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Comparative Study of Hemolytic Substances Produced by Coagulase-Negative *Staphylococcus* Strains

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Hemolytic substances H7, H62, and E56, produced by *Staphylococcus haemolyticus* 7 and 62 and *S. epidermidis* 56, respectively, were purified. H7 and H62 are probably similar on the basis of their isoelectric focusing profiles in 8 M urea and complete immunological identity as revealed by immunodiffusion with rabbit anti-H7 and anti-E56 sera. For E56, we observed seven bands instead of three in isoelectric focusing and only partial immunological identity with H7 and H62. However, H7 and E56 were similar with regard to the following characteristics: hemolytic spectra against different erythrocytes, kinetics of erythrocyte lysis, heat stability, and inhibition by phosphatidylcholine. E56 was not active at a temperature lower than or equal to 25°C, and its activity increased more rapidly with increased temperature compared with H7. For both substances, the complexes obtained by molecular filtration on Ultrogel AcA54 and the purified peptides by reverse-phase high-pressure liquid chromatography showed some hemolytic activity. These results suggest that a particular association or the presence of a given peptide could enhance the activity.

Coagulase-negative (CN) staphylococci are organisms that normally live in harmony with humans, either within the normal flora or in the immediate environment. Diseases caused by these organisms are frequently associated with abnormal circumstances, such as surgical implantation of a foreign body, medical interference with host defenses, or an overwhelming dose of the organism that overcomes the defenses, often through failure of a mechanical device. *Staphylococcus epidermidis* is responsible for most infections and has been associated with urinary tract infections (13), peritonitis as a complication of continuous ambulatory peritoneal dialysis (22), prosthetic valve endocarditis (5), and neonatal necrotizing enterocolitis (20, 21). Infections have also been associated with *S. haemolyticus*, *S. saprophyticus*, *S. hominis*, and *S. capitis* (7).

Delta-like-toxin activity has been reported in most tested clinical isolates of CN staphylococci (10–12, 14, 21). Gemmel and Roberts (12), by examination of 118 CN staphylococcal isolates of human infections, found that many of the strains produced up to six distinct toxins and enzymes, including both alpha and delta hemolysins. Delta-like toxin produced by CN staphylococci has been associated with neonatal necrotizing enterocolitis (20, 21).

A gonococcal growth inhibitor with delta-like-hemolytic activity produced by *S. haemolyticus* 7 has already been purified and characterized (1, 8, 9). This substance is a lipid-associated protein in which the protein component is the active part. The protein component is composed of low-molecular-weight peptides that, in the absence of a dissociating agent, are present as complexes or aggregates. Amino acid sequence analyses have shown that this substance is composed of three peptides of 44 amino acids each with high sequence homology (23). These peptides contain a high proportion of hydrophobic amino acids, and their hydrophobicity profiles are closely related. Many of the properties exhibited by the inhibitor are similar to those reported for the delta toxin of *S. aureus* (16, 24). However, on the basis of their amino acid sequences, the two substances are completely different.

The present work was undertaken to isolate the antigonococcal and hemolytic substances produced by *S. haemolyticus* 62 and *S. epidermidis* 56 and to compare their chemical and biological properties with those of the substance produced by *S. haemolyticus* 7.

MATERIALS AND METHODS

Bacterial strains. *S. haemolyticus* 7 and 62 and *S. epidermidis* 56 were isolated from urogenital flora (3, 18) and used for production of the respective antigonococcal and hemolytic substances H7, H62, and E56. *Neisseria gonorrhoeae* G-10 was the reference target strain (3), and it was cultivated as previously described (9). These strains were kept either in a freeze-dried state or as frozen suspensions at −76°C.

Production and purification of antigonococcal and hemolytic substances. Production and purification of the substances were performed as previously described for H7 (1). We used brain heart infusion semisolid medium for production and methanol extraction, acetone fractionation, dialysis, and chromatography on Ultrogel AcA54 for purification. The protein components were separated from the lipids by chromatography on Ultrogel AcA54 in 4 M urea. Purification of the different substances was monitored by quantitative determination of the antigonococcal activities of the different preparations as already described (18).

In one experiment, the protein components of H7 and E56 were rechromatographed on an Ultrogel AcA54 column (2.5 by 90 cm) in 0.05 M ammonium acetate buffer, pH 6.0. The fractions corresponding to the different molecular complexes were pooled, their molecular weights were estimated by comparison with different proteins with known molecular weights, and their hemolytic activities against horse erythrocytes were determined.

Protein determination. The protein concentration was determined by the method of Lowry et al. (19), with bovine serum albumin as the reference. In some experiments, the method of Bradford using the Bio-Rad assay kit (Bio-Rad Laboratories, Mississauga, Ontario, Canada) was used.

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Hemolytic activity. Quantitative determination of hemolytic activity was performed as already described (9). A hemolytic unit (HU) was defined as the reciprocal of the dilution of the sample that caused 50% lysis compared with the maximal hemolysis produced by 0.05% saponin. Horse or sheep erythrocytes were routinely used. For the hemolytic spectrum experiment, human, mouse, rabbit, and bovine erythrocytes were also used.

The kinetics of erythrocyte lysis were determined by addition of the appropriate amount of the hemolytic substance to 30 ml of a standardized suspension of horse erythrocytes in 0.05 M phosphate-buffered saline (pH 7.0). The preparation was incubated at 37°C, and samples were taken at 5-min intervals. The samples were immediately centrifuged on a microcentrifuge for 2 min, and the optical density of the supernatant was measured at 545 nm.

Polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed with 10% polyacrylamide by the method of Laemmli (17). Isoelectric focusing in 7% polyacrylamide gels with 8 M urea was performed by a modification of the procedure of Wrigley (25) as already described (9). The pH gradient was determined with a pI marker protein kit (Pharmacia Fine Chemicals, Piscataway, N.J.).

HPLC fractionation. Purification of the different peptides of H7 and E56 was performed at room temperature with a Waters 650 high-pressure liquid chromatography (HPLC) apparatus coupled with a Waters 481 Lambda-Max LC spectrophotometer. The column used was a Waters Radial-Pak C_{18} with an RCM-100 radial compression module. The substances (1 mg dissolved in 1.0 ml of 0.1% [vol/vol] trifluoroacetic acid [TFA]) were injected onto the column, which had been previously equilibrated in 0.1% TFA. The mobile phase was composed of solvent A (0.1% TFA) and solvent B (100% acetonitrile, 0.1% TFA). Elution of peptides of H7 was done with a linear gradient from 0 to 50% solvent B in 20 min, from 50 to 70% solvent B in 65 min, and from 70 to 100% solvent B in 30 min. For substance E56, the elution of the peptides was performed with a linear gradient from 50 to 90% solvent B in 80 min. The flow rate was 1 ml/min.

Inhibition of hemolytic activity by phosphatidylcholine. Samples of lipoprotein complexes H7 and E56 containing approximately 30 HU/ml and various amounts of phosphatidylcholine (type V-E from egg yolk; Sigma Chemical Co., St. Louis, Mo.) were incubated at room temperature for 30 min. The hemolytic activity of each preparation was then evaluated against horse erythrocytes as previously described. Three assays were performed for each concentration of phosphatidylcholine, and the average was calculated.

Immunological studies. Antisera to purified H7 and E56 were prepared in rabbits. The purified substances were administered in a multiple emulsion of complete Freund adjuvant (Difco Laboratories, Detroit, Mich.) with 1% Tween 80. Each animal received a total of 1.0 mg of H7 or E56 in 10 dorsal subcutaneous injections of 0.2 ml each. One month later, the animals were boosted by administration for 3 successive days of a suspension containing 1 mg of antigen in 3 ml of phosphate-buffered saline with 1% Tween 80. Intramuscular injection of 0.5 ml of the suspension on day 1 was followed by intravenous injections of 1.0 and 1.5 ml on days 2 and 3, respectively. The rabbits were bled 1 month after the last injection. Immunodiffusion experiments were conducted by the Ouchterlony double-diffusion technique with 1% agarose in phosphate-buffered saline containing 0.05% (wt/vol) sodium azide.

Chemicals. Agarose, acrylamide, *N*,*N*′-methylenebisacrylamide, and urea were electrophoresis reagent purity grade, purchased from Bio-Rad. Ampholines were products of LKB Instruments, Inc., Rockville, Md. Trifluoroacetic acid and acetonitrile were HPLC purity grade from J. T. Baker Chemical Co., Phillipsburg, N.J. Other chemicals were reagent grade from Fisher Scientific Co., Montreal, Quebec, Canada.

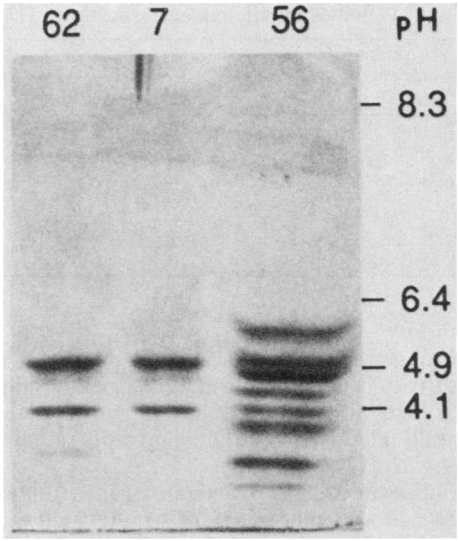

FIG. 1. Isoelectric focusing of H7, H62, and E56 (25 μg each) in a 7% polyacrylamide gel with 8 M urea.

RESULTS

H7, H62, and E56 were purified to homogeneity as estimated by the presence of only one band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (data not shown). A band of similar mobility was found for each substance. The protein component of each substance was further characterized by isoelectric focusing in a polyacrylamide gel with 8 M urea (Fig. 1). H7 and H62 showed the same profile, with two major bands and two minor bands between pH 4.0 and 5.0. E56 had a different profile: four major bands and three or four minor bands between pH 3.0 and 6.0.

In an immunodiffusion experiment with anti-H7 serum, two continuous lines of precipitation were observed between H7 and H62 and one weak line was observed for E56 (Fig. 2A). With anti-E56 serum, H7 and H62 showed a continuous

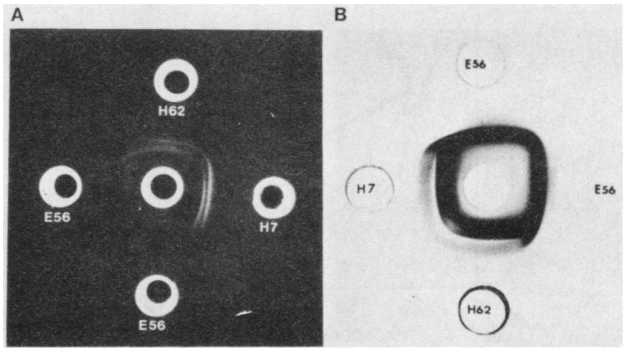

FIG. 2. Immunodiffusion experiment with H7, H62, and E56. Antisera to H7 (A) and E56 (B) were added to the center wells.

TABLE 1. Sensitivities of different erythrocytes to H7 and E56

Erythrocyte source	Hemolytic activity (%)	
	H7	E56
Horse	100	100
Cow	91	25
Human	82	100
Sheep	51	23
Rabbit	23	34
Mouse	58	86

precipitin line and one line of partial identity with E56 (Fig. 2B). These results were confirmed by rocket immunoelectrophoresis (data not shown). Since H7 and H62 appeared to be structurally identical, the following work was performed principally with H7 and E56.

The antigonococcal test revealed that the protein component of E56 had a specific activity of 159, which was similar to the value of 178 already reported for H7 (1). The hemolytic spectra against different erythrocytes are shown in Table 1. H7 and E56 showed some similarity in their hemolytic spectra, although some important differences were also observed. Human and horse erythrocytes were the most susceptible. H7 was much more active on bovine erythrocytes than was E56. E56 was inhibited by phosphatidylcholine but was less susceptible than H7 (Fig. 3). Complete inhibition of hemolytic activity against horse erythrocytes by phosphatidylcholine was observed at 130 μg/ml for E56 and at 80 μg/ml for H7. H7 and E56 showed similar kinetics of erythrocyte lysis with no lag (Fig. 4).

The effects of incubation temperature on the hemolytic activities of both substances are shown in Fig. 5. Hemolytic activity was dependent on temperature; however, this effect was different for each substance. A more rapid rise in specific hemolytic activity as a function of increased incubation temperature was observed for E56 than for H7.

The stabilities of H7 and E56 when incubated at 100°C were compared (Fig. 6). Although the results obtained with E56 varied greatly, complete inactivation of H7 seemed more rapid than that of E56.

The protein components of the hemolytic substances were present as complexes of different molecular weights composed of particular associations of peptides. These complexes were separated by molecular filtration, and their molecular weights and activities were evaluated (Table 2).

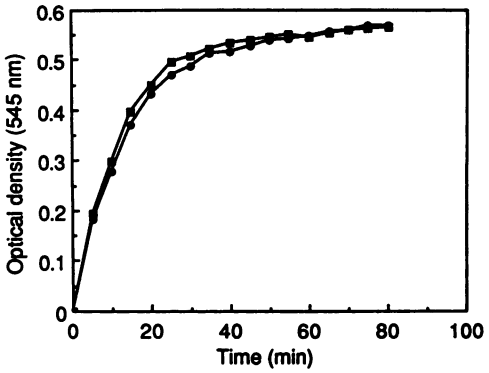

FIG. 4. Kinetics of horse erythrocyte lysis by H7 (■) and E56 (●).

H7 was fractionated in three peaks, and the specific hemolytic activities of these complexes increased with molecular weight. E56 was separated in two peaks whose specific activities were similar to the value obtained for E56 before chromatography.

Purification of the peptides of H7 and E56 was performed by reverse-phase HPLC. H7 was fractionated into three peptides (23). E56 was separated into seven components (Fig. 7). Components E3, E4, E5, and E6 were present in the greatest concentrations. Peptides E4 and E5 were rechromatographed to achieve better purification. The purified peptides of both substances were lyophilized and tested for hemolytic activity (Table 3). Peptide H3 showed significant hemolytic activity. Peptides E1 and E2 had no or only traces of hemolytic activity. Peptides E3, E4, E5, and E6 and a mixture of these peptides showed some activity (11 to 44 HU/mg of protein); however, this was lower than the activity exhibited by E56 before fractionation.

DISCUSSION

The method of purification already described for H7 (1) was also sufficient to purify H62 and E56. These substances are composed of several peptides present as complexes or aggregates. The results of isoelectric focusing in 8 M urea and of immunodiffusion with rabbit anti-H7 and anti-E56 sera suggest that H62 and H7, produced by two different strains of *S. haemolyticus*, are similar. E56 produced by *S. epidermidis* was different, although some epitopes are shared with the other substances, as illustrated by the partial

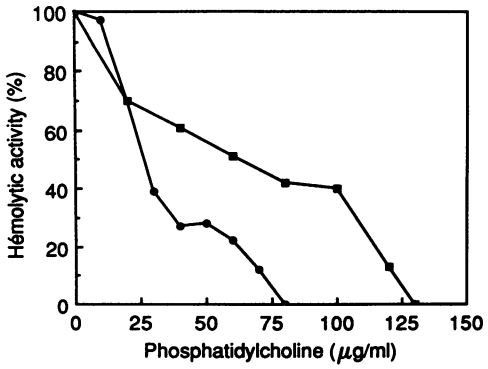

FIG. 3. Effects of various concentrations of phosphatidylcholine on the hemolytic activities of H7 (●) and E56 (■).

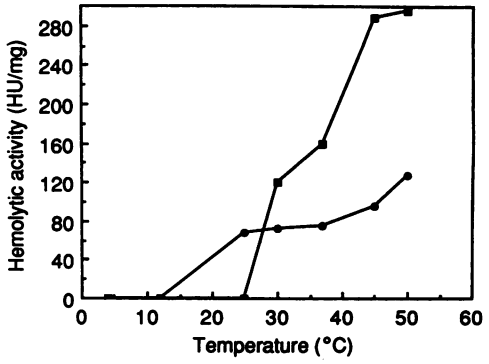

FIG. 5. Effect of incubation temperature on the hemolytic activities of H7 (●) and E56 (■).

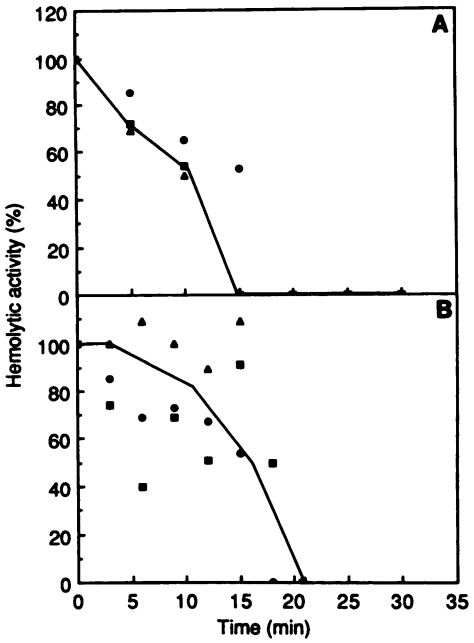

FIG. 6. Stability of H7 (A) and E56 (B) incubated at 100°C. The different symbols correspond to three different experiments.

identity observed in immunodiffusion. Some similarities have already been observed between a bacteriostatic antigonococcal substance produced by *S. epidermidis* 66 and substance H7 (4); however, this substance has not been further characterized.

The results of isoelectric focusing and elution on reverse-phase HPLC suggest that E56 is composed of seven peptides, four of which are present in higher concentrations. Some homology could exist between these peptides, as shown for the three peptides of H7 (23). Amino acid sequence analysis of the peptides that constitute E56 would be necessary to determine precisely the structural relationships among all of these staphylococcal peptides.

The hemolysins described in the literature are generally composed of only one polypeptide and are frequently present as aggregates. Delta toxin and mellitin are peptides of 26 amino acids each (6). Streptolysin O produced by beta-hemolytic streptococci and other hemolysins inactivated by oxygen, such as tetanolysin, cereolysin, listeriolysin, and perfringolysin, are made up of one polypeptide with a molecular weight of 50,000 to 80,000 (2). To be active, most of these polypeptides must be present as complexes.

TABLE 2. Hemolytic activities of different complexes of H7 and E56 obtained by molecular filtration on Ultrogel AcA54

Preparation	Mol wt	Hemolytic activity (HU/mg)[a]
H7 peaks		288
HA	35,500	896
HB	33,800	544
HC	30,000	256
E56 peaks		368
EA	≥70,000	280
EB	35,500	320

[a] Protein concentrations were determined by the Bradford method by using the Bio-Rad assay kit.

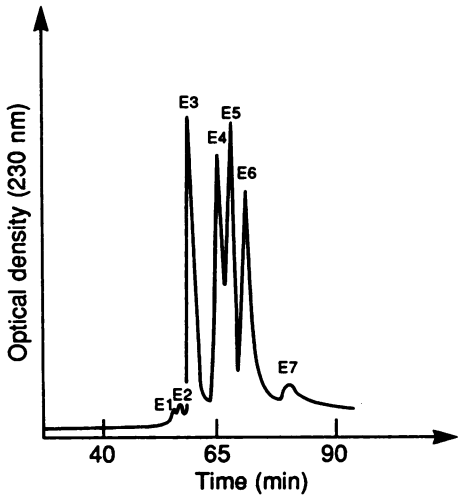

FIG. 7. Separation of E56 components on reverse-phase HPLC. E56 (1 mg) was dissolved in 0.1% TFA and injected into a column equilibrated with 0.1% TFA. Elution was with a linear acetonitrile gradient from 50 to 90% in 80 min with a flow rate of 1 ml/min.

The importance of the formation of a complex or a particular arrangement of the peptides is suggested for H7 by the differences in the hemolytic activities of the different complexes isolated after molecular filtration. The purified peptides must probably reassociate to be active. The presence of one of these peptides could be important, as suggested by the high specific activity of purified peptide H3. However, for E56, no difference in hemolytic activity was observed for the two molecular complexes isolated and the different purified peptides. The mixture of peptides E3, E4, E5, and E6 showed activity similar to that of the separated peptides. It is possible that rearrangement of the peptides in the most active association was not obtained with the experimental conditions used. Some denaturation of the peptides might be induced by the extreme conditions used in the purification of the peptides (acetonitrile in 0.1% TFA) on HPLC.

Previous work on the biological activity of H7 (9) has shown that this toxin had some properties similar to those of the delta toxin of *S. aureus*: high activity against human and horse erythrocytes, inhibition of hemolytic activity by phosphatidylcholine, and relative resistance to heat. E56 had a

TABLE 3. Hemolytic activities of different peptides of H7 and E56

Preparation	Hemolytic activity (HU/mg)[a]
H7	640
Peptide H1	ND
Peptide H2	40
Peptide H3	457
E56	178
Peptide E3	33
Peptide E4	44
Peptide E5	28
Peptide E6	11
E3-E4-E5-E6	21

[a] Protein concentrations were determined by the Bradford method by using the Bio-Rad assay kit. ND, Not determined (the quantity of material obtained was too low).

slight susceptibility to inhibition by phosphatidylcholine, and the incubation temperature had a marked effect on its hemolytic activity. The hemolytic activity increased rapidly when the temperature increased, suggesting a possible surface-active effect of these substances on biological membranes, where hydrophobic interactions would be important (15). The effects of the incubation temperature and some other parameters presented suggest that E56 is different from H7, although they share some biological properties. No lag was observed in the kinetics of erythrocyte lysis; this could mean that the binding of the substances to erythrocytes is rapid.

The importance of these toxins in the pathogenicity of CN staphylococci is not known. Some researchers have reported the presence of delta-like-toxin activity in clinical isolates of CN staphylococci. Gemmel (10, 11), in investigations of 50 clinical isolates of CN staphylococci, has already established a correlation between heat-stable hemolysin activity and cytotoxin activity against fibroblast tissue culture cells. Only one of these toxins, which is produced by *S. epidermidis* and which has been associated with neonatal necrotizing enterocolitis (21), has been recently purified and characterized (A. I. McKevitt, G. B. Bjornson, and D. W. Scheifele, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, B-146, p. 55). The amino acid sequence of this toxin has high homology with that of the delta toxin of *S. aureus*. We cannot exclude the possibility that this peptide is present in E56.

ACKNOWLEDGMENTS

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LITERATURE CITED

1. **Beaudet, R., J. G. Bisaillon, S. A. Saheb, and M. Sylvestre.** 1982. Production, purification, and preliminary characterization of a gonococcal growth inhibitor produced by a coagulase-negative staphylococcus isolated from the urogenital flora. Antimicrob. Agents Chemother. **22**:277–283.
2. **Bhakdi, S., J. Tranum-Jensen, and A. Sziegoleit.** 1985. Mechanism of membrane damage by streptolysin-O. Infect. Immun. **47**:52–60.
3. **Bisaillon, J. G., R. Beaudet, L. Lafond, S. A. Saheb, and M. Sylvestre.** 1981. Interference of *Neisseria gonorrhoeae* growth by aerobic bacterial representatives of the urogenital flora. Rev. Can. Biol. **39**:201–208.
4. **Bisaillon, J. G., L. Lafond, R. Beaudet, S. A. Saheb, and M. Sylvestre.** 1985. Comparison of bacteriostatic and bactericidal inhibitors of *Neisseria gonorrhoeae* growth produced in vitro by urogenital staphylococci. Exp. Biol. **43**:231–242.
5. **Etienne, J., Y. Brun, N. El Solh, V. Delorme, C. Mouren, M. Bes, and J. Fleurette.** 1988. Characterization of clinically significant isolates of *Staphylococcus epidermidis* from patients with endocarditis. J. Clin. Microbiol. **26**:613–617.
6. **Fitton, J. E., A. Dell, and W. V. Shaw.** 1980. The amino acid sequence of the delta hemolysin of *Staphylococcus aureus*. FEBS Lett. **115**:209–212.
7. **Fleurette, J., Y. Brun, M. Bes, M. Coulet, and F. Forey.** 1987. Infections caused by coagulase-negative staphylococci other than *S. epidermidis* and *S. saprophyticus*. Zentralbl. Bakteriol. Mikrobiol. Hyg. Abt. 1 Orig. **16**(Suppl.):195–208.
8. **Frenette, M., R. Beaudet, J. G. Bisaillon, and V. Portelance.** 1988. Site of action of a gonococcal growth inhibitor produced by *Staphylococcus haemolyticus*. J. Med. Microbiol. **26**:199–204.
9. **Frenette, M., R. Beaudet, J. G. Bisaillon, M. Sylvestre, and V. Portelance.** 1984. Chemical and biological characterization of a gonococcal growth inhibitor produced by *Staphylococcus haemolyticus* isolated from urogenital flora. Infect. Immun. **46**: 340–345.
10. **Gemmell, C. G.** 1986. Virulence characteristics of *Staphylococcus epidermidis*. J. Med. Microbiol. **22**:287–295.
11. **Gemmell, C. G.** 1987. Exo-proteins of coagulase-negative staphylococci as possible virulence factors. Zentralbl. Bakteriol. Mikrobiol. Hyg. Abt. 1 Orig. **16**(Suppl.):93–102.
12. **Gemmell, C. G., and E. Roberts.** 1973. Toxins and enzymes of coagulase-negative staphylococci isolated from human infections. J. Hyg. Epidemiol. Microbiol. Immunol. **18**:276–280.
13. **John, J. F., P. K. Gramling, and N. M. O'Dell.** 1978. Species identification of coagulase-negative staphylococci from urinary tract isolates. J. Clin. Microbiol. **8**:435–437.
14. **Kleck, J. L., and J. A. Donohue.** 1968. Production of thermostable hemolysin by cultures of *Staphylococcus epidermidis*. J. Infect. Dis. **118**:317–323.
15. **Kondo, T.** 1976. Mechanism of hemolysis by surface active agents. Adv. Colloid Interface Sci. **6**:139–172.
16. **Kreger, A. S., K. S. Kim, F. Zaboretzky, and A. W. Bernheimer.** 1971. Purification and properties of staphylococcal delta hemolysin. Infect. Immun. **3**:449–465.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Lafond, L., J. G. Bisaillon, S. A. Saheb, R. Beaudet, and M. Sylvestre.** 1981. In vitro antigonococcal activity of urogenital coagulase-negative staphylococci. Can. J. Microbiol. **27**:138–141.
19. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
20. **Scheifele, D. W., and G. L. Bjornson.** 1988. Delta toxin activity in coagulase-negative staphylococci from the bowels of neonates. J. Clin. Microbiol. **26**:279–282.
21. **Scheifele, D. W., G. L. Bjornson, R. A. Dyer, and J. E. Dimmick.** 1987. Delta-like toxin produced by coagulase-negative staphylococci is associated with neonatal necrotizing enterocolitis. Infect. Immun. **55**:2268–2273.
22. **Verbrugh, H. A., and J. Van der Meulen.** 1986. *Staphylococcus epidermidis* and the CAPD host. J. Med. Microbiol. **22**:291–295.
23. **Watson, D. C., M. Yaguchi, J. G. Bisaillon, R. Beaudet, and R. Morosoli.** 1988. The amino acid sequence of a gonococcal growth inhibitor from *Staphylococcus haemolyticus*. Biochem. J. **252**:87–93.
24. **Wiseman, G. M.** 1975. The hemolysins of *Staphylococcus aureus*. Bacteriol. Rev. **39**:317–344.
25. **Wrigley, C.** 1968. Gel electrofocusing. A technique for analyzing multiple protein samples by isoelectric focusing. Sci. Tools **15**:17–22.