.** 1983. Isolation and characterization of erythrogenic toxins. V. Communication: identity of erythrogenic toxin type B and streptococcal proteinase precursor. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **255:**221–233.
7. **Hauser, A. R., and P. M. Schlievert.** 1990. Nucleotide sequence of the streptococcal pyrogenic exotoxin type B gene and relationship between the toxin and the streptococcal proteinase precursor. J. Bacteriol. **172:**4536–4542.
8. **Houston, C. W., and J. J. Ferretti.** 1981. Enzyme-linked immunosorbent assay for detection of type A streptococcal exotoxin: kinetics and regulation during growth of *Streptococcus pyogenes*. Infect. Immun. **33:**862–869.
9. **Huang, T.-T., H. Malke, and J. J. Ferretti.** 1989. Heterogeneity of the streptokinase gene in group A streptococci. Infect. Immun. **57:**502–506.
10. **Iandolo, J. J., and W. M. Shafer.** 1977. Regulation of staphylococcal enterotoxin B. Infect. Immun. **16:**610–616.
11. **Jarvis, A. W., R. C. Lawrence, and G. G. Pritchard.** 1975. Glucose repression of enterotoxins A, B and C and other extracellular proteins in staphylococci in batch and continuous cultures. J. Gen. Microbiol. **86:**75–87.
12. **Kapur, V., M. W. Majesky, L.-L. Li, R. A. Black, and J. M. Musser.** 1993. Cleavage of interleukin 1β (IL-1β) precursor to produce active IL-1β by a conserved extracellular cysteine protease from *Streptococcus pyogenes*. Proc. Natl. Acad. Sci. USA **90:**7676–7680.
13. **Kapur, V., S. Topouzis, M. W. Majesky, L.-L. Li, M. R. Hamrick, R. J. Hamill, J. M. Patti, and J. M. Musser.** 1993. A conserved *Streptococcus pyogenes* extracellular cysteine protease cleaves human fibronectin and degrades vitronectin. Microb. Pathog. **15:**327–346.
14. **Kunji, E. R. S., A. Hagting, C. J. De Vries, V. Juillard, A. J. Haandrikman, B. Poolman, and W. N. Konings.** 1995. Transport of β-casein-derived peptides by the oligopeptide transport system is a crucial step in the proteolytic pathway of *Lactococcus lactis*. J. Biol. Chem. **270:**1569–1574.
15. **Liu, T.-Y., and S. D. Elliott.** 1965. Activation of streptococcal proteinase and its zymogen by bacterial cell walls. Nature (London) **206:**33–34.
16. **Musser, J. M., K. Stockbauer, V. Kapur, and G. W. Rudgers.** 1996. Substitution of cysteine 192 in a highly conserved *Streptococcus pyogenes* extracellular cysteine protease (interleukin 1β convertase) alters proteolytic activity and ablates zymogen processing. Infect. Immun. **64:**1913–1917.
17. **Ohara-Nemoto, Y., M. Sasaki, M. Kaneko, T. Nemoto, and M. Ota.** 1994. Cysteine protease activity of streptococcal pyrogenic exotoxin B. Can. J. Microbiol. **40:**930–936.
18. **Podbielski, A., B. Pohl, M. Woischnik, C. Körner, K.-H. Schmidt, E. Rozdzinski, and B. A. B. Leonard.** 1996. Molecular characterization of group A streptococcal (GAS) oligopeptide permease (Opp) and its effect on cysteine protease production. Mol. Microbiol. **21:**1087–1099.
19. **Regassa, L. B., R. P. Novick, and M. J. Betley.** 1992. Glucose and nonmaintained pH decrease expression of the accessory gene regulator (*agr*) in *Staphylococcus aureus*. Infect. Immun. **60:**3381–3388.
20. **Slack, F. J., J. P. Mueller, M. A. Strauch, C. Mathiopoulos, and A. L. Sonenshein.** 1991. Transcriptional regulation of a *Bacillus subtilis* dipeptide transport operon. Mol. Microbiol. **5:**1915–1925.
21. **Smith, J. L., M. M. Bencivengo, R. L. Buchanan, and C. A. Kunsch.** 1986. Enterotoxin A production in *Staphylococcus aureus*: inhibition by glucose. Arch. Microbiol. **144:**131–136.
22. **Yu, C.-E., and J. J. Ferretti.** 1991. Frequency of the erythrogenic toxin B and C genes (*speB* and *speC*) among clinical isolates of group A streptococci. Infect. Immun. **59:**211–215.