Molecular Characterization of hasC from an Operon Required for Hyaluronic Acid Synthesis in Group A Streptococci

DEMONSTRATION OF UDP-GLUCOSE PYROPHOSPHORYLASE ACTIVITY*

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Hyaluronic acid is a high molecular weight glycosaminoglycan composed of repeating subunits of glucuronic acid and N-acetylglucosamine. It is synthesized by the group A streptococcal membrane-associated enzyme hyaluronate synthase. In previous reports, the locus required for expression of hyaluronic acid and UDP-glucose dehydrogenase, respectively. Since a transcription terminator was not found at the end of hasB, it was the aim of this study to identify the remaining gene(s) in the has operon. By utilizing the Tn1000 method of DNA sequencing and inverse polymerase chain reaction, hasC, the third gene in the has operon was shown to be 915 base pairs in length (304 amino acids) and located 192 base pairs downstream of hasB. Sequence similarities to other genes suggested that hasC encodes UDP-glucose pyrophosphorylase. Overexpression of hasC using isopropyl-1-thio-β-D-galactopyranoside induction of the T7 promoter in the pET translation system allowed for the production of bacterial extracts from Escherichia coli that possessed increased UDP-glucose pyrophosphorylase activity as compared to extracts with vector alone. In addition, expression of HasC resulted in a protein of approximately 36 kDa as shown by SDS-polyacrylamide gel electrophoresis. These data as well as complementation analysis of hasC in an E. coli galA mutant confirmed that hasC encodes UDP-glucose pyrophosphorylase. Finally, since sequence analysis identified a potential rho-independent transcription terminator at the 3-prime terminus of the gene, hasC is the third and probably the final gene in the has operon.

Group A streptococci (Streptococcus pyogenes) are human pathogens that colonize the skin and mucous membranes of their host. They cause localized infections and nonsuppurative sequelae such as post-streptococcal glomerulonephritis and rheumatic fever (1). Prior to 1985, the occurrence of lethal streptococcal infections was quite rare. However, a rise in the number of streptococcal diseases within the past decade has led to increased investigation into the mechanisms involved in the resurgence of disease. One potential explanation for this increased pathogenicity of group A streptococci could be that the bacteria have evolved enhanced virulence determinants that assist in the pathogenesis of the organism. The primary virulence factor for group A streptococci is the M protein. M protein is a coiled-coil dimeric molecule present on the surface of streptococci that has been shown to interfere with phagocytic uptake of the organism by neutrophils and protect the organism from clearance (2). The hyaluronic acid capsule has also been shown to be involved in the pathogenicity of group A streptococci. It has been observed that clinical isolates from outbreaks of rheumatic fever are highly encapsulated as compared to nonpathogenic strains (3, 4). Three percent of isolates from patients with uncomplicated pharyngitis (group I) possess a hyaluronic capsule, whereas 21% of isolates from severe streptococcal infections (group II), and 42% of rheumatic fever isolates (group III) exhibit a capsule. However, in the most frequently isolated group A streptococcal serotype, M1, the frequency of capsule production is even greater: 6% for group I, 22% for group II, and 80% for group III. These data support the hypothesis that the hyaluronic acid capsule is a major virulence determinant for group A streptococci in the human host.

To demonstrate that the hyaluronic acid capsule is necessary for pathogenesis of group A streptococci, Wessels et al. (5, 6) have created acapsular mutants via transposon mutagenesis that exhibited increased sensitivity to phagocytic killing in human blood, whereas the encapsulated wild-type strains survived killing. These same acapsular mutants were also less virulent in mice as compared to the wild-type streptococci. In addition, Wessels and Bronze (7) showed that the hyaluronate capsule is necessary for early colonization of the pharynx and may be required for invasion of group A streptococci from the pharynx to produce a disseminated infection. These results suggested that the hyaluronic acid capsule may provide a selective advantage over the unencapsulated phenotype for group A streptococci.

Hyaluronic acid is a high molecular weight glycosaminoglycan that is synthesized by the alternate addition of UDP-glucuronic acid and UDP-N-acetylglucosamine by the streptococcal membrane-associated enzyme hyaluronate synthase (8). The chromosomal locus that is necessary for the production of the hyaluronic acid capsule (has operon) has recently been identified (9–12) and is conserved in all strains of group A streptococci and encapsulated group C streptococci (6, 13). The has operon was found to consist of at least two genes, hasA and hasB. hasA was shown to encode hyaluronate synthase (11–13). DeAngelis and Weigel (15) have created two monoclonal antibodies raised against synthetic peptides corresponding to portions of HasA. Both antibodies recognized a 42-kDa protein from group A streptococci and a recombinant protein from Escherichia coli containing hasA on a plasmid. hasB was
shown to encode UDP-glucose dehydrogenase (10). Furthermore, a transposon insertion in hasC created a polar effect on hasB expression, providing evidence that the genes are transcribed by the same promoter. Since sequence analysis of DNA downstream of hasA did not reveal any terminator-like sequences (11), the hasA promoter could regulate transcription of the entire operon and thereby produce a polycistronic message. Recently, it was demonstrated by Northern blot analyses that hasA, hasB, and hasC are located on the same mRNA transcript (4.2 kb), thus confirming the observation that the genes are components of an operon (14).

In this report, the sequence of the third gene (hasC) of the has operon is presented. hasC is located 192 nucleotides downstream of hasB and contains a potential rho-independent terminator at the 3-prime end of the gene, thus suggesting that hasC is the last gene in the has operon. Sequence comparisons and T7 overexpression of hasC demonstrated that hasC encodes UDP-glucose pyrophosphorylase, an enzyme which catalyzes the production of UDP-glucose from glucose 1-phosphate and UTP.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The streptococcal strain used in this study (WF51, T18) was grown in CDM (16). E. coli strains used were JM109, BL21(DE3), DPWC, BW26 (17), and DE6v (gift from the E. coli Genetic Stock Center, Yale University; lacZ105, ρ -gal, galU65, relA1, spoI1, thi-1) (18) grown in Luria broth or TYPG (16 g of tryptone, 16 g of yeast extract, 5 g of NaCl, 2.5 g of K2PO4, and 5 g of glucose/liter of H2O; see Ref. 10) medium as indicated. Plasmids used in this study include pGAC144 (10), pGAC312 (this study), pGAC315 (this study), pMOB (17), pBluescript (Stratagene), pET11a (19), pLYS5 (19), and pLYS15 (19).

DNA Purification and Manipulations—Streptococcal chromosomal DNA was purified as per Dougherty and van de Rijn (9). Plasmid DNA was purified from E. coli cultures (3 ml) by the alkaline lysis procedure (20) or Wizard Mini-preps (Promega, Madison, WI) and resuspended in a final volume of 100 μl of TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). DNA restriction enzymes were purchased from Promega and used according to the manufacturer’s suggestions.

Cloning and DNA Sequence of the hasC Gene—The 0.9-kb EcoRI/HindIII fragment of pGAC144 was subcloned into the miniplasmid pMOB for sequencing utilizing the Tn1000 protocol as per Strathmann et al. (17). Briefly, pMOB containing the 0.9-kb EcoRI/HindIII fragment was transformed into the host strain DPWC (which contains Tn1000) and used according to the manufacturer’s suggestions.

Cloning of pGAC144 into the T7 expression vector pGAC312 was achieved as per Studier et al. (19). Briefly, T7 expression (5 ml) was inoculated into a 50-mI culture of E. coli (strain BL21(DE3)pLysE), and the cells were harvested by centrifugation. The cells were resuspended in 15 ml of T7 induction buffer (50 mM potassium phosphate, pH 7.5, 1 mM MgCl2, 0.1 mM dithiothreitol, 10 mM isopropyl β-D-thiogalactopyranoside (IPTG), 50 μg/ml ampicillin) and incubated at 37 °C for 30 min. The enzyme was induced by the addition of IPTG (1 mM final concentration), and the cultures were incubated for an additional 45 min at 37 °C. The cells were harvested by centrifugation and resuspended in 1 ml of T7 induction buffer. After another 45 min at 37 °C, the cells were harvested, and the cell-free extract was mixed with 2 volumes of acetone and incubated at −20 °C for 15 min.

Sequence Analysis—To sequence the 3′ upstream region of hasC from an Operon Required for Hyaluronic Acid Synthesis

hasC from an Operon Required for Hyaluronic Acid Synthesis

The complete sequence of hasC was determined by utilizing the fragment assembly program of the GCG software package (21). BESTFIT and PILEUP, additional GCG programs, were used to align other sequences in the data base with hasC. FOLDRNA was used to determine the secondary structure of hasC.

The abbreviations used were: kb, kilobase; IPTG, isopropyl-1-thio-β-D-galactopyranoside; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

Complementation of E. coli galU Mutations—pGAC315 or pET11a was transformed into the E. coli galU mutant strain DE6v (18), and single colonies were grown in chromogenic medium (3 ml; 1% polyethylene, 0.5% yeast extract, 2% galactose, 0.003% bromthymol blue, 0.5 mM IPTG, 50 μg/ml ampicillin) (22) at 37 °C. Complementation was judged by a color change of the culture supernatant from green to yellow that demonstrated that the plasmid-encoded hasC-complemented mutant produced acid from growth in galactose.

T7 Overexpression of hasC in E. coli—In order to express hasC, chromosomal DNA isolated from WF51 was subjected to PCR using oligonucleotide D-34a (5′-GCCATGACCCAAGTAGCAAGAAG-3′) to incorporate an NdeI site at the 5′-prime end of hasC and oligonucleotide D-37a (5′-GCCAGTCCATATTGTTC-3′) to incorporate a BamHI site at the 3′-prime end for further subcloning into the translation vector pET11a. The 925-bp pair PCR product was subcloned into T4 polynucleotide kinase (Promega) in order to phosphorylate the ends and then subcloned into Smal-digested pBluescript II (KS+) to create pGAC312. Next, hasC was removed from pGAC312 by restriction digestion with NdeI and BamHI and finally subcloned into NdeI/BamHI-digested pET11a to create pGAC315.

pGAC315 was transformed into E. coli strains BL21(DE3), BL21(DE3)pLYS5, and BL21(DE3)pLYS(E) in order to test expression experiments as a plasmid control. pET11a was also transformed into the above strains. T7 overexpression of hasC was achieved as per Studier et al. (19). Briefly, T7 expression (5 ml) was inoculated into a 50-mI culture of E. coli from which a single colony was picked. The culture was grown in 1 ml of Luria broth supplemented with 50 μg/ml ampicillin (22) at 37 °C. Complementation was judged by the Markwell et al. (26) assay using duplicate samples and BSA as standard. A unit of UDP-glucose pyrophosphorylase activity was defined as the amount of enzyme required to produce 1 μmol of NADP/ min at 30 °C.

RESULTS

Cloning and DNA Sequence of the hasC Gene—Further analysis of pGAC144, a clone that contained the 3′-prime region of hasB and approximately 1 kb of additional DNA (10), identified an open reading frame 192 nucleotides downstream of hasB. In order to sequence this open reading frame, the 0.9-kb EcoRI/HindIII fragment of pGAC144 (beginning 130 nucleotides downstream of hasB) was subcloned into the minivector pMOB.
and subjected to Tn1000 sequencing as per Strathmann et al. (17). The open reading frame, designated hasC, was shown to initiate 62 nucleotides downstream of the EcoRI site (Fig. 1); however, the 3-prime end of the gene (up to the HindIII site) did not contain a stop codon. Additional experiments were initiated to determine the sequence of the remaining portion of hasC. Chromosomal DNA was isolated from WF51 (the prototype encapsulated strain of group A streptococci), cloned to form a circular piece of DNA, and subjected to inverse PCR using primers D-10 and D-11 (see "Experimental Procedures"). The 1.4-kb PCR product was then sequenced using hasC primers to complete the sequence of hasC. A termination codon (TAA) was found 39 nucleotides (12 amino acids) downstream of the HindIII site of pGAC144, thus identifying the end of hasC.

hasC was demonstrated to consist of 915 base pairs (304 amino acids, approximately 33.7 kDa) and possesses a potential Shine-Delgarno sequence (Fig. 1; RBS, base pairs 176–184) capable of basepairing with the 3-prime terminus of the streptococcal 16 s rRNA (27). Weak 235 and 210 sites were observed upstream of the hasC start codon; however, primer extension analysis did not identify a transcription start site directly upstream of hasC (data not shown), and Northern analyses demonstrated that hasC was part of the 4.2-kb hasC operon transcript (14). A potential transcriptional terminator was located within the 3-prime end of hasC, spanning an additional 24 base pairs past the hasC termination codon. These data suggested that hasC is the third and last gene in the hasC operon.

Comparison of hasC to Other Bacterial UDP-glucose Pyrophosphorylases—Sequence comparisons using the FASTA program of the Genetics Computer Group software package (21) revealed that hasC shows homology to other genes that encode UDP-glucose pyrophosphorylase. HasC displayed 71.2% identity (init, 1682) to Cps3U from type 3 Streptococcus pneumoniae (28) (Fig. 2), 63.5% identity (init, 667) to GtaB from Bacillus subtilis (29), 57.5% identity (init, 432) to E. coli GalU (30), 53.6% identity (init, 218) to CelA of Acetobacter xylinum (31), and 52.8% identity (init, 178) to Rhizobium meliloti ExoN (32). The lysine residue essential for catalytic activity of UDP-glucose pyrophosphorylase from potato tuber has previously been determined (33). Alignment of the surrounding 9 amino acid residues with HasC and Cps3U demonstrated homology with 6 residues including the catalytic lysine (Fig. 2). Further experimentation is required to determine whether this lysine is involved in catalysis in the streptococcal enzyme.
Fig. 2. Alignment of the HasC and Cps3U amino acid sequences. An optimal alignment of HasC and Cps3U proteins was generated using BESTFIT program from GCG software package. Bars represent identical amino acids; single dots, similarities of <0.5 but >1.0; and double dots, similarities that are >0.5 between the residues. The shaded region indicates the region of identity around the active site lysine with potato tuber enzyme. The catalytic lysine residue defined by Katsube et al. (33).

pET11a were transformed into DEV6, an E. coli K-12 strain that has a mutation in galU and is therefore deficient in UDP-glucose pyrophosphorylase activity (18). After growth in chromogenic medium containing bromthymol blue, the clone containing pGAC315 produced acid from galactose as exhibited by the color change from green to yellow. J M109 (galU) served as a positive control. However, the clone that contained the vector without hasC or untransformed DEV6 were unable to ferment galactose. These observations further indicated that hasC encodes UDP-glucose pyrophosphorylase.

Overexpression of hasC in E. coli—To determine the molecular size of its gene product, hasC was subcloned under the control of the T7 promoter in the pET11a translation vector (see "Experimental Procedures"). For T7 expression, transformants harboring pGAC315 or pET11a were grown in TYPG and induced with 1 mM IPTG. Transformants containing pLyS5 and pGAC315 produced the maximum amount of overexpression as compared to transformants harboring pLeS5 or BL21(DE3) alone (data not shown). Control cells harboring pET11a did not exhibit overexpression of new proteins (Fig. 3; pET11a, lanes 0, 2, and 3) as compared to cells containing pGAC315 (lanes 2 and 3) which demonstrated overexpression of a 36-kDa protein at 2 and 3 h postinduction with IPTG. This band was not enhanced in extracts of cells harboring pGAC315 prior to IPTG induction (Fig. 3, lane 0).

Analysis of UDP-Glucose Pyrophosphorylase Activity—Following the expression of hasC in E. coli, cell extracts were assayed for UDP-glucose pyrophosphorylase activity as described under "Experimental Procedures" in order to confirm that hasC coded for UDP-glucose pyrophosphorylase. In these experiments, BL-21(DE3)(pLyS5) harboring the pET11a expression plasmid did not demonstrate UDP-glucose pyrophosphorylase activity before or after induction. Similarly treated BL-21(DE3)(pLyS5) harboring pGAC315 demonstrated UDP-glucose pyrophosphorylase activity only after IPTG induction (specific activity 0.13 unit/mg of protein). Substitution of UDP-galactose for UDP-glucose in the assays demonstrated no activity in uninduced or IPTG-induced organisms (data not shown).

DISCUSSION

Sequence and homology analyses demonstrated that hasC encoded UDP-glucose pyrophosphorylase, the enzyme that in the presence of UTP catalyzes the reaction of glucose 1-phosphate to UDP-glucose. UDP-glucose is the substrate for the gene product of hasB, UDP-glucose dehydrogenase, responsible for the production of UDP-glucuronic acid in the presence of NAD. Finally, UDP-glucuronic acid is a component of the hyaluronic acid capsule for group A streptococci which is synthesized by the membrane associated enzyme hyaluronate synthase encoded by hasA. All three genes have been shown to be components of the has operon (10, 11, 14). It has previously been shown by Southern analysis that the three genes that comprise the operon are located on the same 8.4-kb XbaI restriction fragment. In addition, Northern blot analyses demonstrated that hasA, hasB, and hasC are contained within a 4.2-kb mRNA transcript. Sequence data revealed that hasC is located 192 nucleotides downstream of hasB. This is a substantial distance for sequential genes in an operon in streptococci where the distance between hasA and hasB is only 37 nucleotides. Potential transcription initiation sites were observed between hasB and hasC; however, primer extension analyses of the entire 192 nucleotide region using multiple primers did not reveal an initiation site (data not shown) providing further evidence that hasC is a constituent of the has operon.

Our previous Northern analysis data (14) and the presence of a potential rho-independent terminator at the 3-prime end of hasC gene (Fig. 1, bold) provides strong evidence that hasC is the last gene comprising the has operon. Characteristics of a strong terminator include dyad symmetry, an inverted repeat containing 6–8 uracil residues, and a stem-loop rich in G and C nucleotides. The stem of the terminator for hasC is 25 nucleotides in length and contains 6 A-T pairs, the loop consists of 5 nucleotides (CACAAGT), and there are 16 G or C nucleotides (Fig. 4). Although the terminator of hasC complies with all of the requirements for a rho-independent terminator, further experimentation is required to confirm that this potential
structure is the transcription termination site of the has operon.

To overexpress hasC in E. coli, the pET-T7 expression system was utilized. This system employed the use of various plasmids to enhance the expression of the gene of interest, especially genes that may be toxic to the cell. For overexpression of hasC, transformants harboring pLysE were found to produce maximal amounts of HasC (Fig. 3), as compared to transformants containing pLysE or BL-21(DE3) alone (data not shown). The higher level of lysozyme produced from pLysE has been shown to interfere with the growth rate of the bacteria and may decrease the amount of transcription after IPTG induction. As with the previous expression of hasB (10) it was important to use low copy number plasmids under tight transcription control for expression of the gene product in E. coli.

The predicted size for HasC was approximately 33.7 kDa which is in good agreement with the product of the T7 overexpression of hasC in E. coli (36 kDa). Variation in the predicted size from the size demonstrated by SDS-PAGE also was seen with other bacterial UDP-glucose pyrophosphorylases. GalU was shown to be 40 kDa (predicted size, 32.9 kDa) (22, 30) and CelA was 30 kDa (predicted size, 30.9 kDa) (31). The size difference between the predicted and the actual size determined by SDS-PAGE could be due to secondary structure of the protein causing the protein to migrate through the gel slower than expected.

Also as with the expression analysis of group A streptococcal UDP-glucose dehydrogenase (HasB) (10), induction of group A streptococcal UDP-glucose pyrophosphorylase in E. coli appears to have led to a greater expression of protein (Fig. 3) than enzyme activity. Analysis of crude extracts indicated that enzyme activity versus protein concentration appeared approximately linear (data not shown) and that no NADPH oxidation was demonstrated. One important consideration is that the enzyme is labile and/or inhibited by components of the assay system. Toward this hypothesis it was determined that both imidazole-HCl and Tricine-NaOH buffers greatly inhibited the streptococcal enzyme (data not shown). Both of these buffers have previously been used for other UDP-glucose pyrophosphorylase assays (24, 25). Finally, the specific activity of various preparations of the streptococcal UDP-glucose pyrophosphorylase varied 2–4-fold when expressed in E. coli (data not shown). This did not appear to be due to granule formation since all of the enzyme activity could be retrieved from the cytoplasmic fraction.

UDP-glucose pyrophosphorylase is found in a wide variety of organisms (plant, animals, and bacteria) and provides UDP-glucose for the biosynthetic pathways of many carbohydrates (sucrose, cellulose, and glycogen) as well as hyaluronic acid. The most detailed work on UDP-glucose pyrophosphorylases is found using the cDNA from potato tuber (34). The cDNA has been cloned and the recombinant protein expressed in E. coli (33). The active site of the enzyme was shown to include 5 key lysine residues, as shown by site-directed mutagenesis and affinity labeling (33). Hossain et al. (22) have demonstrated that prokaryotic UDP-glucose pyrophosphorylases are structurally diverse from the eukaryotic enzyme. Although HasC and other prokaryotic UDP-glucose pyrophosphorylases do not possess sequence homology with the potato tuber enzyme or other eukaryotic pyrophosphorylases, many contain the lysine residue found to be indispensable for enzyme function (Fig. 2, Lys-367 from potato tuber). However, further structural analysis of the catalytic sites of these enzymes are required in order to confirm whether this lysine is necessary for enzymatic activity in the prokaryote UDP-glucose pyrophosphorylases.

In summary, our recent Northern analysis data demonstrating a single 4.2-kb transcript (14) and the observed putative rho-independent terminator at the 3-prime end of hasC would indicate that the has operon is composed of hasA, hasB, and hasC. On going experiments include analysis of regulatory factors that may assist in the control of has operon transcription during exponential phase of growth. Finally, purification of the expressed HasC protein will be required to determine its enzyme kinetics and an analysis of its active site is required for comparison to other prokaryote and plant and animal UDP-glucose pyrophosphorylases to better understand the evolutionary differences between these enzymes.

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REFERENCES

1. Veasy, L. G., Wiedmeier, S. E., Orsmond, G. S., Ruttenberg, H. D., Boucek, M. M., Roth, S. J., Taft, V. F., and Thompson, J. A. (1987) N. Engl. J. Med. 316, 421-427
2. Lancefield, R. C. (1962) J. Immunol. 89, 307-313
3. Johnson, D. R., Stevens, D. L., and Kaplan, E. L. (1992) J. Infect. Dis. 166, 374-382
4. Panzehl, V., and Fischetti, V. A. (1992) J. Exp. Med. 176, 415-426
5. Wessels, M. R., Moses, A. E., Goldberg, J. B., and DiCesare, T. J. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 8317-8321
6. Wessels, M. R., Goldberg, J. B., Moses, A. E., and DiCesare, T. J. (1994) Infect. Immun. 62, 433-441
7. Wessels, M. R., and Bronze, M. S. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 12238-12242
8. Markovitz, A., Cifferi, J. A., and Dorfman, A. (1959) J. Biol. Chem. 234, 2343-2350
9. Dougherty, B. A., and van de Rijn, I. (1992) J. Exp. Med. 175, 1293-1299
10. Dougherty, B. A., and van de Rijn, I. (1993) J. Biol. Chem. 268, 7118-7124
11. Dougherty, B. A., and van de Rijn, I. (1994) J. Biol. Chem. 269, 169-175
12. DeAngelis, P. L., Papacostantinou, J., and Weigel, P. H. (1993) J. Biol. Chem. 268, 14568-14571
13. DeAngelis, P. L., Papacostantinou, J., and Weigel, P. H. (1993) J. Biol. Chem. 268, 19181-19184
14. Crater, D. L., and van de Rijn, I. (1995) J. Biol. Chem. 270, 18452-18458
15. DeAngelis, P. L., and Weigel, P. H. (1994) Biochemistry 33, 9033-9039
16. van de Rijn, I., and Kessler, R. E. (1980) Infect. Immun. 27, 444-448
17. Strathmann, M., Hamilton, B. A., Mayeda, C. A., Simon, M. I., and Meyrowitz, E. M. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1247-1250
18. Roop, R., and Elseviers, D. (1980) J. Bacteriol. 143, 1054-1056
19. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) Methods Enzymol. 185, 60-89
20. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1987) Current Protocols in Molecular Biology, Wiley, New York
21. Deveauux, J., Haeberli, P., and Smithies, O. (1984) Nucleic Acids Res. 12, 387-395
22. Hossain, S. A., Tanizawa, K., Kazuta, Y., and Fukui, T. (1994) J. Biochem. (Tokyo) 115, 983-972
23. Laemmli, U. K. (1970) Nature 227, 680-688
24. Joshi, J. G. (1982) Methods Enzymol. 89, 599-605
25. Fjaerov, E., Fredylund, K., Vallis, S., Huggrat, Y., and Benzman, M. (1991) FEMS Microbiol. Lett. 77, 325-330
26. Markwell, M. A. K., Haas, S. M., sieve, L. L., and Bolbert, N. E. (1978) Ani. J. Bacteriol. 166, 420-422
27. Ludwig, W., Seewaldt, E., Kilpper-Balz, R., Schleifer, K. H., Magrum, L., and Tams, R. A. (1992) J. Exp. Med. 176, 965-972
28. Dillard, J. P., Vineyard, M. W., and Yother, J. (1994) J. Med. Microbiol. 42, 60-89