Preparation and Characterization of Everted Membrane Vesicles from Cells of Staphylococcus aureus

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We developed a method for the preparation of everted membrane vesicles from cells of Staphylococcus aureus. The cells were first treated with ampicillin to weaken the peptidoglycan layer, then the cells were passed through a French press cell. The resulting vesicles were roughly 0.1 μm in diameter, judging from electron microscopic observations. We detected fairly high membrane-bound ATPase activity in the membrane vesicles. We observed respiratory-driven quenching of quinacrine fluorescence, which indicates that inward H⁺ transport took place. These results indicate that the vesicles are everted. We characterized the membrane-bound ATPase. We also detected Na⁺/H⁺ antiport, erythromycin/H⁺ antiport and chloramphenicol/H⁺ antiport activities in the membranes of S. aureus.

Key words everted membrane vesicle; H⁺-ATPase; drug/H⁺ antiporter; S. aureus

Membrane vesicles are very useful for analyses of energy transducing systems in membranes. In Escherichia coli, which is the best characterized microorganism from a biochemical point of view, either right-side-out membrane vesicles or everted (inside-out) vesicles can be prepared rather easily by established methods.1,2 Generally, right-side-out vesicles are suitable for analyses of transport systems which accumulate solutes in the cells, and everted vesicles are suitable for analyses of export systems and enzyme systems that are normally accessible from only the inside of the cytoplasmic membrane. We are interested in the membrane-related energy transducing systems of Staphylococcus aureus, such as H⁺-translocating ATPase (membrane-bound ATPase), ion transport systems and so on. Although a method for the preparation of right-side-out vesicles from S. aureus cells has been developed,3 the preparation of everted membrane vesicles from this microorganism that are suitable for biochemical analyses of the membrane-related energy transducing systems is difficult. The French press method is often utilized to prepare everted membrane vesicles from E. coli cells.4,5 In the case of cells of gram-positive bacteria, however, this method has not been successful because of the presence of a rigid and thick peptidoglycan layer in the cell wall. Thus, we tried to prepare everted membrane vesicles from S. aureus by first weakening the cell wall with ampicillin and then passing the cells through a French press cell. Here we report a method for the preparation of everted membrane vesicles from S. aureus cells that is suitable for analyses of membrane energy transducing systems. We also report on the properties of some of these systems.

MATERIALS AND METHODS

Bacterial Cells and Growth S. aureus 209P was used in this study. Cells were grown in a nutrient broth (0.5% beef extract, 1.5% polypeptide, 0.5% NaCl, 0.5% K₂HPO₄, pH 7.1) at 37 °C under aerobic conditions. Cell growth was monitored turbidimetrically at 650 nm. When necessary, ampicillin was added to the culture medium at various concentrations. Cell shapes were observed by microscopy.

Preparation of Everted Membrane Vesicles Our standard method for the preparation of everted membrane vesicles from S. aureus cells is as follows. Cells of S. aureus were grown to the early exponential phase of growth with shaking. Ampicillin was then added to a final concentration of 0.1 μg/ml and the culture medium was shaken for another 3 h. Cells were harvested, washed twice with a buffer consisting of 10 mM Tris–HCl (pH 8.0), 140 mM KCl, 5 mM MgSO₄, 2 mM β-mercaptoethanol and 10% glycerol, and suspended in the same buffer. The cells were passed through a French press cell at a pressure of 2400 kg/cm². Unbroken cells and cell debris were removed by centrifugation (15000 rpm, 10 min), and membrane vesicles were collected by ultracentrifugation (35000 rpm, 1 h). The membrane vesicles were washed with the buffer (described above) and then suspended in the same buffer. An equal volume of glycerol was added to the vesicle suspension, and the mixture was frozen in liquid nitrogen and stored at –80 °C until used.

Electron Microscopy For the observation by electron microscopy, membrane vesicles were fixed with glutaraldehyde (2% in 10 mM phosphate buffer, pH 7.4) for 2 h and postfixed in osmium tetroxide (1% in 10 mM phosphate buffer, pH 7.4) for 1 h. Fixed samples were washed three times with phosphate buffer, dehydrated through graded ethanol solutions, and embedded in Epon. Ultrathin sections were obtained with a Hitachi ultramicrotome MT 5000, mounted in Formvar grids, and stained with uranyl acetate and lead citrate.6 The specimens were examined with a Hitachi electron microscope H500.

ATPase Activity The standard assay mixture (0.6 ml) consisted of 20 mM Tris–HCl, pH 8.0, 1 mM MgSO₄, and 80 mM Na₂SO₄. When necessary, Na₂SO₄ was replaced with other salts. Membrane vesicles (containing about 5 μg of protein) were added to this mixture and preincubated for 3 min at 37 °C. The reaction was initiated by adding ATP to the mixture to a final concentration of 2 mM. The reaction mixture was incubated at 37 °C for 15 min, then the inorganic phosphate released was determined colorimetrically.6 One unit of activity is defined as which releases 1 μmol of inorganic phosphate per min. When necessary, toluene (2%) was

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mixed with membrane vesicles and incubated for 15 min on ice to permeabilize the membrane.

**Fluorescence Quenching Method** pH gradient formation due to respiration and the antiporter activities were measured by the quinacrine fluorescence quenching method\(^7\) in an assay mixture consisting of 10 mM MOPS–KOH, pH 8.0, 140 mM KCl, 1 µM quinacrine hydrochloride and membrane vesicles (about 0.2 mg of protein). When K\(^+\)/H\(^+\) antiporter activity was examined, K\(^+\) in the above assay mixture was replaced with choline. Either NADH (1 mM) or potassium lactate (5 mM) was added to initiate the respiration.

**Others** Protein contents were determined by the method of Schaffner and Wissmann.\(^9\) Reagents were obtained from commercial sources.

**RESULTS**

As cells of *S. aureus* are surrounded by a rigid and thick peptido glycan layer, it is important to disrupt or weaken this peptido glycan layer before preparing membrane vesicles. We tried to weaken the peptido glycan layer by growing cells in the presence of low concentrations of ampicillin. Since too much ampicillin treatment of *S. aureus* cells resulted in cell lysis, it was necessary to determine the optimum ampicillin concentration and time of treatment. Figure 1 shows the effect of ampicillin concentration on cell growth (or lysis). Ampicillin was added when the OD (optical density) 650 was about 0.3, and cell growth was monitored. At ampicillin concentrations higher than 0.2 µg/ml, cell lysis occurred 4 to 5 h after the addition of ampicillin (Fig. 1). At 0.1 µg/ml ampicillin, cell growth (shown by an increase in OD) stopped 3 h after ampicillin addition, but cell lysis (judged by microscopic observations) didn’t occur. We observed some enlargement of cells at this stage.

We harvested cells at various stages (different ampicillin concentrations and different time lengths after ampicillin addition), and prepared membrane vesicles by passing the cells through the French press cell. We evaluated the formation of everted membrane vesicles by measuring membrane-bound ATPase activity, the formation of a pH gradient across the membrane due to respiration and protein yield in the membrane fraction (Table 1). There was a tendency for higher concentrations of ampicillin (between 0.1 and 0.5 µg/ml) and longer treatments (1.5 to 3 h) to result in a higher specific activity of ATPase in the membrane preparation. However, membrane preparations that showed higher ATPase activity did not necessarily form a larger pH gradient (Table 1). Longer ampicillin treatment or higher ampicillin concentration resulted in a lower pH gradient formation. This suggests that although cells were disrupted, portions of the membrane preparation were not vesicles but fragments. As for membrane yield, a rather constant yield (11 to 13% with respect to protein recovery in the membrane fraction) was obtained from cells treated with 0.1 to 0.2 µg/ml ampicillin.

We also tested the effect of pressure of the French press cell on vesicle formation. A pressure of 2400 kg/cm\(^2\) gave the best results (data not shown).

Based on these results, we set up our standard conditions for the preparation of everted vesicles as described under Materials and Methods.

We confirmed that our membrane samples were really vesicles and not fragments by observation with the electron microscope. As shown in Fig. 2, the great majority of our membrane preparations were vesicles and not fragmented membranes. The diameter of the vesicles was roughly 0.1 µm.

We measured membrane-bound ATPase activity in the absence or presence of toluene to roughly estimate the proportions of everted and right-side-out vesicles. The ATPase activity increased by about 20% when measured in the presence of toluene compared with the activity measured in its absence (data not shown). This indicates that although some portions of the membranes were right-side-out, the great majority were everted.

A membrane-bound Mg\(^{2+}\)-activated ATPase is present in *S. aureus*.\(^{10}\) We characterized the membrane-bound ATPase in our membrane preparation. We confirmed that the ATPase

![Fig. 1. Effect of Ampicillin on the Growth of S. aureus](image)

Cells were grown in a nutrient broth at 37°C under aerobic conditions. Ampicillin was added at the time point indicated by the arrow at the following concentrations: 0 (○), 0.05 µg/ml (●), 0.1 µg/ml (∆), 0.2 µg/ml (▲), 0.5 µg/ml (□).

<p>| Table 1. Comparison of Conditions for Preparation of Everted Membrane Vesicles |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Ampicillin (µg/ml)</th>
<th>Times of harvest(^a) (h)</th>
<th>Membrane ATPase (unit/mg protein)</th>
<th>ΔpH formation (arbitrary %)(^b)</th>
<th>Yield(^c) (protein %)</th>
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\(^a\) After addition of ampicillin, cells were shaken for indicated times at 37°C and then cells were harvested.  
\(^b\) Quinacrine fluorescence quenching % is shown.  
\(^c\) Yield of membrane was evaluated from protein yield in the membrane fraction. Original total cell protein was 100%.
activity was stimulated by Mg$^{2+}$. Maximal stimulation was observed when the concentration ratio of ATP:Mg$^{2+}$ was 2:1 (data not shown), as reported with other ATPases. In addition, the ATPase activity was enhanced by various salts, such as NaCl, Na$_2$SO$_4$, Na$_2$SO$_3$ or NaNO$_3$ (Fig. 3). The largest stimulation was observed with Na$_2$SO$_4$. The cationic species didn’t have a significant effect on the activity (data not shown). Thus, anions are important for stimulation. Among the anions tested, SO$_4^{2-}$ showed the highest stimulation, followed by Cl$^-$, SO$_3^{2-}$ and NO$_3^-$. In the absence of SO$_4^{2-}$, the ATPase activity was maximal at pH 8 to 9 and very low at pH 7 (Fig. 4). On the other hand, fairly high activity was observed from pH 7 to 9 when SO$_4^{2-}$ was present. The ATPase activity was inhibited by several reagents such as NaN$_3$, ZnSO$_4$, dicyclohexylcarbodiimide (DCCD) or 4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonate (SITS). Among the reagents tested, ZnSO$_4$ and NaN$_3$ showed the strongest inhibition (Fig. 5). Inhibition by DCCD or SITS was about 50% at most. Vanadate, bafilomycin, NO$_3^-$ or tetrachlorosalicylanilide (TCS) didn’t show any significant inhibition (data not shown).

The Na$^+/H^+$ antiporter plays a central role in Na$^+$ circulation in some bacteria. This antiporter is especially important for cell growth in the presence of high NaCl. However, it was not known whether a Na$^+/H^+$ antiporter exists in S. aureus. Since S. aureus cells can survive in the presence of high concentrations of NaCl, it seems that this microorganism must possess an antiporter. The fluorescence quenching method and everted membrane vesicles have been utilized to analyze the Na$^+/H^+$ antiporter in several bacteria. As shown in Fig. 6, we detected Na$^+/H^+$ antiport activity by the quinacrine fluorescence quenching method (Fig. 6). However, the activity was low compared with those of E. coli and other bacteria. We also observed Li$^+/H^+$ antiport activity which was diminished by the presence of Na$^+$ (data not shown). Therefore, it seems that Li$^+$ is a substrate for the Na$^+/H^+$ antiporter, as it is in other bacteria. Although we tested it several times, no K$^+/H^+$ antiport activity was detected in the vesicles of S. aureus (data not shown).

Several bacteria possess drug/H$^+$ antiporters for the extrusion of drugs. We tested whether S. aureus possesses such systems using everted vesicles. Among the drugs tested, erythromycin and chloramphenicol showed antiporter activity (Fig. 6). Erythromycin didn’t compete for the chloramphenicol/H$^+$ antiport, and vice versa (data not shown). Thus, it is clear that cells of S. aureus inhibit erythromycin/H$^+$ antiporter and a chloramphenicol/H$^+$ antiporter. No ac-
systems present on the inner surface of the cytoplasmic membrane.

This technique has made the present characterization of the membrane-bound ATPase of *S. aureus* possible. This ATPase appears to be a H⁺-translocating ATPase (F₆,F₄-ATPase). Properties of the ATPase in this organism were very similar to those of the ATPases of *E. coli* and other bacteria.¹¹,²¹ However, stimulation by SO₄²⁻ of the *S. aureus* ATPase is a unique property. Such stimulation is not seen in *E. coli* ATPase. Previously, we reported that the ATPase of *Vibrio para-haemolyticus*, which is a Gram-negative, slightly halophilic marine bacterium, is greatly stimulated by either SO₄²⁻ or Cl⁻.¹²,²¹,²² In some Gram-positive bacteria, stimulation by SO₄²⁻ but not by Cl⁻ has been reported.²³,²⁴ Thus, stimulation by SO₄²⁻, but not by Cl⁻, may be a characteristic of the membrane-bound H⁺-translocating ATPase of gram-positive bacteria. The specific activity of the membrane-bound ATPase of *S. aureus* measured in the presence of SO₄²⁻ was 1.4 unit/mg protein (Fig. 3). Some properties of the membrane-bound ATPase of *S. aureus* had been previously reported.¹⁰ The specific activity of the ATPase reported in that study was about 0.2 unit/mg protein.¹⁰ This value corresponds to our value measured in the absence of SO₄²⁻. In the previous report, the authors prepared the membrane fraction by disrupting *S. aureus* cells by vigorously vibrating them with glass beads. It is not clear whether the resulting membranes were of a vesicular form or not. Some properties of the *S. aureus* ATPase are similar to those of the *V. para-haemolyticus* ATPase, for example, stimulation by SO₄²⁻ and inhibition by ZnSO₄,²² which are not seen with the *E. coli* ATPase. However, tetrachlorosalicylanilide (TCS), which strongly inhibited the *V. para-haemolyticus* ATPase, did not inhibit the *S. aureus* ATPase at all.

We found that there is a Na⁺/H⁺ antiporter in the membrane of *S. aureus*. The Na⁺/H⁺ antiporter is widely distributed in cells ranging from microbial cells to animal cells and is a major system for Na⁺ extrusion.¹² The functions of this antiporter are: 1) extrusion of toxic Na⁺ and Li⁺, 2) establishment of an electrochemical potential of Na⁺ across the membrane, which is the driving force for the Na⁺-coupled active transport of solutes, and so on, 3) intracellular pH regulation under alkaline conditions, and 4) cell volume regulation. Cells of *S. aureus* can survive in the presence of high concentrations of NaCl.¹⁴ Cells of *S. aureus* possess Na⁺-coupled solute transport systems (unpublished observation). *S. aureus* is able to grow even at pH 9. Thus, it is quite reasonable to assume that cells of *S. aureus* possess a Na⁺/H⁺ antiporter. In fact, we found it. However, the activity of the Na⁺/H⁺ antiporter seems to be weak compared with those of *E. coli* and other bacteria.⁷,⁸,¹³,¹⁵ Thus, other Na⁺-extrusion system(s) may be present in *S. aureus* cells. It has been reported that the activity of a Na⁺/H⁺ antiporter (the NaHₐ system) of *E. coli* is enhanced when cells are grown in the presence of high concentrations of NaCl.²³ In the case of *S. aureus*, however, such enhancement was not observed (data not shown). Recently, we have succeeded in cloning a chromosomal gene of *S. aureus* that encodes the Na⁺/H⁺ antiporter. Structural analysis of this gene is now under way.

Using the everted membrane vesicles, we found two drug/H⁺ antiporters, the erythromycin/H⁺ and the chloramphenicol/H⁺ antiporters, in a wild type of *S. aureus* (209P).
Perhaps because the activities of these antiparticles are not high enough, the cells do not show much resistance to these drugs. However, if one can disrupt the genes for these antiparticles, the cells will probably become more sensitive to these antibiotics. Several drug efflux systems have been reported in \textit{S. aureus} or related microorganisms: Smr (QacC),\textsuperscript{26,27} MsrA,\textsuperscript{28} NorA,\textsuperscript{29} QacA,\textsuperscript{30} QacB,\textsuperscript{30} TetK,\textsuperscript{31} and TetL.\textsuperscript{32} Judging from the substrate specificities, it seems that the erythromycin/H\textsuperscript{+} and chloramphenicol/H\textsuperscript{+} antiparticles are different from these systems.

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