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Facile Penetration of the *Staphylococcus aureus* Capsule by Lysostaphin

BRIAN F. KING, MICHAEL L. BIEL, AND BRIAN J. WILKINSON*
Department of Biological Sciences, Illinois State University, Normal, Illinois 61761

The ability of macromolecules to cross the capsular layer of encapsulated microorganisms and interact with their cell walls is important in considerations of the mechanisms of resistance to phagocytosis and of antigen masking in such strains. Lysostaphin was employed as a probe of the penetrability of the *Staphylococcus aureus* capsule. The rates of lysostaphin-induced lysis of encapsulated and unencapsulated *S. aureus* strains were compared. Encapsulated *S. aureus* strains M and Smith diffuse were lysed by lysostaphin at the same rate as their respective unencapsulated counterpart strains M variant and Smith compact. Growth of the M strain in a medium designed to enhance capsule production did not delay the onset or decrease the rate of lysis of the strain compared with organisms grown in normal medium. Cations did not selectively decrease the rate of lysis of the encapsulated strain, but inhibited the lysis of both the M and M variant strains. Peptidoglycan, the presumed lysostaphin target, isolated from both M and M variant strains was digested by lysostaphin at very similar rates. In contrast to whole cells, cations stimulated the rate of lysostaphin digestion of peptidoglycan. It is concluded that the fraction of lysostaphin active in cell lysis, believed to be a glycylglycine endopeptidase with a molecular weight of about 25,000, passes freely through the capsular layer to its target in the staphylococcal cell wall.

In recent studies of the mechanism of resistance to phagocytosis in encapsulated *Staphylococcus aureus* M, the third component of complement (C3) was found to become localized at the staphylococcal cell wall, under the capsule, after incubation of organisms in normal human serum (21). The cell wall localization of C3 implies that the capsule does not act as a barrier to cell wall-directed proteins of high molecular weight, such as immunoglobulins and complement components (21). However, Verbrugh et al. (18) found that less C3 became fixed to the cell surface of strain M than to the unencapsulated M variant strain. The study of Verbrugh et al. (18), therefore, suggests that the capsule may play some role as a barrier to C3 fixation. Also, in encapsulated *Escherichia coli*, Glynn and Howard (6) and Horwitz and Silverstein (7) appear to view the capsule as a barrier preventing access of antibodies and complement components to cell wall lipopolysaccharide.

The sieving properties of bacterial capsules do not appear to have been assessed in the same way as those of bacterial cell walls (12), and there is little information on the role of capsular polysaccharides as general barriers (5). It does appear that the capsules of a variety of bacterial species can exclude bacteriophage particles, which have a head diameter of about 100 nm, and prevent them from interaction with their receptors in the bacterial cell wall (2, 3, 19, 23). Whether the capsule can exclude soluble molecules of lower molecular weight from interaction with cell wall components is unclear.

The present study was undertaken to give some insight on the passage of molecules through the capsular layer of *S. aureus*. We have taken advantage of the fact that the protein preparation lysostaphin contains, as a major component, a glycylglycine endopeptidase with a molecular weight of about 25,000 (16), which specifically lyses staphylococcal cells (13) by hydrolyzing glycylglycine bonds in the polyglycine bridges that form cross-links in the staphylococcal peptidoglycan (10). We hypothesized that if the *S. aureus* capsule acted in some way as a barrier to lysostaphin access to the cell wall, then encapsulated strains should be lysed slower by the enzyme than unencapsulated variants. No difference was found in rates of lysis of encapsulated and unencapsulated strains, and the presumed lysostaphin target of these organisms, peptidoglycan, showed similar susceptibilities to lysostaphin. It is concluded that lysostaphin passes freely through the *S. aureus* capsule layer.

MATERIALS AND METHODS

Organisms. The *S. aureus* strains used in this study were encapsulated strain M and its unencapsulated derivative, M variant, encapsulated strain Smith diffuse and its unencapsulated derivative, Smith compact (19, 20).

Growth and washing of organisms. All organisms used in this study were maintained on tryptic soy agar (Difco Laboratories, Detroit, Mich.) slants at 4°C. Organisms were grown overnight in 20 ml of peptone-yeast extract-glucose-K_2HPO_4 (PYK) broth (20) or staphylococcus no. 110 medium as modified by Yoshida and Ekstedt (25) in 50-ml Erlenmeyer flasks with shaking (200 rpm) at 37°C. The cultures were harvested by centrifugation at 13,000 × *g* for 10 min at 4°C and were washed in an equal volume of cold distilled water.

Isolation of peptidoglycan. Peptidoglycan was isolated from *S. aureus* M and M variant strains by the method of Park and Hancock (9) with the slight modifications of Wilkinson and White (22).

Assay of lysostaphin-induced lysis. The pellets obtained after washing organisms in cold water were suspended in 2 ml of cold 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.5. An appropriate amount (usually 0.1 ml) of this suspension was added to cuvettes containing 4.9 ml of 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.5, with added cations when appropriate, to yield an absorbance at 580 nm (A_{580}) of 0.55 in a Spectronic 20 spectrophotometer (Bausch & Lomb, Inc., Rochester, N.Y.). To these suspensions, 0.1 ml of a stock solution of 200 μg of lysostaphin (Sigma Chemical Co., St. Louis, Mo.; 289 U/mg) per ml in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.5, containing 0.145 M NaCl was added, yielding a final concentration of 5 μg of lysostaphin per ml. The suspension was incubated at the chosen temperature (generally 30°C), and A_{580} readings were taken at intervals. A suspension to which no lysostaphin was added served as a control for autolysis; no autolysis was noted within the normal incubation period.

An appropriate amount of isolated peptidoglycan was added to cuvettes containing 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.5, with or without added cations, to yield an A_{580} of 0.55 and a final volume of 4.9 ml. To this suspension, 0.1 ml of the stock solution of lysostaphin was added, the mixture was incubated at 30°C, and A_{580} readings were taken as described above.

RESULTS AND DISCUSSION

Lysostaphin-induced lysis of encapsulated and unencapsulated *S. aureus* strains. The rates of lysostaphin-induced lysis of *S. aureus* M and M variant strains are shown in Fig. 1A. The strains were lysed at rates very similar to each other at 30 or 10°C in repeated experiments (see Fig. 2), although lysis was much slower at 10°C. Attempts were made to decapsulate *S. aureus* M to see whether capsule removal enhanced the rate of lysis. Three treatments of the M strain in 0.9% (wt/vol) saline at 40°C, each followed by homogenization in a Waring blender, as recommended by Stinson and van Oss (14) for strain Smith diffuse, failed to remove the capsule as observed in India ink preparations. The rates of lysostaphin-induced lysis of the M strain grown in PYK broth and modified staphylococcus no. 110 medium were compared. The modified staphylococcus no. 110 medium is reported to enhance capsule production (25), and it was hoped to make the M strain produce a larger capsule to see whether this delayed lysostaphin-induced lysis. As shown in Fig. 1B, organisms lysed at the same rate whether they were grown in PYK broth or staphylococcus no. 110 medium. In India ink preparations, organisms grown in staphylococcus no. 110 medium appeared more clumped but did not have a capsule noticeably larger than that of PYK broth-grown organisms.

In accord with the findings with M and M

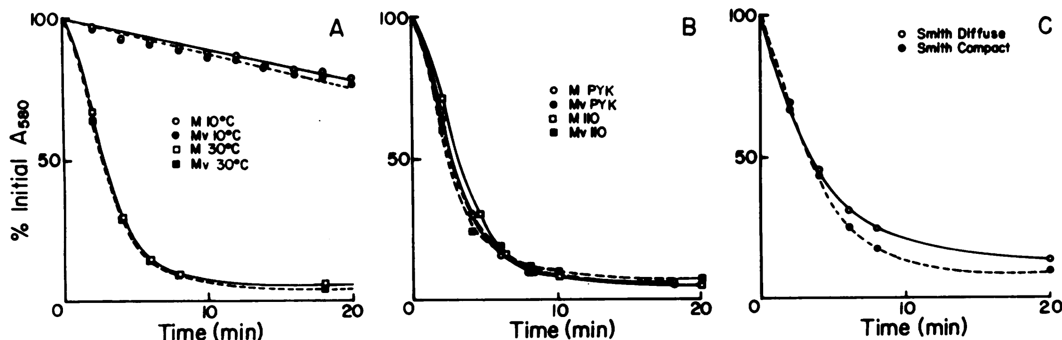

FIG. 1. *Lysostaphin-induced lysis of S. aureus. (A) Lysostaphin-induced lysis of M and M variant (Mv) strains at 10 and 30°C; (B) lysostaphin-induced lysis at 30°C of M and M variant strains grown in PYK broth and staphylococcus no. 110 medium; (C) lysostaphin-induced lysis of Smith diffuse and Smith compact strains at 30°C.*

variant strains, no difference in the rates of lysis of encapsulated *S. aureus* strain Smith diffuse and its unencapsulated counterpart Smith compact was found (Fig. 1C) in experiments conducted at 30°C.

Effect of cations on the rates of lysostaphin-induced lysis of encapsulated and unencapsulated *S. aureus*. *S. aureus* capsular polysaccharides are polyanions (8) and are expected to bind cations (5). The divalent cations Mg^{2+} and Ca^{2+} have been shown to "stiffen" polysaccharides (24). The effects of cations on lysostaphin-induced lysis were compared in encapsulated M and unencapsulated M variant strains (Fig. 2). Lysostaphin-induced lysis of both M and M variant strains was strongly inhibited by 100 mM Mg^{2+} or Ca^{2+} (Fig. 2A and B). The M variant strain was inhibited to a greater extent than was the M strain at 100 mM concentrations of these cations. The monovalent cation, Na^{+}, did not inhibit lysis as much as the divalent cations (Fig. 2C). Interpretation of these results is difficult. It appears that divalent cations interfere in some way with lysostaphin-induced lysis of whole cells. Perhaps this occurs by stabilizing the membrane of digested cells rather than inhibiting lysostaphin activity, since digestion of peptidoglycan by the enzyme is stimulated by these cations (see below).

Lysostaphin digestion of peptidoglycan from *S. aureus* M and M variant strains and the effect of cations. The foregoing evidence indicated that the capsule posed no significant barrier to lysostaphin access to cell wall, since no difference in the rate of lysis was noted between encapsulated and unencapsulated strains. However, interpretation of the results could be complicated if the target of lysostaphin, presumably peptidoglycan, was more susceptible to the enzyme in the encapsulated strain. Accordingly, peptidoglycan was isolated from both M and M variant strains, and its lysostaphin susceptibility was assessed. Peptidoglycan from strains M and M variant was lysed at very similar rates by lysostaphin (Fig. 3). Thus, the similar rates of lysis of M and M variant strain cells cannot be attributed to a more lysostaphin-susceptible peptidoglycan in the M strain. When the effects

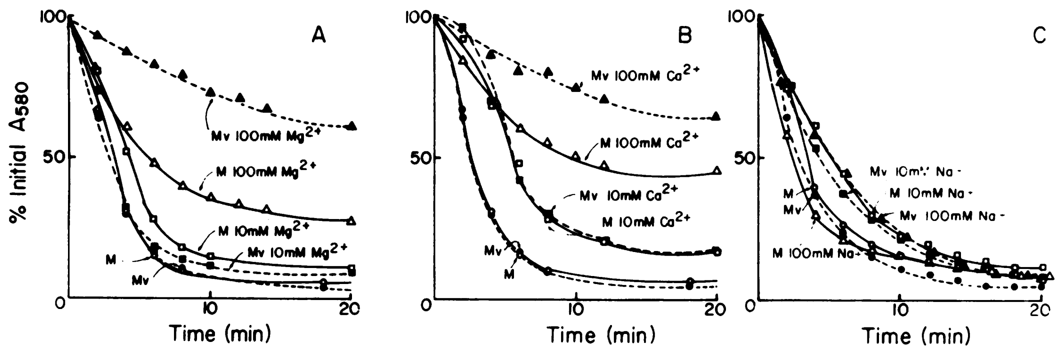

FIG. 2. *Effects of cations on the lysostaphin-induced lysis of M and M variant (Mv) strains. In each case the control is lysis in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride; pH 7.5. (A) Mg^{2+}; (B) Ca^{2+}; (C) Na^{+}. The experiments were conducted at 30°C.*

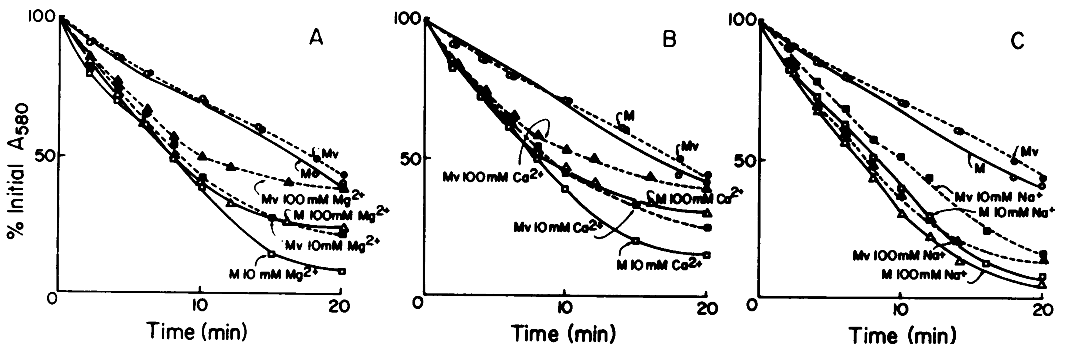

FIG. 3. *Effects of cations on the lysostaphin-induced lysis of peptidoglycan isolated from M and M variant (Mv) strains. In each case the control is lysis in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.5. (A) Mg^{2+}; (B) Ca^{2+}; (C) Na^{+}. The experiments were conducted at 30°C.*

of cations on lysostaphin digestion of peptidoglycan were studied, in contrast to effects observed in whole cells, Mg^{2+}, Ca^{2+}, and Na^{+} stimulated the rate of peptidoglycan digestion. Thus, cations appear to stimulate lysostaphin activity on isolated peptidoglycan.

It is conceivable that the lysis which occurs on treatment of *S. aureus* with lysostaphin is a result of the direct action of the enzyme, plus a contribution from the autolysins of the organism, which may be triggered by lysostaphin. This might complicate an interpretation of the results, especially if the unencapsulated strains were tolerant staphylococci with low autolytic activity (11). This potential complication does not appear likely since heat-killed M and M variant strains (19), which thus had no autolytic activity, were lysed by lysostaphin at very similar rates. Also, none of the strains used appear to be tolerant staphylococci since their nafcillin minimum inhibitory and minimum bactericidal concentrations were close (11; D. A. Anderson and B. J. Wilkinson, unpublished data).

Polysaccharide capsules external to the cell wall can be expected to act in varying degrees as diffusion barriers, molecular sieves, and adsorbents (5). The concentration of polysaccharide in capsules appears to be on the order of 1.5 to 2%, which is of the same magnitude as the concentration of agar used in normal laboratory media (5). It appears that there is only a small decrease in diffusion in gels of this concentration compared with rates in solution (5). Of more significance are the pores and channels in the three-dimensional gel network through which molecules must diffuse. The pores in 1 and 2% agar have been estimated to be 95 and 44 nm, respectively (1, 5). This estimation is consistent with the apparent exclusion of bacteriophage by the *S. aureus* capsule (19). The exact dimension of the lysostaphin molecule is not known, but it is expected to be much smaller than those pores, since an enzyme of similar molecular weight has been shown to have a diameter of 4.2 nm (4). Additionally, polyanionic bacterial capsules may immobilize soluble polycations such as lysozyme (15). However, this does not appear to be a factor in the case of lysostaphin, since lysis of encapsulated strains is noted.

These studies, then, are consistent with the idea that the *S. aureus* capsule is a highly porous structure. This is supported by the finding that encapsulated *S. aureus* strains bind large amounts of immunoglobulin G, presumably through cell wall-associated protein A (B. F. King and B. J. Wilkinson, unpublished data). Besides being important in studies of the mechanisms of resistance to phagocytosis, such considerations may also have implications for the mechanism of the phenomenon of antigen masking in encapsulated microorganisms (17; B. J. Wilkinson, manuscript in preparation). In summary, this study has shown that the *S. aureus* capsule is not a diffusion, a permeability, or an adsorption barrier to cell wall-directed lysostaphin.

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