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Identification and Preliminary Characterization of *Vibrio cholerae* Outer Membrane Proteins

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Outer membrane proteins of *Vibrio cholerae* were purified by sucrose density centrifugation and Triton X-100 extraction at 10 mM Mg^{2+}. The proteins were separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. *V. cholerae* outer membrane proteins presented a unique pattern when compared with the patterns of other gram-negative rods. There were 8 to 10 major bands (M_r 94,000 to 27,000), with most of the protein located in band 5 (M_r ~45,000), which thus appears to be the major structural protein of the outer membrane. Lipid and carbohydrate were associated with band 6.

Outer membranes of gram-negative bacilli are composed primarily of proteins, lipids, and lipopolysaccharide. The outer membranes of members of the *Enterobacteriaceae* and *Pseudomonadaceae* (8, 10, 18, 19, 26, 28, 34, 36) have been characterized, but little has been reported on the outer membrane of *Vibrio cholerae* (23, 37). *V. cholerae* differs from members of the *Enterobacteriaceae* and *Pseudomonadaceae* by having a lipopolysaccharide which lacks 2-keto-3-deoxyoctonate (29-31).

Rough cholera strains are avirulent (16), and recent mutant studies suggest that other cell surface changes may play a role in the virulence of *V. cholerae* (6). We studied the outer membrane proteins of *V. cholerae* strain CA401, a virulent wild-type strain.

Slightly modified methods, similar to those used for isolation of outer membranes of *Enterobacteriaceae*, were successful in obtaining outer membrane proteins of *V. cholerae*. Characterization of these proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis revealed 8 to 10 major proteins, with more than 90% of the outer envelope protein appearing in a band having an M_r of ~45,000.

In this paper we present a useful method for isolating outer membrane proteins of *V. cholerae*, assign numbers to outer membrane proteins, and discuss some of the unique features of the *V. cholerae* outer envelope.

MATERIALS AND METHODS

Bacterial strain. *V. cholerae* CA401 is a classical Inaba strain isolated in 1953 in Calcutta, India (22), which has been extensively characterized (3-6, 35). Stock cultures were kept lyophilized or frozen at −70°C in brain heart infusion broth plus 15% glycerol. Working stocks were kept on meat extract agar slants (2) at 4°C for no longer than 3 weeks.

Cell culture and harvesting. *V. cholerae* was routinely grown in the semisynthetic medium (Syncase) of R. A. Finkelstein and C. E. Lankford (Bacteriol. Proc., p. 43, 1955) to mid-logarithmic growth phase and was occasionally grown in other media for comparison: complex medium, meat extract broth (2), and defined minimal A medium (27). Cultures were grown aerobically by inoculating approximately 20 ml of medium with 2 ml of an overnight culture in the same medium and incubating it at 35 to 37°C with shaking (180 to 200 rpm) in a New Brunswick shaking waterbath until mid-logarithmic phase. Ten milliliters of this culture was used to inoculate 400 ml of the same medium in a 2-liter Erlenmeyer flask and incubated at 37°C, 180 to 200 rpm, until mid- to late logarithmic phase (absorbance at 600 nm was 0.4 to 0.6). To compare growth phases of CA401, Syncase-grown cells in early log, mid-log, and stationary phases were also studied. Cells were harvested by centrifugation at 5,000 to 6,000 × *g* for 20 min. This step and all subsequent steps were performed at 4°C unless otherwise indicated.

Cells were washed once with 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer containing 10 mM $MgCl_2$. The cell pellet was then frozen and stored at −70°C until the membrane preparations were made.

Preparation of total envelopes. Frozen pellets were thawed and suspended in HEPES buffer containing 20% (wt/vol) sucrose. Approximately 3 μg of DNase (Sigma Chemical Co., St. Louis, Mo.) per ml was added to the suspension, and it was incubated for 30 min at 4°C. The cell suspension was then either treated with ultrasonic oscillations or passed through a French press at approximately 15,000 lb/in^2. Cell debris and unbroken cells were removed by centrifugation at 5,000 × *g* for 20 min. The cell lysate was diluted with an equal volume of HEPES buffer to lower the sucrose concentration and layered onto a sucrose step gradient containing 1 ml of 65% (wt/vol) sucrose and 5 ml of 15% (wt/vol) sucrose in HEPES

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buffer, and the tubes were centrifuged for 1 h at 38,000 rpm in a Beckman SW41 rotor. The dense orange layer at and slightly below the 65%-15% interface was collected as the envelope fraction and either used in assays or further fractionated. This total envelope fraction could be frozen and stored at −70°C before use in the cytochrome *c* determinations.

Separation of outer and inner membranes. The total envelope fraction (approximately 2.0 ml) was layered onto 10.0 ml of a linear sucrose density gradient of 45 to 60% (wt/vol) sucrose in HEPES buffer and centrifuged for approximately 24 h at 38,000 rpm in a Beckman SW41 rotor. An orange layer, near the center of the tube, and a grayish-white layer, about 0.5 cm below the orange layer, were collected by aspiration. These fractions were either washed once in HEPES buffer (by centrifugation at 45,000 rpm in a Beckman Ti 50 rotor for 1 h) and labeled crude inner and outer membranes or treated with Triton X-100 as described below. These fractions could be stored frozen at −70°C until gel electrophoresis or cytochrome *c* assays were performed.

Triton X-100 extraction. Triton X-100 extraction was carried out according to Diedrich et al. (9), using a final concentration of 2% Triton X-100 (Bio-Rad Laboratories, Los Angeles, Calif.). The Triton-soluble proteins in the supernatant were precipitated with 2 volumes of cold 95% ethanol, incubated at −20°C for 1 to 2 h, and pelleted by centrifugation for 30 min at 12,000 × *g*. Both the Triton-soluble, ethanol-insoluble and the Triton-insoluble pellets were then resuspended in 60 mM sodium phosphate buffer (pH 7.2) at a protein concentration of approximately 1 mg/ml. These fractions were either assayed for cytochrome *c* or stored frozen at −70°C until gel electrophoresis was performed.

Analytical procedures. Protein was determined by the method of Lowry et al. (25) with bovine serum albumin as the standard. Assay for the presence of cytochrome *c* was done according to Matsushita et al. (26), using the extinction coefficient for cytochrome *c* as in reference 21.

Polyacrylamide gel electrophoresis. Two polyacrylamide gel electrophoresis systems were used: (i) an SDS-urea slab gel system (a modification of the Laemmli [24] system) and (ii) a discontinuous buffer system with tube gels (7). The slab gel system had a separation gel of 11.5% acrylamide, 0.21% bisacrylamide, 0.1% SDS, and 3% urea in a 0.375 M Tris-hydrochloride buffer, pH 8.8. It was polymerized with 40 μl of *N,N,N′,N′*-tetramethylethylenediamine (TEMED) and 0.5 ml of a 10% solution of ammonium persulfate per 100 ml of gel. The stacking gel (approximately 1 cm deep) was 3% acrylamide, 0.08% bisacrylamide, and 0.1% SDS in 0.125 M Tris-hydrochloride, pH 6.8. It was polymerized with 10 μl of TEMED and 0.1 ml of 10% ammonium persulfate per 10.0 ml of stacking gel. The gels were 0.75 mm thick and either 10 or 20 cm long. The electrophoresis buffer was 0.0255 M Tris-hydrochloride and 0.192 M glycine (pH 8.3), with the upper buffer having SDS added to a final concentration of 0.1%. The samples were diluted 1:2 with the sample buffer (which contained 12.5% glycerol, 1.25% SDS, and 1.25% 2-mercaptoethanol in 0.25 M Tris-hydrochloride buffer [pH 6.8] with bromphenol blue added as a tracking dye), solubilized by boiling for 5 min, and applied to gels to obtain 20 to 30 μg of protein per lane. The gels were run with an ISCO model 493 power source, using constant voltage set at 60 V until the tracking dye had entered the separation gel and then increased to 160 V until the tracking dye reached the bottom of the gel (about 4 h total for the short gel and approximately 16 h for the longer gel). The gels were stained for protein with Coomassie blue R250 (Sigma), destained according to Fairbanks et al. (11), and dried onto Whatman no. 3 filter paper, using a Bio-Rad slab gel drier. The dried gels were scanned with a Joyce-Loebl Chromoscan 201 gel scanner with a 520-nm filter.

Slab gels were also stained with the cationic carbocyanine dye 1-ethyl-2-[3-(1-ethylnaphtho(1,2*d*)-thiazolin-2-ylidene)-2-methylpropenyl]-naphtho(1,2*d*) thiazolium bromide (Stains-All; Eastman Kodak Co., Rochester, N.Y.) by a modification of the procedure of Green et al. (17). Before staining, gels were fixed and washed extensively for 24 h in 25% isopropanol in 3% acetic acid to remove SDS. When no more SDS was detected (SDS produced foam or bubbles when shaken), gels were stained overnight in the dark with a working solution of stain. Working stain contained 10 ml of stock (10 mg of Stains-All in 10 ml of formamide, made fresh daily), 10 ml of formamide, 30 ml of isopropanol, 1.0 ml of 3.0 M Tris-hydrochloride (pH 8.8), and 0.1 ml of 2-mercaptoethanol, with distilled water to 200 ml. They were destained slowly with 15% isopropanol in 0.1 M Na_2HPO_4 (pH approximately 9.2) plus 200 μl of 2-mercaptoethanol per 400 ml of solution. The gels were observed at frequent intervals for color, since the protein stained best at high pH and the lipopolysaccharide stained best at a lower pH. Gels were stored in the dark and could be restained if the color faded too rapidly.

RESULTS

Preparation of total envelope. *V. cholerae* cells were broken either by passage through a French press or by sonication, but good separation of outer membranes in subsequent procedures occurred only when the French press was used. Plasmolysis of *Vibrio* cells before breakage was necessary for optimal separation of inner and outer membrane fractions. Use of a step gradient when separating total envelope from cytosol was a key step, and this method allowed us to collect a concentrated total membrane fraction which had not been packed into a pellet.

Separation of inner and outer membranes. The most effective procedure for separation of inner and outer membranes was a linear 45 to 60% sucrose gradient in HEPES buffer. After centrifugation, the inner membrane (top band) formed a layer approximately one-half the distance down the tube (density, 1.18) and was orange-pink in color. About 0.5 cm below the orange band, a second band (density, 1.23; outer membrane) grayish-white in color was seen. The two bands were easily visualized and could be

collected by manual aspiration. However, complete separation of outer membranes was not accomplished. Cytochrome *c* determinations showed contamination of the lower band by the upper to be 10% or more (Table 1). Additionally, the orange color associated with the upper band was visible in the material from the lower band, even after washing.

Triton X-100 extraction. Triton X-100 did not completely solubilize inner membrane proteins from total envelope fractions of *V. cholerae*, although it does so with *Escherichia coli* and *Pseudomonas aeruginosa* (9, 28, 32, 33). However, Triton X-100 treatment of the crude outer membrane fraction (obtained from linear sucrose gradients) solubilized inner membrane proteins and left only 0.3% contamination estimated by cytochrome *c* content (Table 1).

The concentration of Mg^{2+} during the Triton X-100 extraction is critical (33). Various concentrations of Mg^{2+} were tested for their ability to solubilize inner membrane proteins while retaining the insolubility of outer membrane proteins. The optimal Mg^{2+} concentration was between 1.0 and 10.0 mM (Fig. 1), and 10.0 mM Mg^{2+} was used throughout the remainder of these experiments.

SDS-polyacrylamide gel electrophoresis. Alkaline tube gels (7) did not resolve *V. cholerae* membrane proteins well, and a slab gel system was used routinely. Figure 2 shows a photograph of such a gel, demonstrating the patterns obtained with *V. cholerae* CA401 cytosol (A), Triton-soluble inner membrane (B), Triton-insoluble outer membrane (C), and molecular weight markers (D and E). Outer membrane proteins have been numbered for convenience. Cytosol and inner membrane fractions showed patterns consistent with those of other gram-negative organisms. More than 50 different peptide bands were present in the cytosol fraction, and more than 30 different bands were seen in the inner membrane fraction. The outer membrane fraction showed 7 to 9 major bands with Coomassie blue stains. The most prominent band (band 5) accounted for approximately 85 to 90% of the isolated protein and had an approximate M_r of 45,000. Band 5 ran as a doublet when smaller amounts of protein were applied. When the outer membrane fraction was solubilized in SDS only (no mercaptoethanol) or was heated only to 50°C, most of band 5 migrated at a high apparent molecular weight, ≥100,000. Incubation of broken cells with lysozyme before membrane isolation had no effect on the pattern of outer membrane proteins.

Lane F of Fig. 2 contained purified cholera toxin (obtained from R. A. Finkelstein, University of Missouri, Columbia; 14, 15) treated in the same manner as the other preparations. The large band with an apparent M_r of 21,000 is subunit A_1. Subunit B migrated with the tracking dye.

Table 2 lists the bands of the outer membrane fraction with their approximate molecular weight (M_r) values and their staining characteristics with Stains-All dye. M_r values for the bands ranged from 93,000 to 27,000. Band 6 (M_r 35,000) appeared purple to blue (depending on pH), indicating the presence of glycoprotein. Band 6 stained yellow or orange at low pH, indicating the presence of lipoprotein.

Effect of growth conditions on polypeptide pattern. Outer membrane proteins from

TABLE 1. *Comparison of densities and cytochrome c contents of membrane fractions of V. cholerae*

Envelope prepn	Density (g/ml)	Cytochrome *c* (nmol/mg of protein)	% Contamination
Total		2.74	
Crude inner	1.17	0.99	
Crude outer	1.23	0.11	11.1
Triton-soluble inner		2.30	
Triton-insoluble outer		0.007	0.3

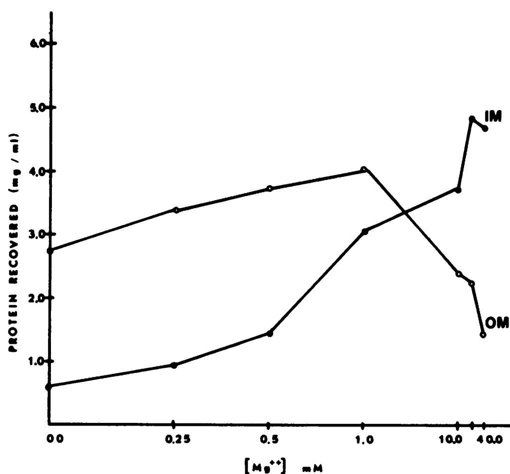

FIG. 1. *Effect of magnesium ion concentration on protein recovery. Identical samples of V. cholerae CA401 cultures were washed in 50 mM HEPES buffer (pH 7.4) with various concentrations of* Mg^{2+} *(0, 0.25, 1.0, 10.0, 20.0, and 40.0 mM). The pellets were then processed as usual except that the test buffers replaced the 50 mM HEPES-10 mM* Mg^{2+}*. The final outer membrane (OM) and inner membrane (IM) protein pellets were suspended in 1.0 ml of 60 mM* PO_4 *buffer (pH 7.2), and total protein was determined (25). Protein recovered was plotted against the concentration of* Mg^{2+} *used.*

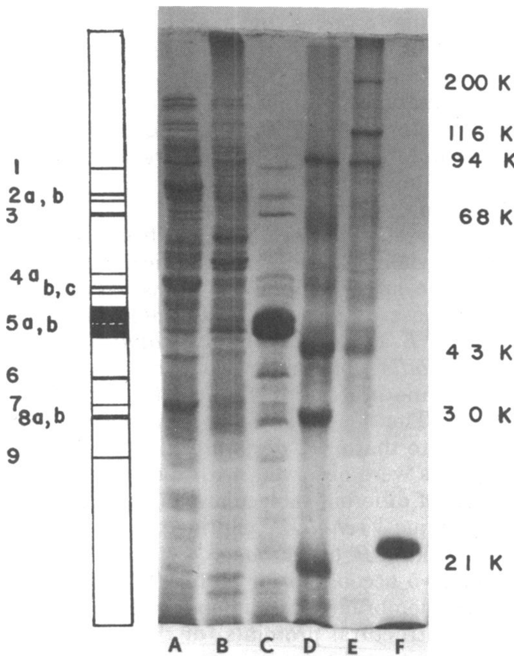

FIG. 2. *SDS-polyacrylamide gel electrophoresis of V. cholerae strain CA401 proteins. Protein compositions of V. cholerae cytoplasm, inner membrane, and outer membrane are shown in lanes A, B, and C, respectively. A schematic diagram of outer membrane proteins is given at the left, with the major bands identified. Bio-Rad molecular weight markers are in lanes D and E as follows: lane D contains phosphorylase B (M_r 94,000), bovine serum albumin (M_r 68,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000), and soybean trypsin inhibitor (M_r 21,000), and lane E contains myosin (M_r 200,000), β-galactosidase (M_r 116,500), phosphorylase B, bovine serum albumin, and ovalbumin; the right-hand side shows the positions of these marker proteins. Lane F contains purified cholera toxin; under the reducing and denaturing conditions used, only subunit A_1 (M_r ~21,000) is visualized.*

V. cholerae CA401 grown in complex broth medium, Syncase, and defined minimal medium were isolated and electrophoresed (Fig. 3). The peptide patterns of the major bands from the cells grown in either the complex broth or Syncase were identical, but the pattern from the cells grown in minimal broth showed differences. A new major protein, which ran slightly behind band 2, appeared concurrently with an absence of or decrease in bands 2, 4a, and 4b.

The influence of stage of growth on outer membrane proteins was examined by using outer membrane preparations from strain CA401 (grown in Syncase medium) at early log growth phase, middle log growth phase, and stationary phase. The preparation from early-log-phase cells (Fig. 3) showed an increase in band 2a and an increase in the number of minor bands in the high-molecular-weight range. Stationary-phase cells gave a pattern similar to that of mid-log-phase cells.

TABLE 2. *Characteristics of the outer membrane proteins of V. cholerae CA401*

Band[a]	M_r	Color with Stains-All	% Total protein
1	93,000	Red	0.7
2a, b	81,000	Red doublet	1.1
3	74,000	Red	2.4
4a	52,000	Not visualized	1.5
b	49,000	Not visualized	0.1
c	47,000	Not visualized	0.1
5a, b	42,000–51,000[b]	Red doublet	91.7
6	35,000	Yellow-red with bluish center	1.2
7	32,000	Red	0.1
8a, b	30,000	Red doublet	0.7
9	27,000	Red	0.4
Tracking dye-lipoprotein[c]	<21,000	Orange-yellow	—[d]

[a] Bands 7 and 9 appear faintly.

[b] When smaller amounts of protein were run, the major band appeared at M_r 45,000, with a smaller band at a higher M_r.

[c] Bromphenol blue was the tracking dye. Low-molecular-weight proteins ran faster than bromphenol blue. A lipoprotein of low molecular weight was seen in gels stained with Stains-All.

[d] —, Not determined, as tracking dye interfered with scan.

DISCUSSION

Highly purified *V. cholerae* outer membrane proteins were reproducibly isolated without using EDTA. The latter point is important, since several workers have shown that use of EDTA in isolating outer membrane proteins of *P. aeruginosa* and *Proteus mirabilis* led to altered structure and/or early release of some outer membrane proteins (20, 26). *V. cholerae* is sensitive to EDTA (1). Furthermore, a recent study of *V. cholerae* outer membranes which used EDTA (23) showed only 3 to 6 outer membrane proteins as contrasted to more than 10 membrane proteins described here.

Although the densities of crude inner and outer membrane fractions were similar to those of other gram-negative organisms, we could not get good separation of the two membranes on

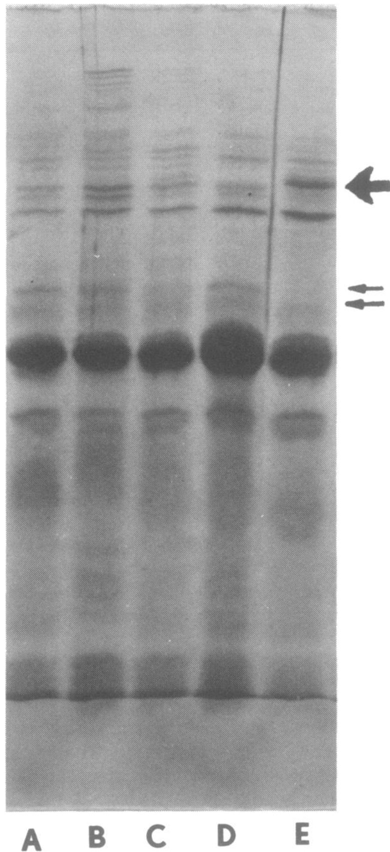

FIG. 3. *SDS-polyacrylamide gel electrophoresis of V. cholerae outer membrane proteins comparing different growth conditions. V. cholerae was grown to early logarithmic growth phase (B), middle logarithmic growth phase (C), or stationary phase (D) in Syncase medium, and outer membrane proteins were isolated and electrophoresed; V. cholerae was also grown in meat extract broth (A) or defined minimal medium (E) (note that growth in defined medium resulted in a new major outer membrane protein [large arrow] and loss of bands 4a and 4b [small arrows]).*

sucrose gradients. Adhesion sites (10) between the membranes in *V. cholerae* might interfere with mechanical separation of the membranes, or the chemical composition of outer membrane components could differ from those of previously studied gram-negative rods. Another explanation could be that the secretory behavior of *V. cholerae* might result in linking inner and outer membranes more tightly than in most gram-negative rods.

Unlike *E. coli* or *P. aeruginosa* total envelopes, the total envelope of *V. cholerae* could not reliably be fractionated into inner and outer membrane proteins by simple Triton X-100 treatment. Triton X-100 solubilized some, but not all, inner membrane proteins. Unusual vesicle formation which limited exposure of the inner membrane to Triton X-100 could have occurred. Alternatively, this failure again might involve an increased number of adhesion sites between the two membranes.

The peptide pattern observed from the SDS-polyacrylamide gel electrophoretic analysis of *V. cholerae* outer membrane proteins was different from those patterns found in enteric bacteria, such as *E. coli*, *Salmonella typhimurium*, and *P. mirabilis*, which show two to four major outer membrane proteins in the M_r 30,000 to 40,000 range. The *V. cholerae* pattern was broadly similar to that of *P. aeruginosa*, in that 8 to 10 peptides were observed, with four to six major bands of different molecular weights. However, the unique *V. cholerae* peptide pattern differed from that of *P. aeruginosa*.

Band 5 accounted for 85 to 90% of the total outer membrane protein and is probably the major structural protein(s) of the outer membrane. When the sample was applied in lesser concentrations, band 5 separated into two bands, and thus appears to contain at least two related peptides. It may be similar to the major outer membrane band of *E. coli*, which was later resolved into three to five bands (10).

When the outer membrane fraction was solubilized without mercaptoethanol or at temperatures below 50°C, most of band 5 migrated at a higher molecular weight, indicating that it may exist as a polymer in vivo. The matrix protein of *E. coli* has been shown to form an aggregate in solution (10).

Fernandes and co-workers (12, 13) suggested that cholera toxin is possibly membrane bound or assembled at the membrane level before secretion. Under the denaturing conditions used in these experiments, cholera toxin would exist in subunits as A_1 (approximately M_r 21,000), A_2 (approximately M_r 6,000), and B (approximately M_r 11,000). In our gel system, the A_2 unit would not be detected due to its small size, the B subunit would migrate with (or slightly ahead of) the tracking dye, and the A_1 subunit should be seen at approximately M_r 21,000. Even though peptides of M_r 21,000 and below were poorly resolved, a faint band running at about M_r 21,000 was detected in both inner and outer membrane protein samples. When more concentrated samples were run, a band in the M_r range 20,000 to 23,000 was seen. This band may represent the A_1 subunit of toxin.

The bands seen in the high M_r range (bands 1, 2, and 3) may be involved in nutrient uptake.

Cells grown in glucose-salts medium showed a new peptide which ran slower than band 2a. *P. aeruginosa* has proteins of high molecular weight inducible by cultivation in iron-limited media, and an increase in band D (M_r ~50,000) occurs when glucose is the sole carbon source (28).

Band 6 exhibited unusual staining with Stains-All dye. Either several substances migrate similarly, or band 6 contains a glycolipoprotein. Since lysozyme treatment did not affect the staining observed in band 6, it is not due to fragments of lipopolysaccharide attached to outer membrane protein.

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