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An approach to the selection of membrane mutants of *Staphylococcus aureus* based on pH sensitivity

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SUMMARY

Mutants of *Staphylococcus aureus* that are incapable of growth at pH 5.2 but grow normally at pH 7.0 have been isolated. Several of these mutants have protoplasts that are osmotically fragile when compared to wild-type protoplasts. Another group of mutants have an alteration in the lipid composition of their membranes. This mutant-screening procedure, based on sensitivity to pH, may be useful in the isolation of membrane mutants.

One approach to the study of structure and function of cell membranes is the use of mutants with defective membranes. Several investigators have isolated membrane mutants by selecting for impairments in specific functions, such as lipid biosynthesis[1-3] or transport of small molecules[4]. Another more general approach to isolate membrane mutants is to select for sensitivity to certain environmental stresses, such as low pH or high ionic strength, that might directly affect membrane properties. The membranes of these mutants could then be studied to determine the nature of the defect and its effect on various membrane processes. We have devised a procedure to isolate mutants of *Staphylococcus aureus* that are unable to grow at a relatively low pH. The sensitivity of such mutants to low pH could be due to an inactivation of one or more membrane-associated enzymes or an increase in the permeability of the membrane to hydrogen ions.

Staphylococcus aureus 63, a strain auxotrophic for histidine and arginine, was grown in chemically defined Medium A[5]. When the cells reached mid-log stage (absorbance at 660 nm = 5.4), 1.0 ml of the culture was centrifuged for 10 min at 6000 × *g* and washed in 50 ml of 0.05 M Tris–maleate buffer, pH 6.0. The pellet was resuspended in 20 ml of the same buffer containing 2.0 mg *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

(Aldrich Chemical), and the suspension was incubated for 30 min at 37 °C and stored at 4 °C overnight. The cells were diluted and plated onto agar plates containing Medium B (1% Bacto peptone, 1% yeast extract, 0.5% NaCl, 0.04% Na_2HPO_4) at pH 7.0 and incubated overnight at 37 °C. The colonies from these master plates were then replicated onto pairs of plates containing Medium B at pH 5.2 and 7.0. After an overnight incubation at 37 °C, colonies were selected that grew at pH 7.0 but not at pH 5.2. Colonies of the pH 5.2-sensitive mutants were streaked on pH 7.0 agar medium to obtain single colonies, which were also tested on the pH 5.2 and pH 7.0 agar medium. Approximately 0.3% of the colonies originally plated were found to be incapable of growth at pH 5.2. Twelve mutants were selected for further study. The nutritional requirements and the antibiotic sensitivity of the pH 5.2-sensitive mutants were tested. All of the mutants were auxotrophic for histidine and arginine, as is the parent strain. The mutants and wild type were also grown on agar medium in the presence of discs impregnated with various antibiotics, and the sensitivity of each mutant to the antibiotics was found to be identical to that of the wild type.

It seemed possible that, if there were structural defects in the membranes of the pH 5.2 -sensitive mutants, protoplasts prepared from them might also be sensitive to other environmental stresses, such as low ionic strength. To test this, wild-type and mutant cells were converted to protoplasts in a medium of high ionic strength. Then the protoplasts were diluted into solutions of lower ionic strengths to compare the osmotic fragility of the protoplasts of the mutants with that of the wild type.

Mutant and wild-type cells were grown at pH 7.0 in Medium B to mid-log phase. Cells were harvested, washed in 0.05 M Tris–0.145 M NaCl, pH 7.5, and resuspended in the same buffer to an absorbance of 6 at 660 nm. A 1.0-ml aliquot of this suspension was added to 4.8 ml of 2.4 M NaCl–48 mM $MgCl_2$, followed by 0.2 ml buffer containing 13.6 μg lysostaphin (Mead–Johnson), and the mixture was incubated for 20 min at 37 °C. Schuhardt *et al.*[6] have shown that 98% of the cells of *S. aureus* incubated with lysostaphin under these conditions are converted to protoplasts. Aliquots of the suspension of protoplasts were diluted 5-fold into solutions of decreasing osmolarity and the absorbance values were determined. The values obtained were corrected for differences in initial cell concentration determined before the addition of lysostaphin. Protoplasts of two mutants tested in this manner (C_7 and C_{15}) were osmotically fragile and lysed even at concentrations of NaCl (1.2 M) sufficient to completely stabilize the wild type (Fig. 1A). Protoplasts of most of the mutants (*e.g.* C_{13}, B_5 and B_9), however, were as stable as wild-type protoplasts (Fig. 1B).

It was also of interest to study the lipid composition of the low pH-sensitive mutants because it has been observed that several Gram-positive organisms contain a higher proportion of positively charged phospholipids when grown in acidic media than cells grown in neutral or basic media. In *S. aureus* the proportion of positively charged lysylphosphatidylglycerol is higher in cells harvested at pH 4–5 than at pH 7, primarily because the phosphatidylglycerol content is decreased[5]. These two lipids are the major phospholipids in *S. aureus*. In speculating on the function of lysylphosphatidylglycerol, Houtsmuller and Van Deenen[7] implied that an increase in the proportion of the positively charged lipids

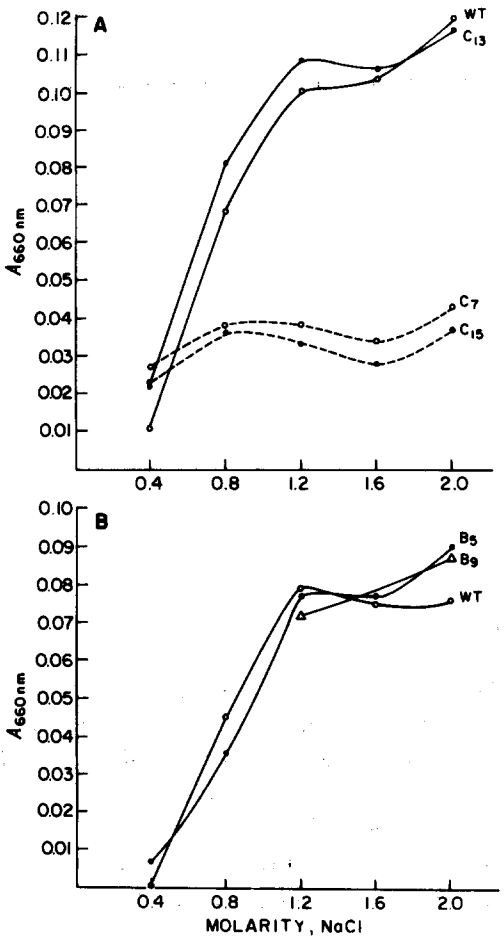

Fig. 1. Lysis curves of wild type (WT) and low pH-sensitive mutants. For experimental details see text.

might serve as a mechanism to allow growth at low pH. Using a model bilayer system, Hopfer *et al.*[8] provided support for the hypothesis that an increase in the proportion of lysylphosphatidylglycerol results in an increase in positive surface charge on the membrane, thus reducing the permeability of the membrane to protons. These studies showed that phospholipid bilayers composed of positively charged lysylphosphatidylglycerol were much less permeable to protons than bilayers prepared from acidic lipids such as phosphatidylglycerol. More recently Haest *et al.*[9] have confirmed these findings using intact cells of *S. aureus* and liposomes as a model system.

In order to study further the possible function of lysylphosphatidylglycerol in control of proton permeability of the membrane, the lipid content of the low pH-sensitive mutants of *S. aureus* was examined. Cells of the wild type and mutants were grown in 200 ml of Medium B to early log stage. Each culture was centrifuged and the pellet was resuspended in either 200 ml of pH 5.2 buffer (0.047 M citric acid, 0.13 M Na_2HPO_4,

TABLE I

RATIO OF LIPID LYSINE TO TOTAL LIPID PHOSPHATE FROM WILD-TYPE AND MUTANT CELLS INCUBATED AT pH 5.2 OR pH 7.0

Organism	*μmoles lipid lysine/μmole lipid phosphate*	
	pH 5.2	*pH 7.0*
Wild type	0.34*	0.16
B_2	0.30	0.19
B_4	0.36	0.15
B_{16}	0.12	0.038
C_7	0.31	0.15
C_{12}	0.29	0.12
C_{13}	0.31	0.20
C_{15}	0.21	0.084
C_{17}	0.32	0.13
C_{22}	0.28	0.17
B_5	0.051	0.016
B_9	0.07	0.015

* Average of values obtained from 4 experiments.

0.011 M glucose) or pH 7.0 buffer (0.018 M citric acid, 0.21 M Na_2HPO_4, 0.011 M glucose). The suspension was incubated on a rotary shaker for 1 h at 37 °C, and the cells were then collected by centrifugation and washed with 20 ml of 0.9% NaCl, pH 2. The cell pellet was suspended in 8 ml methanol; 16 ml chloroform were added and the mixture was stirred 10–17 h at room temperature to extract the lipids. The mixture was filtered through glass wool, washed with 5.0 ml 0.9% NaCl, pH 2, and the upper layer removed. The chloroform layer was washed again with 8 ml methanol and 5 ml 0.9% NaCl, pH 2, then evaporated to dryness on a rotary evaporator.

The total lipids were chromatographed on neutral silicic acid plates in two solvent systems: hexane–diethyl ether–formic acid (90:75:1.5, by vol.) and chloroform–methanol–water (180:65:4, by vol.). None of the mutants studied showed any significant qualitative alteration in neutral lipid or phospholipid content.

The ratio of lysylphosphatidylglycerol to the total phospholipid content of the pH-sensitive mutants was then determined. An aliquot of lipid (1 mg) from the wild type and each mutant was hydrolyzed with 1.0 ml 6 M HCl for 17 h at 110 °C. The hydrolysates were evaporated to dryness, dissolved in 1 ml water, and extracted three times with 1 ml diethyl ether to remove free fatty acids. The lysine content of the hydrolysate, which is indicative of the amount of lysylphosphatidylglycerol, was determined using a Beckman amino acid analyzer. (Thin-layer chromatography of the lipid extracts before hydrolysis revealed no contamination by protein). The total lipid phosphate was determined by the procedure of Bartlett[10]. The ratios of lipid lysine to total lipid phosphate for mutants and wild type incubated at pH 5.2 and 7.0 are shown in Table I. The relative amounts of lysylphosphatidylglycerol are higher at pH 5.2 than at pH 7.0 in the wild type and in all the mutants. It is

evident that the majority of the mutants incapable of growth at pH 5.2 have the same ratio of lipid lysine to lipid phosphate as the wild type. This, of course, was not unexpected since there are many possible causes for pH sensitivity. However, in two of the mutants B_5 and B_9, the amount lysylphosphatidylglycerol is markedly reduced at both pH 5.2 and 7.0. It is interesting to note that, despite this alteration in lipid content, protoplasts of these two mutants were similar in osmotic stability to the wild type (Fig.1B).

If the loss of ability to grow at pH 5.2 were related to the low lysylphosphatidylglycerol content in these mutants, then revertants that regain the ability to grow at pH 5.2 should have the normal content of lysylphosphatidylglycerol. In order to isolate spontaneous revertants, B_5 and B_9 were grown in Medium B buffered with pH 7.0 buffer for 16–20 h; 0.1 ml was then inoculated into Medium B buffered with pH 5.2 buffer. After 24 h, cells were diluted and plated onto agar plates containing Medium B with pH 5.2 buffer. It was confirmed that colonies that grew on these plates were revertants by further plating at pH 5.2 and 7.0. One revertant capable of growth at pH 5.2 as well as pH 7.0 was found for each mutant. Both revertants were auxotrophic for histidine and arginine and had the same antibiotic sensitivity and growth rate as the wild type, B_5 and B_9.

Cells of the mutants, revertants and wild type were incubated at pH 5.2 and 7.0 and the lipids were extracted to determine the ratio of lipid lysine to total lipid phosphate. The procedure was the same as above except that lysine was determined using an enzymatic assay[5]. As shown in Table II, each of the revertants has a high proportion of lysylphosphatidyl-

TABLE II

RATIO OF LIPID LYSINE TO LIPID PHOSPHATE FROM WILD-TYPE, MUTANT AND REVERTANT CELLS INCUBATED AT pH 5.2 AND pH 7.0

Organism	*μmoles lipid lysine/μmoles lipid phosphate*	
	pH 5.2	*pH 7.0*
Wild type	0.27	0.14
B_5	0.071	0.073
B_5R	0.33	n.d. *
B_9	0.053	0.014
B_9R	0.28	0.13

* Not determined.

glycerol, as does the wild type. These data indicate that the ability to grow at pH 5.2 is linked to the high lysylphosphatidylglycerol content in the wild type, since the revertants have regained both, presumably in a single-step reversion. It may be, however, that a low content of lysylphosphatidylglycerol does not necessarily result in sensitivity to low pH. The lesions in B_5 and B_9 may affect a process relevant to both lysylphosphatidylglycerol content and ability to grow at low pH, so that the two are linked but do not necessarily have a direct, causal relationship. Moreover, it is known that under certain conditions wild-type cells can be grown at pH 5.2 without a concomitant increase in lysylphosphatidyl-

glycerol content (C. Kent, as cited in Gould and Lennarz[5]). This indicates that the mechanism that allows growth of cells at low pH is more complex than a simple adjustment of the ratio of basic to acidic phospholipids.

In any event, this simple approach for selection of mutants unable to grow at low pH has permitted the isolation of three classes of mutants. The first class of mutants, although sensitive to low pH, is similar to the wild type with respect to both stability of protoplasts and lipid content. In contrast, the protoplasts of the second class of low pH-sensitive mutants are osmotically fragile, whereas the lipid content of the third class is altered. Since both protoplast stability and lipid composition are directly related to the cytoplasmic membrane, it may be that this procedure for isolating pH-sensitive mutants, when coupled with other screening procedures, will be applicable to the isolation of a variety of membrane mutants.

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