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ADAPTIVE CHANGES IN CARDIOLIPIN CONTENT OF *STAPHYLOCOCCUS AUREUS* GROWN IN DIFFERENT SALT CONCENTRATIONS

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Abstract: Adaptive changes in cardiolipin content were examined in *Staphylococcus aureus* 209P using the ^{32}P pulse-labeling method. Cardiolipin synthesis showed increased adaptation when cells grown in normal medium were transferred into high NaCl containing medium. When *S. aureus* cultured in 10% NaCl medium was transferred back to normal medium, cardiolipin concentration decreased to the normal level within 3 hours. The catabolic rate of cardiolipin in the cells was much slower in the 5% NaCl medium than in normal medium. The cardiolipin synthetase activity was examined by isolated membrane fraction from *S. aureus* grown both in normal and 10% NaCl medium. The activity was higher by two-fold in membrane fractions from cells cultured in 10% NaCl-containing medium than in membranes from cells cultured in normal medium.

Staphylococcus aureus is known to be relatively resistant in culture medium of high NaCl concentration. This feature differs from other bacteria and is distinct from NaCl-dependent halophilic bacteria. This property has been applied to isolate the organism selectively from other bacteria. It has been assumed that the organism has a specific membrane barrier system or transport system or both against higher salt environments. However, the mechanism of salt tolerance has not yet been clarified. In a previous report from this laboratory (1), it was shown that the phospholipid composition of the membrane was altered by increased NaCl concentrations in the culture medium. The conclusion of the previous report was that cardiolipin content increased and that phosphatidylglycerol (PG) and lysylphosphatidylglycerol (L-PG) content decreased as NaCl concentration increased in the medium.

The present study was undertaken on *S. aureus* to further examine the adaptive changes in cardiolipin (CL) content resulting from media alterations of NaCl concentration.

MATERIALS AND METHODS

Microorganism and cultivation: *Staphylococcus aureus* 209P (FDA) was grown in a semisynthetic medium (1) supplemented with NaCl at final concentrations

of 145 mM (normal), 950 mM (5%) or 1900 mM (10%). These bacterial cell culture media are referred henceforth as normal, 5% NaCl or 10% NaCl cells, respectively. Stationary-phase organisms were inoculated at a concentration of 1% (v/v) into experimental Monod-type tubes containing the growth media. Tubes were incubated at 37°C with aeration by gentle shaking, and the growth was followed by measuring the absorbance at 650 nm with a Shimadzu Bausch & Lomb spectrophotometer. The cells were labeled in medium containing ^{32}P orthophosphate (92 μC-5 μC/ml).

Extraction and analysis of phospholipids: Lipids were extracted with $CHCl_3$-MeOH (2 : 1, v/v) from packed whole cells, followed by purification by the Folch's partition dialysis procedure (2, 3). Purified lipids thus obtained were separated and identified on two-dimensional thin layer chromatography by the method described previously (4). The individual phospholipid contents were determined by measuring inorganic phosphorus in scraped individual spots after perchloric acid digestion. Experimental methods for examining the effects of medium change on lipid composition are described under the Results.

Assay for cardiolipin systhesis: Membrane fractions were prepared by using L-11 enzyme (Staphylolytic enzyme generously offered by Prof. S. Kotani, University of Osaka) on the middle logarithmic growth phase *S. aureus* cultured in normal medium and in 10% NaCl medium. After the complete lysis of cell walls, the protoplasts were suspended in hypotonic buffer, and the membrane fractions were collected by centrifugation at 24,000×g for 20 minutes. Membrane pellets were washed three times with 0.05M Tris-HCl, (pH 7.2), to remove the cytoplasmic components. ^{32}P-labeled PG was used as substrate for assaying CL synthesis activity. The assay was based on measuring the transfer of ^{32}P from PG to CL after incubation of the enzyme-substrate mixture by the method of De Siervo and Salton (6).

RESULTS

Incorporation of ^{32}P into individual phospholipids by pulse-labeling after changing the medium: *S. aureus* grown in high NaCl medium contained abundant cardiolipin compared to normal *S. aureus*. The experiment was designed to examine the synthesis changes of individual phospholipids after the transfer of *S. aureus* from the normal medium to the high NaCl medium. *S. aureus* were incubated in ^{32}P-free normal medium up to the late logarithmic growth phase, and then the cells were transferred to normal and 5% NaCl medium that contained ^{32}P (3.5 μC/ml) (Fig. 1-A). Incubation at 37°C was continued, and the cells were removed at one or two hours after the medium change. The lipids were extracted for assay of ^{32}P incorporation to the phospholipid (Fig. 1-B). It was clearly shown that cells in the normal medium proliferated effectively and incorporated ^{32}P into the phospholipids. After two hours incubation, the ^{32}P incorporation into CL reached 8% of total incorporation. On the other hand, in the group transferred from the normal medium to 5%

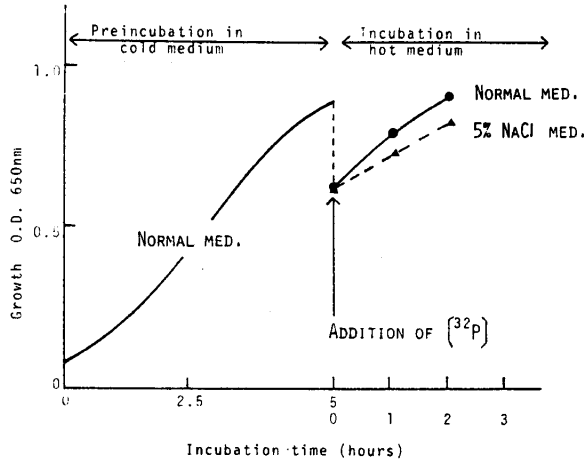

Fig. 1-A. Experimental design for phospholipid synthesis after a medium change. *S. aureus* 209P were first cultured in normal medium up to the late log phase. They were then transferred to normal or 5% NaCl medium, each containing 3.5 μC/ml of ^{32}P. Growth was followed by absorbance changes at 650 nm.

NaCl medium, the proliferation rate was somewhat slower, and less ^{32}P was incorporated than in normal medium. The relative ^{32}P incorporation rate into CL, however, was accelerated from 3% at one hour to 20% at two hours incubation. It was clearly shown that the synthesis of CL in the cells transferred to the high NaCl medium quickly increased, due possibly to enviromental adaptation.

Prelabeled phospholipids after changing the medium: Cells labeled through several generations by incubation for 5 hours in normal medium with ^{32}P (5 μC/ml) were divided into two groups: one group of cells was resuspended in normal medium and the other group was resuspended in 5% NaCl medium. Both groups were incubated for one to three hours to follow the radioactivity changes of the

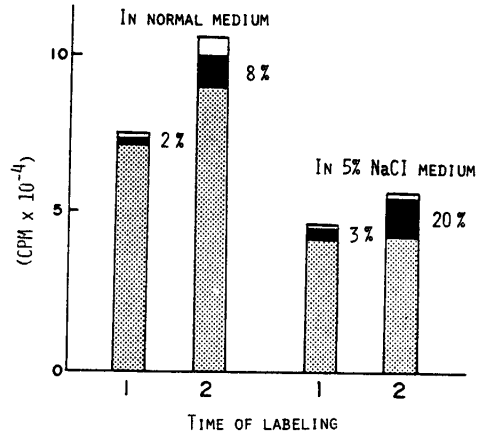

Fig. 1-B. Incorporation of ^{32}P into individual phospholipids by pulse-labeling after changing the growth medium. Percentage indications show the ratio of counts in cardiolipin to that of total phospholipids. □, lysylphosphatidylglycerol; ■, cardiolipin; ▨, phosphatidylglycerol.

individual phospholipids. As shown in Fig. 2, both groups showed a remarkable PG turnover after transfer to the new medium (normal-to-normal, normal-to-5% NaCl medium). The radioactivity of PG showed a peak at 30 minutes and then rapidly decreased. The formation of this peak was probably due to incorporation from pooled ^{32}P in the cells. However, CL and L-PG reached a peak slower than PG and then gradually lost radioactivity at a much slower rate in normal medium, which indicated the presence of a synthesis pathway from PG to CL and from PG to L-PG. Cardiolipin in the 5% NaCl medium showed a different turnover rate from L-PG, and its loss of radioactivity was slower and considerable radioactivity was still present after a further incubation of three hours.

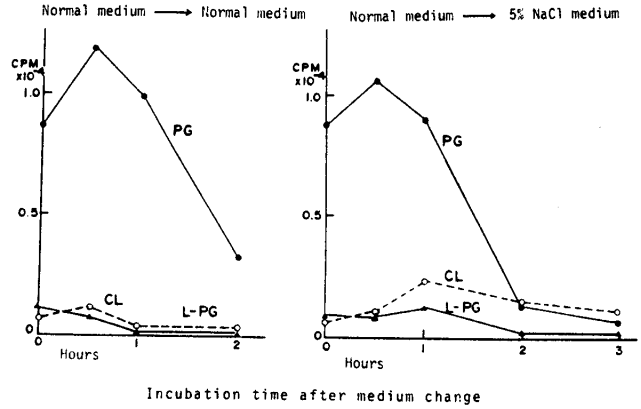

Fig. 2. Catabolic rates of individual phospholipids of *S. aureus* 209P after medium changes. PG, phosphatidylglycerol; CL, cardiolipin; L-PG, lysylphosphatidylglycerol.

Phospholipid composition after medium change: Changes of phospholipid composition were examined after the transfer of 10% NaCl *S. aureus* back to the normal medium to examine the adaptive catabolism of cardiolipin. Cells grown to the middle logarithmic phase in the 10% NaCl medium were transferred and cultured in the normal medium. The phospholipid analysis disclosed that the control group showed 50.3% CL after a further incubation of 3 hours, while the experimental group in normal medium showed 29.7% of CL after 1.5 hour and 15.6% at three hours incubation. PG and L-PG contents were increased indicating adaptive changes compared to the normal *S. aureus* lidid pattern (Table 1).

Cardiolipin synthesis by membrane fraction: The cardiolipin synthesizing activity of membrane fragments was studied. The membrane fractionations were prepared from normal and 10% NaCl *S. aureus* using L-11 enzyme. An

TABLE 1 ALTERATIONS IN THE MAJOR PHOSPHOLIPID COMPOSITION OF *S. aureus* 209P INDUCED BY CHANGING THE CULTURE MEDIUM FROM 10% NACL-TO-NORMAL

Growth medium	Starting specimen*	Experimental group		Control
		Normal	Normal	10% NaCl
Incubation time (hour)	0	1.5	3	3
Growth (wet wt. mg/ml)	0.80	1.80	3.05	2.50
Phosphatidic acid	4.0	4.1	4.8	4.0
Lysylphosphatidyl glycerol	4.5	9.0	11.6	4.3
Phosphatidylglycerol	38.0	50.5	59.0	35.3
Cardiolipin	52.4	29.7	15.6	50.3

* Starting specimen was computed after bacterial proliferation up to the middle log phase in 10% NaCl medium following washing. Composition expressed as percentages of the total phospholipid.

TABLE 2 CARDIOLIPIN SYNTHESIS BY MEMBRANE FRACTIONS OBTAINED FROM *S. aureus* 209P CULTIVATED IN NORMAL MEDIUM AND 10% MEDIUM.

Membrane fraction	Triton X-100	n moles CL produced from PG
Normal	−	10
	+	36
10% NaCl	−	10
	+	60

Assay condition: ^{32}P phosphatidylglycerol, 0.3 mM; Tris-HCl, 0.05 M (pH 7.2); total volume, 1.0 ml; incubation for 1 hour at 37°C; Triton X-100, 0.25%; assay tubes contained 100 μg protein. Membrane fractions were prepared from normal and 10% NaCl *S. aureus* by using enzyme L-11.

appropriate amount of ^{32}P-PG for obtaining a final concentration of 0.3 mM was suspended in 0.05 M Tris-HCl buffer (pH 7.2) and sonically oscillated to transparency. One hundred μg of membrane protein were added and adjusted to a final volume of 1.0ml. As shown in Table 2, it was clear that CL synthesis activity in 10% NaCl *S. aureus* was about two-fold higher than that of normal *S. aureus* in the presence of Triton X-100.

DISCUSSION

The salt resistance of *S. aureus* differs from other bacteria. *S. aureus* can proliferate in a wide range of NaCl concentrations and is distinct from halophilic bacteria which are dependent on NaCl. From previous observations of *S. aureus* cultured in high NaCl medium, an enhancement of barrier function has been suggested in the membrane due to the increase of cardiolipin (1). Lipid contents have been reported to vary depending upon the cultural conditions. Among these conditions total lipid has been found to increase in

E. coli when temperature decreases (7) and that in *S. aureus* the cell lipid content was found to decrease as the culture time lengthened (9). However, the total lipid content of *S. aureus* cultivated in higher NaCl medium showed no significant change (1). On the other hand, there are a number of reports showing that the membrane lipid pattern of bacteria changes in response to stress (8–14). For instance, when the culture is prolonged in either gram-positive or gram-negative bacteria, CL increases (8–10), and PG and CL increase under more aerobic conditions (12). In *S. aureus* cultured in high NaCl medium, a marked increase of CL occurred in membrane lipid composition (1). In any case, these reports have indicated that lipid changes adapt to environmental situations.

A question that arises is whether such a CL increase under high NaCl concentration is due to increased synthesis or to decreased catabolism, or both. The synthesis of CL arose adaptively after the quick change from normal medium to 5% NaCl medium. The duration of synthesis by pulse-labeling was two hours after the medium change. When cells were transferred from normal to normal medium, the population of the cells corresponded exactly to one generation, and the incorporation of ^{32}P into CL was 8% of total radioactivity. In the change of medium from normal to 5% NaCl, the cell population was a half-generation but ^{32}P incorporation into CL increased to 20% of total cpm. This clearly indicates that an acceleration of CL synthesis occurred. On the other hand, catabolism of CL was suppressed compared with the turnover of other individual phospholipids. It is concluded that the combination of the synthesis-acceleration and catabolism-inhibition raises the CL content when cells are transferred to a new enviroment.

The synthesis of cardiolipin in *E. coli* has been reported by Kanfer and Kennedy (15), Houtsmuller and Van Deenen (16) and Kanemasa, Akamatsu and Nojima (4). It is generally accepted that cytidine is necessary for CL synthesis (17–21). However, Brundish, Shaw and Baddiley (22) suggested the possibility that two molecules of PG could be converted directly to CL on the basis of chemical studies. Stanacev and Stuhne-Sekalec (23) reported a CL synthesis pathway in which PG works as an acceptor and donor of the phosphatidyl moiety under catalysis of phospholipase D. De Siervo and Salton (6) and Short and White (24) demonstrated the presence of a pathway from PG to CL without any involvement of phospholipase D in the membrane of *Micrococcus lysodeikticus* and *S. aureus*.

In the present report, it is demonstrated that the activity of CL synthesis in 10% NaCl *S. aureus* was twice that of normal. The finding coincides with the result of the pulse-labeling experiment. Microorganisms generally adapt easily to different environmemt, and when reversed to the original state, they

quickly return to their previous behaviour. When 10% NaCl *S. aureus* having CL at about 50% of total lipid was reversed to the normal condition, the CL content decreased to 15% in two generations, showing practically a normal pattern which confirmed the adaptive phenomenon.

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