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Ultrastructural and Intracellular Chemical Changes of a Novel Halophilic Strain V430 of *Staphylococcus saprophyticus* under $CaCl_2$ Stress

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Abstract Observation of the ultrastructural and intracellular chemical changes of $CaCl_2$-tolerant strains is important both for understanding their adaptation mechanism under high salt stress and for providing theoretical basis of their application in treating high-$CaCl_2$ wastewaters. A novel strain V 430 isolated by us has been successfully used to decrease the chemical oxygen demand (COD) concentration of diosgenin wastewater from 20 g l^{-1} to less than 0.5 g l^{-1}. For this study, strain V430 was incubated in culture media of different $CaCl_2$ concentrations (up to 9.0%). Strain V430 cells incubated in media of high $CaCl_2$ concentration excreted extracellular substances and accumulated intracellular Ca^{2+} and K^+ and free amino acids. The levels of intracellular cations and free amino acids increased with increase in $CaCl_2$ concentration of the medium. The increase in total free amino acids was mostly due to accumulation of glutamic acid. The strain cells under 9.0% $CaCl_2$ stress took up K^+ in a short time, while accumulation of Ca^{2+} proceeded over the whole growth process.

Keywords *Staphylococcus saprophyticus* · $CaCl_2$-tolerant · Ultrastructure · Cations · Free amino acid

Introduction

Treatment of hypersaline wastewaters with high chemical oxygen demand (COD) loads has been a major challenge for environmental scientists, due to the difficulty of screening and application of halophilic bacteria to biodegrade the wastewaters. For instance, the wastewater of diosgenin production is a representative of hypersaline wastewaters. Diosgenin is extracted from *Dioscorea zingiberensis* and has been used to synthesize steroids for medicines such as pregnenolone and progesterone. The wastewater of diosgenin production has low pH (less than 2) and high concentrations of chloride ion (280–700 mM)

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and refractory organics (COD up to 10–30 g l^{-1}) [1]. Hydrochloric acid is commonly used for the hydrolysis of *D. zingiberensis*, as the crucial step to extract diosgenin. CaO is usually added for neutralization, and the main salt in the wastewater is therefore calcium chloride. Great effort has been made to biodegrade the wastewaters. A novel strain V 430 isolated by us has been successfully applied to decrease the COD concentration from 20 g l^{-1} to less than 0.5 g l^{-1}. However, the adaptation mechanism of the strain under $CaCl_2$ stress was poorly understood. Studying the mechanism is important for application of the strain in environmental engineering practice.

Ordinary bacterial cells could not maintain the water activity of their cytoplasm higher than that of the surrounding brine because this would lead to a rapid loss of water to the environment [2]. Therefore, the unique properties of microorganisms living in hypersaline environment were related to their adaptation to fluctuations in external osmotic pressure (osmoadaptation) with the development of specific functions [3–6].

However, molecule accumulation of microorganisms differs for different species. For instance, halophilic archaea and acetogenic anaerobes could accumulate large amounts of salts (KCl) [7–10]. Other bacteria may accumulate organic compatible solutes within their cytoplasm to balance the osmotic pressure of the medium. Compatible solutes detected in halophilic and halotolerant microorganisms include glycerol [11–13], sugars and sugar derivatives (sucrose, trehalose, and glucosylglycerol) [14, 15], amino acids and derivatives [16–19], glycine betaine and ectoine [20, 21].

The predominant salt type for previous studies on halophilic and halotolerant bacteria has been NaCl. Studies on NaCl-tolerant microorganisms commonly indicated that the intracellular ionic concentrations were similar to those of the surrounding medium. In general, the ionic composition of the cytoplasm differs greatly from that of the medium, which in most cases contains NaCl as the main salt. In addition to NaCl, effects of molar concentrations of KCl on the intracellular environment were studied by some authors [7–10]. However, little has been known about the biological properties of $CaCl_2$-tolerant strains up till now. In this study, the microbiological behavior of the novel $CaCl_2$-tolerant strain V430 of *Staphylococcus saprophyticus* was characterized. Changes in cell surface and internal structures, intracellular cation contents, and free amino acid contents of the strain incubated in media of high $CaCl_2$ concentration were examined to understand the adaptation mechanism of the strain under high $CaCl_2$ stress.

Materials and Methods

Bacterial Strains and Culture Conditions

Strain V430 of *Staphylococcus saprophyticus*, with 16S rDNA sequences in GenBank accession number DQ355389, was isolated from the activated sludge of wastewater treatment system of a diosgenin production plant at Shiyan city, Hubei province, central China. Isolation was performed under anaerobic conditions at 30°C and pH 7.5 in liquid medium (named medium 1): 0.2 g l^{-1} $MgSO_4 \cdot 7H_2O$, 0.5 g l^{-1} KH_2PO_4, 1.5 g l^{-1} K_2HPO_4, 0.5 g l^{-1} NH_4Cl, and 90 g l^{-1} $CaCl_2$, and 1 ml microelement liquor [2 g l^{-1} $FeCl_3 \cdot 6H_2O$, 2 g l^{-1} $CoCl_2$, 0.5 g l^{-1} $MnCl_2$, 0.03 g l^{-1} $CuCl_2$, 0.05 g l^{-1} $ZnCl_2$, 0.05 g l^{-1} $NiCl_2 \cdot 6H_2O$, 1.0 g l^{-1} ethylenediaminetetraacetic acid (EDTA)] was dissolved into 1 l diosgenin wastewater (COD=1 g l^{-1}). The defined medium consisted of medium 1, with an appropriated addition of some amino acids except glutamic acid. The peptone medium (named medium 2) has the following composition: 10 g of peptone l^{-1}, 5 g of beef paste l^{-1},

18 g l^{-1} $CaCl_2$, and 5 g l^{-1} NaCl. In some experiments, $CaCl_2$ concentrations were adjusted from 0.2 to 9.0%. Cell growth was monitored turbidimetrically by measuring the optical density at 600 nm.

Ultrastructural Analysis

Scanning Electron Microscope

Cells grown until the stable phase in medium 2 with 0.2 to 9.0% $CaCl_2$ were collected by filtration through bacterial filters, fixed with 2.5% glutaraldehyde in 100 mmol l^{-1} phosphate buffer (pH 7.1) for 2 h at 4°C, washed with phosphate buffer, dehydrated with increasing concentrations of ethanol, postfixed with 2% OsO_4 in 100 mmol l^{-1} cacodylate buffer at pH 7.4, and then coated with gold for analysis with a JSM-6700F scanning electronic microscope (SEM).

Transmission Electron Microscope

Cells grown until stable phase in medium 2 containing 0.2 to 9.0% $CaCl_2$ were collected by filtration through bacterial filters, fixed with 2.5% glutaraldehyde in 100 mmol l^{-1} phosphate buffer (pH 7.1) for 2 h at 4°C, washed with phosphate buffer, dehydrated with increasing concentrations of ethanol, postfixed with 2% OsO_4 in 100 mmol l^{-1} cacodylate buffer at pH 7.4. The cells were then washed, dehydrated in acetone, and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate and observed under a H-7000FA transmission electronic microscope (TEM).

Analysis of Intracellular Cation Contents

Intracellular cation contents of cells grown until stable phase in medium 2 with 0.2, 1.8, 5.4, and 9.0% $CaCl_2$ were measured first. Stable-phase cells from the 0.2% $CaCl_2$ medium were then incubated under 9.0% $CaCl_2$ for 30, 60, 90, and 120 min, respectively. The biomass was harvested by filtration through bacterial filters and rapidly washed three times with 5 ml deionized water. The cells on filters were immersed in 10 ml of deionized water and boiled for 10 min. The extract was cooled and collected by centrifugation (12,000×*g* for 20 min). The supernatant was used for cation analysis by inductively coupled plasma-mass spectrometry (ICP-MS). The cation content was expressed as a percentage of dried biomass. The data were obtained by two duplicate assays, with standard deviations around 8%.

Analysis of Intracellular Free Amino Acid Contents

Intracellular free amino acid contents of cells grown until the stable phase in medium 2 with 0.2, 1.8, 5.4, and 9.0% $CaCl_2$, were measured first. The stable-phase cells from the 0.2% $CaCl_2$ medium were then incubated under 9.0% $CaCl_2$ for 30, 60, 90, and 120 min to measure the intracellular free amino acids contents under salt stress. The biomass was harvested by filtration through bacterial filters and rapidly washed three times with 5 ml deionized water. The cells were dissolved in 3 ml 10% perchloric acid and vibrated on multifunctional Mixcraft (WH-951) for 10 min, and then 500 mmol l^{-1} NaOH was added to adjust pH to 7.0. The combined extract was collected by centrifugation (12,000×*g* for 20 min). The supernatant was used for total amino acid analysis with an amino acid analyzer (Hitachi 830-50). The measurements were made in duplicate, with standard deviation around 10%.

Results

$CaCl_2$ Tolerance

Growth capability of bacterial strain V430 under different $CaCl_2$ stress can be seen from Fig. 1. The strain V430 has remarkable ability to grow in the presence of 0–12.6% $CaCl_2$ mediums. Cells grown in the presence of 0.0 to 9.0% $CaCl_2$ has higher optical density ($OD_{600\ nm}$) than cells grown in the presence of 10.8 to 12.6% $CaCl_2$. Therefore, strain V430 cells have the ability to grow well in the presence of 0.0 to 12.6% $CaCl_2$, and the $CaCl_2$ level for optimal growth in medium 1 is between 0.0 and 9.0%.

Ultrastructural Change

SEM and TEM observation revealed morphology and configuration changes of cells under different $CaCl_2$ concentrations (Figs. 2 and 3). The surface of cells was relatively smooth (Fig. 2a), when the $CaCl_2$ concentration was 0.2%. However, when strain V430 cells were grown in 9.0% $CaCl_2$ medium, some secretions obviously occurred on the cells' surface (Fig. 2b). TEM images of cells also indicated that there were dramatic extracellular secretions on the surface of the cells (Fig. 3b).

Intracellular Cation Contents

The intracellular cation contents of Strain V430 under different $CaCl_2$ concentrations were tested and presented in Table 1 and Fig. 4. In the strain V430 cells, intracellular accumulation of Ca^{2+}, K^+, and Mg^{2+} increased with the increase in growth medium salinity (Table 1). The Ca^{2+} concentration was 10.9 mg g dry $cell^{-1}$ when the $CaCl_2$ concentration of medium 2 was 0.2%. But the concentration increased 2.2-, 8-, and 13-fold for $CaCl_2$ concentration of 1.8, 5.4, and 9.0%, respectively. Another cation with notable concentration increase was K^+, 1.94 times higher at 9.0% $CaCl_2$ concentration compared with that at 0.2%. The intracellular K^+/Ca^{2+} ratio decreased with increasing $CaCl_2$ concentration in the growth medium, from 1.11 in 0.2% $CaCl_2$ medium to less than 0.5 under $CaCl_2$ stress. The relatively low intracellular Mg^{2+} content was also greatly affected by $CaCl_2$ concentration. The Mg^{2+} content at $CaCl_2$ concentration of 9.0% was 3.8-fold higher than that at 0.2%.

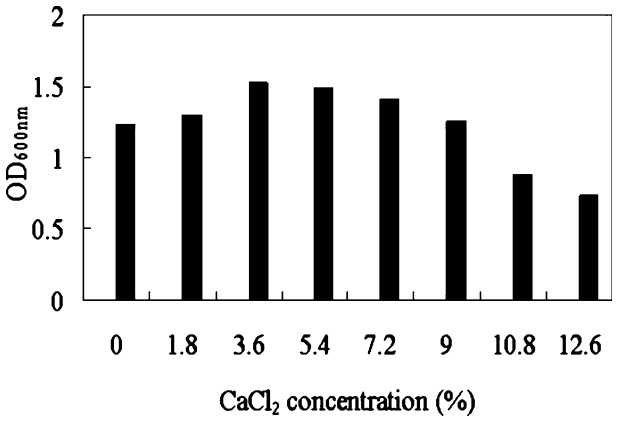

Fig. 1 Growth capability of bacterial strain V430 under different $CaCl_2$ stress

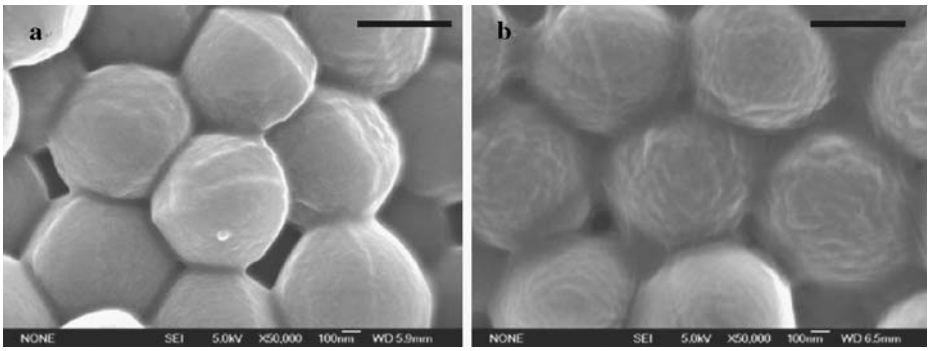

Fig. 2 SEM images of V430 cells. **a** control (0.2% $CaCl_2$); **b** under $CaCl_2$ stress (9.0% $CaCl_2$). The *scale bar* is 0.5 μm

The changes in intracellular Ca^{2+} and K^+ contents with time under 9.0% $CaCl_2$ stress were shown in Fig. 4. The contents of intracellular Ca^{2+} and K^+ in 0.2% $CaCl_2$ medium was 5.0 and 8.0 mg g dry $cells^{-1}$, respectively and increased to 10.7, 17.2, 23.1, 25.4 mg g dry $cells^{-1}$, and 19.7, 38.9, 43.1, 43.2 mg g dry $cells^{-1}$ under 9.0% $CaCl_2$ stress, respectively, for 30, 60, 90, and 120 min. In other words, the contents of intracellular Ca^{2+} and K^+ of strain V430 under 9.0% $CaCl_2$ stress increased with increase in stress time. The rate of the intracellular K^+ content increase was higher than that of Ca^{2+}. Therefore, the strain V430 cells under 9.0% $CaCl_2$ stress were able to take up K^+ in a relatively short time. By contrast, the accumulation of Ca^{2+} proceeded over the whole growth period.

Intracellular Free Amino Acid Contents

Glutamic acid was the predominant species of amino acids, accounting for 46.9% of the total amino acid content (Table 2). Aspartic acid was also abundant in the pool. As shown in Table 2, Glutamic acid increased substantially with increase in $CaCl_2$ amount added to

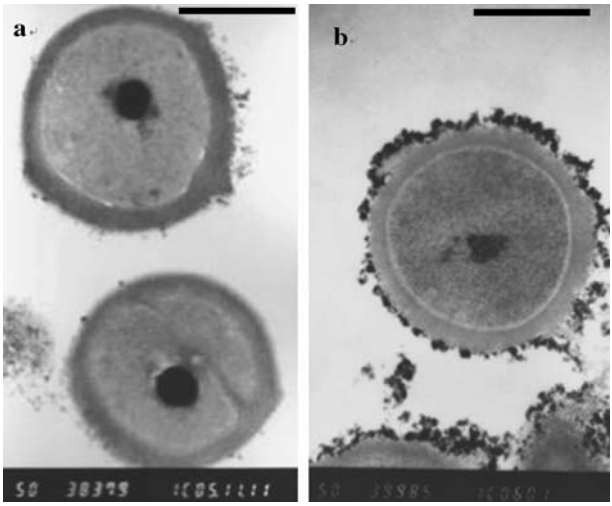

Fig. 3 TEM images of V430 cells. **a** control (0.2% $CaCl_2$); **b** under $CaCl_2$ stress (9.0% $CaCl_2$). The *scale bar* is 0.5 μm

Table 1 Intracellular cation content of strain V430 cells in stable phase incubated for about 38 h in culture media with different $CaCl_2$ concentrations.

$CaCl_2$ (%)	Cations (mg g dry cells^{-1})			
	K^+	Ca^{2+}	Mg^{2+}	K^+/Ca^{2+}
0.2	12.1	10.9	0.4	1.1
1.8	15.8	34.5	1.2	0.46
5.4	25.9	98.2	1.4	0.26
9.0	35.8	153.3	1.9	0.23

The standard deviations of duplicate measurements of the cation contents are around 8%.

the medium. Relative to controls in 0.2% $CaCl_2$ medium, the levels of glutamic acid in the intracellular pools for cells under 1.8, 5.4, and 9.0% $CaCl_2$ stress increased 1.4-, 3.9-, and 4.8-fold, respectively. Glutamic acid accounted for 53.4% of the total amino acid pool when cells were grown under 9.0% $CaCl_2$ stress. As the salt concentration was increased from 0.2 to 9.0% $CaCl_2$, isoleucine, leucine, proline, and valine increased 283-, 44.8-, 26.6-, and 6.7-fold, respectively. For other amino acids, no significant changes were observed. The effect of 9.0% $CaCl_2$ stress on the intracellular amino acid pool at 30, 60, 90, and 120 min is shown in Fig. 5. The total intracellular free amino acid content could be accumulated at 120 min to 168.4 mg g dry cells^{-1}, up to 73% of the content of stable-phase cells incubated in 9.0% $CaCl_2$ medium.

Discussion

The outer surface of the cell was permanently exposed to high salt stress and thus must have undergone adaptive changes [6]. SEM and TEM observation on the ultrastructure of strain V430 of *S. saprophyticus* under different $CaCl_2$ stress provided critical information on the cells' surface and structure. When the strain V430 cells were incubated in 9.0% $CaCl_2$ medium, some extracellular secretions obviously occurred on the surface of the cells. This was considered a physiological phenomenon due to secretion of some bacteria under high salt stress [22, 23]. It is still unclear whether these extracellular exopolymer secretions may act as organic ligands to protect the cells from damage of high salinity stress [24]. However, the excretions on the cells' surface in the presence of 9.0% $CaCl_2$ medium (Fig. 2b and Fig. 3b) were obviously more than those in 0.2% $CaCl_2$ medium. Whether this

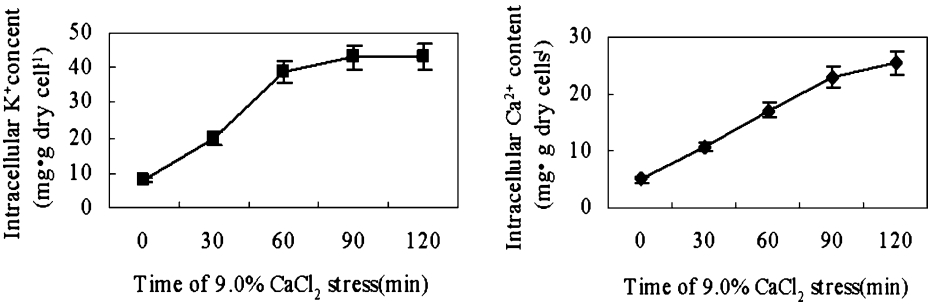

Fig. 4 Changes of intracellular Ca^{2+} and K^+ content of V430 cells with time under 9.0% $CaCl_2$ stress

Table 2 Compositions of intracellular free amino acids of bacterial strain V430 under different $CaCl_2$ stress.

Component (mg g dry cell^{-1})	$CaCl_2$ solution concentration (%)			
	0.2	1.8	5.4	9.0
Aspartic acid	9.8	14.9	22.7	25.1
Threonine	N.D.	N.D.	N.D.	N.D.
Serine	0.7	N.D.	N.D.	N.D
Glutamic acid	21.0	49.3	103.7	122.0
Hydroxide praline	N.D.	N.D.	N.D.	N.D.
Proline	0.6	1.2	12.2	15.4
Glycine	0.3	0.3	0.4	0.5
Alanine	1.2	2.4	7.1	7.2
Cystine	1.6	0.8	0.5	0.3
Valine	0.9	1.1	4.7	7.1
Methionine	1.4	2.6	3.3	4.2
Isoleucine	0.1	1.5	10.8	14.2
Leucine	0.2	0.3	5.5	6.9
Tyrosine	4.6	3.5	2.6	2.3
Phenylalanine	2.2	1.9	1.7	1.6
Lysine	5.2	6.1	17.5	19.2
Histidine	0.3	0.6	2.0	2.2
Tryptophan	N.D.	N.D.	N.D.	N.D.
Arginine	1.0	1.1	1.0	0.5
Total	50.8	87.5	195.7	228.7

The standard deviations of duplicate measurements are around 10%.

N.D. Not detected

increased secretions at high salinity is a specific salt-adaptation mechanism for this strain, needs more detailed investigation. It is necessary to more accurately define the composition of the bacterial strain V430 in future studies.

The halophilic bacteria previously studied by other authors were capable of taking up K^+ as the major cation to entreat the high salt environment [7–10]. Strain V430 was different from these bacteria in maintaining internal Ca^{2+} and K^+ contents (especially Ca^{2+}) when they were incubated in 9.0% $CaCl_2$ medium to stage phase. The intracellular

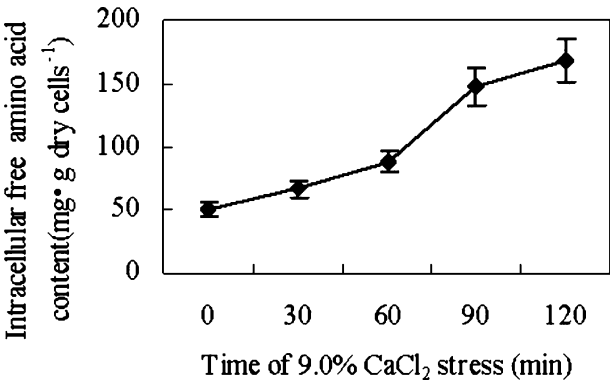

Fig. 5 Change of intracellular free amino acid contents of strain V430 with time under 9.0% $CaCl_2$ stress

Ca^{2+} and K^{+} contents were markedly increased when cells were incubated in 9.0% $CaCl_2$ medium.

It was reported that Gram-positive bacteria has a higher content of free amino acids mainly made of glutamic acid [25, 26] under control (low-salt) conditions. In this study, the glutamic acid content of total free amino acids is relatively high, up to 51.1 mg g dry $cell^{-1}$ under low salt stress (0.2% $CaCl_2$), showing the compositional characteristics Gram-positive bacteria typically have. The accumulation of proline or other free amino acids was observed in some microorganisms exposed to high salinity. Proline was found to act as an osmoregulator in *Staphylococcus aureus* [25]. Gram-positive bacteria were reported to accumulate the neutral amino acid proline [25–27] in response to increasing salt stress. Increase of glutamic acid was osmotically induced in numerous Gram-negative bacteria [28]. However, in this study, aspartic acid, proline, isoleucine, lysine, valine, leucine, and alanine were accumulated in strain V430 of *S. saprophyticus*, as the main osmoprotectant to make the strain acclimated to high $CaCl_2$ conditions, which is different from the behavior of ordinary Gram-positive bacteria. Although alanine has been found to be an inducible compatible solute in *Streptomycetes indigenous* [17], the occurrence of isoleucine, lysine, valine, and leucine as inducible compatible solutes in *S. saprophyticus* has not been reported in previous studies. Therefore, in terms of response to salt stress, strainV430 of *S. saprophyticus* has several specific features that have not been found in other Gram-positive bacteria. The accumulation of intracellular free amino acids under high salt stress could be the result of either synthesis or uptake from the medium. The results of this study indicate that the increase in total free amino acids was mostly due to accumulation of glutamic acid in the presence of high salt stress. Therefore, only the manner of glutamic acid accumulation was discussed. Strain V430 was grown until the stable phase in defined medium (with other amino acids except glutamic acid) with different $CaCl_2$ concentrations. When strain V430 cells was grown in 9.0% $CaCl_2$ defined medium, there was still an obvious increase in the glutamic acid pool. Consequently, the accumulation of glutamic acid was primarily the result of synthesis in the strain cells.

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