Effect of KCl and NaCl on Uptake of Proline in *Staphylococcus aureus*

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Abstract

Staphylococcus aureus, the most salt-tolerant food-borne pathogen, produces enterotoxins which may cause symptoms such as vomiting, diarrhea, nausea, and cramps. Since this bacterium has been able to grow at extremely high osmolarity, its identity in foods with low water activity values such as salted or dried foods is of great concern. In this study, the growth of *S. aureus* at high osmolarity has been studied and the doubling time of *S. aureus* grown at TSB medium containing 15% NaCl has been found to be increased to 4~5 hours. The stimulation of proline uptake after exposure of cells to high concentration of both extracellular KCl and sucrose was not increased. Stimulation of proline uptake at these environment only occured when 25mM NaCl was present in transport buffer. In additional experiments, the time required to reach mid-logarithmic phase in defined medium of high osmolarity found to be reduced by the presence of glycine betaine, proline, and choline.

Key word : Osmoregulation, Staphylococcus aureus, proline uptake.

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Introduction

Staphylococcal food poisoning has been associated with a variety of foods and is one of the most common causes of reported food-borne illness[1]. *Staphylococcus aureus* is a facultative anaerobic, Gram-positive, spherical organism(0.5~1.0 μm in diameter) and is usually arranged in grapelike irregular cluster. This bacterium grows between 10 and 45℃(optimum : 30~37℃) and at pH values between 4.2 and 9.3(optimum : pH 7.0~7.5)[2]. It has been reported that *S. aureus* is able to grow well in media which has water activity values below 0.86[3]. Thus, staphylococcal poisoning is a particular problem within foods of low water activity values.

S. aureus is ubiquitous and produces a variety of extracellular enzymes and toxins according to strains. A common type of food poisoning is caused by heat-resistant enterotoxins which are also stable to the action of human gut enzymes. Many strains of *S. aureus* produce enterotoxins, which, if ingested via contaminated foods, may produce flu-like symptoms including abdominal pains, vomiting, nausea, cramps and diarrhea[4]. Outbreaks of staphylococcal food poisoning are

associated with processed red meats, poultry products, and cream-filled bakery products[5]. Although *S. aureus* has represented distinguished problems from other food-borne pathogens by its ability to grow at low water activity, little is known about the osmoregulation by this organism. Recent studies have indicated that the compatible solutes such as glycine betaine and proline are accumulated by transport systems of this organism at high osmolarity[6-7]. However, the effect of KCl and sucrose at varied concentrations for the osmoregulation of this organism has never been reported. This research was carried out to gain better understanding of the osmoregulatory mechanisms utilized by *S. aureus* for growth at high osmolarity such as KCl and sucrose.

Materials and Methods

Bacterial strain and culture conditions

S. aureus ATCC 12600 was obtained from the American Type Culture Collection. This strain is a proline auxotroph and requires micromolar concentrations of proline for growth[8]. Cells were maintained on 1.5% agar Trypticase Soy Broth (TSB, BBL Microbiology Systems, Cockeysville, Maryland) at 5℃. Cells were cultured at 37℃ either in 250mℓ TSB medium or in 500mℓ defined medium(DFM) on a rotary shaker(150 rpm). The composition of basal defined medium is shown in Table 1. The vitamin and amino acid solutions were sterilized by passage through 0.22 μm-pore GSWP047 membrane filters(Millipore Corporation, Bedford, MA). The glucose and salt solutions were sterilized by autoclaving at 121℃ for 25 minutes. The glucose, amino acids, vitamins and salts were obtained from Sigma Chemical Co.(St. Louis, Mo.). Cultures with or without 15% NaCl were incubated at 37℃ on a gyratory shaker, and growth was monitored at 650nm using a spectrophotometer(Spectronic 20, Milton Roy Co., Rochester, N.Y.). Growth rate has been expressed as doubling time.

Table 1. Composition of basal defined medium

Ingredient	Amount[a]
Glucose	2.50g
Salts	
K_2HPO_4	3.50g
KH_2PO_4	1.00g
Na_3-citrate-$2H_2O$	0.20g
$MgSO_4$	0.05g
$(NH_4)_2SO_4$	0.05g
Vitamins	
Thiamine	1 mg
Niacin	1.2 mg
Biotin	0.005mg
Ca-pantothenate	0.25 mg
Amino acids	
L-glutamic acid	100mg
L-serine	30mg
L-methionine	2mg
L-tyrosine	50mg
L-alanine	60mg
L-lysine	50mg
L-threonine	30mg
L-phenylalanine	40mg
L-histidine	20mg
L-tryptophan	10mg
L-isoleucine	30mg
L-valine	80mg
L-leucine	90mg
L-arginine	50mg
L-cystine	20mg
Glycine	50mg

a : Final concentration per liter.

Cell were harvested at the mid-logarithmic phase of growth by centrifugation at 9,000×g for 10 minutes at 5℃. Cells were washed twice using 50mℓ of 50mM potassium phosphate buffer (pH 7.5), resuspended in 5mℓ of the same buffer containing 40mM glucose, and maintained on ice for 5 minutes until used in the transport experiments.

Transport experiments

Transport experiments were performed by a filtration method. Cells were preincubated at 37℃ for 5 minutes in transport buffer(50mM K_2HPO_4, pH 7.5, 25mM NaCl, 40mM glucose, and 100μg of chloramphenicol per mℓ) at a final concentration of approximately 250μg of total cellular protein per mℓ. The chloramphenicol was used in these experiments to detect the activity of pre-existing proline transport systems, not resulted from de novo synthesis of the transport systems. Next, [^{3}H]-proline was added to the cellular suspension. The specific activity of proline was 1,000cpm/nmol and the final concentration of proline was 1mM. Upon the addition of [^{3}H]-proline, cell suspensions were agitated at 37℃ at 150rpm. At various time intervals(30, 60, and 90 seconds), 1-ml aliquots were removed and assayed for proline uptake by filtration as described below.

For the filtration experiments, 1-mℓ aliquots of cell suspensions were rapidly passed through a 0.22-μm-pore GV filter(Millipore Corporation, Bedford, MA.). The entire filtration was completed in 2 to 3 seconds. Filters were then washed twice with 1-mℓ of unlabeled transport buffer. Next, filters were transferred to scintillation vials and allowed to dry overnight. Samples were counted in 5-mℓ of scintillation solution with a Beckman model LS1701 scintillation spectrometer.

Protein determination

The protein concentration in cell suspension was determined by a modification of the method of Lowry et al[9]. Before the Folin reaction, the cell suspensions were heated at 90℃ in 1N NaOH for 10 minutes in order to obtain complete solubilization. The bovine serum albumin standard was treated in the same manner since this treatment reduces the intensity of color development[10].

Results and Discussion

An elevation of osmolarity of the microbial environment generally retards microbial growth rate and metabolic activity[11]. This study shows, *Staphylococcus aureus*, the most salt-tolerant food borne pathogen, was capable of growth in TSB medium supplemented with 15% NaCl. Media(50-mℓ in side-armed flasks) were inoculated with 5mℓ of a culture grown overnight at 37℃ in TSB medium. As shown in Figure 1, growth of strain ATCC 12600 in TSB medium was possible even when the NaCl concentration was 15%. However, the doubling time of 15% NaCl grown cells was much longer(approximately 4 to 5 hours) than that of cells grown in TSB medium containing no added NaCl. The *Staphylococcus aureus* grown at TSB medium had very short doubling time(approximately 45 minutes).

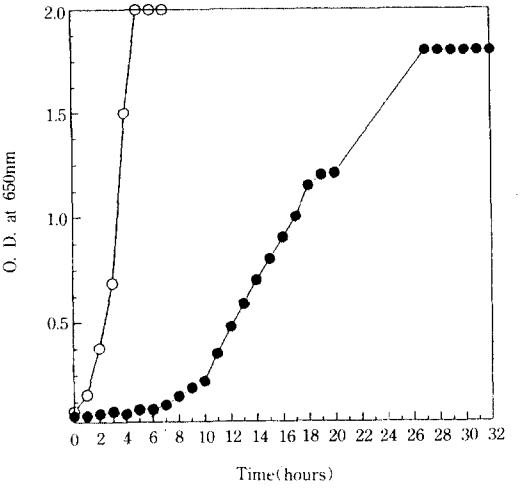

Fig. 1. Growth curve of *Staphylococcus aureus* grown at TSB medium with or without 15% NaCl.
○−○ *S. aureus* grown at TSB medium with no added 15% NaCl.
●−● *S. aureus* grown at TSB medium supplemented with 15% NaCl.

The studies of staphylococcal osmoregulation by Measures[12], Koujima et al[13], and Anderson and Witter[14] revealed that proline was a major organic solute accumulated by *S. aureus* at high osmolarity(10% NaCl). Furthermore, these studies indicated that proline accumulation by the cell was carried out by transport and not by synthesis. The data presented in Figure 2 were obtained when high concentrations of KCl other than NaCl was added to the transport media with or without 25mM NaCl. Cells were incubated for 30 sec, 60 sec, and 90 seconds in transport buffer containing KCl concentration varied between 0 to 2.0 M. The stimulation of proline uptake measured after exposure of cells to high concentrations of extracellular KCl has not been increased. Interestingly, upon the addition of 25 mM NaCl to the high KCl concentrations, the levels of proline uptake were elevated. The maximum proline uptake occurred after cells were incubated for 90 seconds in transport buffer containing 1M KCl and 25mM NaCl.

The same results were shown when high concentrations of sucrose other than NaCl were added to the transport buffer to increase the osmolarity. Figure 3 shows proline uptake was not stimulated when cells were incubated in transport buffer containing sucrose concentations varied between 0 to 2.0 M. Thus, the stimulation of proline uptake detected only by the addition of 25mM NaCl. The finding that high concentrations of both KCl and sucrose did not stimulate the proline uptake, which has been known as a compatible solute for this bacterium, indicates that the osmoregulation of this extremely osmotolerant Gram-positive coccus may not be dependent on the osmotic strength of medium, but on the presence of NaCl. This implies the proline transport systems in this bacterium may be Na-dependent as in proline transport in E. coli. The studies by Stewart et al[15] showed that a 4~5 fold stimulation of proline uptake occurred upon addition of KCl to *E. coli* washed and incubated in a sodium-containing buffer. When the buffer was replaced with Na^+-free buffer, the rate of proline uptake remained very low and was not stimulated by KCl[15]. Other studies with halophilic bacteria[16] and ruminal bacteria[17] also reported that the amino acid transport into these bacteria was achieved by a chemical gradient of sodium. The proline uptake stimulated by sucrose was 2 to 3-fold higher than the proline uptake by KCl. This result seems to be due to the specificity

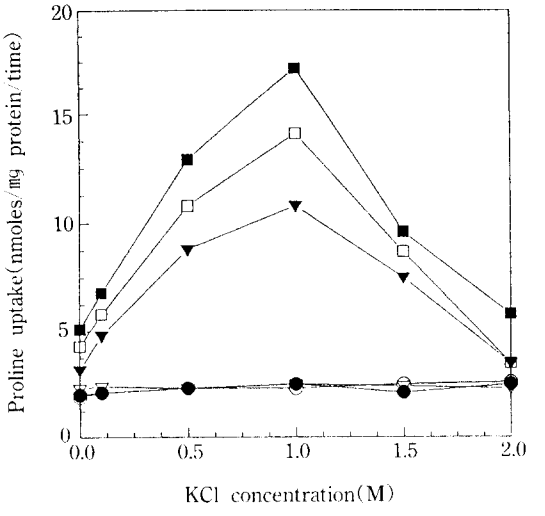

Fig. 2. Proline uptake by *S. aureus* as a function of KCl concentration. Cells were suspended in transport buffer at a concentration of 240μg of total cellular protein per mℓ. Proline uptake is expressed as nanomoles per milligram of total cellular protein per each time. Cells were incubated for 30 sec, 60 sec, and 90 seconds in transport buffer with or without 25mM NaCl.
(Symbols : ○ No NaCl, 30 sec, ● No NaCl, 60 sec, ▽ No NaCl, 90 sec, ▼ 25mM NaCl, 30 sec, □ 25mM NaCl, 60 sec, ■ 25mM NaCl, 90 sec)

of sucrose to the transport system.

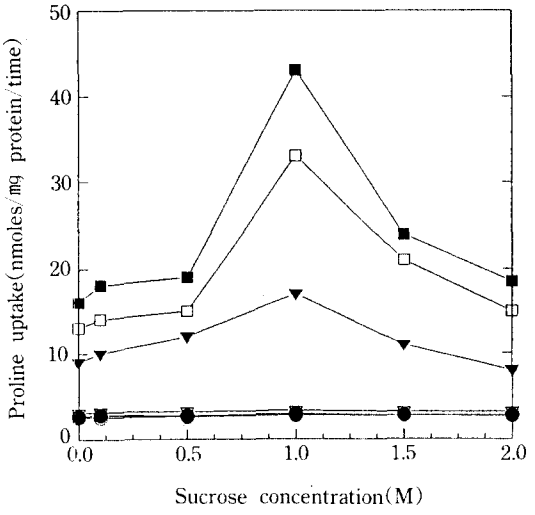

Fig. 3. Proline uptake by *S. aureus* as a function of sucrose concentration. Cells were suspended in transport buffer at a concentration of 260μg of total cellular protein pel mℓ. Proline uptake is expressed as nanomoles per milligram of total cellular protein per each time. Cells were incubated for 30 sec, 60 sec, and 90 seconds in transport buffer with or without 25mM NaCl.
(Symbols : ○ No NaCl, 30 sec, ● No NaCl, 60 sec, ▽ No NaCl, 90 sec, ▼ 25mM NaCl, 30 sec, □ 25mM NaCl, 60 sec, ■ 25mM NaCl, 90 sec)

The recent study by Miller et al.[8] showed that proline and glycine betaine both play important roles in the osmoregulation of *S. aureus*. This study revealed that both compounds are accumulated at high concentration(e.g., molar) within *S. aureus* when cells were grown at high osmolarity. Because these compounds were accumulated to high concentrations within cells grown at high osmolarity, the effects of these compatible solutes along with choline on the growth rate of cells cultured in defined medium containing 5% NaCl were examined. As shown in Figure 4, when defined medium containing 5% NaCl was supplemented with 5mM proline, 5mM glycine betaine or 5mM choline, the time required to reach at mid-logarithmic phase was reduced. The time was substantially shorter when glycine betaine was added to the growth medium. Also, reduction of the time by choline shows that the choline, a precursor of glycine betaine, could be a new possible compatible solute for this organism. The pathway for glycine betaine synthesis via ethanolamine and choline has been proposed for spinach and sugar beets[18].

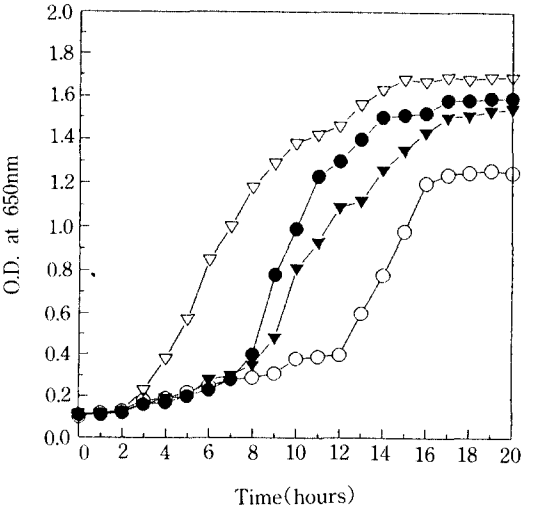

Fig. 4. Growth curve of *S. aureus* at DFM medium contalining 5% NaCl. Media (50mℓ in side-armed flasks) were inoculated with 5mℓ of a preculture grown overnight at 37℃ in DFM medium. Growth was monitored at 650 nm.
(Symbols : ○ DFM containing 5% NaCl and 5μM proline. ● DFM containing 5% NaCl and 5mM proline. ▽ DFM containing 5% NaCl, 5μM proline and 5mM betaine. ▼ DFM containing 5% NaCl, 5μM proline and 5mM choline.)

The dependence of this bacterium on the transport of these compatible solutes for its osmoregulation implies that these solutes could serve

as a source of osmoregulator within the natural environment. Proline and glycine betaine, which are plant metabolites, could be available in vegetable foods. Phosphatidylcholine(lecithin) may serve as an abundant source of choline which may be released through the action of phospholipase D, an enzyme in plant tissue[19]. Glycine betaine has also been found within animal tissues. For examples, it is synthesized by kidney cells[20]. Phosphatidyl choline is also abundant in animal tissue and could also theoretically serve as a source of glycine betaine within meat products. Regarding proline, this amino acid may potentially be released in the free form within protein rich foods through the action of extracellular proteinases produced by contaminating bacteria[4]. Through this understanding, it may be possible in future studies to develop novel strategies for the control of this food-borne pathogen within low water activity foods.

요 약

포도상구균은 식중독을 유발하는 미생물로 이 균이 오염된 식품을 섭취하였을 시 구토, 설사, 메쓰꺼움, 위경련 등의 증세를 일으키게 된다. 이 균은 다른 식중독을 유발하는 미생물들과는 달리 삼투압이 높은 환경에서도 성장이 가능함으로 water activity 값이 낮은 염장식품이나 건조식품에서까지 문제가 되고 있다. 따라서 본 연구에서는 실제 고농도 환경에서 세포가 어떻게 성장하며, 이 세균의 compatible solute로 알려진 물질들을 medium에 첨가해 주었을 때 세포성장이 어떻게 변하는지에 대해 조사 해 보았다. 15% NaCl를 포함한 TSB medium에서 성장시킨 *S. aureus*의 경우 doubling time 은 약 4~5 시간으로 증가하는 현상을 보였다. 또 KCl이나 Sucrose를 사용하여 외부삼투압을 올려준 후 proline의 uptake정도를 측정해본 결과, 25mM NaCl이 존재 할 경우에만 고농도의 환경에서 proline 을 축적시켰다. Proline, glycine betaine, choline을 defined growth medium에 넣어준 결과 mid-logarithmic phase에 이르는 시간이 단축되었고 이는 glycine betaine, proline, choline의 순으로 나타나 choline 또한 가능한 compatible solute가 될 수 있음을 암시해 주었다.

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