JOURNAL OF BACTERIOLOGY, Feb. 1980, p. 914-927
0021-9193/80/02-0914/14$02.00/0
Vol. 141, No. 2

# Separation and Properties of the Cytoplasmic and Outer Membranes of Vegetative Cells of *Myxococcus xanthus*

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We have developed methods for separating the cytoplasmic and outer membranes of vegetative cells of *Myxococcus xanthus*. The total membrane fraction from ethylenediaminetetraacetic acid-lysozyme-treated cells was resolved into three major fractions by isopycnic density centrifugation. Between 85 and 90% of the succinate dehydrogenase and cyanide-sensitive reduced nicotinamide adenine dinucleotide oxidase activity was found in the first (I) fraction ($\rho$ = 1.221 g/ml) and 80% of the membrane-associated 2-keto-3-deoxyoctonate was found in the third (III) fraction ($\rho$ = 1.166 g/ml). The middle (II) fraction ($\rho$ = 1.185 g/ml) appeared to be a hybrid membrane fraction and contained roughly 10 to 20% of the activity of the enzyme markers and 2-keto-3-deoxyoctonate. No significant amounts of deoxyribonucleic acid or ribonucleic acid were present in the three isolated fractions, although 26% of the total cellular deoxyribonucleic acid and 3% of the total ribonucleic acid were recovered with the total membrane fraction. Phosphatidylethanolamine made up the bulk (60 to 70%) of the phospholipids in the membrane fractions. However, virtually all of the phosphatidylserine and cardiolipin were found in fraction I. Fraction III appeared to contain elevated amounts of lysophospholipids and contained almost three times the amount of total phospholipid as compared with fraction I. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis resolved approximately 40 polypeptides in the total membrane fraction. Two-thirds of these polypeptides were enriched in fraction I, and the remainder was enriched in fraction III. Fraction II contained a banding pattern similar to the total membrane fraction. Electron microscopy revealed that vegetative cells of *M. xanthus* possessed an envelope similar to that of other gram-negative bacteria; however, the vesicular appearance of the isolated membranes was somewhat different from those reported for *Escherichia coli* and *Salmonella typhimurium*. The atypically low bouyant density of the outer membrane of *M. xanthus* is discussed with regard to the high phospholipid content of the outer membrane.

*Myxococcus xanthus* is a gram-negative gliding bacterium that exhibits a primitive form of multicellular development. In the laboratory, developmental behavior can be induced by placing cells at high density on agar lacking sufficient nutrients for vegetative growth. Within a few hours of nutrient deprivation, the cells begin to aggregate towards discrete foci, forming macroscopic raised mounds called fruiting bodies. Within these fruiting bodies, a portion of the population differentiates into resistant myxospores (46, 55).

We have been interested in the possibility that cell surface components may be specifically involved in the developmental process. In particular, these components may provide receptors for chemotactic substances, may be involved in gliding motility, may play a role in physical interaction between cells, or may provide an as yet undefined means of communication between cells. To pursue this line of inquiry, it was necessary to separate and isolate the membranes of vegetative cells since there is no information available on the composition and properties of the isolated cytoplasmic and outer membranes of *M. xanthus*.

In this paper, we describe a procedure for separating the cytoplasmic and outer membranes of vegetative cells of *M. xanthus*, and we present data showing (i) the macromolecular composition, (ii) selected enzyme activities, (iii) phospholipid classes, (iv) polypeptide composition, and (v) morphology of the cytoplasmic and outer membranes.

The techniques described here have been applied to developing cells and several aggregation-deficient mutants (unpublished data) and will be the subject of a future publication.

## MATERIALS AND METHODS

**Bacteria and media.** Vegetative cells of *M. xanthus* MD-2 (37) were used in this study. Cells were grown in a medium containing 2% (wt/vol) Casitone (Difco)–10 mM potassium phosphate (pH 7.6)–8.3 mM magnesium sulfate (Casitone medium). Stock cultures were maintained in Casitone medium and transferred daily. To reduce variability, new stock cultures were started weekly from frozen, glycerol-myxospores (16).

**Growth and radioactive labeling.** Cultures were routinely grown at 31°C in 250-ml Erlenmyer flasks containing 40 ml of Casitone medium with vigorous agitation on a gyratory shaker (350 rpm; New Brunswick model G24). Cultures were harvested during logarithmic growth at $4 \times 10^8$ to $5 \times 10^8$ cells per ml (150 to 200 Klett units).

Radioactive labeling was carried out in Casitone medium. For routine labeling of membrane fractions, cells were grown for five to six generations in a medium containing 0.175 μCi of [$^{14}$C]acetate (2.5 μCi/μmol) per ml. For dual labeling experiments, cells were grown in a medium containing 3.7 μCi of [$^3$H]acetate (150 μCi/μmol) per ml for five generations before the addition of the second radioactive compound. Labeling procedures for specific determinations are described under the appropriate headings.

**Membrane isolation and fractionation.** Vegetative cells were collected by pouring cultures over one-third volume crushed ice in 50-ml centrifuge tubes, followed by centrifugation at 12,000 × *g* for 10 min. The cell pellet was resuspended and washed once in an equal volume of ice-cold distilled water. The washed cell pellet was resuspended to $5 \times 10^9$ cells per ml in 0.75 M sucrose–10 mM Tris-acetate (pH 7.8). Egg white lysozyme (Aldrich) was added to a final concentration of 300 μg/ml, followed by the addition of 100 mM EDTA (pH 7.8) to a final concentration of 0.5 mM. This mixture was incubated on ice for 12 to 15 h with stirring. The EDTA–lysozyme-treated cells were collected by centrifugation at 27,000 × *g* for 15 min and gently resuspended in an equal volume of cold distilled water by repeated pipetting. Incubation on ice for 30 min resulted in 99% conversion of the rod-shaped cells into round, phase-dark spheroplasts. The spheroplasts were reisolated by centrifugation at 12,000 × *g* for 10 min, the supernatant suspension was reserved, and the pellet was resuspended in 2 volumes of cold 5 mM EDTA (pH 7.8). Osmotic lysis was allowed to proceed on ice with stirring for 1 h before the supernatant suspension, which contained from 2 to 5% of the outer membrane, was added. After an additional hour of incubation, the membrane fraction was isolated by centrifugation (166,000 × *g* for 2 h). The membrane pellet was gently dispersed in 1 to 2 ml of 10 mM Tris-acetate (pH 7.8) with a 1-ml syringe fitted with an 18-gauge needle. The viscosity of the suspension was reduced by dilution with an equal volume of 10 mM Tris-acetate (pH 7.8), 5 mM $MgCl_2$, and 166 μg of DNase per ml (Sigma type I), followed by incubation on ice with intermittent mixing for 30 min or until the membranes were completely resuspended. (DNase was omitted when DNA determinations were performed. The omission of DNase resulted in slightly poorer resolution of the membrane fractions, but had no effect on the buoyant densities of the fractions.) Magnesium was removed by dialysis for 14 to 18 h against 10 mM Tris-acetate–3 mM EDTA (pH 7.8). The membrane fraction was reisolated by centrifugation (166,000 × *g* for 2 h) and resuspended in 25% (wt/wt) sucrose–5 mM EDTA (pH 7.8) with a 1-ml syringe fitted with progressively smaller-gauge needles, i.e., 18, 23, 26. This preparation, which contained both the cytoplasmic and outer membranes, was designated the total membrane (TM) fraction. The TM fraction was diluted to approximately 1 mg of membrane protein per ml and subjected to isopycnic density centrifugation in an SW 41 rotor (Beckman) as described by Osborn et al. (35). Gradients were fractionated by puncturing the bottom of the tubes and collecting drops (5 drops per fraction). Larger preparations, requiring more than three SW 41 gradients, were fractionated by puncturing the sides of the tubes with a coarse needle and collecting the visible membrane bands in a syringe.

**Analytical procedures.** Protein was determined by a modification of the microassay method of Bradford (6). The addition of 1 drop of 0.1 N NaOH per 5 to 20 μl of sample before the addition of the dye reagent greatly facilitated solubilization of the membrane proteins. Without this modification, large blue flakes were often seen in the cuvettes. Bovine serum albumin was used as a standard.

Phospholipids were extracted from whole cells by a modification of the method of Vorbeck and Marinetti (49). A washed cell pellet was resuspended in 10 volumes of methanol (volume/wet weight of cells) and refluxed at 65°C for 5 min. After cooling, chloroform was added to give a chloroform-methanol ratio of 2:1 (vol/vol), and extraction with stirring continued for 2 h at room temperature in a nitrogen atmosphere. Insoluble material was removed by filtration through a glass fiber filter under nitrogen pressure. Nonlipids were removed by solvent partitioning; 0.2 volume of 0.3% NaCl was added to the filtrate, the mixture was then vigorously agitated for 2 min on a Vortex mixer, and the phases were separated by centrifugation. The chloroform-methanol layer was transferred to a round-bottomed flask and taken to a paste on a rotary evaporator at 40°C. The paste was taken up in a small volume of chloroform-methanol (2:1), transferred to a 15-ml conical centrifuge tube, and taken to a paste under a stream of nitrogen. Residual water was removed from the sample by repeated resuspension and evaporation in chloroform under a stream of nitrogen, care being taken never to take the sample to complete dryness. Isolated membrane fractions were extracted for 2 h in 20 volumes of chloroform-methanol (2:1) with subsequent steps following those for whole cells.

Total lipid phosphorus was determined by the ultramicroassay of Bartlett (2) except that samples ($2.5 \times 10^{-3}$ to $4.0 \times 10^{-2}$ μmol of lipid phosphorus) were combusted in 0.2 ml of 70% perchloric acid rather than sulfuric acid and hydrogen peroxide. Also, phospholipid samples were taken to dryness before combustion.

Phospholipid classes were separated by thin-layer chromatography; 0.2 to 1.0 μmol of extracted phospho-

lipid was applied to Silica Gel G plates (Brinkmann Sil G-25, 20 by 20 cm glass plates, 0.25-mm silica gel without gypsum) and developed in a two-dimensional solvent system: (i) chloroform-methanol-water (95:35:5, vol/vol/vol) (ii) diisobutylketone-acetic acid-water (80:50:10, vol/vol/vol) by the method of Skipski and Barclay (43). The identification of specific phospholipid classes was facilitated by comparison with standards and by the use of group-specific spray reagents. Sprays used in this study were (i) ninhydrin (43) for detection of amino groups, (ii) Dragendorff spray (50) for detection of choline-containing phospholipids, (iii) periodate-Schiff spray (41) for detection of alpha-glycols, (iv) diphenylamine spray (43) for the detection of neutral glycolipids, and (v) ammonium molybdate spray (48) for the detection of phospholipids.

For quantitating the classes of phospholipids in *M. xanthus*, cells were grown for six generations in medium containing 1.9 $\mu$Ci of $H_3{}^{32}PO_4$ (carrier free) per ml. Extracted $^{32}$P-phospholipids were applied to silica gel plates, and the plates were developed as described above. Phospholipid spots, detected by autoradiography (31), were scraped off and counted as described under counting procedures. The amount of radioactivity in each spot was recorded as a percentage of the total radioactivity recovered from the plates. Over 95% of the radioactivity applied to the plates was recovered in the isolated phospholipid spots.

Lipopolysaccharide (LPS) was estimated by determining the amount of 2-keto-3-deoxyoctonate (KDO) in the membrane fractions. KDO was determined by the thiobarbituric acid method (52) as modified by Osborn et al. (35). Approximately 2 mg of membrane protein was required to give reproducible KDO determinations. The amount of KDO present in the total membrane fractions was greatly reduced upon dialysis after DNase treatment. Consequently, in membrane preparations where KDO was determined, dialysis was omitted; instead, DNase-treated membrane fractions were diluted eightfold with 10 mM Tris-acetate–3 mM EDTA (pH 7.8) and reisolated by centrifugation. The washed membranes were then incubated in a small volume of the above buffer and EDTA at 4°C for 12 to 14 h and reisolated as described for the dialyzed membrane fractions. The treatment had little effect on the subsequent separation of the membrane fractions and resulted in much improved recovery of KDO. The reason for the loss of KDO after dialysis is unknown.

The DNA and RNA content of the isolated membrane fractions was measured as acid-precipitable radioactivity from cells labeled with [$^{14}$C]thymidine or [$^{14}$C]uridine. Cultures, prepared as described for dual labeling, were grown for one generation (4 h) in medium containing 0.09 $\mu$Ci of [$^{14}$C]thymidine (54 $\mu$Ci/$\mu$mol) per ml or 0.375 $\mu$Ci of [$^{14}$C]uridine (49 $\mu$Ci/$\mu$mol) per ml. Isolated membrane fractions were precipitated with cold 10% trichloroacetic acid for 1 h on ice, and the precipitate was retained on glass fiber filters. The filters were washed with 0.2 N HCl followed by absolute ethanol. The filters were then dried and assayed for radioactivity as described under counting procedures. When cells were labeled with [$^{14}$C]thymidine, 100% of the acid-precipitable $^{14}$C radioactivity in the total membrane fraction was present in DNase-sensitive material. When cells were labeled with [$^{14}$C]uridine, approximately 15% of the acid precipitable $^{14}$C radioactivity in the total membrane fraction was in RNase A-sensitive material, with about 30% in DNase-sensitive material. We have assumed that the remaining RNase-insensitive material represents rRNA since, in whole cells, virtually all [$^{14}$C]uridine label incorporated after a one-generation pulse and DNase treatment is extracted as RNA (B. A. Smith and M. Dworkin, unpublished data).

**Gel electrophoresis.** The polypeptide composition of the isolated membrane fractions was analyzed by sodium dodecyl sulfate (SDS)-polyacylamide gel electrophoresis with the discontinuous buffer systems of Laemmli (28). The separation gel consisted of a 10-cm (0.75-mm thick), 12 to 18% linear acrylamide gradient. The addition of glycerol to the stock acrylamide solution (30% acrylamide, 0.8% bis-acrylamide, 23.8% glycerol) improved the resolution of the polypeptides. Glycerol was omitted in the stock solution for the stacking gel (28). Protein solubilization was accomplished by suspending the membrane fractions in final sample buffer (28) followed immediately by a 2.5-min incubation in a boiling-water bath.

The solubilized membrane preparations, approximately 20 $\mu$g of protein per slot, were applied, and electrophoresis was carried out at room temperature at 15 mA of constant current until 1.5 h after the tracking dye had left the bottom of the gel (total running time was approximately 6 h). Gels were stained overnight in Coomassie brilliant blue stain (51) and destained for 5 to 15 h in 7.5% acetic acid–5% methanol with gentle agitation. Gels were dried for 1 h on a Hoeffer gel dryer before photographing. Molecular weights were estimated by comparing relative mobilities to a standard curve prepared with beta-galactosidase (130,000), bovine serum albumin (68,000), ovalbumin (43,000), lactate dehydrogenase (36,000), and lysozyme (14,000) (51). For quick visualization of LPS in the membrane fractions, gels were stained for carbohydrate by the periodate-Schiff method for acrylamide gels (58). In this case glycerol was omitted from the stock acrylamide solution. This omission resulted in a slight decrease in resolution of the polypeptides.

**Radioisotope counting procedures.** Radioactivity was measured in a Beckman LS-230 liquid scintillation counter. The scintillation fluid was toluene-based 0.01% POPOP [1,4-bis-(5-phenyloxazolyl)-benzene]–0.5% PPO (2,5-diphenyloxazole). For aqueous samples, scintillation fluid was mixed 2:1 (vol/vol) with Triton X-100. Sucrose gradient fractions (usually 20 $\mu$l) were mixed with 0.8 ml of distilled water before the addition of scintillation fluid (6 ml). Samples containing chloroform or other organic solvents were taken to dryness in the scintillation vial before the addition of scintillation fluid. Scrapings from thin-layer chromatography plates were transferred to counting vials and resuspended in 6 ml of scintillation fluid containing Triton X-100.

**Electron microscopy.** Isolated membrane fractions were fixed overnight at 4°C in cacodylate-buffered 2% glutaraldehyde and post-fixed for 1 h in 1% osmium tetroxide, pH 7.2. Samples were stained for 1 h en bloc with aqueous 1% uranyl acetate and

dehydrated in increasing concentrations of ethanol. Epon 812 was the final embedding medium. A double stain was employed consisting of aqueous 2% uranyl acetate followed by lead citrate. Thin sections were examined with a Siemens Elmiskop I electron microscope.

**Enzyme assays.** NADH oxidase activity was measured in incubation mixtures containing 50 mM Tris-hydrochloride (pH 7.5), 0.12 mM NADH, and the membrane fraction (30 to 100 μg of protein) in a total volume of 1.0 ml. The rate of decrease in absorbance at 340 nm at 22°C was measured with a Zeiss PMQ II spectrophotometer coupled to a Sergent Welch model SR-L recorder. Cyanide sensitivity was measured in reaction mixtures containing 10 mM NaCN.

NADH dehydrogenase (EC 1.6.99.3) activity was measured in incubation mixtures containing 50 mM Tris-hydrochloride (pH 7.5), 0.12 mM NADH, 0.5 mM $K_3Fe(CN)_6$, and the membrane fraction (20 to 50 μg of protein) in the total volume of 1.0 ml. The rate of decrease in absorbance at 340 nm at 22°C was measured as described for NADH oxidase.

Succinate dehydrogenase (EC 1.3.99.1) was measured as described by Osborn et al. (35) with 10 to 50 μg of membrane protein.

Phospholipase A (EC 3.1.1.4) and lysophospholipase (EC 3.1.1.5) activity was measured as the conversion of [2-$^{14}$C]glycerol-labeled phospholipid to water-soluble products. To obtain substrate for the reaction, cultures of MD-2 were labeled for six generations in medium containing 0.19 μCi of [2-$^{14}$C]glycerol (16.4 μCi/μmol) per ml, and the phospholipids were extracted as described above. Ninety-three percent of the chloroform–methanol-extractable radioactivity was present in phospholipids as judged by thin-layer chromatography; the remainder of the radioactivity was associated with a large yellow spot which represented a heterogeneous group of pigments that were coextracted with the phospholipids. A portion of the crude phospholipid extract was saponified by the method of Brockerhoff (8); over 94% of the label (discounting the 7% found in the pigments) was recovered in the aqueous phase. No attempt was made to remove the yellow pigments from the phospholipids because they were judged not to constitute a significant fraction of the labeled compounds.

Phospholipase assays were performed in 15-ml conical glass centrifuge tubes. Incubation mixtures contained 80 nmol of crude phospholipid ($2.6 \times 10^5$ cpm/nmol), 30 μl of methanol, 90 μl of Triton X-100, 300 μl of 50 mM Tris-acetate (pH 7.8), 75 mM $CaCl_2$, 200 mM KCl, and 100 to 300 μg of membrane protein. The substrate, in chloroform, was added first and taken to dryness under a stream of nitrogen. Methanol and Triton X-100 were then added, and the phospholipids were dispersed by vigorous agitation on a Vortex mixer before the addition of the remaining components. The reaction mixtures were kept on ice before transfer to a 31°C water bath. At 0, 15, and 30 min of incubation, 150-μl samples were removed, and the reaction was stopped by transfer to 30-ml centrifuge tubes containing 2.5 ml of ice-cold chloroform-methanol (2:1, vol/vol). Solvent partitioning was accomplished by the addition of 0.5 ml of 0.3% NaCl, followed by 20 s of agitation on a Vortex mixer and brief centrifugation. A portion of the aqueous phase (0.8 ml) was transferred to a scintillation vial and counted as described under radioisotope counting procedures.

## RESULTS

**Isolation of the membrane fractions.** Fractionation of isopycnic sucrose density gradients produced the radioactive profile shown in Fig. 1. The three major membrane fractions were isolated by pooling fractions under each peak, indicated by the bars in Fig. 1 and diluting threefold with 10 mM Tris-acetate (pH 7.8) followed by centrifugation at 166,000 × *g* for 2.5 h. In some cases it was more convenient to isolate the membrane fractions by puncturing the sides of the tubes and withdrawing bands (see Materials and Methods). This procedure, if done with care, had little effect on cross-contamination of the three fractions. Membrane bands collected in this manner were reisolated by dilution and centrifugation as described for the pooled individual fractions. The membrane pellets were resuspended in 20 to 200 μl of dilution buffer and stored at 4°C. The isolated fractions, designated I, II, III, and the TM fraction were used to obtain the data presented in this section. Determinations were normally performed within 24 h of isolation; however, the fractions could be stored for longer periods frozen at −20°C in 25% glycerol with little effect on subsequent determinations; exceptions to this were NADH oxidase, which showed considerable loss of activity, and phospholipids, which showed considerable oxidation.

**Chemical composition of the isolated membrane fractions.** The macromolecular composition of the membrane fractions is shown

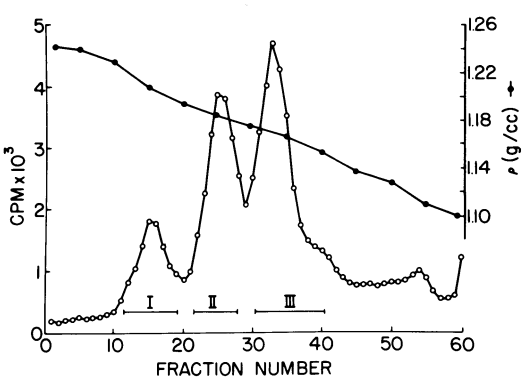


FIG. 1. *Isopycnic density centrifugation of the TM fraction of M. xanthus. Cells were grown in Casitone medium containing 0.175 μCi of [$^{14}$C]acetate per ml, and the total membrane fraction was isolated as described in the text. Fractions I, II, and III were obtained by pooling fractions from the areas of the gradient designated by the bars.*

in Table 1. The results shown are the average of three to five individual experiments. The internal consistency of each experiment was monitored by the distribution of succinate dehydrogenase activity and LPS content. Routine monitoring of LPS content in the membrane fractions was done by subjecting the fractions to SDS-polyacrylamide gel electrophoresis followed by Schiff-periodate staining as described in Materials and Methods. Except in rare instances, periodate–Schiff-positive material was located exclusively in the II, III, and TM fractions (data not shown).

There is about $3 \times 10^{-10}$ mg of protein per cell in *M. xanthus* MD-2. By the methods described, approximately 21% of this protein was recovered with the total membrane fraction. The distribution of protein between fractions I and III, which proved to be the cytoplasmic and outer membrane, respectively (see below), was roughly equivalent. Fraction II, which proved to be unresolved cytoplasmic and outer membrane, contained approximately 15% of the total recovered membrane protein. KDO, an eight-carbon sugar specific to LPS, has been used in numerous studies as the definitive marker of the outer membrane (1, 5, 22, 23, 35, 39, 40, 44). KDO was present in *M. xanthus* in concentrations of approximately $5 \times 10^{-12}$ μmol/cell. Approximately 40% of the total cellular KDO was isolated with the total membrane fraction and was found to be greatly enriched in fraction III. In the absence of DNase, 26% of the total cellular DNA (approximately $2 \times 10^{-8}$ μg/cell [59]) was isolated with the TM fraction. This percentage, which is several times greater than values found with other gram-negative bacteria, might reflect the gentle methods used to isolate the membranes (i.e., osmotic lysis was employed to disrupt the spheroplasts rather than sonic oscillation [5, 35]) in combination with the relatively high concentration of lysozyme (300 μg/ml) used to form spheroplasts (42). Fractionation of isopycnic-density gradients revealed that 83% of the DNA isolated with the TM membrane fraction banded at a density of 1.13 g/ml and therefore was not isolated with the membrane fractions (data not shown). The apparent low density of the DNA was presumably due to the low salt gradients used to isolate the membrane fractions (10, 33). The amount of cellular RNA (approximately $5 \times 10^{-8}$ μg/cell [B. A. Smith and M. Dworkin, unpublished data]) isolated with the total membrane fraction was negligible (3% of the total cellular RNA) when compared with the amount of DNA isolated. However, the total cellular RNA isolated with the TM fraction was approximately twice that found in *S. typhimurium* (35). The TM fraction of *M. xanthus* contained a high phospholipid-to-protein ratio, about 1.2 μmol of lipid phosphorus per mg of membrane protein. The bulk of this lipid appeared to be in fraction III (47%); fraction I had 18%. However, fraction I did contain virtually all of the chloroform–methanol-extractable pigments. The distribution of phospholipids and the considerable amount of pigment associated with the cytoplasmic membrane might be partially responsible for the atypical densities of the cytoplasmic and outer membrane (see Discussion).

**Enzyme activities associated with the isolated membrane fractions.** The distribution of enzyme activities in the isolated membrane fractions is shown in Table 2. NADH oxidase has been used in several studies as a marker of the cytoplasmic membrane (5, 18, 22, 23, 35, 40). Somewhat to our surprise the NADH oxidase activity was present in all the membrane fractions. However, as shown in Table 2, cyanide sensitivity of NADH oxidase was confined to fraction I (76% inhibition). Since NADH oxidase was measured as oxidation of NADH with oxygen as the terminal electron acceptor, we ex-

TABLE 1. *Macromolecular composition of isolated membrane fractions*[a]

| Fraction | Buoyant density (g/ml) | Recovery of protein (% total) | | Recovery of KDO (% total) | | Recovery of DNA (% total) | | Recovery of RNA (% total) | | Recovery of phospholipids (% total) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Cellular protein | Membrane protein | Cellular KDO | Membrane KDO | Cellular DNA | Membrane DNA | Cellular RNA | Membrane RNA | Cellular lipid | Membrane lipid |
| TM | | 21 | (100) | 40 | (100) | 26 | (100) | 3 | | >95 | (100) |
| I | 1.221 | | 47 | | 8 | | 3 | | ND[b] | | 18 |
| II | 1.185 | | 15 | | 12 | | 3 | | ND | | 35 |
| III | 1.166 | | 38 | | 80 | | 11 | | ND | | 47 |

[a] Values listed are the average of three to five individual experiments. Actual amounts of the macromolecular species, listed as "percent of total cellular" in this table, are as follows: protein, $3 \times 10^{-10}$ mg/cell; KDO, $5 \times 10^{-12}$ μmol/cell; DNA, $2 \times 10^{-8}$ μg/cell (59); RNA, $5 \times 10^{-8}$ μg/cell (B. A. Smith and M. Dworkin, unpublished data); phospholipid, $9 \times 10^{-11}$ μmol/cell. All measurements in this legend are subject to approximately 20% error.

[b] ND, Not determined.

TABLE 2. *Enzyme activities of isolated membrane fractions*[a]

| Enzyme | Total activity membrane bound (%) | Sp act[b] | | | |
|---|---|---|---|---|---|
| | | TM | I | II | III |
| NADH oxidase | >95 | 0.030 | 0.038 | 0.027 | 0.030 |
| NADH oxidase + 10 mM NaCN | >95 | 0.036 | 0.009 | 0.031 | 0.032 |
| NADH dehydrogenase | 65 | 0.900 | 0.420 | 1.227 | 1.201 |
| Succinate dehydrogenase | >95 | 0.115 | 0.271 | 0.061 | 0.012 |
| Phospholipase A + lysophospholipase | 90 | 2.720 | 2.850 | 3.880 | 2.430 |

[a] Enzyme activities listed are the average of three to five individual experiments.

[b] Specific activities of NADH oxidase, NADH dehydrogenase, and succinate dehydrogenase are expressed in micromoles of substrate oxidized per minute per milligram of protein. Phospholipase A and lysophospholipase activity is expressed as nanomoles of lipid phosphorus converted to water-extractable form per minute per milligram of protein.

amined the possibility that fraction III might contain an NADH dehydrogenase activity whose terminal electron acceptor was other than oxygen. Results of NADH dehydrogenase assays on the membrane fraction with potassium ferricyanide as the electron acceptor showed that indeed fraction III exhibited a very high NADH dehydrogenase activity. Succinate dehydrogenase activity has also been used as a marker of the cytoplasmic membrane (1, 5, 11, 17, 18, 22, 23, 35, 44). As shown in Table 2, this activity appeared to be significantly enriched in fraction I. This observation, coupled to the cyanide sensitivity of NADH oxidase activity and the distribution of KDO (Table 1), led us to conclude that fraction I represented the cytoplasmic membrane and fraction III represented the outer membrane, with fraction II representing an unresolved combination of cytoplasmic and outer membrane. Enzymes involved in phospholipid breakdown (Table 2) did not appear to follow the distribution found in other gram-negative bacteria. To date, all bacteria tested have shown significant enrichment of detergent-resistant phospholipase A and lysophospholipase activity (14) in the outer membrane (5, 35, 40). In *M. xanthus*, detergent-resistant phospholipase A and lysophospholipase activity appeared to be distributed equally in the membrane fractions.

**Phospholipid composition of whole cells and membrane fractions.** The phospholipid composition of *M. xanthus* has not been previously reported. In this study several methods of extraction were tested (4, 36, 49). All of the methods tested gave comparable results with regard to the classes of phospholipids extracted; however, a slight modification of the method of Vorbeck and Marinetti (49) yielded the most complete extraction ($9 \times 10^{-11}$ μmol of lipid phosphorus per cell). Classes of phospholipids of whole cells and membrane fractions, separated by thin-layer chromatography and detected by autoradiography, are shown in Fig. 2. As with phospholipid extraction, several one-dimensional and two-dimensional solvent systems were tested to find a procedure to maximize resolution of phospholipid classes (9, 30, 40, 54). The solvent system finally employed was a combination of the mixture of Scott et al. (40) in the first dimension and the second dimension mixture as described by Lepage (30). For routine monitoring of phospholipid classes, the first-dimensional solvent was adequate to resolve the six major phospholipids in whole cells. For quantitative analysis, however, the two-dimensional system was required. Table 3 lists the percentages of phospholipid classes found in each membrane fraction and whole cells. The table indicates the percentage of $^{32}P$ in individual spots, and therefore the values do not represent mole fractions of all phospholipids. As with several other gram-negative bacteria (35, 40, 53), phosphatidylethanolamine comprised the bulk of the phospholipid in whole cells (76%) and isolated membrane fractions. Additional phospholipid classes were found in the membrane fractions, e.g., phosphatidylserine and lysophosphatidylethanolamine. The accumulation of the additional lipid classes in the membrane fractions is probably due to the protracted isolation procedure. This would allow time for intermediates in phospholipid synthesis and breakdown to accumulate (see Discussion). Many attempts were made to identify the unknown phospholipids (unknown [UK] 1 and 2, Fig. 2 and Table 3). Both species gave negative reactions for choline and did not react with the periodate-Schiff reagent or the diphenylamine reagent. However, UK 1 showed a ninhydrin-positive reaction. This led us to suspect that UK 1 was a derivative of phosphatidylethanolamine or phosphatidylserine. Phosphomonomethyl- and phosphodimethylethanolamine (kindly supplied by W. R. Finnerty) were tested as well as lysophosphatidylserine, all with negative results.

**SDS-polyacrylamide gel electrophoresis**

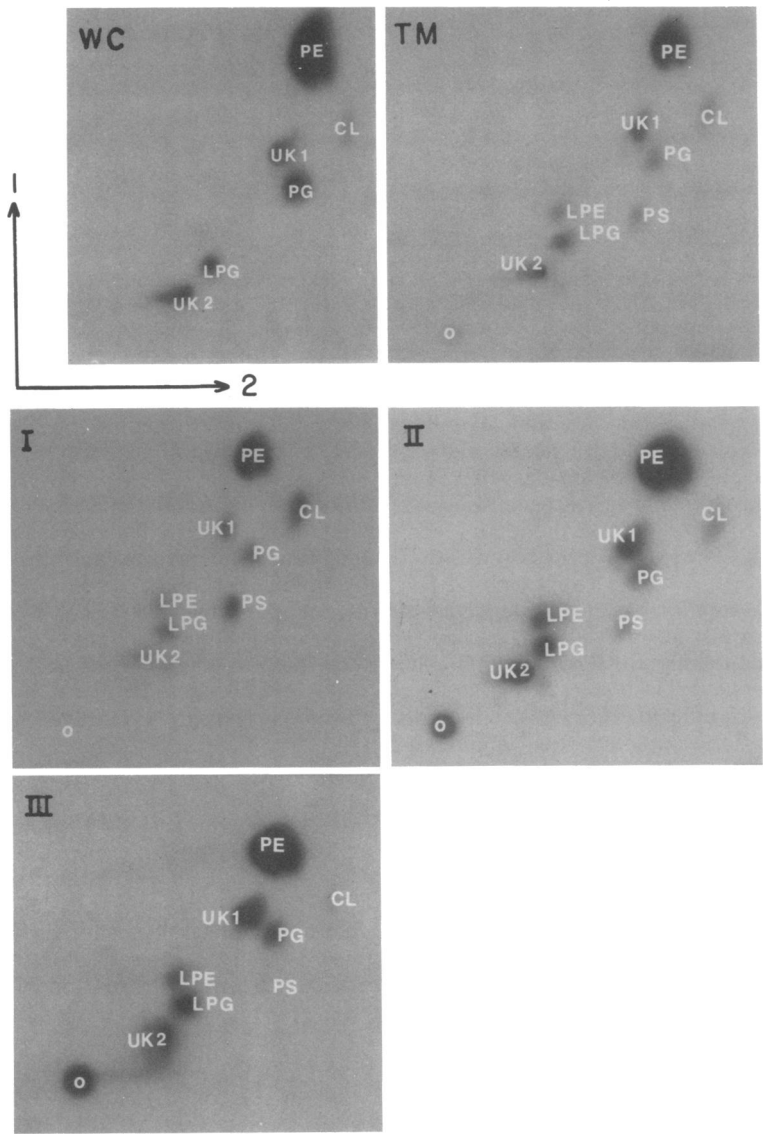


FIG. 2. *Autoradiograms of $^{32}P$-phospholipids from whole cells, spheroplasts, and membrane fractions from cells grown in Casitone medium containing 1.9 μCi of $^{32}P$ per ml. The lipid classes were separated by thin-layer chromatography and identified by the use of standards and group-specific spray reagents as described in the text. Whole cells (WC), TM fraction, membrane fractions I (cytoplasmic membrane), II (unresolved cytoplasmic and outer membrane), III (outer membrane).*

**of membrane fractions.** Polypeptides of the membrane fractions were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 3). The cytoplasmic membrane (fraction I) contained approximately 30 to 35 polypeptide bands. The outer membrane (fraction III) contained approximately 26 polypeptide species. Fraction II appeared to have a banding pattern similar to that of the TM fraction. The amounts of protein applied to the gel from fractions I, II, and III are not equal but represent the relative amounts of protein recovered from the isolated fraction; hence, fraction II represents approximately one-half of the protein compared to fractions I and III. The strongly stained band in a position corresponding to a molecular weight of 14,000 is believed to be lysozyme which associates with membranes during cell wall digestion (35, 42); TM fractions from cells disrupted by sonic oscillation do not show this band. Under the con-

ditions employed, 8 to 10 major protein bands appeared to be significantly enriched in the outer membrane as judged by their relative intensity of staining in the TM fraction. However, the two major protein species of the outer membrane of *E. coli* (38) and *S. typhimurium* (35) (molecular weights, 32,000 and 44,000, respectively) appeared to be absent in *M. xanthus*.

**Electron microscopy.** Thin sections of whole cells, spheroplasts, and isolated membrane fractions are shown in Fig. 4. The envelopes of whole cells have the trilaminar appearance of typical gram-negative bacteria (3, 12) in which a peptidoglycan layer is sandwiched between the cytoplasmic and outer membrane. The micrograph of a spheroplast suspension shows a spheroplast in which the outer membrane has pulled away from the cytoplasmic membrane. The small vesicles shown in the panel are very similar to outer membrane vesicles seen by Kulpa and Leive in *S. typhimurium* (27). The total membrane fraction appears to contain both vesiculated and non-vesiculated double-track membranes. Membranes from fractions I and II appear to be less vesiculated than those in the TM fraction. Fraction III shows predominantly closed or C-shaped open vesicles characteristic of the outer membranes of several other gram-negative bacteria (18, 38, 40) although somewhat smaller in size.

TABLE 3. *Distribution of $^{32}$P-labeled phospholipid in isolated membrane fractions*[a]

| Phospholipid | Phospholipid classes (% total phospholipid recovered)[b] | | | | |
|---|---|---|---|---|---|
| | Whole cells | TM | I | II | III |
| Phosphatidylethanolamine | 76.0 | 65.6 | 61.0 | 61.0 | 64.0 |
| Phosphatidylglycerol | 9.0 | 3.5 | 4.9 | 3.0 | 2.5 |
| Cardiolipin | 1.1 | 2.7 | 9.3 | 1.5 | 0.4 |
| Phosphatidylserine | 0.0 | 2.0 | 6.5 | 1.0 | 0.2 |
| Unknown 1 | 7.4 | 7.8 | 4.8 | 8.8 | 10.0 |
| Unknown 2 | 3.3 | 8.4 | 5.4 | 9.1 | 7.8 |
| Lysophosphatidylglycerol | 2.8 | 5.7 | 4.6 | 6.6 | 5.4 |
| Lysophosphatidylethanolamine | 0.0 | 3.1 | 3.4 | 4.1 | 7.7 |
| Origin | 0.0 | 1.2 | 0.1 | 4.9 | 7.7 |

[a] Values listed are from one experiment and represent the $^{32}$P radioactivity scraped from thin-layer plates whose autoradiograms appear in Fig. 2. Comparable results were obtained in a second and third experiment (not shown). The distribution of total phospholipids in the membrane fractions is shown in Table 1.

[b] Values are recorded as percent $^{32}$P radioactivity recovered as a particular phospholipid class and hence do not represent the mole fractions of each phospholipid class.

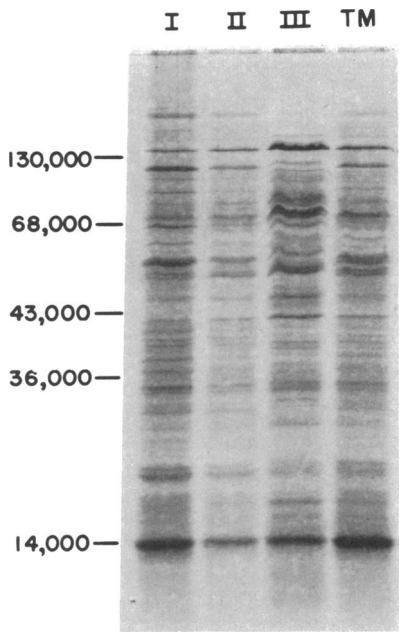


FIG. 3. *SDS-polyacrylamide gel electrophoresis of the membrane fractions from M. xanthus. Solubilized membrane fractions were subjected to electrophoresis on a 10-cm (0.75-mm-thick) linear 12 to 18% acrylamide gradient and stained with Coomassie brilliant blue stain as described in the text. TM fraction, membrane fractions I (cytoplasmic membrane), II (unresolved cytoplasmic and outer membrane), III (outer membrane).*

## DISCUSSION

The procedure described herein permits the separation of the cytoplasmic and outer membranes of *M. xanthus*. The two membranes were separated by taking advantage of their different buoyant densities essentially as described by Osborn et al. (35) for *S. typhimurium*. However, for *M. xanthus*, new methods had to be devised to digest the cell wall, obtain spheroplasts, and isolate the TM fraction. Efficient spheroplast formation required a long incubation with lysozyme-EDTA at low temperatures (12 to 14 h at 4°C). Shorter incubations at ambient temperatures or at 31°C resulted in spheroplast formation, but in this case the spheroplasts were small and refractile. Also, the conversion was asynchronous and subsequent membrane separation was poor. Spheroplasts were routinely lysed by osmotic shock in the presence of EDTA. Sonic disruption of the spheroplasts was not rigorously compared with osmotic lysis. Artifactual membrane hybrids have been observed during sonic oscillation (47). Thus, this treatment was avoided. The total membrane fraction was orig-

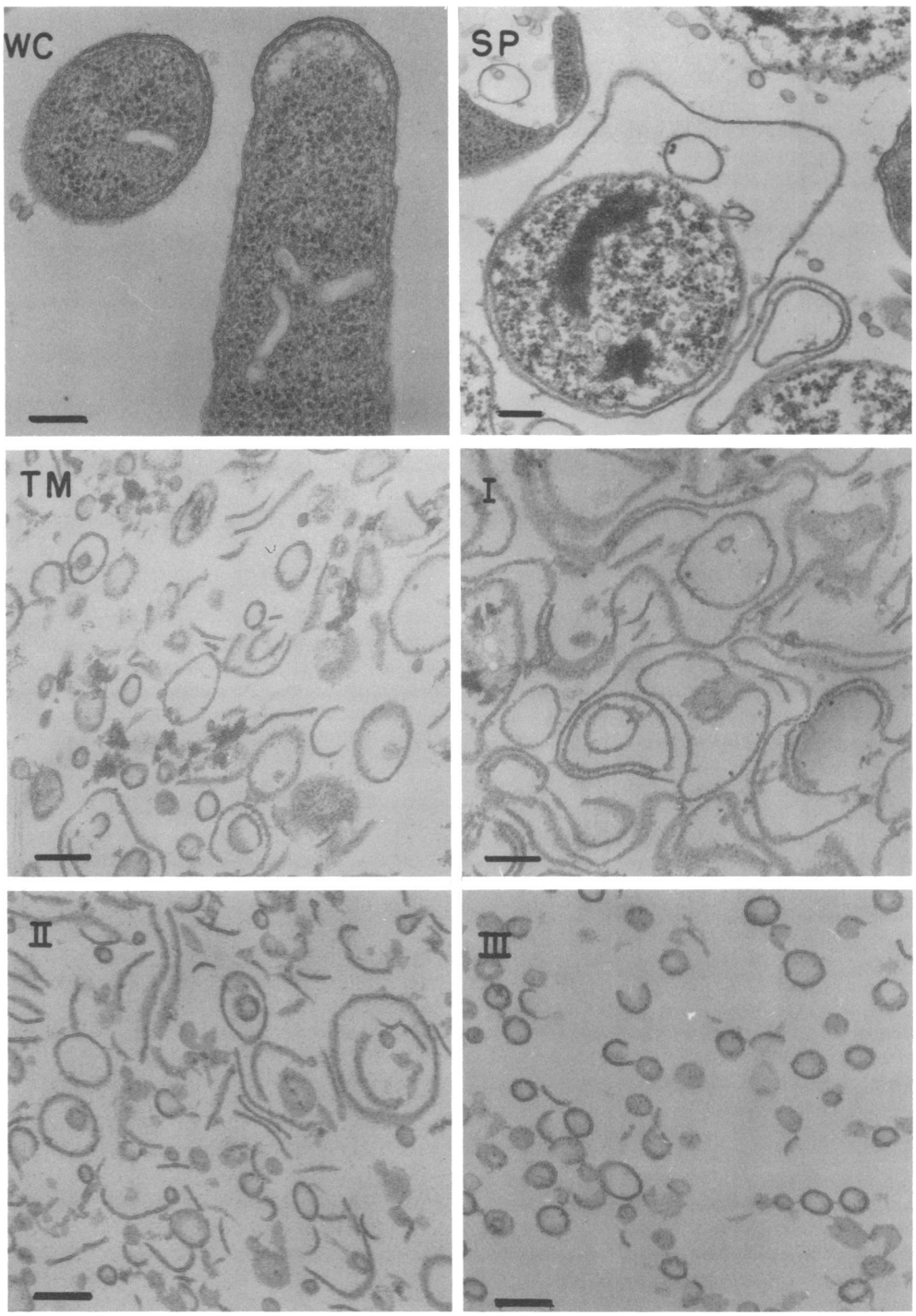


FIG. 4. *Electron microscopy of whole cells, spheroplasts, and membrane fractions of M. xanthus. Cells were grown in Casitone medium. Samples for whole cells (WC) were removed from the growth medium and washed once in distilled water before fixation. Spheroplasts (SP) were formed by lysozyme-EDTA treatment for 14 h as described in the text. The treated cells were then isolated by centrifugation and resuspended in distilled water for 10 min before reisolation and fixation. Membrane fractions (I, II, III) were collected from the density gradients with a coarse needle and syringe and reisolated by centrifugation. Samples were fixed and embedded as described in the text. The bar in each panel represents 0.15 μm. Whole cells (WC), spheroplasts (SP), membrane fraction I (cytoplasmid membrane), II (unresolved cytoplasmic and outer membrane), III (outer membrane).*

inally subjected to isopycnic density centrifugation the same day that it was isolated; however, it was soon discovered that several hours of incubation on ice after the initial isolation improved separation. This observation subsequently led to the DNase treatment followed by dialysis lasting about 14 h. Whereas the DNase treatment and dialysis could be omitted (as in experiments to determine DNA and KDO content, respectively), the 14-h incubation at 4°C postisolation was necessary for good separation. This effect may be due to a gradual hydration of tightly closed vesicles and hybrid membrane fragments formed during high-speed centrifugation. In addition, the incubation may allow the action of proteases or other membrane-associated enzymes to disrupt connection between cytoplasmic and outer membranes. Membrane separation was also greatly influenced by the mode of resuspension of the TM pellet both after the initial isolation and for resuspension in 25% sucrose-EDTA before isopycnic centrifugation. Large (5 to 10 ml) syringes appeared to have an adverse effect on membrane separation; large pressure changes were generated, and it was not easy to gauge the force applied to the preparation. The use of a 1-ml tuberculin syringe instead apparently set an upper limit on how much pressure could be applied, and its use resulted in preparations with reproducible properties.

Isopycnic density centrifugation of the TM fraction resulted in fractions enriched in the cytoplasmic and outer membrane (fractions I and III, respectively, in Fig. 1) as well as a "hybrid" fraction (fraction II). Fraction II could be resolved into two fractions by a second isopycnic density centrifugation. In this case, the sucrose gradient was expanded in the region of the densities at which fraction II originally banded. The resulting two fractions showed identity with the original fractions I and III. The purity of the two fractions, however, was less than that of the original fractions I and III (data not shown). The hybrid band could be eliminated entirely by using a step gradient composed of one-fifth 50% sucrose, three-fifths 43.4% sucrose, and one-fifth 30% sucrose in the presence of 5 mM EDTA. In this case, isopycnic density centrifugation deposited the outer membrane at the junction between the 30% and 43.4% sucrose and the cytoplasmic membrane between 43.4% and 50% sucrose (data not shown). This procedure resulted in less pure but acceptable separation. However, since purer preparations could be obtained with the original 25 to 50% sucrose gradients (described by Osborn et al. [35]), this method was preferred for the initial characterization of the membranes.

The macromolecular composition of the membrane fractions (Table 1) showed similarities and differences between *M. xanthus* and other gram-negative bacteria. The percentage of cellular protein in the total membrane fraction of *M. xanthus* is relatively high when compared with *Escherichia coli* (39) and *Salmonella typhimurium* (35). However, this was probably due to the method of membrane isolation and to the fact that rather large amounts of lysozyme remain tightly bound to the isolated membranes (see Fig. 3). About 40% of the LPS, as measured by KDO, was recovered in the TM fraction (as compared to whole cells). The loss of 60% of the LPS appeared to be unavoidable. This loss was probably due, at least in part, to the relatively high concentrations of EDTA used throughout the membrane isolation and fractionation procedure (29). However, EDTA (0.5 mM) was necessary for the efficient action of lysozyme, and higher concentrations (5 mM) were necessary for osmotic lysis of the spheroplasts and subsequent membrane separation. Mechanical methods of cell disruption (e.g., sonic oscillation or French pressure cell) which would have eliminated the need for lysozyme and EDTA were avoided both because of the possible formation of membrane hybrids (as mentioned earlier) and also the possible loss or inactivation of extrinsic (peripheral) membrane proteins. The loss of LPS did not appear to entail the loss of specific outer membrane proteins (26, 27, 56), i.e., we could not detect any gross decrease in specific membrane proteins in stained acrylamide gels when an envelope fraction from sonically disrupted cells was compared with our TM fraction (data not shown).

When DNase treatment was not employed, the DNA content of the TM fraction was high (26% of the total cellular DNA) when compared to that reported in *S. typhimurium* (1%) (35). As with the protein content, this was probably a reflection of the mode of disruption of the spheroplasts. Most of the DNA isolated with the TM fraction was not recovered in the gradient-derived membrane fractions because the DNA migrated independently and had a low bouyant density compared with the membrane fractions. We believe that the DNA isolated with the total membrane fraction is artifactually associated, possibly through electrostatic binding with lysozyme-membrane fragments (42) and that this complex is dissociated on passage through the sucrose density gradient. The RNA present in the total membrane fraction (3% of the total cellular RNA) is most probably rRNA since much of it (approximately 85%) is RNase A resistant.

Perhaps the most striking feature of the outer

membrane of *M. xanthus* is its high phospholipid content. In most gram-negative bacteria the phospholipid content is greatly reduced in the outer membrane (11, 18, 23, 35, 40, 53). The high phospholipid content may be responsible for the low buoyant density of the outer membrane, although this is probably not the only factor (see below). The high phospholipid content of the outer membrane may be a factor in this organism's increased sensitivity to the lipophilic antibiotics actinomycin D (15), rifampin, novobiocin, and thiostrepton (C. Manoil, personal communication). Nikaido (34) has produced convincing evidence that the sensitivity of gram-negative bacteria to lipophilic antibiotics is due to the ability of these antibiotics to traverse the outer membrane. This, in turn, appears to be related to the presence of exposed phospholipid bilayer regions which probably exist in the outer membrane of *M. xanthus*.

The enzyme activities of the membrane fractions (Table 2) indicated that the enzymes associated with oxidative phosphorylation (cyanide-sensitive NADH oxidase and succinate dehydrogenase) were located in fraction I. Fraction III appeared to be enriched in a cyanide-resistant NADH oxidase activity. It is unlikely that the cyanide-insensitive NADH oxidase activity in this fraction is involved in energy-yielding processes; rather, it is most probably an NADH dehydrogenase involved in the reduction of an as yet unspecified substrate within the membrane. Consistent with this conclusion, fraction III had high NADH dehydrogenase activity (Table 2). The distribution of cyanide-sensitive NADH oxidase and succinate dehydrogenase activities coupled to the distribution of KDO (Table 1) led us to conclude that fraction I represented the cytoplasmic membrane, and fraction III represented the outer membrane, with fraction II representing an unresolved combination of cytoplasmic and outer membrane. We were puzzled by the distribution of phospholipase A and lysophospholipase activities (Table 2). In all gram-negative bacteria examined, these activities appear almost exclusively in the outer membrane (5, 35, 40). Our results show that these activities are relatively uniformly distributed in the membrane fractions (Table 2). This result is somewhat paradoxical in view of the distribution of lysophospholipids in the membrane fractions (see Table 3 and below). However, comparable activities of these enzymes in the cytoplasmic and outer membranes could be partially responsible for the high precentage of phospholipid found in the outer membrane (Table 1).

There were six classes of phospholipids in freshly isolated whole cells (Fig. 3, Table 2). Phosphatidylethanolamine comprised the major class (76% of the total lipid phosphorus). This high proportion of phosphatidylethanolamine is also a characteristic of enteric bacteria (53, 35). With regard to other myxobacteria, Kleinig (25) has found that 72% of the lipid phosphorus of *M. fulvus* is present as phosphatidylethanolamine. He has also found a compound "X" that is similar to our UK 1 with respect to its ninhydrin reactivity. In addition, Kleinig found that 22% of the phospholipids of *M. fulvus* were alk-1-enyl acyl derivatives. Our results from base hydrolysis of extracted phospholipids (see phospholipase assay in Materials and Methods) suggest that such a high precentage of these derivatives is not possible in *M. xanthus*.

In contrast to the six phospholipid classes found in whole cells, the membrane fractions contained a minimum of eight phospholipid classes (Fig. 2, Table 3). Fraction I contained phosphatidylserine and an increased percentage of cardiolipin and lysophosphatidylethanolamine. By comparison to fraction I, fraction III has almost no phosphatidylserine, reduced amounts of cardiolipin, and substantial increases in the percentages of lysophosphatidylethanolamine, lysophosphatidylglycerol, two unknown components, and nonmigratory material (see Fig. 2 and Table 3). The presence of additional phospholipid classes in the membrane fractions tends to indicate that phospholipid metabolism continued during membrane isolation and fractionation.

SDS-polyacrylamide gel electrophoresis of the membrane fraction revealed that the total membrane fraction consisted of about 30 to 35 distinct polypeptide species (Fig. 3). The electrophoretic pattern of polypeptides in the membrane fractions was not similar to those found for the common enteric bacteria (35, 38). This is perhaps not too surprising since myxobacteria have radically different nutritional requirements (7, 37), modes of motility (19), and life cycles (55). With regard to the polypeptide composition of the membrane fractions, Inouye et al. (20, 21) have made an extensive examination of both soluble proteins and total membrane proteins during development of *M. xanthus* (20) and have described the synthesis and self-assembly of protein S, a soluble protein that is exported to the surface of myxospores (21). Comparisons between our polyacrylamide gel profiles and those of Inouye et al. (20) were not possible because of significant methodological differences in obtaining the TM fraction and in gel electrophoresis.

Electron microscopic examination of thin sections of whole cells, spheroplasts, and membrane

fractions (Fig. 4) revealed that the whole cells and spheroplasts of *M. xanthus* resembled those of other gram-negative bacteria (3, 12, 24). However, the isolated membrane fractions appeared to be somewhat different in that the membranes appeared generally less vesiculated. The membrane vesicles of fraction III most closely resembled published electron micrographs of the outer membranes of other gram-negative bacteria. The vesicles were small and bounded by a comparatively electron dense membrane and were frequently open. However, the outer membrane vesicles of *M. xanthus* were about one-third smaller in diameter than those observed in *E. coli* (39), *S. typhimurium* (35), and *Proteus mirabilis* (18). The small size of the vesicles might be due to the prolonged EDTA treatment during isolation; the outer membranes of *Pseudomonas aeruginosa*, after EDTA treatment, look very similar to the outer membranes of *M. xanthus* both in size and general morphology (32).

The finding that the outer membrane of *M. xanthus* was less dense than the cytoplasmic membrane was highly unusual but not unique. We are aware of one other organism, *Acinetobacter* sp. (40), that shows this characteristic. However, *Acinetobacter* sp. does not show a pronounced difference in buoyant densities of the cytoplasmic and outer membrane, and this condition of reversed densities is dependent upon growth conditions (40). It is currently believed that the carbohydrate side chains of LPS are responsible for the higher density of the outer membrane (13); and there is ample evidence to support this contention (26, 56, 27). However, it is not clear that LPS or more specifically the carbohydrate side chains determine, in a gross sense, the density of the outer membrane; membranes from *E. coli* depleted of 70% of their LPS (57) will still separate efficiently on the basis of buoyant density differences, and outer membranes from glucose-deficient (Rd) strains of *S. typhimurium* show virtually no difference in buoyant density over control Ra (complete core) strains (35). In the case of *M. xanthus* it is reasonable that the loss of 60% of the LPS during membrane isolation would affect the buoyant density of the outer membrane. Nevertheless, we feel that in *M. xanthus* the high phospholipid content (among other more complex factors [45]) plays the most important role in determining the density of the membranes. Another factor that may contribute to the large difference in the relative density of the two membranes in *M. xanthus* is the presence of a heterogeneous group of yellow pigments which are located in the cytoplasmic membrane (P. E. Orndorff and M. Dworkin, unpublished data). These pigments are relatively hydrophilic (M. Dworkin, unpublished data) and would tend to increase the buoyant density of the cytoplasmic membrane relative to the lipid-rich outer membrane. With regard to the influence of pigments upon the density of membranes, it should be noted that the cytoplasmic and outer membranes of another highly pigmented gliding bacteria, *Flexibacter elegans*, have been described (22). The outer membrane of this organism has an unusually low buoyant density. The authors have speculated that this condition is due to the highly lipophilic flexirubin pigments which are located in the outer membrane. The pigment composition of *M. xanthus* is poorly characterized at present. However, the influence of these molecules on the fundamental structure of the membranes would be substantial.

We are currently examining the membrane protein composition of cells in the aggregation phase of development and hope these studies will aid in identifying cell surface components involved in intercellular communication and cooperation during fruiting body formation.

## ACKNOWLEDGMENTS

This research was supported by Public Health Service research grant GM-19957 from the National Institutes of Health. P.E.O. is a predoctoral trainee supported by Public Health Service training grant CA-09138 from the National Institutes of Health.

We gratefully acknowledge the expert technical assistance of Donna Ritzi in performing the electron microscopy.

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