The Role of Proline in Salt-tolerance Mechanism of *Staphylococcus aureus*

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ABSTRACT *Stapylococcus aureus* has a high salt tolerance and grows well in a high concentration of sodium chloride. For this, some mechanisms are needed to cope with high external osmotic pressure. It has been reported that osmoregulation is effected by the accumulation of proline in cells. We investigated in details the dynamics of proline in osmoregulation, using a synthetic medium. As a result, it was elucidated that proline was not only essential for cell construction, but also was needed in a large amount for osmoregulation in high salt environment; for cell construction, only a small amount of proline as 20 μM was needed, however, for cell growth in high salinity, a large amount of proline as much as 700 μM was to be added to the medium containing 10 % NaCl. Moreover, when a sufficient amount of proline was not made available in the medium, *S. aureus* accumulated glutamine to barely compensate proline for osmoregulation. In conclusion, proline definitely plays a major and glutamine a minor role in osmoregulation in high salt environment.

Key words: *Staphylococcus aureus,* Salt-tolerance, Osmoregulation, Proline

Introduction

It has been known that Staphylococcus grows well in the presence of a high concentration of sodium chloride (2). Two mechanisms are known to be involved in the adaptation of this organism to a high salt concentration. One is a mechanism for changing the composition of membrane phospholipids to suppress Na^+-permeability in high salt environment. The phospholipids of *S. aureus* growing in normal salt environment consist mainly of phosphatidylglycerol, lysylphosphatidylglycerol and a few percent of cardiolipin, whereas in high salt environment, cardiolipin increased to 50% or more, serving as a barrier to Na^+-permeability and maintaining intracellular homeostasis of monovalent cations (3, 6, 7, 11). The other is a regulatory mechanism for high external osmotic pressure (4, 5, 8, 12). When *S. aureus* was transferred from a normal salt environment to a high salt environment, the cells increased their internal osmotic pressure by a loss of water due to shrinkage of the cells followed by a prompt uptake and accumulation of proline, inducing an influx of water into the cell bodies (8). This regulatory mechanism is an important reaction proceeding to a change in cell membrane for adaptation to a high salt environment.

In the present study, we investigated in details the dynamics of proline contributing to osmoregulation, using a synthetic medium.

Materials and Methods

Strain and culture method used

S. aureus 209P (FDA strain) was used throughout this study. A liquid synthetic medium

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of Pattee and Neveln (10) was used for shaking culture at 37℃. Middle-log phase growth of the organisms adapted to the synthetic medium was used throughout the experiment. The growth rate was monitored by the increase in optical density at 650 nm, using a Spectronic 20A (Shimadzu, Tokyo, Japan).

Analytical methods

The bacteria were grown in the synthetic medium until the growth reached to late-log phase. One half amount of the culture was used for weight determination of the bacterial cells and the other half for amino acid analysis.

The dry weight of bacterial cells was determined after drying the pellet (8). The intracellular water content was determined by a phosphate dilution method (4).

For amino acid analysis, 100 ml of the culture were centrifuged at 10,000×g for 10 min. The pellet obtained was used for analysis of intracellular amino acid content and the supernatant for determination of residual amino acid content in the medium (8). For analysis of intracellular amino acids, 5 ml of cold 6 % trichloroacetic acid (TCA) was added to the pellet. This mixture was stirred for 2 hr to extract intracellular free amino acids, and recentrifuged at 12,000×g for 20min. The supernatant was used for analysis of free amino acid content. For analysis of residual amino acids, cold TCA was added to the supernatant obtained by centrifugation of the culture to a final concentration of 6 %. Amino acid content was determined with an automated amino acid analyzer (JEOL, TOKYO, Japan).

Results.

Amino acid requirements for growth

The requirement of amino acids was assessed by bacterial growth in single amino acid-limited synthetic media. Table 1 shows the requirements of individual amino acids for the growth of *S. aureus* 209P. The values were expressed as percentage of turbidity of growth (12 hour-culture) in single amino acid-limited synthetic medium to that of stationary phase growth (12 hour-culture) in a complete synthetic medium.

Table 1. Requirement of amino acid for growth of *S. aureus* 209P

Eliminated amino acid	Growth(% of OD_{650nm})
control	100
aspartic acid	105
lysine	104
threonine	102
alanine	99
histidine	99
serine	94
tryptophane	83
glycine	75
methionine	63
leucine	50
tyrosine	48
isoleucine	17
proline	6
glutamic acid	2
arginine	2
phenylalanine	2
cystine	1
valine	0

The results showed that although a slight growth was obtained in isoleucine-limited medium, there was no growth in the synthetic media, in which proline, glutamic acid, arginine, phenylalanine, cystine or valine was individually omitted. Therefore, these amino acids were considered essential for the growth of *S. aureus* 209P.

Influence of the amount of proline on the growth of S. aureus in normal and high salt media

Fig. 1 shows the growth curves of *S. aureus* in proline-defined synthetic media under normal salt concentration (physiological concentration). The maximum amount of growths increased along with an increase in the amount of proline to 10, 15, 20, 100 and 700 μM, respectively. However, the patterns of the

growth curves showed that about 20 μM proline was a necessary and sufficient amount needed for cell construction.

Fig. 2 shows the growth curves of the organisms in synthetic media containing 10 % salt and varying amount of proline. The duration of the lag phase was extended because the cells should be adapted to the high salt condition, but the growth was enhanced along with an increase in the amount of proline added to the medium. The maximum of the growth curves indicated that 700 μM proline was a sufficient amount for bacterial growth in the presence of 10 % salt.

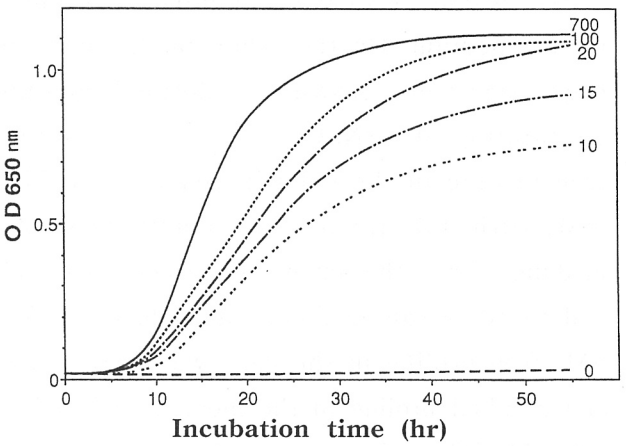

Fig.1 Growth curves of *S. aureus* in proline -defined synthetic media. Numbers in figure represent μM of proline added to the medium.

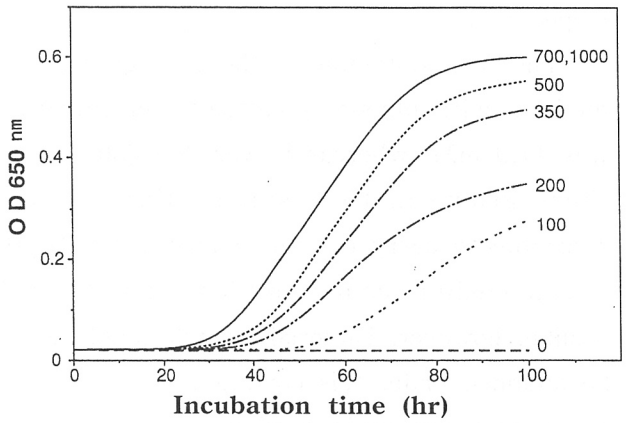

Fig.2 Growth curves of *S. aureus* in proline -defined synthetic media containing 10% NaCl. Numbers are the same as stated in Fig.1.

Analysis of amino acids in cells growth in synthetic media with normal and high salt concentrations and the residual amino acids in the cultured media

Experiments were carried out with synthetic media containing 700 μM proline (70 μmoles in 100 ml culture system) and normal salt or 10 % salt concentration. In case of the synthetic medium containing 10 % NaCl, additional experiments were carried out with 500 μM and 200 μM proline (Table 2). The cultures reaching O.D. indicated in the Table 2 were used for analysis of amino acids in the cells and in the media. The amino acid contents were expressed as absolute amounts of proline in 100 ml culture system and densities of proline, glutamine and glutamic acid.

In the medium containing normal salt concentration, the cells accumulated 36.9 mM proline in spite of 700 μM proline initially present

Table 2. Major free amino acid contents in cells and synthetic media after cultivation

NaCl added into medium	Add. proline μmol* (μM)	Growth OD650nm (Incub.time)	Dry weight of cells mg*	Intracellular water content μl*		Content of amino acid					
						Proline		Glutamine		Glutamic acid	
						μmol*	mM	μmol*	mM	μmol*	mM
Non NaCl	70 (700)	0.75 (18hr)	29.65	50.40	cell	1.86	36.9	0.60	11.9	1.74	34.5
					sup	47.05	0.5	1.05	0.0	54.14	0.5
10%NaCl	70 (700)	0.50 (70hr)	13.03	11.46	cell	18.99	1657.1	4.20	366.5	2.43	212.0
					sup	25.80	0.3	7.20	0.1	40.50	0.4
10%NaCl	50 (500)	0.45 (73hr)	11.78	10.37	cell	15.75	1518.8	1.02	387.7	2.07	199.6
					sup	15.75	0.2	6.15	0.1	40.50	0.4
10%NaCl	20 (200)	0.32 (90hr)	9.08	7.99	cell	7.89	983.7	5.04	630.8	1.50	187.7
					sup	4.20	0.0	5.40	0.1	43.50	0.4

* Data show values of 100ml culture system

in the medium with 0.5 mM proline remaining in the medium. On the other hand, when the organisms were grown in a defined medium containing 10 % NaCl and 700 μM proline, free proline in the cells increased to 1657.1 mM, with 0.3 mM proline remaining in the medium. When the amount of proline to be added to 10 % salt medium was reduced to 500 μM, free proline in the cells was 1518.8 mM and residual proline in the medium reduced to 0.2 mM. When the amount of proline was further reduced to 200 μM, free amino acids in the cells and residual amino acids in the medium further decreased to 983.7 mM and trace, respectively.

The free glutamine in the cells grown in a medium with physiological salt concentration was 11.9 mM, whereas it was 360-630 mM in those grown in 10 % salt medium. It was desirable to carry out an experiment with 10 % salt medium containing less than 200 μM proline, however, bacterial growth could hardly be obtained under this condition. Consequently, no analysis was made possible.

Discussion

When *S. aureus* was grown in a high salt medium, it has, unlike the halophilic bacteria, an adaptational ability to maintain the homeostasis of intracellular cations (3, 6, 7, 11). For this, there must be some mechanisms for dealing with the difference between intracellular and extracellular osmotic pressure. Most of the bacteria have rather high intracellular osmotic pressure, however, there is a need of delicate osmoregulation for their growth under a extreme increase of external osmotic pressure.

Koujima et al. (8) investigated a sequential osmoregulatory mechanism of *S. aureus* grown in a high salt environment and reported that the cells increased their internal osmotic pressure by an efflux of water due to shrinkage of the cells and then induced an influx of water into the cell bodies by a prompt uptake and accumulation of proline from the medium.

Since no detailed study using a synthetic medium has been made, we carried out the experiments with synthetic media to elucidate the amounts of proline required and the dynamics of proline.

Our studies on the growth curves and the analysis of amino acids in the cells and in the medium indicated that 20 μM of proline was needed for cell growth in a normal salt environment, whereas 700 μM was needed when *S. aureus* was grown in 10% salt medium because proline was accumulated above 1,500 mM for osmoregulation in addition to the cell construction (8). The specific accumulation of proline may be attributed to the high water-solubility of proline (14 M at 37℃) and its physicochemical property that is in non-polar status giving no excessive charges (9).

Anderson and Witter (1) stated that glutamine played a role in osmoregulation much more than proline did, as *S. aureus* that was grown in a high salt medium accumulated more glutamine than proline as free amino acids in the cells.

As for glutamine, it was hardly accumulated in the cells grown in the medium containing normal salt concentration, while 360-630 mM glutamine that was converted from glutamic acid was accumulated in those grown in a high salt medium. In the present experiment using a synthetic medium, the accumulated proline in the cells decreased and the amount of glutamine merely increased as the amount of proline added to the medium was reduced. We could observe the slightly complementary activity of glutamine.

Summarizing the various facts obtained, we may conclude that proline definitely plays a major role and glutamine a minor role in osmoregulation in high salt environment.

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ブドウ球菌の耐塩機構におけるプロリンの役割

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要旨　黄色ブドウ球菌は10%以上の高食塩下でもよく増殖し得るという耐塩性を有する。そのための一つの機構として、われわれは、細胞膜を構成するリン脂質組成中のカルジオリピンを著増させることによって、外部からの食塩流入を抑制して細胞内一価カチオンのホメオスタシスを維持することを明らかにした。もう一つの機構としては、外部の高浸透圧に対処するために、一時的に急激な水分放出を行い、ひき続いて培地中のプロリンを細胞内に取り込み蓄積することによって浸透圧調節を行っていることも明らかにした。

このたびの研究では、黄色ブドウ球菌における浸透圧調節に果たすプロリンの動態を、合成培地を用いて定量的に解析した。その結果を要約するとつぎのごとくである。プロリンは細胞構築上の必須アミノ酸の一つであるが、その量は20μM程度の微量で充分である。しかし10%食塩含有培地という高浸透圧下での増殖のためには、700μMの大量を必要とし、これを細胞内に1600μM以上蓄積して外部高浸透圧に対処する。プロリンが充分量存在しない場合は、グルタミンを取り込むことによってプロリンの役割を代償する。

キーワード：黄色ブドウ球菌、耐塩性、浸透圧調節、プロリン