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Effects of Temperature and Sodium Chloride Concentration on the Phospholipid and Fatty Acid Compositions of a Halotolerant *Planococcus* sp.

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The phospholipid headgroup composition and fatty acid composition of a gram-positive halotolerant *Planococcus* sp. (strain A4a) were examined as a function of growth temperature (5 to 35°C) and NaCl content (0 to 1.5 M) of the growth medium. When the growth temperature was decreased, the relative amount of mono-unsaturated branched-chain fatty acids increased. When *Planococcus* sp. strain A4a was grown in media containing high NaCl concentrations, the relative amount of the major fatty acid, $C_{a15:0}$, increased. The relative amount of anionic phospholipid also increased when the NaCl concentration of the growth medium was increased. The increase in anionic phospholipid content resulted from a decrease in the relative mole percent content of phosphatidylethanolamine and an increase in the relative mole percent content of cardiolipin.

It has been well established that most microorganisms alter their membrane lipid composition in response to changes in temperature (see references 5, 30, 32, and 42 for reviews). Such alterations are believed to result in membranes with physical characteristics which permit proper membrane function within a particular temperature range. For example, it has been suggested that an appropriate level of membrane fluidity must be maintained by bacteria in response to temperature change (45). Further studies (20, 30), however, indicate that the measured average membrane fluidity level of the bacterial membrane may not be as important as the need for a minimal amount of fluid lipid domain within the membrane.

In addition to a mechanism for temperature-induced alteration of membrane lipid composition in bacteria, additional regulatory systems may be induced by variations of other environmental parameters. For example, growth in the presence of high concentrations of NaCl has been shown to result in the alteration of the phospholipid headgroup composition and fatty acid composition of two halophilic gram-negative bacteria (12, 24, 36, 37) and two halotolerant gram-positive staphylococci (21, 25). Such changes in bacterial lipid composition in response to increased NaCl concentrations may also result in membranes with altered physical or thermal characteristics. It has, in fact, been suggested that the NaCl-induced alterations of membrane lipid composition may be important in controlling the ionic permeability of halotolerant or halophilic bacteria (21, 25, 36, 37).

When the gram-negative halophilic bacteria *Pseudomonas halosaccharolytica* and *Vibrio costicola* are cultured in media with elevated NaCl concentrations, the phosphatidylethanolamine content has been shown to decrease whereas the phosphatidylglycerol content increases (12, 24, 36, 37). Conversely, phosphatidylglycerol content has been shown to decrease with a corresponding increase in cardiolipin content when the gram-positive halotolerant staphylococci are cultured in media containing high NaCl concentrations (21, 25). Because of the above studies, it has been suggested that the phospholipid headgroup alterations of gram-positive halotolerant bacteria differ from those of gram-negative halophilic bacteria when grown in media containing high NaCl concentrations (37). However, the markedly different phospholipid headgroup compositions of the gram-positive halotolerant staphylococci, which typically lack detectable levels of phosphatidylethanolamine, and the gram-negative halophilic bacteria (9, 29) make such a comparison difficult.

In this study, the effects of growth temperature and NaCl concentration on the phospholipid and fatty acid compositions of a gram-positive halotolerant *Planococcus* sp. (strain A4a) are described. The halotolerant and psychrotolerant nature of *Planococcus* sp. strain A4a permitted analyses over wide ranges of both temperature and NaCl concentrations. In addition, unlike the gram-positive halotolerant staphylococci of previous studies (21, 25), *Planococcus* sp. strain A4a was found to contain significant levels of phosphatidylethanolamine. When *Planococcus* sp. strain A4a was grown in media supplemented with high concentrations of NaCl, a significant decrease in phosphatidylethanolamine content and an increase in cardiolipin content were detected. The increase in cardiolipin content was similar to that previously reported for the gram-positive halotolerant staphylococci (21, 25); however, the decrease in phosphatidylethanolamine content represents a phospholipid alteration reported previously only for gram-negative halophilic bacteria (12, 24, 36, 37).

MATERIALS AND METHODS

Organism and culture conditions. The bacterium used in this study was *Planococcus* sp. strain A4a (ATCC 35671), a halotolerant psychrotolerant bacterium isolated from saline Antarctic Dry Valley soil (33). Cells were grown in 6.4 liters of 5TYE-Na media (33) containing different concentrations of added NaCl (0.0, 1.0, or 1.5 M) at 5, 20, or 35°C. The inoculum (5%, vol/vol) consisted of cells grown for 36 h at room temperature in 5TYE-Na media of the same NaCl concentration. Cultures were incubated on a gyratory shaker within a Psychrotherm Incubator (New Brunswick Scientific

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Co., Inc., Edison, N.J.). Growth was monitored turbidometrically at 660 nm with a Klett-Summerson colorimeter.

Preparation of lipid extracts. Total lipids were extracted by the technique of Bligh and Dyer (4) as modified by Kates (22), except that protoplasts of *Planococcus* sp. strain A4a were prepared before extraction. Cell densities ranged from 1 to 3 × 10^8 cells per ml at the time of harvest. Cells were harvested at the early-logarithmic, mid-logarithmic, and stationary phases of growth by centrifugation at 5,200 × *g*. Cells were washed once with 10 mM potassium phosphate buffer (pH 7.5) and resuspended in 10 mM potassium phosphate buffer (pH 7.5) containing 15 mM $MgCl_2$ and 0.4 M sucrose. The cell concentration was approximately 0.3 g (wet weight) per ml. Lysozyme (Grade I from egg white; Sigma Chemical Co., St. Louis, Mo.) was added to give a final concentration of approximately 300 μg/ml. The suspension was incubated at 37°C for 30 min. Protoplast formation was evaluated by phase-contrast microscopy. Protoplast suspensions were extracted for total lipid in the dark with butylated hydroxytoluene (0.1%, wt/vol) included in the extraction solvents as an antioxidant. Total lipid extracts were concentrated to dryness under nitrogen and suspended in solutions of chloroform-methanol (2:1) containing butylated hydroxytoluene (0.05%, wt/vol) at a final concentration of approximately 10 to 20 mg of lipid per ml. Lipid extracts were stored under nitrogen at −20°C until use.

Phospholipid identification. Phospholipids were separated by thin-layer chromatography. Glass plates coated with 0.25-mm-thick Silica Gel H (Supelco Inc., Bellefonte, Pa.) were heat activated at 110 to 120°C for 1 to 2 h immediately before use. Plates were cooled to room temperature within a desiccator cabinet, and total lipid extract was applied to the plates under a stream of nitrogen. Plates were developed in chambers presaturated for 30 min with solvent. Several one-dimensional solvent systems were employed: A, chloroform–methanol–water (65:25:4); B, diisobutyl ketone–glacial acetic acid–water (40:25:5); C, chloroform–acetone–methanol–glacial acetic acid–water (6:8:2:2:1); D, chloroform–methanol–ammonia (28 to 30%) (65:25:5); E, chloroform–methanol–2-propanol–KCl (0.25%)–triethylamine (30:9:25:6:18). The following detection reagents were used: molybdenum blue reagent (46), periodate-Schiff reagent (22), α-naphthol reagent (22), Dragendorfrff reagent (22), iodine vapor (22), and ninhydrin reagent. Ninhydrin reagent was prepared by combining 10 ml of 1% (wt/vol) $CdCl_2$, 5 ml of glacial acetic acid, and 100 ml of 0.3% (wt/vol) ninhydrin in acetone. Thin-layer plates were sprayed with a fine mist of ninhydrin reagent and heated to 100°C for approximately 5 to 10 min. Phospholipid identification was based on the relative mobilities of the various components in each solvent system as compared with phospholipid standards as well as by the reactivity with specific detection reagents. Phospholipid identification was further confirmed by mild alkaline deacylation of phospholipids followed by paper chromatography as described by Kates (22). Water-soluble products of mild alkaline deacylation were examined by paper chromatographic analysis on Whatman no. 1 paper. Isopropanol–water–ammonia (28 to 30%) (7:2:1) was used as the solvent system. Phospholipid standards were subjected to the same mild alkaline deacylation procedure and paper chromatographic separation. Paper chromatograms were sprayed with ninhydrin reagent or dipped in the phosphate detection reagent, salicylsulfonic acid–ferric chloride (22), to reveal deacylation products.

Quantitative analysis of phospholipids. The relative amounts (mole percent) of the phospholipid components of *Planococcus* sp. strain A4a were determined by phosphorus analysis. A modification of the Bartlett phosphorus determination (2) was performed as described below.

Separation of phospholipid components was achieved by two-dimensional thin-layer chromatography. The first dimensional separation was performed by using solvent system A. After first-dimensional separation, plates were placed in a desiccator cabinet for 30 to 45 min under a nitrogen atmosphere. Plates were then turned 90° and developed in the second dimension by using solvent system C. After development of the second solvent system, the plates were allowed to dry overnight at room temperature.

Phospholipids were revealed by spraying thin-layer chromatography plates with a fine mist of cupric acetate (3%, wt/vol) in sulfuric acid (8%, wt/vol). Plates were sprayed until translucent and were then heated to approximately 170°C for 30 min. Phospholipids appeared dark brown on a white background.

Each phospholipid spot (50 to 400 nmol of phosphorus) was scraped into a test tube with a Teflon-lined screw cap, and a modification of the Bartlett phosphorus analysis (2) was performed. A slurry was prepared by the addition of 0.5 ml of water and two drops of H_2O_2 (30%, wt/vol). Tubes were heated to 95°C on a heating block, and the contents were mixed intermittently until the solution cleared and the silica gel turned white (approximately 2 h). The tubes were allowed to cool to room temperature and 3.45 ml of ammonium molybdate reagent (ammonium molybdate [0.22%, wt/vol] in sulfuric acid [5.2%, wt/vol]) was added followed by the addition of 0.15 ml of Fiske and Subbarow reagent (sodium bisulfite, anhydrous [15%, wt/vol]; 1-amino-2-naphthol-4-sulfonic acid [0.25%, wt/vol]; sodium sulfite, anhydrous [0.5%, wt/vol]). Fiske and Subbarow reagent was prepared fresh daily and filtered before use. The tubes were mixed by vortexing and placed into a boiling water bath for 10 min. After cooling, the contents of the tubes were transferred to 12-ml conical centrifuge tubes and centrifuged at 450 × *g* for 5 to 10 min with a clinical tabletop centrifuge (International Equipment Co., Needham Heights, Mass.). Supernatants were removed and absorbance was measured at 830 nm with a Bausch & Lomb Spectronic 21 spectrophotometer.

A standard curve was constructed by using 0 to 500 nmol of KH_2PO_4 in the presence of Silica Gel H thin-layer chromatography plate scrapings. Control plate scrapings were treated in an identical manner to those containing phospholipid phosphorus. The limit of phosphorus detection by this method was approximately 20 nmol. All glassware used for phosphorus determinations were cleaned with sulfuric acid–dichromate solution (sodium dichromate [4.6%, wt/vol] in H_2SO_4 [11 M]) and rinsed several times with distilled water.

Fatty acid methyl ester analysis. Fatty acid methyl esters were prepared from total lipid extracts by the method of Morrison and Smith (35); however, boron trichloride–methanol reagent (Supelco) was used instead of boron trifluoride–methanol. Methyl esters were extracted with pentane and examined by gas-liquid chromatography and mass spectrometry.

Fatty acid methyl esters were identified by gas-liquid chromatography before and after mild hydrogenation by the method of Appelqvist (1) and by gas-liquid chromatography linked to mass spectrometry. Gas-liquid chromatographic analysis was performed by using a Varian 4600 gas chromatograph with an accompanying Vista 401 data station. Fatty acid methyl ester separation was performed by using

TABLE 1. Identification of the four phospholipids of *Planococcus* sp. strain A4a[a]

Phospholipid	Indicator reagent[b]						Deacylated product
	I	N	D	A	P	M	
Cardiolipin	+	−	−	−	−	+	bis(Glycerophosphoryl)glycerol
Phosphatidylglycerol	+	−	−	−	+	+	Glycerylphosphorylglycerol
Phosphatidylethanolamine	+	+	−	−	−	+	Glycerylphosphorylethanolamine
Unidentified (*X*)	+	−	−	−	−	+	Not determined

[a] Identification was based on reaction with indicator reagents: +, positive reaction; −, negative reaction.
[b] Indicator reagents: I, iodine vapor; N, ninhydrin; D, Dragendorff reagent; A, α-naphthol reagent; P, periodate-Schiff reagent; M, molybdenum blue reagent.

two column systems. A stainless steel column (6 ft by 0.125 in. [ca. 182.88 by 0.32 cm]) packed with SP2100 (3%) on 100/120 Supelcoport (Supelco) was heated from 150 to 225°C at 4°C per min. The injector port was maintained at 200°C, and the flame ionization detector was maintained at 250°C. Helium carrier gas was used at 30 ml/min. The second column used was a stainless steel column as before, packed with ethylene glycol adipate (10%) on 80/100 Chromasorb WAW (Supelco). This column was operated isothermally at 150°C with a helium flow rate of 30 ml/min. The injector port and detector temperatures were as indicated above. Known amounts of standard methyl esters were used to quantify esters of fatty acids in the range of carbon numbers 12 through 18.

Gas-liquid chromatography linked to mass spectrometry was performed with a Hewlett Packard 5840A gas chromatograph linked to a Hewlett Packard 5985 GC/MS (ion source: 200°C, 70 eV). Separation of methyl esters was achieved by using a SE-54 glass capillary column (30 m by 0.2 mm). The column was heated from 125 to 300°C at 10°C per min. Identification was accomplished by the analysis of mass spectra of each of the methyl esters according to the interpretive guidelines described by Ryhage and Stenhagen (43) and Tornabene et al. (49). Retention time for each methyl ester was compared with methyl ester standards.

Lipid dry weight determination. The percentage of total cellular dry weight which was lipid was determined for cells grown to the mid-logarithmic stage of growth in 5TYE-Na medium containing either 0.0 or 1.5 M NaCl at 5, 20, or 35°C. Cells were harvested as described above but were washed twice in 10 mM potassium phosphate buffer (pH 7.5). The harvested cells were divided into four portions. Two portions were used for the determination of total cellular dry weight. These cell pellets were suspended in 5 ml of 10 mM potassium phosphate buffer and dried to constant weight at 70°C in a vacuum oven (Forma Scientific Co., Marietta, Ohio). Dry weight determinations for buffer alone were also performed, and these values were subtracted from those determined for the total cellular suspensions. The remaining cells were extracted for lipid as described above; however, butylated hydroxytoluene was not added to the extracting solvents. Also, during extraction the final methanol-water phase was extracted one additional time with chloroform. The chloroform extracts were combined and dried to constant weight under nitrogen.

The percentage of the dry weight of total lipid which was phospholipid was determined. Portions of total lipid extract were separated by two-dimensional thin-layer chromatography as described above. Phospholipids were visualized and analyzed for phosphorus. The dry weight of phospholipid was estimated by calculating the molecular weights of the three major phospholipids. This calculation was based on headgroup structure and the average fatty acid molecular weight (as determined by gas chromatography). The unidentified phospholipid, designated X, was assigned a molecular weight of 700 for these calculations. It was assumed that phospholipid X possessed a molecular weight similar to that of phosphatidylglycerol or phosphatidylethanolamine and contained 1 mol of phosphorus per mol of phospholipid.

Chemicals. All chemicals used were of reagent grade. Cardiolipin, phosphatidylglycerol, and phosphatidylethanolamine were purchased from Sigma Chemical Co. Fatty acid methyl ester standards were purchased from Supelco Inc., Sigma Chemical Co., and Applied Science Inc., State College, Pa.) All organic solvents were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis., and were of spectrophotometric grade and "Gold Label" quality.

RESULTS

Phospholipid composition. Thin-layer chromatography revealed the presence of four phospholipids. Three phospholipids were identified as cardiolipin, phosphatidylglycerol, and phosphatidylethanolamine (Table 1). The fourth phospholipid, designated X, was a minor component (less than 10 mol%) and was not identified.

Fatty acid composition. Fifteen fatty acid methyl ester components were detected by gas-liquid chromatography. The methyl esters were of primarily anteiso and iso branched-

TABLE 2. Fatty acid composition of *Planococcus* sp. strain A4a as a function of NaCl concentration and temperature at the early-logarithmic stage of growth[a]

Temp (°C)	NaCl (M)	Fatty acid (%)					
		$C_{a15:0}$	$C_{i16:1}$	$C_{i16:0}$	$C_{i+a17:1}$	$C_{i+a17:0}$	Other[b]
5	0.0	45.4	21.6	3.3	18.3	4.5	6.9
5	1.0	63.0	9.4	2.6	14.1	3.1	7.8
5	1.5	61.0	8.5	3.1	19.0	1.9	6.5
20	0.0	45.1	20.8	3.4	15.6	6.4	8.7
20	1.0	56.4	12.8	2.8	17.6	4.2	6.2
20	1.5	64.6	7.8	3.0	14.0	2.7	7.9
35	0.0	45.8	14.7	8.6	8.6	12.7	9.6
35	1.0	55.8	9.6	5.7	8.3	9.8	10.8
35	1.5	68.7	2.2	6.5	2.2	7.5	12.9

[a] Fatty acids are abbreviated such that the number of carbon atoms precedes the colon and the number of double bonds follows the colon. The symbols "a" and "i" represent the anteiso-branched structure and the iso-branched structure, respectively. Cells were grown at 5, 20, or 35°C in 5TYE-Na medium containing 0.0, 1.0, or 1.5 M NaCl. The fatty acid methyl ester content is expressed as the relative weight percent of the total.
[b] Values represent the combined relative weight percent of the following fatty acids: $C_{a13:0}$, $C_{i14:0}$, $C_{14:0}$, $C_{i15:0}$, $C_{15:0}$, $C_{16:0}$, $C_{17:0}$, $C_{18:1}$, and $C_{18:0}$. These fatty acids were each present at concentrations below 2.2% except that the relative weight percent content of $C_{i14:0}$ and $C_{i15:0}$ ranged from 2.2 to 4.5% and 0.7 to 3.6%, respectively.

TABLE 3. Fatty acid composition of *Planococcus* sp. strain A4a as a function of NaCl concentration and temperature at the mid-logarithmic stage of growth[a]

Temp (°C)	NaCl (M)	Fatty acid (%)					
		$C_{a15:0}$	$C_{i16:1}$	$C_{i16:0}$	$C_{i+a17:1}$	$C_{i+a17:0}$	Other[b]
5	0.0	46.9	22.0	1.6	19.9	3.0	6.6
5	1.0	62.0	11.5	1.8	15.6	2.5	6.6
5	1.5	64.0	8.6	2.2	18.1	1.2	5.9
20	0.0	46.3	19.8	4.4	14.5	6.0	9.0
20	1.0	53.4	15.0	2.8	17.4	3.1	8.3
20	1.5	64.5	8.3	2.9	13.6	2.1	8.6
35	0.0	46.3	15.5	8.3	9.0	10.4	10.5
35	1.0	53.0	11.6	5.8	8.5	6.9	14.2
35	1.5	67.3	2.5	7.1	1.8	7.2	14.1

[a] Same as in footnote *a*, Table 2.

[b] Values represent the combined relative weight percent of the following fatty acids: $C_{a13:0}$, $C_{i14:0}$, $C_{14:0}$, $C_{i15:0}$, $C_{15:0}$, $C_{16:0}$, $C_{17:0}$, $C_{18:1}$, and $C_{18:0}$. These fatty acids were each present at concentrations below 2.2% except that the relative weight percent content of $C_{i14:0}$ and $C_{i15:0}$ ranged from 2.4 to 5.6% and 1.1 to 5.4%, respectively.

chain acids which comprised up to 99% by weight of the total fatty acid composition (Tables 2 to 4). Gas-liquid chromatography on ethylene glycol adipate resolved $C_{a15:0}$ and $C_{i15:0}$ fatty acid methyl esters. The four fatty acid methyl ester species, $C_{a17:0}$, $C_{i17:0}$, $C_{a17:1}$, and $C_{i17:1}$, were not sufficiently resolved on this column for quantitation; however, mass spectrometry, gas chromatography, and hydrogenation procedures showed all four fatty acid methyl esters to be present. The relative weight percentages of $C_{a17:0}$ and $C_{i17:0}$ fatty acids (and the mono-unsaturated branched-chain acids of carbon number equal to 17) were combined and the data are presented as $C_{i+a17:0}$ (and $C_{i+a17:1}$).

The major fatty acid component was identified by gas-liquid chromatography and mass spectrometry as $C_{a15:0}$. Two major mono-unsaturated species were also detected by gas-liquid chromatography. Mass spectrometry indicated that these components contained one double bond and corresponded in molecular weight to $C_{16:1}$ and $C_{17:1}$. The presence of one double bond in each component was confirmed by mild hydrogenation reactivity. The fully saturated products of hydrogenation comigrated with the methyl esters of $C_{i16:0}$ and $C_{i+a17:0}$ and not with the corresponding straight-chain methyl esters. Therefore, these fatty acid components each possessed one methyl branch as well as one double bond per acyl chain.

Effects of growth stage on the relative phospholipid composition. The relative mole percent content of phosphatidylethanolamine remained essentially unchanged as a function of growth stage (Fig. 1A). The relative mole percent content of cardiolipin, however, increased at the transition from the mid-logarithmic to the stationary phase of growth at 20 and 35°C (Fig. 1B). The relative mole percent content of phosphatidylglycerol decreased when the cells entered the stationary phase of growth at 20 and 35°C (Fig. 1C). The relative mole percent content of phospholipid X remained below 10 mol% for all of the growth conditions examined (Fig. 1D).

Effects of growth temperature on the relative phospholipid composition. The relative mole percent levels of cardiolipin and phosphatidylethanolamine were significantly affected by growth temperature (Fig. 1A and B). For example, phosphatidylethanolamine was present at high levels in cells grown at 35°C (>40 mol% in cells grown in media not supplemented with NaCl). The relative mole percent levels of phosphatidylethanolamine at 5 and 20°C were lower than those detected at 35°C; this was observed for every growth stage and NaCl concentration examined (Fig. 1A).

While the relative phosphatidylethanolamine content of *Planococcus* sp. strain A4a was reduced at the lower growth temperatures, the relative mole percent content of cardiolipin was correspondingly increased. This was most clearly evident at the early-logarithmic phase of growth (Fig. 1B). Phosphatidylglycerol levels did not display temperature-dependent alterations (Fig. 1C).

Effects of NaCl concentration on the relative phospholipid composition. Increasing the NaCl concentration resulted in significant changes in the relative amounts of phosphatidylethanolamine and cardiolipin (Fig. 1A and B). The relative mole percent content of phosphatidylethanolamine decreased significantly as a function of increased NaCl concentration in the growth medium (Fig. 1A). This effect was observed at all three growth temperatures examined and at all three stages of growth. The decrease in the relative mole percent content of phosphatidylethanolamine was greatest at 35°C; levels declined from 37.2 to 43.4 mol% of the total phospholipid of cells grown in a medium containing no added NaCl to 9.8 to 10.0 mol% of the total phospholipid of cells grown in a medium containing 1.5 M NaCl (Fig. 1A). The relative decrease in phosphatidylethanolamine content detected with increased NaCl concentration was balanced by an increase in the mole percent content of cardiolipin. For example, at 35°C the relative amount of cardiolipin increased from 9.4 to 22.5 mol% of the total phospholipid of cells grown in a medium containing no added NaCl to 32.7 to 51.5 mol% of the total phospholipid of cells grown in a medium containing 1.5 M NaCl (Fig. 1B).

Fatty acid methyl ester analysis. The predominant fatty acid at all conditions of growth was $C_{a15:0}$ (Tables 2 to 4). The relative amount of this fatty acid was affected by the NaCl concentration of the growth medium. The relative amounts of the two mono-unsaturated branched-chain fatty acids $C_{i16:1}$ and $C_{i+a17:1}$ varied as a function of both temperature and NaCl concentration as described below.

Fatty acid composition as a function of growth stage. The fatty acid composition of *Planococcus* sp. strain A4a did not vary significantly with growth stage (Tables 2 to 4).

Effects of growth temperature on the relative fatty acid composition. When the temperature of growth of *Planococcus* sp. strain A4a was increased, the relative weight percent

TABLE 4. Fatty acid composition of *Planococcus* sp. strain A4a as a function of NaCl concentration and temperature at the stationary stage of growth[a]

Temp (°C)	NaCl (M)	Fatty acid (%)					
		$C_{a15:0}$	$C_{i16:1}$	$C_{i16:0}$	$C_{i+a17:1}$	$C_{i+a17:0}$	Other[b]
5	0.0	43.6	21.2	4.3	14.8	3.3	12.8
5	1.0	60.6	12.9	2.3	13.7	1.9	8.6
5	1.5	63.3	9.9	1.7	13.4	1.6	10.1
20	0.0	43.5	18.3	6.6	13.5	6.3	11.8
20	1.0	49.1	14.9	3.3	16.4	2.9	13.4
20	1.5	64.0	9.3	2.1	12.0	1.0	11.6
35	0.0	43.7	12.9	11.2	7.1	10.3	14.8
35	1.0	55.6	11.1	5.1	8.6	6.6	13.0
35	1.5	66.8	2.1	7.9	1.6	7.1	14.5

[a] Same as in footnote *a*, Table 2.

[b] Values represent the combined relative weight percent of the following fatty acids: $C_{a13:0}$, $C_{i14:0}$, $C_{14:0}$, $C_{i15:0}$, $C_{15:0}$, $C_{16:0}$, $C_{17:0}$, $C_{18:1}$, and $C_{18:0}$. These fatty acids were each present at concentrations below 1.8% except that the relative weight percent content of $C_{i14:0}$ and $C_{i15:0}$ ranged from 5.3 to 8.0% and 0.5 to 5.0%, respectively.

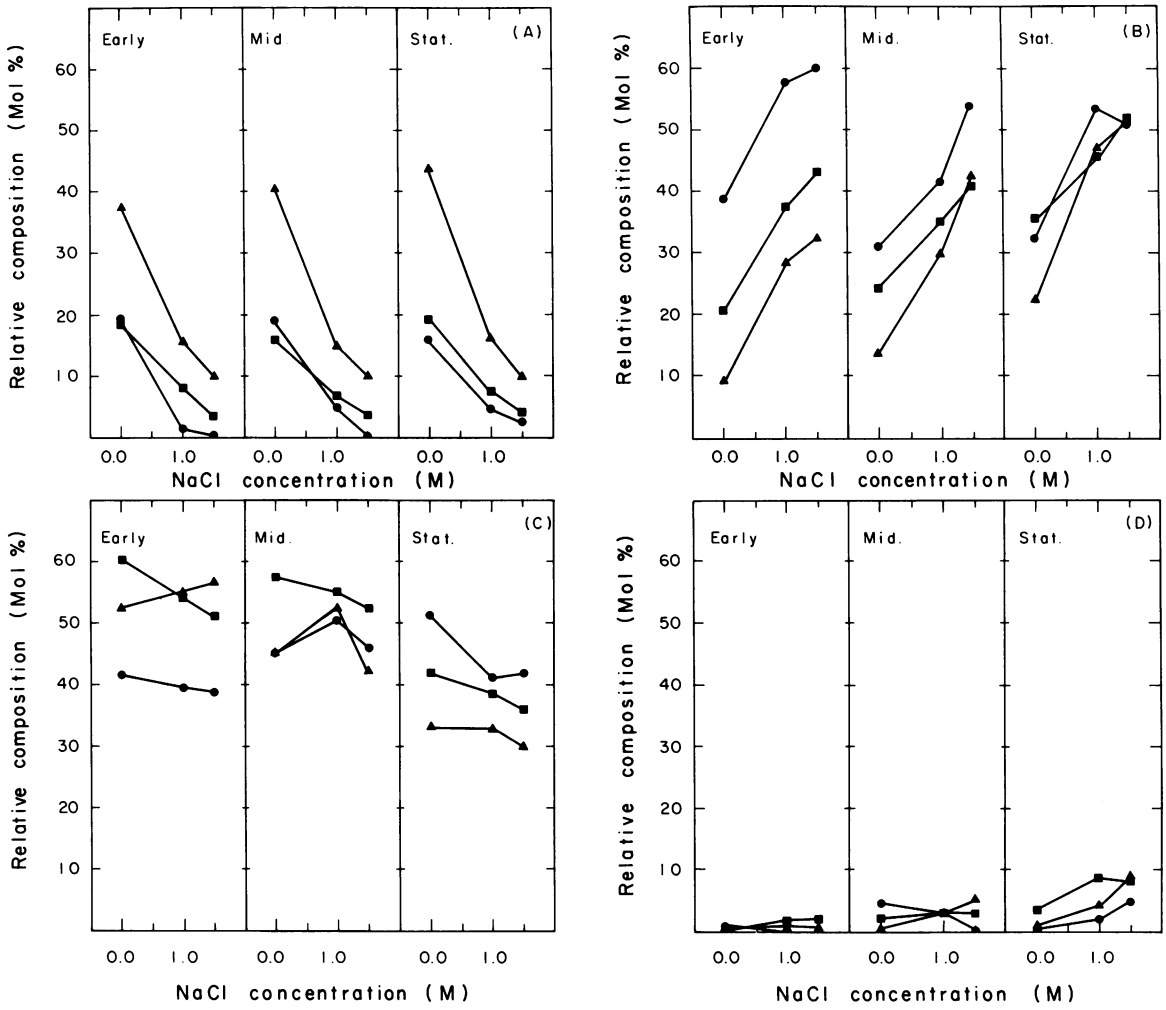

FIG. 1. Relative mole percent composition of the four phospholipids of *Planococcus* sp. strain A4a as a function of NaCl concentration, growth temperature, and stage of growth. A, phosphatidylethanolamine; B, cardiolipin; C, phosphatidylglycerol; D, unidentified phospholipid (X). Cells were harvested at the early-logarithmic stage of growth (Early), the mid-logarithmic stage of growth (Mid.), and the stationary stage of growth (Stat.). Symbols: ▲, 35°C; ■, 20°C; ●, 5°C.

content of the mono-unsaturated fatty acids decreased. Both $C_{i16:1}$ and $C_{i+a17:1}$ levels decreased with a corresponding increase in the levels of $C_{i16:0}$ and $C_{i+a17:0}$ (Tables 2 to 4 and Fig. 2). However, the relative weight percent content of the major fatty acid, $C_{a15:0}$, appeared to be unaffected by temperature (Tables 2 to 4 and Fig. 2).

Effects of NaCl concentration on the fatty acid composition. The fatty acid composition of *Planococcus* sp. strain A4a was altered when cells were grown in media containing different concentrations of NaCl (Tables 2 to 4 and Fig. 2). The relative weight percent content of $C_{a15:0}$ increased as the NaCl concentration of the growth medium was increased. For example, the relative amount of $C_{a15:0}$ increased from 43.5 to 46.9% (wt/wt) in cells grown in a medium containing no added NaCl at 5, 20, and 35°C to 61.0 to 68.7% (wt/wt) in cells grown in a medium containing 1.5 M NaCl at 5, 20, and 35°C. This relative increase in $C_{a15:0}$ content resulted in a general decrease in the relative levels of the other major fatty acid components (e.g., $C_{i16:1}$, $C_{i16:0}$, $C_{i+a17:1}$, and $C_{i+a17:0}$) as shown in Tables 2 to 4 and Fig. 2.

Effects of growth conditions on the total lipid content. The total lipid content of *Planococcus* sp. strain A4a ranged between 4.5 and 8.2% (wt/wt) of the cellular dry weight (Table 5). The phospholipid content ranged between 62.4 and 88.3% (wt/wt) of the total lipid and was found to increase with increased growth temperature and decrease with increased NaCl concentration (Table 5).

DISCUSSION

In this study it has been shown that the phospholipid and fatty acid composition of *Planococcus* sp. strain A4a were significantly affected by the stage of growth, temperature of incubation, and NaCl content of the growth medium.

The predominant alteration of phospholipid composition detected as a function of growth stage was an increase in the relative mole percent content of cardiolipin at the transition from mid-logarithmic phase to stationary phase at growth temperatures of 20 and 35°C (Fig. 1B). A similar phospholipid alteration has been detected in other gram-positive and gram-negative bacteria (5, 6, 39, 44).

The predominant alteration in fatty acid composition detected in response to decreased growth temperature was an increase in the relative weight percent of mono-unsaturated branched-chain fatty acids (Tables 2 to 4 and Fig. 2).

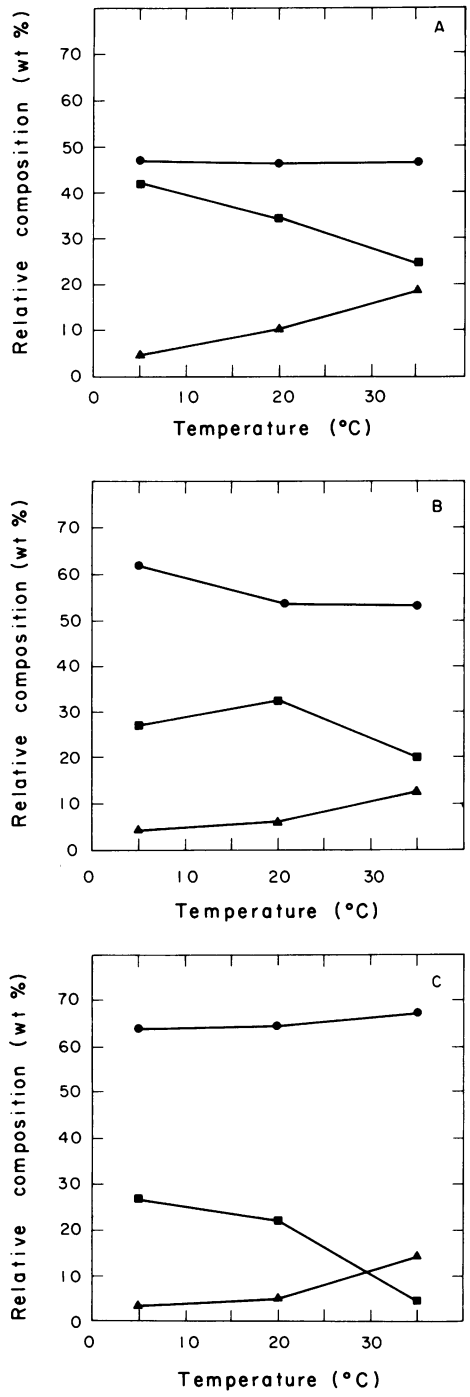

FIG. 2. The relative weight percent composition of the major fatty acid components of *Planococcus* sp. strain A4a at the mid-logarithmic stage of growth as a function of temperature. A, cells grown in 5TYE-Na medium containing no added NaCl; B, cells grown in 5TYE-Na medium containing 1.0 M NaCl; C, cells grown in 5TYE-Na medium containing 1.5 M NaCl. Symbols: ●, $C_{a15:0}$; ■, $C_{i16:1}$ and $C_{i+a17:1}$; ▲, $C_{i16:0}$ and $C_{i+a17:0}$.

This increase may be indicative of a temperature-regulated mechanism that modifies membrane thermal properties (5, 30, 32, 42).

The phospholipid headgroup composition of *Planococcus* sp. strain A4a was also altered when the growth temperature was varied. An increase in the relative mole percent content of phosphatidylethanolamine was detected at increased growth temperatures with a corresponding decrease in the relative mole percent content of cardiolipin (Fig. 1A and B). Similar trends in phospholipid headgroup composition as a function of growth temperature have been detected in *Bacillus caldotenax* (13) and *Bacillus megaterium* (41). Phosphatidylethanolamine content has been reported to decrease, however, within a thermophilic bacillus (47) and within a strain of *B. megaterium* (7) when the growth temperature was increased. It appears from these studies that alterations of phospholipid headgroup composition in response to temperature change may differ markedly in character among bacterial strains. It is therefore difficult to describe the effects of such alterations in general terms.

When *Planococcus* sp. strain A4a was grown in media containing increased concentrations of NaCl, the relative weight percent content of the major fatty acid, $C_{a15:0}$, increased (Tables 2 to 4 and Fig. 2). An increase in the relative level of $C_{a15:0}$ in response to increased NaCl concentration has been noted in other gram-positive halotolerant bacteria such as *Staphylococcus epidermidis* (25) and *Staphylococcus aureus* (21).

The phospholipid headgroup composition of *Planococcus* sp. strain A4a was also significantly altered when cells were grown in media containing different concentrations of NaCl. The relative amount of anionic phospholipid increased when the NaCl concentration of the growth medium was increased (Fig. 1). This increase was due to a decrease in the relative mole percent content of phosphatidylethanolamine and an increase in the relative mole percent content of cardiolipin. In a previous study of the effect of sea salt concentration on the lipid composition of *Planococcus citreus*, an increase in lysocardiolipin content was detected at elevated sea salt concentrations (48). The results of this previous study are difficult to interpret, however, since it is not clear whether the lysocardiolipin was a natural membrane component of *P. citreus* or a breakdown product of cardiolipin produced during lipid extraction procedures.

An increase in the relative amount of anionic phospholipid (or the negative charge per mole of phospholipid) in response to increased NaCl concentrations has been detected in both gram-positive halotolerant bacteria (21, 25) and gram-negative halophilic bacteria (12, 24, 36, 37). In the halotolerant staphylococci, the increase in anionic charge per mole of phospholipid results from an increase in cardiolipin content (21, 25). Unlike the gram-positive planococci, the halotolerant staphylococci examined in these previous

TABLE 5. Total lipid composition of *Planococcus* sp. strain A4a as a function of temperature and NaCl concentration[a]

Temp (°C)	NaCl (M)	Total lipid[b]	Phospholipid[c]
5	0.0	6.71 ± 0.15	80.0 ± 0.38
5	1.5	7.56 ± 0.24	62.4 ± 3.42
20	0.0	5.67 ± 0.19	83.1 ± 1.53
20	1.5	5.25 ± 0.09	64.4 ± 4.40
35	0.0	8.15 ± 0.14	88.3 ± 0.16
35	1.5	4.49 ± 0.09	77.1 ± 5.00

[a] Cells were grown in 5TYE-Na medium containing either no added NaCl or 1.5 M NaCl at 5, 20, or 35°C.

[b] The total lipid dry weight percentage of cellular dry weight was determined in duplicate. Results represent the average, and ± indicates the range.

[c] The weight percent of total lipid content which was phospholipid was determined by phosphorus analysis as described in the text. The analysis was performed in duplicate. Results represent the average, and ± indicates the range.

studies (21, 25) did not contain detectable levels of phosphatidylethanolamine. Phosphatidylethanolamine, however, is present in significant levels within gram-negative halophilic bacteria such as *Pseudomonas halosaccharolytica* and *Vibrio costicola* (12, 24, 36, 37). The increase in relative anionic phospholipid content in response to increased NaCl concentrations in these two gram-negative halophiles results from a decrease in phosphatidylethanolamine content and an increase in phosphatidylglycerol content (12, 24, 36, 37). It is of interest that the decrease in phosphatidylethanolamine content detected in *Planococcus* sp. strain A4a was similar to results previously reported for gram-negative halophilic bacteria, whereas the increase in cardiolipin content detected was similar to the phospholipid headgroup alteration that is characteristic of the gram-positive halotolerant staphylococci.

An increase in relative anionic phospholipid content may represent a general mechanism of adaptation to high concentrations of NaCl by both gram-positive halotolerant bacteria and gram-negative halophilic bacteria. It has been suggested that the increased relative mole percent levels of anionic phospholipid may modify the ionic permeability of the halophilic or halotolerant bacterial membrane (21, 25, 36, 37). Experimental evidence to support this is found in studies conducted with model liposome systems and bacterial cells (11, 17, 38).

The alteration of phospholipid headgroup composition may also provide a means of modifying the cation composition at the membrane environment of the halotolerant or halophilic bacterium in the presence of high concentrations of NaCl. For example, the binding affinity of anionic phospholipid for divalent cations is approximately 1,000 times greater than that for monovalent ions such as Na^+ or K^+ (14, 15). It is therefore possible that an increase in the relative amount of anionic phospholipid may provide a means of maintaining an appropriate level of divalent cations at the membrane surface under conditions of high NaCl concentrations. In this context, it should be noted that the phospholipids of extremely halophilic bacteria are unusually acidic (3, 23, 26), and it is believed that these phospholipids may serve as binding sites for divalent cations such as Mg^{++} (23, 27, 40). Divalent cation binding function has also been proposed for the phospholipids of nonhalophilic bacteria (34). For example, when *Bacillus subtilis* was grown under conditions of magnesium ion limitation, the level of phosphatidylethanolamine decreased significantly and the level of phosphatidylglycerol increased (34). It was suggested that anionic phospholipids may function to maintain appropriate levels of magnesium ions at the membrane surface of this bacterium (34). The relative amount of anionic phospholipid has also been shown to increase in *Escherichia coli* when cells are grown in media limiting for magnesium (10). Other anionic bacterial cell envelope components have been implicated for this function as well. For example, it has been proposed that the teichoic acids of many gram-positive bacteria may function to provide divalent cations (such as Mg^{++}) to the membrane environment during conditions of divalent cation limitation (8, 16, 18, 19, 28, 31).

In summary, the phospholipid and fatty acid compositions of *Planococcus* sp. strain A4a were altered when the NaCl concentration of the growth medium or the temperature of growth was varied. In response to changes in growth temperature, the alteration of fatty acid composition was consistent with a temperature-regulated mechanism for membrane fluidity modulation. When the NaCl concentration of the growth medium was increased, the relative amount of anionic phospholipid increased in this bacterium. The increase in anionic phospholipid content resulted from an increase in cardiolipin content and a decrease in phosphatidylethanolamine content. This is the first demonstration of an NaCl-dependent decrease in phosphatidylethanolamine content within gram-positive halotolerant bacteria.

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