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Effect of medium salinity on amino acid and phospholipid composition of two halophilic *Bacillus* species from Saudi Arabia

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ABSTRACT

The cell envelope amino acids of two moderately halophilic *Bacillus* isolates (BST & BSF) varied according to the salinity of the medium. Cystine and proline were affected mostly. In both isolates growing in the presence of 6 and 18% NaCl there was more dicarboxylic amino acids than basic amino acids which makes the cell envelope proteins acidic. The concentrations of the cell-associated cations (Na^+, K^+ and Mg^{2+}) were high in both isolates and varied according to the NaCl concentration. The two isolates contained glucosamine and muramic acid in their cell walls. The amounts of these two sugar derivatives, however, varied with the NaCl concentration. Thin-layer chromatography of phospholipids revealed the presence of cardiolipins, phosphatidylglycerols and phosphatidylethanolamines in the two isolates, irrespective of the salinity of the medium. Phosphatidylglycerols and the phosphatidylethanolamines increased with increasing NaCl concentration of the growth medium. Lysophosphatidylglycerols were detected only in the 6%-grown BST cells. Unidentified phospholipids designated X1 (in isolates BSF & BST), X2 (in isolate BST) and X3 (in isolate BSF) were also detected; the concentrations of X1 and X3 were salinity dependent.

INTRODUCTION

Moderately halophilic bacteria grow at a salt concentration of 0.4–4.0 M NaCl, and they should make extensive modification of their chemical structure as an adaptation to the extreme environment.

Previous studies on a number of moderate halophiles have shown that organisms grown at higher salinities have greater proportions of negatively charged phospholipids in their membranes (Kanemasa *et al.* 1972; Komaratat & Kates 1975; Ohno *et al.* 1976, 1979; Kushner *et al.* 1983; Kogut & Russell 1984; Miller 1985, 1986) and have an excess of dicarboxylic amino acids over basic amino acids (Kushner *et al.* 1964; Kushner & Onishi 1966; Lanyi 1974; Hipkiss 1980). The above findings have led these authors to conclude that the excess negative charge is needed to balance the increasing positive charge brought about by the increasing NaCl concentration. The cell-bound cations of the moderately halophilic bacteria

have been shown to change upon changing the NaCl concentration of the growth medium (Masui & Wada 1973; Shindler *et al.* 1977). This change would increase the positive charge to a degree enough for structural stability.

Chemical studies of the envelopes of extremely halophilic rods and cocci showed that they lack muramic acid (Kushner *et al.* 1964; Koncewics 1972). There are no studies on Gram positive spore-forming rods. The major objective of the present study was to investigate the effect of salinity on amino acid and lipid composition of two moderately halophilic spore-forming Gram positive *Bacillus* species (BST & BSF) isolated from a salt basin in Saudi Arabia (Salamah 1988).

MATERIALS AND METHODS

ORGANISMS AND GROWTH CONDITIONS

Two moderately halophilic bacterial isolates (BST & BSF) were obtained from Al-Kaseem Natural Salt Basin, Saudi Arabia and identified as two different species of the genus *Bacillus* (Salamah 1988).

The isolates were grown in 1000 ml batches of the medium described by Sehgal & Gibbons (1960) except that the concentration of NaCl in the medium was either 6% or 18%. The growth flasks were shaken at 120 rpm for 16 h at 30°C. The cells were harvested by centrifugation, washed twice with either 6% or 18% NaCl in distilled water and stored at −20°C until use.

ISOLATION OF CELL ENVELOPES

Frozen cells (wet weight between 3 and 4 g) were resuspended in 20 ml of 10 mM Tris-hydrochloride buffer (pH 8·0) containing 1 mM DL-dithiothreitol, 1 mM EDTA, and 6 or 18% NaCl. Pancreatic RNase and DNase were added to the suspension which was then passed three times through a French pressure cell at 15,000 to 20,000 lb/in^2. This and all subsequent steps were carried out at 4°C. After centrifugation of the lysate at 1300 × *g* for 10 min, the pellet of unbroken cells was discarded. The supernatant was centrifuged at 45,000 × *g* for 30 min, and the resulting supernatant was discarded and the pellet was washed with 5 ml of the breakage buffer. The purity of the envelope preparation was asserted and its protein concentration was determined by the method of Lowery *et al.* (1951) with the modification of Herbert *et al.* (1971) and stored at −20°C (Salamah & Charnetzky 1986).

AMINO ACID ANALYSIS OF THE CELL ENVELOPES

For amino acid determination, envelopes were hydrolysed under nitrogen in 6 N HCl for 24 h at 110°C as recommended by Kushner & Onishi (1966). The samples were filtered, neutralized by 50% NaOH and diluted 5 times with loading buffer pH 2.2. A volume of 40 ml of each diluted sample was injected into an LKB-Pharmacia amino acid analyser. The salt arising from neutralization did not interfere with this analysis.

DETERMINATION OF GLUCOSAMINE AND MURAMIC ACID

Portions (20–25 mg) of the cell wall samples were hydrolysed, filtered and neutralized as described above. The filtrates were then evaporated to dryness over a boiling

water bath and finally resuspended in 1 ml of water. The glucosamine and muramic acid contents of the above samples were determined as described by Stewart-Tull (1968) using known concentrations of glucosamine and muramic acid (Fluka) as standards.

DETERMINATION OF THE IONIC CONTENT

The cells were grown in the presence of two different salt concentrations as described above. Each sample was pelleted by centrifugation in a 5 ml polycarbonate centrifuge tube. The minerals were then extracted with 10% (wt/vol) hot trichloroacetic acid as described by Shindler *et al.* (1977). A Perkin-Elmer 3030 atomic absorption spectrophotometer was used for the emission analysis of Na^+, K^+ and Mg^{2+}.

EXTRACTION AND PURIFICATION OF LIPIDS

The cells were harvested and washed with 6 or 18% NaCl. The lipids were extracted with chloroform-methanol (2 : 1, v/v) and purified by passing through a Sephadex G-25 column as described by Hanson & Phillips (1981).

SEPARATION OF PHOSPHOLIPIDS

The phospholipids were separated from other lipid classes by dissolving 150 to 200 mg of the purified total lipids and passing them through an acid-treated Florisil column to obtain the fraction of phospholipids as described by Hanson & Phillips (1981).

THIN-LAYER CHROMATOGRAPHY OF PHOSPHOLIPIDS

The phospholipids were separated and identified by thin-layer chromatography using Winlab Silica Gel 60 F254 plates (0·25 mm thick; fluorescent). A unidimensional system was developed with chloroform : methanol : water (70 : 25 : 4, v/v). For two-dimensional separation, the first solvent was chloroform : methanol : 7 M ammonia (65 : 30 : 4, v/v) and the second consisted of chloroform : acetic acid : methanol : water (170 : 25 : 25 : 6, v/v). After development, the phospholipid spots were located by the exposure of the plates to ultra violet light and by spraying with molybdenum, ninhydrin, Dragendorff's and periodate-Schiff's reagents. The phospholipid spots were then identified by comparing their mobilities with known phospholipid standards (Winlab). The above studies were repeated three times using three different envelope preparations.

RESULTS

AMINO ACID ANALYSIS OF THE CELL ENVELOPE

The results of the amino acid analysis of the cell envelopes are shown in Table 1. The amounts of amino acids at 6% NaCl were different from those at 18% NaCl. Cystine and proline were present in the cell envelope of the 18%-grown but not in

Table 1. Amino acid content of the envelopes of the isolates BSF and BST (μmol per mg protein) grown in the presence of 6% or 18% NaCl

Amino acid	*Bacillus* BSF		*Bacillus* BST	
	6% NaCl	18% NaCl	6% NaCl	18% NaCl
Arginine	0·090	0·052	0·043	0·052
Histidine	0·321	0·170	0·165	0·212
Lysine	0·286	0·000	0·225	0·323
Ammonia	1·112	0·983	0·860	1·112
Phenylalanine	0·173	0·085	0·104	0·125
Tyrosine	0·142	0·060	0·087	0·118
Leucine	0·531	0·278	0·347	0·399
Isoleucine	0·712	0·730	0·304	0·292
Methionine	0·100	0·086	0·102	0·087
Cystine	0·017	0·000	0·000	0·016
Valine	0·411	0·199	0·260	0·342
Alanine	0·730	0·564	0·556	0·539
Glycine	0·292	0·159	0·156	0·208
Proline	0·009	0·000	0·000	0·204
Glutamic acid	0·697	0·405	0·478	0·512
Serine	0·213	0·139	0·121	0·173
Threonine	0·234	0·112	0·139	0·187
Aspartic acid	0·816	0·524	0·850	0·747

the 6%-grown BST cells. Lysine, cystine and proline were present in the cell envelope of the 6%-grown but not in the 18%-grown BSF cells. For both isolates, and at both NaCl concentrations, there was an excess of dicarboxylic amino acids (aspartic and glutamic acids) over basic amino acids (lysine, arginine and histidine).

DETERMINATION OF GLUCOSAMINE AND MURAMIC ACID

Both isolates had muramic acid and glucosamine in their cell walls (Table 2). Increasing the concentration of NaCl from 6 to 18% led to the decrease of muramic acid and glucosamine of isolate BSF, whereas it led to their increase in isolate BST.

EFFECT OF MEDIUM NaCl CONCENTRATION ON CELL-ASSOCIATED CATIONS

Increasing medium NaCl caused an increase of the cell-associated cations (Na^+, K^+ and Mg^{2+}) of isolate BSF (Table 3). The NaCl increase, however, caused an increase

Table 2. Hexosamine and muramic acid contents in the cell-wall preparations of isolates BST and BSF grown in the presence of 6 or 18% NaCl

Isolate	NaCl concentration (%)	Amount (μg/g cell-wall wet weight)	
		Muramic acid	Glucosamine
BSF	6	12·49	14·59
	18	7·49	7·47
BST	6	6·88	7·62
	18	13·48	21·61

Table 3. Cell-associated cations of the isolates BSF and BST grown in the presence of 6 or 18% NaCl

Isolate	NaCl concentration (%)	(Cell-associated cations (μg/g wet weight)		
		Na^+	K^+	Mg^{2+}
BSF	6	509·07	29·03	9·36
	18	598·13	33·33	41·63
BST	6	531·73	43·95	25·84
	18	474·80	92·09	6·24

of the cell-associated K^+ and a decrease of the cell-associated Na^+ and Mg^{2+} for isolate BST.

PHOSPHOLIPID ANALYSIS

The unidimensional thin-layer chromatography of phospholipids revealed the presence of three phospholipids in both isolates. These phospholipids were identified as cardiolipins, phosphatidylethanolamines and phosphatidylglycerols. Isolate BST grown in the presence of 6% NaCl contained, in addition, lysophosphatidylglycerols, whereas an unknown phospholipid designated X1 was present in the 6% NaCl-grown BSF and BST cells. As indicated by the spot densities, phosphatidylglycerols and phosphatidylethanolamines seemed to increase in the presence of higher NaCl concentrations. The two-dimensional chromatographic analysis of the cell envelope of isolate BSF grown in the presence of 6% or 18% NaCl revealed the same phospholipid classes identified by the unidimensional chromatographic analysis in addition to a phospholipid (designated X3) present only in the 18% NaCl-grown cells. The two-dimensional chromatographic analysis of the phospholipids of isolate BST grown in the presence of 6% or 18% NaCl revealed the same phospholipid classes identified by the unidimensional chromatographic analysis in addition to a phospholipid (designated X2) present in the 6% and 18% grown BST cells.

DISCUSSION

The moderate halophilic bacteria exhibit adaptation to changes in the salt concentrations in their growth media (Novitsky & Kushner 1975; Ohno *et al.* 1979; Kogut & Russell 1984; Vreeland *et al.* 1984; Miller 1985). The results presented in this paper deal with the salt adaptation of two moderately halophilic *Bacillus* isolates described earlier (Salamah 1988).

The amino acid analysis of the cell envelopes show interesting changes in response to changing the NaCl concentration. These changes are represented by the decrease or increase of most of the amino acids present in the cell envelope. It is interesting to note that cystine and proline behave conversely in the two isolates. The increase of dicarboxylic amino acids (aspartic and glutamic acids) over basic amino acids (lysine, arginine and histidine) demonstrates the acidic nature of the cell envelope of these isolates. The negative charges of the amino acids are required to neutralize the positive sodium ions. The present results confirm those recorded on

cell envelope penetrations of *Halobacterium* (Kushner & Onishi 1966; Steensland & Larsen 1969; Kushner 1978).

The amino acid lysine is present in the peptidoglycan of Gram positive bacteria. However, it was absent from the 18%-grown BSF cells. The analysis of the peptidoglycan repeating units (N-acetylglucosamine and N-acetylmuramic acid) show that the two glycans increase upon increasing the NaCl concentration for isolate BST, whereas they decrease for BSF. The decrease of the glycans in strain BSF might explain the lack of lysine in their cell envelope upon growth in 18% NaCl. The presence of peptidoglycans in these two moderately halophilic *Bacillus* isolates, requiring high NaCl concentrations for growth, is of interest because the Gram positive halophilic cocci lack it (Novitsky & Kushner 1975; Kushner 1978; Miller 1985).

High concentrations of Na^+ were noticed in the cell envelopes of the two isolates. It has been found that the Na^+ and K^+ form ionic bonds with the acidic groups of the cell envelopes (Masui & Wada 1973, Shindler *et al.* 1977; Miller 1986). The high concentrations of Na^+ and, to a lesser extent, K^+ in the cell envelopes of the present isolates were probably required to neutralize the negative charges brought about by the dicarboxylic amino acids, so that the cell rigidity may be maintained under these conditions.

The presence of phosphatidylethanolamines, phosphatidylglycerols and cardiolipins in the cells of the present *Bacillus* isolates is in agreement with earlier observations (Rilfors *et al.* 1978). The above increase of phosphatidylethanolamines and phosphatidylglycerols in response to increased NaCl concentration would increase the negative charge of the cells in order to balance the excess cationic charge (Na^+) at the membrane surface as has been found by other authors (Steensland & Larsen 1969; Ohno *et al.* 1979; Kogut & Russell 1984; Vreeland *et al.* 1984; Miller 1986).

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تأثير ملوحة البيئة على تركيب الأحماض الأمينية والفوسفوليبيدات لنوعين محبين للملوحة من جنس باسيلاّس عُزلا من المملكة العربية السعودية .

علي عبدالله السلامة
وحدة الميكروبيولوجيا
قسم النبات بكلية العلوم ، جامعة الملك سعود ،
ص . ب . ٢٤٥٥ ، الرياض ١١٤٥١ ،
المملكة العربية السعودية .

خلاصة

تختلف الأحماض الأمينية الموجودة في غلاف خلايا عزلتين (BSF; BST) محبتين للملوحة من جنس باسيلاس *Bacillus* وفقا لملوحة البيئة . وكان أكثر الأحماض الأمينية تأثرا بتركيز الصوديوم السستين (cystine) والپرولين (proline) . لقد وجد أن الأحماض ثنائية المجموعة الكربوكسيلية لكلا العزلتين المنماتين بوجود ٦٪ أو ١٨٪ كلوريد صوديوم أكثر من الأحماض الأمينية القاعدية ، مما جعل بروتينات الغلاف الخلوي حمضية التفاعل . وكانت تراكيز الكاتيونات الخلوية (الصوديوم ، الكالسيوم ، المغنيسيوم) لكلا العزلتين عالية وتختلف باختلاف تركيز كلوريد الصوديوم . وتحتوي العزلتان على جلوكوزامين وحمض ميورامك في الجدار الخلوي ، إلا أن كمية هذين المشتقين السكريين تختلف باختلاف تركيز كلوريد الصوديوم . وأثبت التحليل الكروماتوجرافي للفوسفوليپيدات وجود فوسفاتيديل جليسيرولات وفوسفاتيديل إيثانولامينات وكارديوليپينات في كلا العزلتين بصرف النظر عن ملوحة البيئة . ولقد ازداد تركيز كل من فوسفاتيديل إيثانولامينات وفوسفاتيديل جليسيرولات على أثر زيادة كلوريد الصوديوم في بيئة النمو . كما وجدت ليسوفوسفاتيديل جليسيرولات في خلايا العزلة BST المنماة في وجود ٦٪ كلوريد صوديوم ووجدت فوسفوليپيدات غير معروفة أشير إليها بالرموز X1 (في العزلتين BSF ، BST) ، X2 (في العزلة BST) ، X3 (في العزلة BSF) . ولوحظ أن تركيز X1 و X3 يعتمد على درجة الملوحة .