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Isolation of Spheroplast Membranes and Stability of Spheroplasts of *Bacillus stearothermophilus*

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Spheroplasts were prepared by lysozyme digestion of the cell wall and ruptured by suspension in 0.15 M NaCl, followed by centrifugation at 30,900 × *g* for 35 min, and by a final suspension in 0.05 M NaCl for 12 to 16 hr at 5 C. The membrane ghosts were washed four times in tris(hydroxylmethyl)aminomethane (Tris) magnesium buffer and once in distilled water. The intact membranes resembled empty sacs with narrow slits in which the cytoplasm was extruded. A 92% recovery of cell membrane was obtained with all membrane preparations. The spheroplasts do not require a stabilizing medium to keep them from rupturing, and they are stable for 2 to 3 hr when exposed to a temperature of 65 C. The membrane content of the cell increases with age of culture (mid-log, 16.5%; late-log, 17.0%; and stationary, 17.6%) and temperature of growth (55 C, 16.5%; and 65 C, 17.8%), and it is unaffected by composition of the growth medium. The ratio of the protein to lipid content of the membrane increases with the complexity of the medium, age of culture (mid-log, 3.65; late-log, 3.91; and stationary, 4.15), and temperature of growth (55 C, 3.65; and 65 C, 5.22). The ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) content of the membranes was 9.0 to 13.7% and 0.3 to 0.8%, respectively. Reducing sugar (determined as glucose) amounts to 0.9 to 1.0% of the membrane weight and did not significantly vary for the different membrane preparations. Medium composition, age of culture, and temperature of growth have no significant effect on the amount of each amino acid in the membrane. Aspartic acid, glutamic acid, alanine, leucine, and lysine are present in the greatest amount and represent 12.9 to 14.1%, 10.4 to 11.3%, 9.6 to 10.3%, 7.7 to 8.8%, and 7.6 to 8.5% of the membrane peptide, respectively. Prior to the rupture of the spheroplasts, 25.0, 15.7, and 50.0% of the protein, RNA, and DNA, respectively, is lost. In potassium phosphate-magnesium buffer without sucrose, 90% of the protein and RNA and 95% of the DNA is lost from the spheroplasts. In the presence of sucrose, the leakage of RNA and DNA is similar to that observed for spheroplasts suspended in Tris magnesium buffer; however, the leakage of protein is 2.4 times greater.

It is now generally agreed that the capacity of thermophilic bacteria to grow at high temperatures is caused by their ability to synthesize cell constituents that have a greater heat stability than the corresponding components synthesized by mesophiles. The literature in this field has been extensively reviewed by Gaughran (14), Allen (2), Koffler (19), Farrell and Rose (10), Campbell and Pace (7), and Friedman (12). Most of the studies have focused on the effect of temperature on growth and nutritional requirements of thermophiles or the thermal stability of macromolecules such as enzymes, flagella, deoxyribonucleic acid (DNA), soluble ribonucleic acid (RNA), ribosomal RNA, and ribosomes. Brock (5) has suggested that the molecular mechanism of thermophily is more related to the stability of the membrane than to any one macromolecule; however, detailed studies on the cytoplasmic membrane of thermophiles are incomplete and have been concerned mainly with the membrane lipid. Heilbrunn (17) and Bělehrádek (3) have postulated that the heat resistance of an organism is related to the melting temperature of the cell lipid. The temperature at which the lipids melt (degree of saturation) sets the maximal growth temperature. Gaughran (13) has reported a high degree of saturation of the lipids of a thermophile and that the lipids are relatively constant both in quantity and degree of saturation as the growth

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temperature is increased. Long and Williams (20), however, have shown that when the growth temperature of a thermophile is changed from 37 to 55 C the lipid content of the vegetative cells is decreased by 34.1%. Cho and Salton (8) reported that the membrane lipid of *Bacillus stearothermophilus* is composed of over 50% of both C_{15} and C_{17} branched chain fatty acids.

The generally accepted definition of a protoplast (4) requires that the spherical, osmotically sensitive body be free of all cell wall components. Since chemical analysis of the membranes show the presence of glucosamine, we shall use the term "spheroplast" and "spheroplast membrane" to describe these bodies and the membrane ghost, respectively. In this paper, we describe the isolation and chemical composition of spheroplast membranes of an obligately thermophilic strain of *B. stearothermophilus*. The stability of the spheroplasts was also determined.

MATERIALS AND METHODS

The organism used in this investigation was *B. stearothermophilus* NCA 1503-4R (31, 39).

Media and growth conditons. The media used were a MCHF medium (36), which is a minimal medium supplemented with 0.1% casein hydrolysate (Mann Research Laboratories, New York, N.Y.), 2% Trypticase (BBL), 0.5% yeast extract (Difco), 0.5% fructose [TYF, (37)], and dilute-TYF medium in which the concentration of Trypticase, yeast extract, and fructose is 0.5, 0.25, and 0.25%, respectively.

A 2,800-ml Fernbach flask, containing 1 liter of TYF, MCHF, or dilute-TYF medium, was inoculated with 12- to 15-hr cells from a Trypticase agar plate and shaken on a New Brunswick model G-25 gyratory incubator shaker for 2 hr at 55 C. The cell inoculum was aseptically poured into a 14-liter fermentor vessel, containing 12 liters of TYF, MCHF, or dilute-TYF medium. The fermentor vessel was agitated (300 rev/min) and aerated (5,000 cc of filter-sterilized air per min) at 55 or 65 C in a New Brunswick fermentor (model FS-314). Growth was measured in a Bausch & Lomb Spectronic-20 colorimeter at 525 nm. In each of the media, the optical density at mid-log, late-log, and stationary phase of growth was 0.35 (2.2×10^8 cells/ml), 0.56 (4.1×10^8 cells/ml), and 0.80 (1.0×10^9 cells/ml), respectively.

Protein determination. Membranes (2 to 6 mg) or cells (10 to 15 mg) were placed in calibrated conical test tubes, dissolved in 0.1 ml of 1 M NaOH, and brought to 10 ml with distilled water. Samples from spheroplast and membrane wash supernatant fluids were diluted in distilled water. Protein was determined by the procedure of Lowry et al. (21) by use of bovine serum albumin (Mann Research Laboratories) as a standard.

DNA and RNA determination. Membranes (2 to 6 mg) or cells (10 to 15 mg) were placed in calibrated conical centrifuge tubes containing 5 ml of 0.5 N $HClO_4$. The tubes were heated at 90 C in a water bath for 20 min, cooled, and centrifuged at $3,000 \times g$ for 15 min. The supernatant fluids were decanted and used for the determination of RNA (9) and DNA (6). The spheroplast and membrane wash supernatant fluids were diluted with an equal volume of 1.0 N $HClO_4$ before the determination of RNA and DNA. Salmon sperm DNA and yeast RNA (Mann Research Laboratories) were used as standards.

Phosphorus determination. The phosphorus content of membranes (0.5 to 1.6 mg) was determined using the procedure described by Sutow and Welker (31).

Sugar determination and chromatography. Membranes (10 to 15 mg) were placed in an acid-washed Pyrex tube (18 by 150 mm) and suspended in 2 ml of 0.5 N H_2SO_4. The tube was flushed with nitrogen, sealed, and heated to 105 C for 8 hr. The contents of the tube were neutralized with $Ba(OH)_2$, and the precipitate was removed by centrifugation. The sample was deionized by passage through a column (0.9 by 4.0 cm) of Amberlite MB-3 (mixture of Amberlite IR-120 and IRA-410), lyophilized, and stored over P_2O_5 in a vacuum desiccator. The dried sample was dissolved in 10.0 or 0.5 ml of distilled water for the determination of reducing groups (18) and for analysis by ascending paper chromatography (36), respectively. Reducing sugars and amino sugars were detected on developed chromatograms by the procedure described by Welker and Campbell (36) and by spraying with 0.2% ninhydrin in *n*-butyl alcohol, respectively.

Lipid extraction. Lipids were extracted from dried membranes (10 to 20 mg) or cells (30 to 40 mg) using a modification of the procedure described by Folch, Lees, and Sloane-Stanley (11). The extraction was performed with 10 to 15 ml of chloroform-methanol (2:1, v/v) in a Tenbroeck tissue grinder at ambient room temperature. The fine precipitate was removed by filtration through chloroform-methanol washed Whatman No. 1 filter paper. The extract was shaken with 0.2 volume of distilled water in a 100-ml separatory funnel and maintained at ambient room temperature for 16 to 18 hr to allow separation of the phases. The upper aqueous phase was carefully removed with a pipette, and the precipitate at the interface was collected by suction filtration through a sintered glass filter (fine porosity). The solvent layer containing the lipid was overlaid with 0.2 volume of 0.05 M $CaCl_2$ and allowed to stand for 30 min. The $CaCl_2$ layer was discarded, and the washing procedure was repeated once more with $CaCl_2$ and once with distilled water. The washed extract was evaporated in a stream of nitrogen, dissolved in 10 to 15 ml of chloroform-methanol, filtered through glass wool, and placed into a tared weighing bottle. The solvent was evaporated as before, and the residue was dried over P_2O_5 in a vacuum desiccator. The weight of the extracted lipid was determined gravimetrically.

Amino acid and amino sugar analysis of membranes. Membranes (4 to 6 mg) were placed in an acid-washed Pyrex ampoule and suspended in 2 ml of constant boiling HCl. The ampoule was flushed with nitrogen, sealed, and heated at 105 C for 8 hr. The contents of the ampoule were filtered through Whatman no. 1 filter paper, taken to dryness by lyophilization, and stored over P_2O_5 in a vacuum desiccator. The hydrolysate was taken up in 10 ml of 0.2 N sodium citrate

buffer, *p*H 2.2 (sample diluter), and analyzed on the Beckman Spinco amino acid analyzer.

Electron microscopy. Spheroplasts and membranes were fixed by using the procedure described by Schlessinger, Marchesi, and Kwan (28). The fixed material was dehydrated in a graded series of ethyl alcohol and propylene oxide and embedded in an Epon-Araldite mixture. Sections were cut on an LKB microtome, mounted on uncoated copper grids, and stained with uranyl acetate (2% in 75% ethyl alcohol). Shadowed material was prepared by placing a droplet of an aqueous suspension of spheroplasts or membranes on a Formvar-coated copper grid, air-dried, and shadowed with nichrome. The grids were examined in a Hitachi HS-7 electron microscope at an initial magnification of 7,500 times.

RESULTS AND DISCUSSION

Isolation of membranes. Cells were harvested by centrifugation in a Sorvall RC2-B refrigerated centrifuge equipped with a KSB-R continuous flow system. Cells from 13 liters of culture were suspended in 320 ml of 0.04 M Tris hydrochloride (*p*H 7.5), 0.01 M $MgCl_2$ buffer (Tris magnesium buffer), and centrifuged at 6,870 × *g* for 10 min. The cells were suspended in 240 ml of Tris magnesium buffer and centrifuged at 6,870 × *g* for 10 min. After this washing procedure was repeated once more, the cells were suspended in 750 ml of Tris magnesium buffer. The washed cells were incubated with 50 μg of lysozyme per ml (Mann Research Laboratories) for 10 to 20 min at 37 C with occasional agitation. The conversion of rods to spheroplasts was followed with a phase-contrast microscope. The spheroplasts were centrifuged at 7,970 × *g* for 15 min, suspended in 140 ml of Tris magnesium buffer, and centrifuged at 7,970 × *g* for 15 min. After this procedure was repeated twice more, the spheroplasts were suspended in 140 ml of 0.15 M NaCl, centrifuged at 30,900 × *g* for 35 min, and suspended in 500 ml of 0.05 M NaCl. Rupture of the spheroplasts occurs after 12 to 16 hr at 5 C. The membranes were centrifuged at 17,300 × *g* for 15 min and suspended in 80 ml of Tris magnesium buffer. After this washing procedure was repeated three more times with Tris magnesium buffer and once with distilled water, the membranes were suspended in 10 to 15 ml of distilled water, lyophilized, and stored over P_2O_5 in a vacuum desiccator at ambient room temperature.

An electron micrograph of a spheroplast membrane of *B. stearothermophilus* 1503-4R is shown in Fig. 1. All of the shadowed membrane preparations examined in the electron microscope consisted of membranes which had split in half. Sometimes the two halves are still attached by a narrow membrane connection. Phase-contrast microscopy of membrane suspensions as well as light microscopy of membrane suspensions stained with Victoria blue (25) showed that 98% of the membranes were intact. The membrane ghosts resemble empty sacs with narrow slits in which the cytoplasm was extruded. The membrane suspensions are relatively free of membrane fragments.

A thin section of a spheroplast membrane pellet is shown in Fig. 2. All of the membranes appear intact, except where the section was cut through the site of the rupture (*see arrows*). The membrane ghosts are free of electron-dense cell wall and contain a scattering of ribosomes. The presence of a relatively dense mat of ribosomes in some of the membrane ghosts could be a result of their physical entrapment at the time of spheroplast rupture or to the presence of intact spheroplasts in the membrane preparation.

A comparison of the total lipid content of the cell and the spheroplast membrane indicated that 8% of the lipid was lost during the isolation procedure. The loss of lipid could be explained as lipid not associated with the membrane, to the loss of small fragments from the outer membrane, or to a loss of the intracytoplasmic membrane system (1). The spheroplasts of *B. stearothermophilus* are relatively insensitive to changes in osmotic pressure and do not need a stabilizing medium to keep them from rupturing. An electron micrograph of an intact spheroplast is shown in Fig. 3. The spheroplasts can only be ruptured by suspension in 0.15 M NaCl, followed by centrifugation at 30,900 × *g* for 35 min, and a final suspension in 0.05 M NaCl at 5 C for 12 to 16 hr. Only 10 to 20% of the spheroplasts rupture if this procedure is changed.

If the *p*H of the Tris magnesium buffer is adjusted to 6.3, a phage lytic enzyme can be used to remove the cell wall (39). No spheroplasts are produced when cells are incubated with lysozyme or phage lytic enzyme in the presence of Tris magnesium buffer minus $MgCl_2$ or Tris magnesium buffer plus 10^{-2} M ethylenediaminetetraacetate (EDTA). If $MgCl_2$ (0.01 M) is added to the 0.15 M NaCl, the spheroplasts will not rupture. Membranes from spheroplasts washed in Tris buffer minus $MgCl_2$ were morphologically identical to the membranes prepared from spheroplasts washed in Tris magnesium buffer. These results indicate that Mg^{++} is necessary for the conversion of *B. stearothermophilus* cells to spheroplasts and that the presence of Mg^{++} during the preparation and washing of the spheroplasts does not account for their stability. Several investigators have reported that for mesophiles Mg^{++} prevents the breakage of spheroplast or protoplast ghosts into small fragments (15, 16, 23, 33–35). Salton and Freer (26), however, have

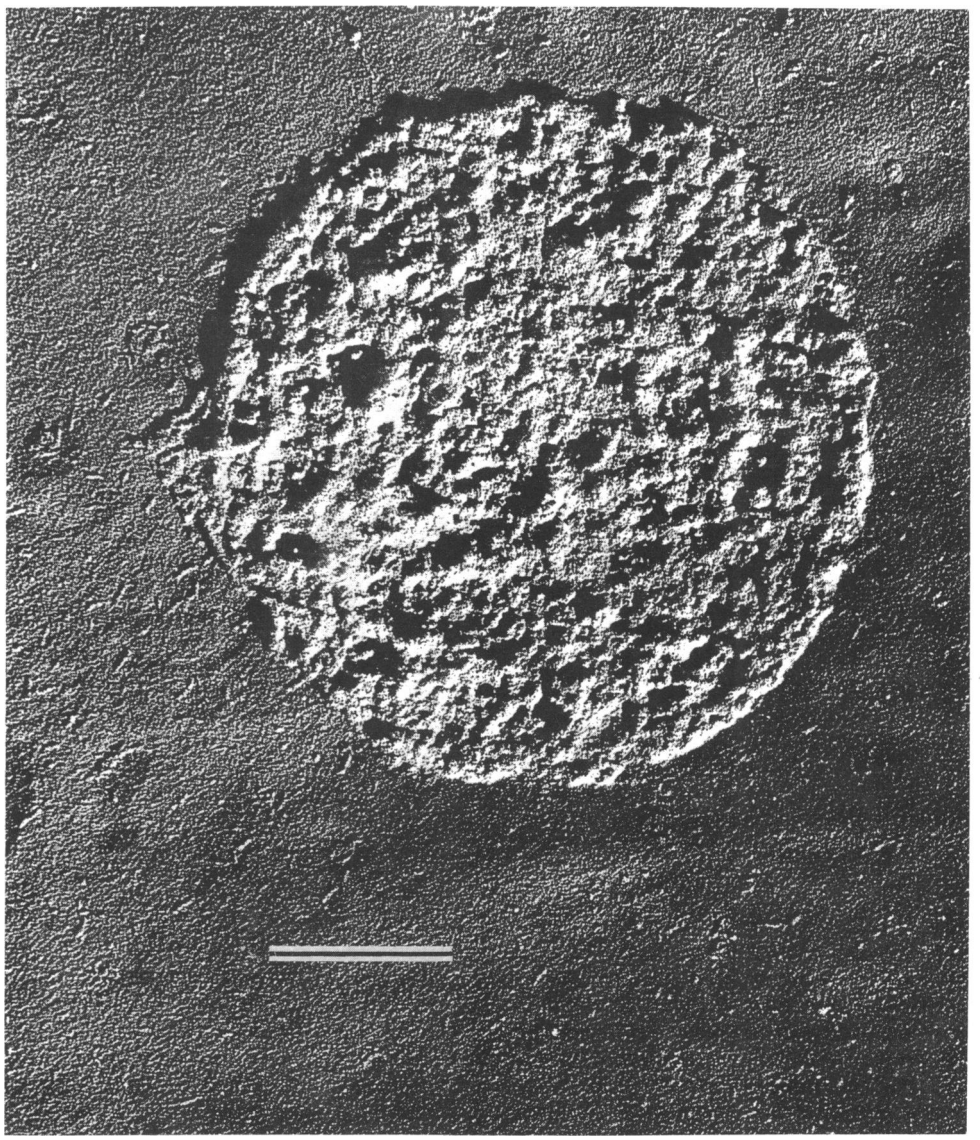

FIG. 1. *Electron micrograph of a membrane of B. stearothermophilus NCA 1503-4R. Shadow-cast with nichrome.* × *24,600. The bar indicates 1.0 μm.*

reported that it was not necessary to add Mg^{++} to Tris buffer during the isolation of membranes from mesophilic bacteria.

Suspensions of spheroplasts or protoplasts of most other mesophilic bacteria will rupture in hypotonic media or upon mechanical manipulation, even in the presence of osmotic stabilizers (22, 23, 34). Spheroplasts were prepared from a mesophile, *B. amyloliquefaciens* F (38), using the same procedure, so that a comparison of spheroplast stability could be made. It was found that during the lysozyme incubation step the resulting spheroplasts began to rupture within 5 to 10 min, and complete rupture was observed after 20 min. An electron micrograph of *B. amyloliquefaciens* F cells which were incubated with lysozyme for 10 min is shown in Fig. 4. If sucrose (0.25 M) is added prior to lysozyme incubation, osmotically sensitive spheroplasts are formed. Sucrose-stabilized spheroplasts of *B. amyloliquefaciens* F, when exposed to a temperature of 50 C or higher, will immediately burst. Spheroplasts

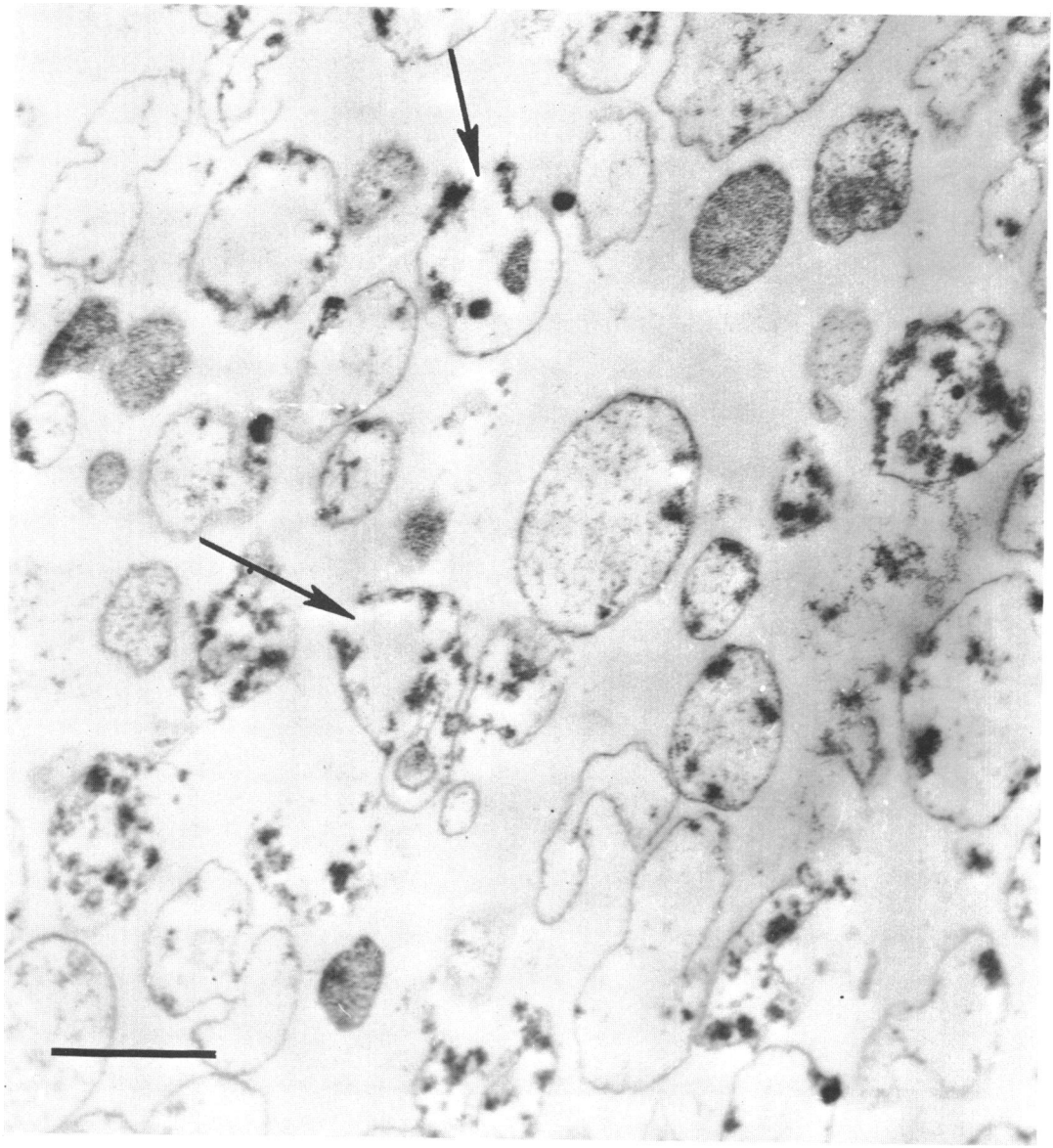

FIG. 2. *A thin section of a membrane pellet stained with uranyl acetate. Arrows, membranes which were sectioned through the site of rupture.* × *22,200. The bar indicates 1 μm.*

of *B. stearothermophilus* NCA 1503-4R, when exposed to a temperature of 65 C, were stable for 2 to 3 hr. These results indicate that spheroplasts of thermophiles are relatively more stable to changes in osmotic pressure and at elevated temperatures than the corresponding structure of mesophiles. We think that the osmotic stability of the thermophile spheroplast is directly related to its heat stability. Abram (1) reported that elongated intact or nearly intact "protoplasts" could be prepared from water suspensions of ruptured cells of thermophilic bacilli but not from mesophilic organisms. Results from this laboratory indicate that when the cell wall of a thermophilic organism is removed enzymatically, the resulting protoplasts or spheroplast is always spherical.

Effect of growth conditions on the membrane content of B. stearothermophilus cells. Membranes isolated from mid-log phase cells grown at 55 C in MCHF, dilute-TYF, and TYF medium

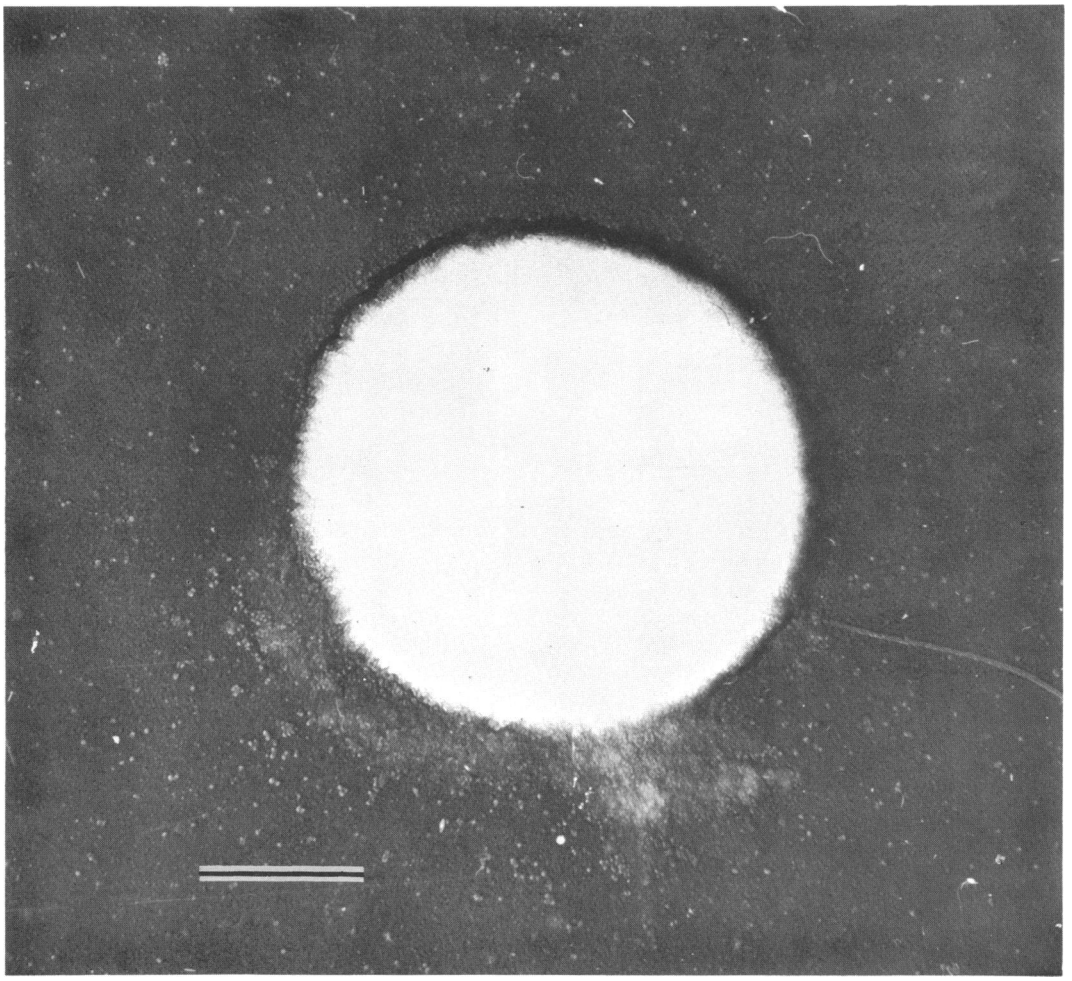

FIG. 3. *Electron micrograph of a spheroplast of B. stearothermophilus NCA 1503-4R. Shadow-cast with nichrome.* × *21,600. The bar indicates 1 μm.*

amounted to 16.3, 16.8, and 16.5% of the cell weight, respectively (Table 1). Although the membrane content of the cells does not significantly vary, the mean generation time in the three media was quite different (MCHF, 90 min; dilute-TYF, 50 min; and TYF, 30 min). Membranes isolated from mid-log phase cells grown in TYF medium at 55 and 65 C amounted to 16.5 and 17.8% of the cell weight, respectively. Membranes isolated from mid-log, late-log, and stationary-phase cells grown in TYF medium at 55 C amounted to 16.5, 17.0, and 17.6% of the cell weight, respectively. A definite increase in membrane content is found in cells from older cultures and from cells grown at 65 C. Salton and Freer (26) reported that membranes isolated from another strain of *B. stearothermophilus* grown in a complex medium at 55 C for 9 hr (stationary phase) amounted to 16.2% of the cell (dry weight). The difference between our values and that reported by Salton and Freer is probably due to a strain difference or to the different growth conditions and membrane isolation and quantitation procedures employed.

Effect of growth conditions on the chemical composition of membranes isolated from. B. stearothermophilus. The protein content of the membranes is not significantly affected by the medium in which the cells were grown; however, the lipid content of membranes isolated from cells grown in MCHF, dilute-TYF, and TYF medium was 20.8, 19.7 and 17.8%, respectively (Table 2). The protein content of membranes isolated from mid-log, late-log, and stationary-phase cells was 65, 72, and 74%, respectively. The lipid content of membranes isolated from mid-

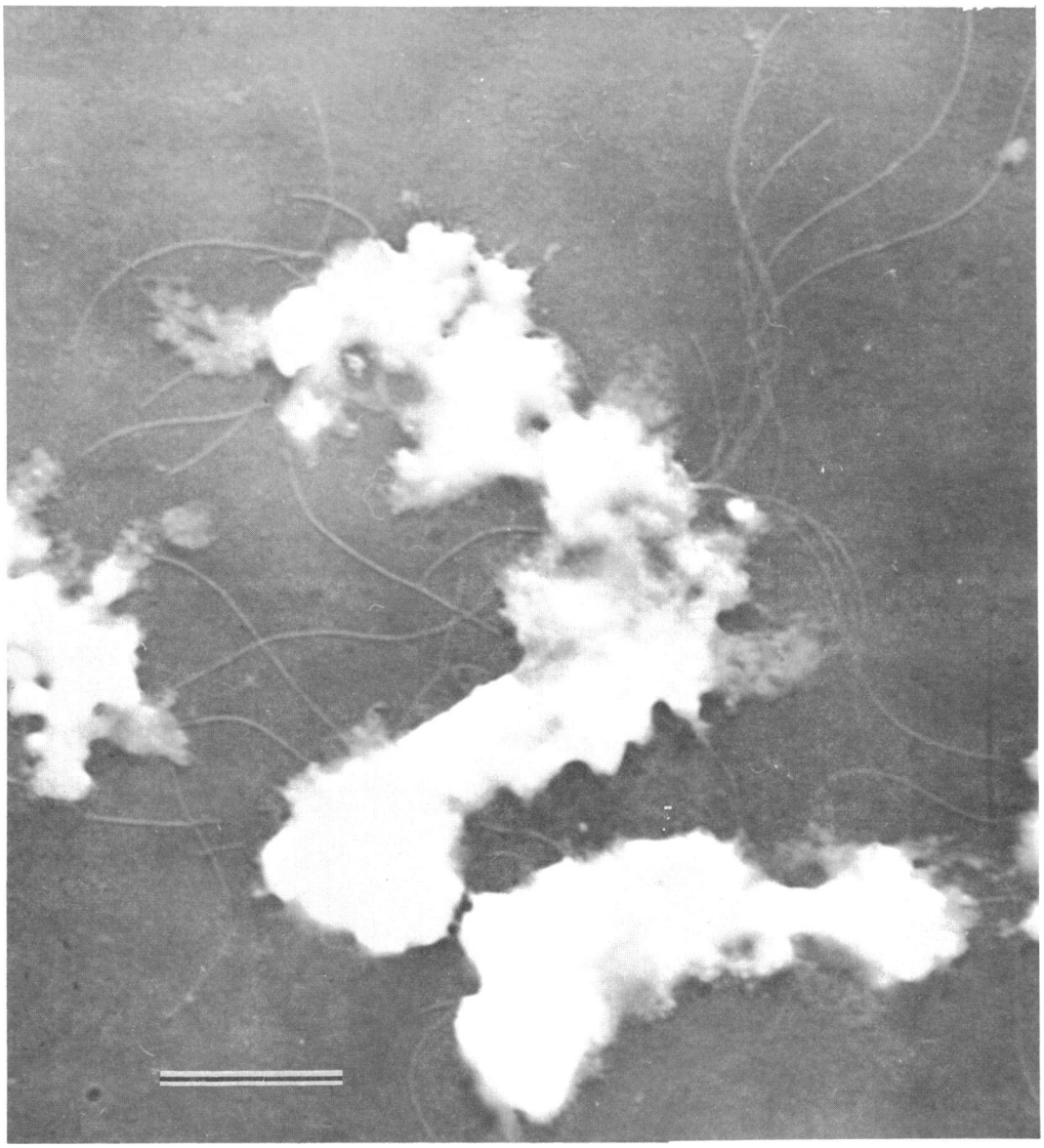

FIG. 4. *Electron micrograph of B. amyloliquefaciens F cells after incubation with lysozyme. Procedure used was the same as that described for the isolation of membranes of B. stearothermophilus. Shadow-cast with nichrome.* × *24,975. The bar indicates 1 μm.*

log, late-log, and stationary-phase cells was 17.8, 18.4, and 17.8%, respectively. Salton and Freer (26) reported the lipid content of membranes isolated from another strain of *B. stearothermophilus* grown for 9 hr (stationary phase) at 55 C in a complex medium as 25.6%. A 10-degree increase in growth temperature (55 to 65 C) resulted in a 4.6% increase and a 26.8% decrease in the protein and lipid content, respectively, of membranes isolated from mid-log phase cells grown in TYF medium. Long and Williams (20) reported a 34.1% decrease in the membrane lipid content of vegetative cells of *B. stearothermophilus* when the growth temperature was changed from 37 to 55 C. Gaughran (13), on the other hand, indicated that the amount of lipid in the cells of obligate thermophiles is relatively constant as the temperature of growth is raised. Variations in the protein and lipid content of bacterial membranes within one species are probably the result

TABLE 1. *Effect of growth conditions on the membrane content of B. stearothermophilus*

Medium	Stage of growth[a]	Temp	Membrane[b]	
		C	%	SD[c]
MCHF	Mid-log	55	16.3	±0.2
Dilute-TYF	Mid-log	55	16.8	±0.2
TYF	Mid-log	55	16.5	±0.2
TYF	Late-log	55	17.0	±0.1
TYF	Stationary	55	17.6	±0.1
TYF	Mid-log	65	17.8	±0.1

[a] Growth was measured in a Bausch & Lomb Spectronic-20 colorimeter at 525 nm. The optical density at mid-log, late-log, and stationary phase of growth was 0.35 (2.2 × 10^8 cells/ml), 0.56 (4.1 × 10^8 cells/ml), and 0.80 (1.0 × 10^9 cells/ml), respectively.

[b] The dry weight of the membranes was determined gravimetrically, and the results are expressed as a percentage of the dry weight of the cells from which they were isolated.

[c] Standard deviation.

TABLE 2. *Effect of growth conditions on protein and lipid content of membranes of B. stearothermophilus*

Medium	Stage of growth[a]	Temp	Protein[b]	Lipid[b]	Ratio of protein-lipid
		C			
MCHF	Mid-log	55	690.3	208.1	3.32
Dilute-TYF	Mid-log	55	690.1	197.1	3.50
TYF	Mid-log	55	650.0	178.2	3.65
TYF	Late-log	55	720.0	184.3	3.91
TYF	Stationary	55	740.1	178.2	4.15
TYF	Mid-log	65	680.0	130.3	5.22

[a] Growth was measured in a Bausch & Lomb Spectronic-20 colorimeter at 525 nm. The optical density at mid-log, late-log, and stationary phase of growth was 0.35 (2.2 × 10^8 cells/ml), 0.56 (4.1 × 10^8 cells/ml), and 0.80 (1.0 × 10^9 cells/ml), respectively.

[b] Values expressed as micrograms of membrane per milligram.

of a combination of factors such as the growth conditions, analytical procedures, and membrane isolation and washing procedures (27). The protein to lipid ratio increases with the complexity of the medium (MCHF, 3.32; dilute-TYF, 3.50; and TYF, 3.65), age of culture (mid-log, 3.65; late-log, 3.91; and stationary, 4.15), and temperature of growth (55 C, 3.65; and 65 C, 5.22). A 10-degree increase in the growth temperature results in the greatest change in the protein to lipid ratio. The data presented in this paper indicate that the protein and lipid content of membranes isolated from *B. stearothermophilus* NCA 1503-4R is dependent upon age of culture, medium composition, and temperature of growth. Shockman et al. (30) have reported that the chemical composition of membranes isolated from *Streptococcus faecalis* varies significantly with age of the culture and medium composition. In contrast, Salton and Freer (26) showed that growth conditions had no effect on the membrane composition of several gram-positive bacteria.

The RNA content of membranes isolated from mid-log phase cells grown in MCHF, dilute-TYF, and TYF medium is 9.0, 10.9, and 13.7%, respectively (Table 3). The DNA content of the membranes is 0.3, 0.6, and 0.8% in the three media. Membranes from mid-log phase cells contained 13.7% RNA and 0.8% DNA. Membranes from late-log and stationary-phase cells each contained 11.0% RNA and 0.5% DNA. A 10-degree increase in growth temperature (55 to 65 C) had no effect on the RNA content of the membrane, but the DNA content was lowered by 37.9%. The phosphorus values include both the phospholipid and nucleic acid phosphorus. The RNA content of the membrane can be lowered to a value of 0.8 to 3.0% by treatment with 10^{-3} M EDTA or by eliminating $MgCl_2$ from the Tris magnesium buffer after the lysozyme step. The results presented in this paper indicate that the 9.0 to 13.7% RNA remaining with the spheroplast membranes represents ribosomal RNA. The amount of reducing sugar (determined as glucose) in the membrane was 0.9 to 1.0% and

TABLE 3. *Effect of growth conditions on RNA, DNA, and phosphorus content of membranes of B. stearothermophilus*

Medium	Stage of growth[a]	Temp	RNA[b]	DNA[b]	Phosphorus[b]
		C			
MCHF	Mid-log	55	89.6	3.3	14.9
Dilute-TYF	Mid-log	55	108.6	5.6	16.6
TYF	Mid-log	55	136.8	7.9	18.2
TYF	Late-log	55	109.9	5.0	18.2
TYF	Stationary	55	111.0	4.7	17.7
TYF	Mid-log	65	134.0	4.9	15.3

[a] Growth was measured in a Bausch & Lomb Spectronic-20 colorimeter at 525 nm. The optical density at mid-log, late-log, and stationary phase of growth was 0.35 (2.2 × 10^8 cells/ml), 0.56 (4.1 × 10^8 cells/ml), and 0.80 (1.0 × 10^9 cells/ml), respectively.

[b] Values expressed as micrograms of membrane per milligram.

did not significantly vary for the different membrane preparations. The sugars identified in acid hydrolysates using multiple ascending paper chromatography were (in descending order of amount) ribose, glucose, and glucosamine. The presence of ribose can be attributed to the relative high RNA content of the membranes. Salton and Freer (26) detected glucose in the membranes of another strain of *B. stearothermophilus*. Under identical hydrolysis conditions, standard glucosamine (Mann Research Laboratories) is hydrolyzed to glucose. These results suggest that glucosamine and not glucose is present in the membranes of *B. stearothermophilus* 1503-4R. A qualitative determination of the membrane sugar, however, will have to be made on purified cytoplasmic membrane material.

Comparison of the amino acid analyses of the spheroplast membrane preparations indicates that the amount of each amino acid does not significantly vary with medium composition, age of culture, or temperature of growth (Table 4). The sum of the amino acids accounts for 89.4 to 90.0% of the weight of membrane protein (Table 2), except for the membranes isolated from mid-log (95.7%) and stationary-phase (89.2%) cells grown in TYF medium at 55 C. Aspartic acid, glutamic acid, alanine, leucine, and lysine are present in the greatest amounts and represent 12.9 to 14.1%, 10.4 to 11.3%, 9.6 to 10.3%, 7.7 to 8.8%, and 7.6 to 8.5% of the membrane peptide, respectively. Shockman et al. (30) have shown that relatively high levels of aspartic acid, arginine, glycine, and alanine were found in membranes of *S. faecalis* which were prepared by using a high lysozyme to cell ratio. We do not believe that our membrane preparations are contaminated with lysozyme because we are using 1,000 times less lysozyme than used by these investigators; our arginine and glycine values are relatively low; and the same amino acid composition is found for membranes prepared by using phage lytic enzyme (N. E. Welker, *unpublished data*). No calf-cystine, muramic acid, or α,ϵ-diaminopimelic acid were detected in the spheroplast membrane preparations. The tryptophan content of the membrane was not determined. The presence of glucosamine in the membrane preparations suggests a contamination with cell wall substance. It is possible, however, that glucosamine is also a membrane component.

Leakage of protein, RNA, and DNA from spheroplasts. In view of the unusual stability of *B. stearothermophilus* spheroplasts in the absence of

TABLE 4. *Amino acid and amino sugar content of membranes of B. stearothermophilus*[a]

Component	MCHF 55 C mid-log	Dilute-TYF 55 C mid-log	TYF 55 C mid-log	TYF 55 C late-log	TYF 55 C stationary	TYF 65 C mid-log
Lysine	5.66	5.24	5.90	5.80	5.97	5.21
Histidine	1.10	1.21	1.15	1.25	1.12	1.11
Ammonia	1.63	1.78	1.67	1.87	1.81	1.69
Arginine	3.92	3.09	3.44	3.79	3.97	3.29
Aspartic acid	9.59	10.79	9.79	9.59	9.12	9.05
Threonine[b]	3.62	3.69	3.98	3.80	3.95	3.50
Serine[b]	3.42	3.84	3.81	3.76	3.92	3.49
Glutamic acid	7.30	7.15	7.99	7.98	7.80	7.50
Proline	2.68	2.67	2.96	2.96	2.78	2.89
Glycine	3.48	3.48	3.63	3.87	3.90	3.48
Alanine	6.99	6.46	6.91	6.87	6.91	6.99
Valine	2.76	2.34	2.69	2.89	2.97	2.87
Methionine	2.41	2.75	2.68	2.75	2.66	2.90
Isoleucine	1.92	1.88	2.01	1.97	1.79	1.92
Leucine	5.28	6.07	5.87	5.82	5.43	5.28
Tyrosine	2.99	2.78	2.98	2.95	2.81	2.76
Phenylalanine	2.81	2.98	2.77	2.86	2.89	2.98
Glucosamine	0.36	0.41	0.36	0.36	0.39	0.36
Total	67.92	68.61	70.59	71.14	70.19	67.27
Peptide Substance[c]	58.4	59.0	60.7	61.2	60.4	57.9

[a] Membranes (4 to 6 mg) were hydrolyzed for 8 hr in constant boiling HCl at 105 C and analyzed on a Beckman Spinco amino acid analyzer. Values expressed as grams of component per 100 g of membrane.

[b] No corrections were made for the decomposition of threonine and serine.

[c] Total amino acids reduced by 14% (approximate correction for water loss in peptide bond formation).

a stabilizing agent, such as sucrose or polyethylene glycol (PEG), we were interested in determining if cellular components were leaking from the spheroplasts. The leakage of protein, RNA, and DNA during the spheroplast isolation and washing procedure is presented in Table 5. Prior to the rupture of the spheroplasts, 25.0, 15.7, and 50.0% of the protein, RNA, and DNA, respectively, are lost. Early spheroplast rupture (less than 0.05%) does not account for the relatively high loss of these cellular constituents. The greatest leakage of protein, RNA, and DNA occurs upon removal of the cell wall and the first spheroplast wash and when the spheroplasts are suspended in 0.15 M NaCl. Sucrose and PEG 4,000 (average molecular weight 3,000 to 3,700) were used as stabilizing agents in combination with Tris magnesium buffer, 0.067 M potassium-sodium phosphate (*p*H 7.5), 0.01 M $MgCl_2$ buffer (PSPM buffer), or 0.067 M potassium phosphate (*p*H 7.5), 0.01 M $MgCl_2$ buffer (PPM buffer). Stable spheroplasts were produced when Tris magnesium buffer was replaced by either of these buffers. Sucrose and PEG were tested over a range of 0.15 1.5 M and 0.5 to 7.5%, respectively. Sucrose or PEG in Tris magnesium buffer inhibited the conversion of cells to spheroplasts. Only 40% of the cells were converted to spheroplasts with PSPM buffer plus 0.5 to 0.8% PEG. Sucrose (0.15 to 0.25 M) or PEG (0.5 to 0.8%) in PPM buffer had no effect on the conversion of cells to spheroplasts. Sucrose (0.25 M) was selected as the stabilizing agent because it had the least effect on the conversion of cells to spheroplasts. Table 6 shows the effect of sucrose on the leakage of protein, RNA, and DNA from spheroplasts suspended in PPM buffer. In the absence of sucrose, 90% of the protein and RNA and 95% of the DNA is lost from the spheroplasts. In the presence of sucrose, the leakage of RNA and DNA is similar to that observed for spheroplasts suspended in Tris magnesium buffer (Table 5). The leakage of protein, however, was 2.4 times greater than that observed with spheroplasts suspended in Tris magnesium buffer. Sucrose or PEG-stabilized spheroplasts can be produced from cells of *B. amyloliquefaciens* F when Tris magnesium buffer is replaced with either PSPM buffer or PPM buffer. The results in this paper

TABLE 5. *Leakage of protein, RNA, and DNA from spheroplasts*[a]

Isolation step	Protein	RNA	DNA
	mg	*mg*	*mg*
Lysozyme	40.0	4.2	0.4
First spheroplast wash	63.2	5.6	0.5
Second spheroplast wash	30.0	1.6	0.3
Third spheroplast wash	30.0	1.8	0.1
Suspension of spheroplasts in 0.15 M NaCl	71.2	9.6	0.3
Per cent of total[b]	25.0%	15.7%	50.0%

[a] Spheroplasts were isolated from mid-log phase cells grown in TYF medium at 55 C, using the procedure described in the text.

[b] Percentage of the total is calculated from the protein, RNA, and DNA content of the cells from which the spheroplasts were isolated.

TABLE 6. *Effect of sucrose on the leakage of protein, RNA, and DNA from spheroplasts suspended in potassium phosphate buffer*[a]

Isolation step	Protein		RNA		DNA	
	Sucrose	No sucrose	Sucrose	No sucrose	Sucrose	No sucrose
	mg	*mg*	*mg*	*mg*	*mg*	*mg*
Lysozyme	138.0	222.0	5.5	27.2	0.5	1.9
First spheroplast wash	133.2	145.2	7.0	8.0	0.6	1.1
Second spheroplast wash	84.0	26.0	1.8	1.6	0.4	0.3
Third spheroplast wash	134.0	18.0	1.9	1.5	0.1	0.1
Suspension of spheroplasts in 0.15 M NaCl	65.2	5.2	10.5	1.6	0.2	0.2
Per cent of total[b]	60.1%	90.0%	17.8%	90.0%	55.0%	95.0%

[a] Spheroplasts were isolated from mid-log phase cells grown in TYF medium at 55 C. The spheroplast isolation procedure is the same as that described in the text, except that the Tris magnesium buffer is replaced by 0.067 M potassium phosphate (*p*H 7.5), 0.01 M $MgCl_2$ buffer. The sucrose concentration is 0.25 M.

[b] Percentage of the total is calculated from the protein, RNA, and DNA content of the cells from which the spheroplasts were isolated.

indicate that the stability of *B. stearothermophilus* spheroplasts is relatively unaffected by the buffer in which they are suspended; however, the leakage of protein, RNA, and DNA is greater when the spheroplasts are suspended in PPM buffer than in Tris magnesium buffer. In this connection, Salton and Freer (26) reported that carotenoid materials are lost when bacterial membranes are prepared and washed in phosphate buffer.

Our results indicate that the spheroplast membranes isolated from cells of *B. stearothermophilus* represent the intact outer cytoplasmic membrane. If all the lipid in this organism is membrane lipid, then the 8% loss of lipid probably represents the intracytoplasmic membrane system. It is possible, however, that the intracytoplasmic membranes have a chemical composition quite different from the outer membrane. Information is available about the chemical composition of membranes isolated from mesophiles such as *B. megaterium* (35), *Micrococcus lysodeikticus* (16, 26), *Staphylococcus aureus* (24, 32), *S. faecalis* (30), *B. licheniformis* (26), *Sarcina lutea* (26), *Listeria monocytogenes* (15), and *M. dentrificans* (29). In general, these reports indicate that the membranes represent 10 to 20% of the dry weight of the cells, and contain 15 to 36%, 42 to 70%, and 0.6 to 20% lipid, protein, and carbohydrate, respectively. The gross chemical composition of membranes of *B. stearothermophilus* does not significantly differ from these values reported for mesophiles, although thermophile spheroplasts are considerably more stable than the corresponding bodies of mesophiles. We have shown that membranes of *B. stearothermophilus* and *B. amyloliquefaciens* F can be dissolved by sodium dodecyl sulfate into membrane subunits (H. Bodman and Welker, Bacteriol. Proc., p. 107, 1966; N. E. Welker, *unpublished data*). A comparison of the chemical and physical properties of mesophile and thermophile membrane subunits should reveal some difference(s) which could explain the heat stability of thermophile spheroplasts.

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