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MEMBRANES FROM *MYXOCOCCUS FULVUS* (*MYXOBACTERALES*) CONTAINING CAROTENOID GLUCOSIDES

I. ISOLATION AND COMPOSITION

H. KLEINIG

Institut für Biologie II der Universität, Lehrstuhl für Zellbiologie, Freiburg i.Br. (Germany)

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SUMMARY

1. The isolation and chemical composition of an electronmicroscopically homogeneous membrane fraction from the gliding bacterium *Myxococcus fulvus* (Gram-negative) was described.

2. The membrane fraction was characterized by the presence of *a*-, *b*,- and *c*-type cytochromes, menaquinone, NADH oxidase, NADH dehydrogenase, succinate dehydrogenase. Ubiquinones which are generally found in Gram-negative bacteria were not present.

3. Phospholipids accounted for 25% of the membrane dry weight. Phosphatidylethanolamine was the main component (72% of total phospholipid). Phosphatidylglycerol, phosphatidylinositol, phosphatidic acid, and an unknown phospholipid were also present. About 22% of total phospholipids were identified as alk-1-enyl–acyl derivatives.

4. The carotenoid glucoside myxobacton ester was localized in the membrane (0.14% of membrane dry weight). The carotenoid glucoside to menaquinone molar ratio was 0.233. This ratio was found for the membrane fraction and for whole bacteria.

INTRODUCTION

Carotenoids are common components of the membranes of many bacteria, where they are thought, *e.g.* to be protectors against photodynamic destruction (for review see ref. 1). The pigments of the gliding myxobacteria (Gram-negative) are mainly carotenoid glycosides (glucosides and rhamnosides) as has been recently reported[2–4]. The special function of these glycosides as well as their biosynthesis, the site of which might also be on the plasma membrane, are not known. For *in vitro* studies, a carotenoid glucoside-containing membrane fraction has been isolated from a member of the myxobacteria *Myxococcus fulvus*. In this communication, the isolation procedure and the chemical characterization of this membrane fraction is described.

This is also the first report on isolated membranes from gliding bacteria. Electron micrographs of the envelope from these organisms reveal the tripple-layered structure typical for Gram-negative bacteria. There is, however, evidence for a special organization of this envelope, *e.g.* a patch-like arrangement of the peptidoglycan layer[5].

MATERIALS AND METHODS

Organism and culture condition

Myxococcus fulvus strain *Mx f2* was grown at 30 °C in 2-l New Brunswick fermentor in Casitone liquid medium as described[4]. Exponentially growing cells (about 1400 mg dry cells per l culture) were harvested by centrifugation and washed once with Tris–$MgCl_2$ buffer (20 mM Tris–HCl (pH 7.4) containing 2 mM $MgCl_2$). All subsequent operations were carried out at 0–4 °C.

Membrane preparation

Washed cells (1400 mg on a dry weight basis) were resuspended in 60 ml Tris–$MgCl_2$ buffer and sonicated in 10-ml portions for 3 min with a MSE sonifier in an ice bath. Most of the cells were broken after this treatment as revealed by phase contrast microscopy. The sonicated suspension was diluted 5-fold with Tris–$MgCl_2$ buffer and centrifuged for 20 min at 13000 rev./min in a WKF 8 × 36 rotor (maximum *g* force about 16000). The pellet contained unbroken cells and large debris overlayered by a whitish layer of wall material, which also contained some cell debris and large vesicles as revealed by electron microscopy. This centrifugation step was repeated once again. The red supernatant was then centrifuged for 120 min at 40000 rev./min in the WKF 8 × 36 rotor (maximum *g* force about 150000). The uncolored supernatant was discarded and the pellet, called crude membranes, was resuspended with a tight fitting Dounce homogenizer in 20 mM Tris–HCl (pH 7.4) containing 0.05 % EDTA (disodium salt) and centrifuged again for 120 min at 40000 rev./min. Large amounts of uncolored cytoplasmic contaminants, especially ribosomes and RNA, were removed by this purification step (about 50 % of the fraction on a dry weight basis). Ribosomes and RNA could also be removed by washing the fraction with 1.5 M KCl and a subsequent RNAase treatment. The EDTA method, however, was routinely used. The membrane pellet was then resuspended in Tris–$MgCl_2$ buffer and layered on a linear sucrose gradient (15–65 %, w/w) and centrifuged over night at 24000 rev./min in a WKF 3 × 35 swing out rotor (maximum *g* force about 75000). The red membranes were collected in one single compact band in the gradient at a density of 1.17–1.18. This gradient centrifugation again removed appreciable amounts of uncolored nonmembraneous material (about 40 % of the fraction on a dry weight basis). The membranes were collected from the gradient by careful pipetting, diluted with Tris–$MgCl_2$ buffer and finally centrifuged for 120 min at 40000 rev./min. The resulting pellet, the purified membrane fraction, was used for the biochemical investigations. About 12 % of the original cell dry weight were recovered in this fraction.

Chemical determinations

The membranes were extracted for lipids according to Folch *et al.*[6]. Proteins, nucleic acids, and lipopolysaccharides were precipitated with 10 % trichloroacetic acid and washed twice. Nucleic acids and lipopolysaccharides were then extracted from the precipitate with hot trichloroacetic acid (5 %, at 90 °C).

DNA and RNA were determined by using the diphenylamine[7] and the orcinol[8] reaction, respectively. Because the lipopolysaccharide sugars gave also a positive reaction with orcinol, RNA and lipopolysaccharide were hydrolyzed with 0.5 M H_2SO_4 for 3 h at 100 °C and the sugars analyzed subsequently by thin-layer chromatography

(cellulose layers, solvent *n*-butanol–pyridine–water (3:2:1, by vol.). Within the crude membranes appreciable amounts of ribose were found, whereas in the purified membranes no ribose could further be detected. The lipopolysaccharide was identified by its characteristic sugar composition (galactose, glucose, mannose, xylose, 3-*O*-methylxylose, rhamnose[9,10]). Quantitative determination of the sugars was made by the phenol–H_2SO_4 method[11].

Proteins were determined by means of a nitrogen determination method[12]. The protein content of the untreated membranes was measured by the method of Lowry *et al.*[13]. By this method, a value was obtained which was constantly too high, by a factor of 1.78, as compared with the values obtained by the nitrogen determination and on a dry weight basis. The values were then corrected by this factor.

Phosphorus was determined by the method of Gerlach and Deuticke[14].

Lipid analysis

Neutral lipids and phospholipids were separated upon a silica gel column. Neutral lipids were eluted with diethyl ether–acetone (1:1, v/v), phospholipids were eluted with chloroform–methanol (1:1, v/v) and methanol. Total neutral lipids were estimated by weight.

The main carotenoid glucoside, myxobacton ester (1′-glucosyloxy-3′,4′-didehydro-1′,2′-dihydro-β,ψ-caroten-4-one monoester, Fig. 2) was isolated and identified as previously described[2]. Quantitative determination was made photometrically at 480 nm (ε = 142000, solvent acetone).

The menaquinone was isolated by thin-layer chromatographic techniques[15] and identified by its absorption spectrum. Quantitative determination was done photometrically at 249 nm (ε = 18 600 in *n*-hexane[16]). The quinone was free of impurities as revealed by thin-layer chromatography on silica gel and visualization with Rhodamine–Tinopal which reagent is known to be very sensitive for lipids[17]. Lipoquinones appear as dark spots after spraying with Rhodamine–Tinopal when exposed to ultraviolet light (at about 360 nm). The quantitative values were confirmed after reduction of the quinone with $NaBH_4$ in ethanol. Ubiquinones were assayed on thin-layer plates as described above for menaquinone using known ubiquinones as reference substances. The limit of detection of ubiquinones was less than 0.5% of the menaquinone content in the sample.

Phospholipids were separated by two-dimensional thin-layer chromatography[17] and identified by comparison with standards. The individual phospholipids were further identified by successive chemical hydrolysis according to Pries *et al.*[18]. The water-soluble glycerophosphate esters were separated and identified chromatographically and electrophoretically according to Dawson *et al.*[19] using cellulose thin layers instead of paper. The amount of alk-1-enyl–acyl and alkyl–acyl analogues was determined according to Pries *et al.*[18] as recently reported[20].

Enzyme assays

The following enzymatic activities of electron transport were assayed at 25 °C in 0.05 M phosphate buffer (pH 7.8) as described by Boll[21]: NADH dehydrogenase with 2,6-dichlorophenolindophenol or ferricyanide as electron acceptor, and succinate dehydrogenase. Specific activities were defined as change of μmoles of reactant per min and g protein.

Qualitative measurement of cytochromes

Cytochromes were identified by reduced (dithionite) *minus* oxidized difference spectra and reduced + CO *minus* reduced difference spectra of membrane suspension obtained by means of a Cary Model 14 spectrophotometer.

Electrophoresis

Acrylamide-gel electrophoresis of delipidized membrane proteins was performed using the sodium dodecyl sulfate system of Shapiro *et al.*[22]. All membrane proteins were quantitatively solubilized with 1 % sodium dodecyl sulfate.

Electron microscopy

Membranes were fixed with cacodylate-buffered (0.05 M, pH 7.2) 3 % glutaraldehyde for 3 h in the cold. After washing in buffer postosmication was done with 2 % OsO_4 in the same buffer for 1 h at 0 °C. The specimen were dehydrated through graded ethanol solutions in the cold and embedded in Epon. Ultra thin sections (stained with uranyl acetate and lead citrate) were observed with a Siemens Elmiskop 101 electron microscope. Negative staining of the membrane suspension was performed with 2 % phosphotungstic acid, adjusted to pH 7.2.

RESULTS

The isolation procedure outlined above avoids enzymatical treatment of bacteria or membranes. Desintegration of the bacteria with glas beads gave a very similar membrane preparation as the short sonication procedure with, however, a very low yield. The sonication method was therefore favored. The final sucrose gradient centrifugation step yielded one red-colored membrane band at a density of 1.17–1.18. The ultrastructural morphology of the membrane fraction is shown in Fig. 1. Small vesicles and open fragments are present which consist of a single unit membrane.

An absorption spectrum of a membrane suspension is depicted in Fig. 2. Light scattering is reduced by solubilization with 0.2 % sodium dodecyl sulfate. The spectrum of the carotenoid glucoside myxobacton ester (in ethanol) is also shown, and the relationship is clearly visible. The fourth spectrum is made from a membrane preparation of mutant *M11* of *M. fulvus Mx f2* which lacks carotenoids. The peak at 410 nm (arrow) is due to cytochrome *c*. This peak shifts to 418 nm on reduction with dithionite. The other soret bands could not be resolved in these spectra because of the presence of the carotenoid.

The chemical composition of the membrane fraction is shown in Table I. 63.5 % of the total dry weight are proteins, 29.1 % are lipids. The proteins split on polyacrylamide gels into a few major and several minor bands, as is generally observed with membrane proteins (*e.g.* ref. 23 concerning membranes from *Escherichia coli*). Phospholipids, which are referred to in more detail below, account for 87 % of total lipids. Glycolipids were not detected. Nonpolar lipids comprise fatty acids, menaquinone and about 6 further unidentified components.

M. fulvus contains about 50 different carotenoid pigments with myxobacton ester as the main component (80 % of total carotenoids)[4]. Although all these pigments are quantitatively located within the membrane, the content in the membrane is quite low compared with other membrane constituants. Myxobacton ester accounts

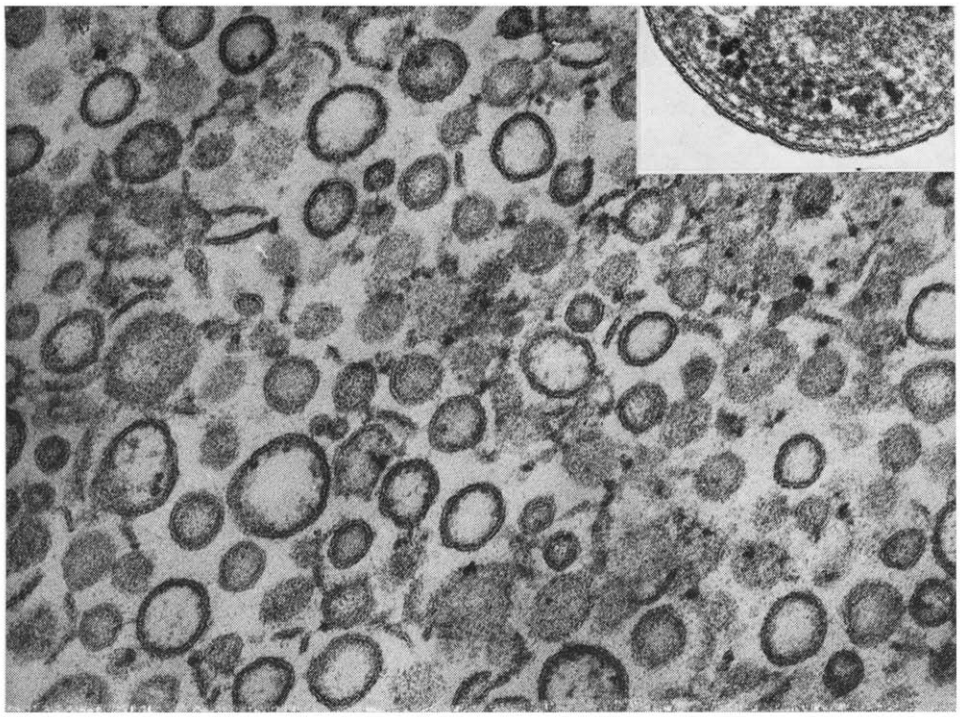

Fig. 1. Thin section through the membrane fraction from *M. fulvus* showing vesicles and open fragments which consist of a single unit membrane. The insert presents a part of a whole bacterium with its triple-layered envelope. Fixation with glutaraldehyde *plus* OsO_4. × 100000.

TABLE I

CHEMICAL COMPOSITION OF THE MEMBRANE FRACTION FROM *M. fulvus*

Compound	*Wt %*
Protein	63.5 ± 1.5
Phospholipid	25.3 ± 1.1
Nonpolar lipid	3.8 ± 0.6
Carotenoid glucoside (myxobacton ester)	0.14 ± 0.02
Carbohydrate	6.4 ± 0.4
Organic bound phosphate (nonlipid-bound)	0.8 ± 0.1
DNA	<0.8
RNA	<0.8

for only 0.14% of the total membrane weight. The carotenoid content increases in the late logarithmical growth phase by a factor of about 3. As can be seen from the molar ratios listed in Table II, the membrane contains more menaquinone than carotenoid glucoside. The carotenoid to menaquinone molar ratio of 0.233 is about the same in the membrane fraction as in whole bacteria.

9.1 μmoles menaquinone per 1 g membrane protein was found in *M. fulvus*. This relatively high value compared with other data (*e.g.* 2 μmoles in *Proteus rettgeri*[24], 5.4 μmoles in *Bacillus megaterium*[25]) is an indication for the purity of the membrane

fraction concerning cytoplasmic contaminants. This is also indicated by the very low content of nucleic acids.

Carbohydrates account for 6.4% of the membrane dry weight (Table I). Galactose, glucose, mannose, xylose, 3-*O*-methylxylose, rhamnose and traces of hexosamines were found all being constituants of the lipopolysaccharide of *M. fulvus*. The small amount of non-phospholipid phosphate is mainly lipopolysaccharide-bound.

True constituants of the cytoplasmic membrane from bacteria are the members of the electron transport chain. Besides the menaquinone mentionned above the

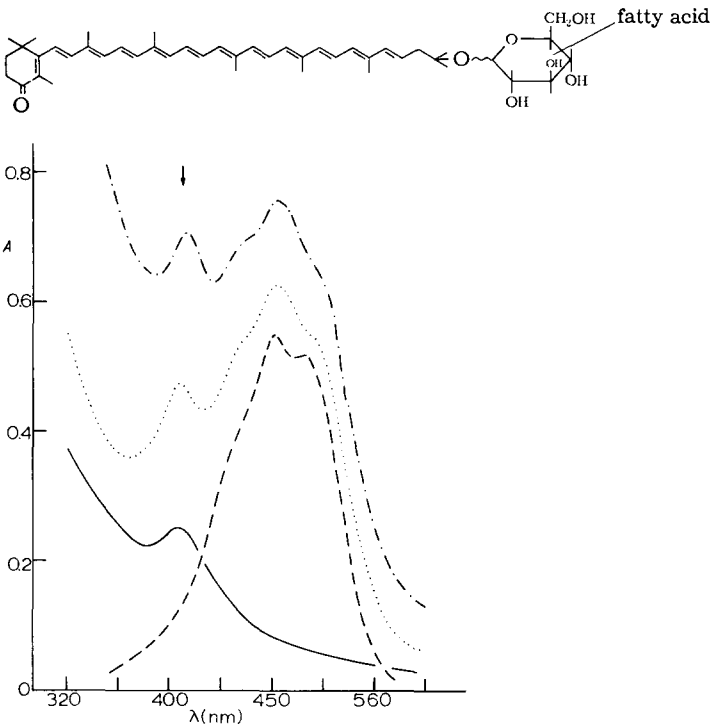

Fig. 2. Structure formula of myxobacton ester and absorption spectra. –·—·–, membrane suspension from *M. fulvus* in Tris–$MgCl_2$ buffer; ·····, the same suspension after addition of 0.2% sodium dodecyl sulfate; — — —, isolated myxobacton ester in ethanol as solvent; ———, membrane suspension in Tris–$MgCl_2$ buffer + 0.2% sodium dodecyl sulfate from a mutant (*M 11*) which lacks carotenoids. The arrow indicates a peak from cytochrome *c*.

TABLE II

MENAQUINONE, MYXOBACTON ESTER, PHOSPHOLIPID AND PROTEIN RATIOS OF THE MEMBRANE FRACTION FROM *M. fulvus*

	Molar ratio	*μmoles/g protein*
Myxobacton ester/phospholipid	0.0038 ± 0.0005	
Menaquinone/phospholipid	0.0163 ± 0.0013	
Myxobacton ester/menaquinone	0.233 ± 0.020	
Menaquinone/protein		9.1 ± 0.7

presence of several further participants in the electron transport was tested. The membranes contain *a*-, *b*-, and *c*-type cytochromes, as revealed by spectroscopical methods, whereas *o*-type cytochrome could not be detected. The reduced *minus* oxidized difference spectrum of the membrane suspension exhibited absorption peaks at 552 and 522 nm which represent the α and β peaks, respectively, of a *c*-type cytochrome, and at 562 and 530 nm representing the α and β peaks of a *b*-type cytochrome. The γ peaks of *b*- and *c*-type cytochromes were mixed together and showed a maximum at 423 nm. Absorption peaks at 600 and 442 nm were typical for an *a*-type cytochrome.

Some specific enzyme activities are shown in Table III. The activities are comparable with the activities obtained from other bacterial systems (*e.g.* ref. 26). Also, the characteristically high activity observed with the NADH dehydrogenase, when ferricyanide is used as electron acceptor, is known from the literature (*e.g.* refs 26 and 27).

This is the first study of phospholipid composition of myxobacteria. The results of the analyses are presented in Tables IV and V. The main compound is phosphatidylethanolamine, which contains about 14 % of alk-1-enyl–acyl derivatives. From unpublished results, it seems that alk-1-enyl–acyl analogues are common in these bacteria. A detailed survey of the phospholipid composition of this bacterial group will be given later. Phosphatidylglycerol, traces of phosphatidic acid, a compound tentatively identified as phosphatidylinositol, and an unknown phospholipid are also present in the membranes of *M. fulvus*. Each of these lipids shows a specific pattern of ether analogues (Table V). Diacyl derivatives are predominant in phosphatidylethanolamine, phosphatidylglycerol, and phosphatidylinositol, whereas the by far major part of compound "X" could not be converted in a water-soluble glycero-

TABLE III

ENZYME ACTIVITIES OF THE MEMBRANE FRACTION FROM *M. fulvus*

Activity	*μmoles reactant/min per g protein*
NADH oxidase	66
NADH dehydrogenase	
2,6-dichlorophenolindophenol	116
ferricyanide	846
Succinate dehydrogenase	214

TABLE IV

PHOSPHOLIPID COMPOSITION OF THE MEMBRANE FRACTION FROM *M. fulvus*

Compound	*Mole %*
Phosphatidylethanolamine	71.6 ± 2.8
Phosphatidylglycerol	8.6 ± 1.3
Phosphatidylinositol	7.3 ± 1.3
Compound "X"	7.7 ± 1.5
Phosphatidic acid	0.7 ± 0.3
Application point	4.1 ± 1.2

TABLE V

DIACYL, ALK-1-ENYL–ACYL, AND ALKYL–ACYL PHOSPHOGLYCERIDES OF THE MEMBRANE FRACTION FROM *M. fulvus*

Values are given as mole % of individual phosphoglycerides.

Compound	*Diacyl*	*Alk-1-enyl–acyl*	*Alkyl–acyl*
Phosphatidylethanolamine	84.2 ± 4.3	14.2 ± 3.7	1.6 ± 0.6
Phosphatidylglycerol	96.5 ± 1.5	1.3 ± 0.3	2.2 ± 0.6
Phosphatidylinositol	71.1 ± 6.5	6.5 ± 2.1	22.4 ± 4.5
Compound "X"*	Traces	Traces	>95.0

* Possibly, Compound "X" is not a alkyl–acyl phosphoglyceride (see text).

phosphate ester by alkaline and acid hydrolysis. Compound "X" is ninhydrine positive, and the nitrogen to phosphorus ratio is 2:1. The chromatographic properties of "X" are similar to lysophosphatidylethanolamine. Basic amino acids (lysine or ornithine) could not be identified on thin-layer plates after acid hydrolysis. Ethanolamine, however, seems to be present. These data would be consistent, *e.g.* with the structure of ceramide phosphorylethanolamine described by Labach and White[28] from an anaerobic bacterium. Further structural analysis of "X" is in progress.

DISCUSSION

Recently, several reports on the isolation of outer and inner membranes from Gram-negative bacteria have been published[23,29–32]. The membranes from *E. coli*[23,29,31,32] and *Salmonella typhimurium*[32] were separated into two or more discrete bands by a final density centrifugation on sucrose gradients. In the present investigation on *M. fulvus* membranes, only one fraction could be obtained on the gradient at a density of 1.17–1.18. The cytoplasmic membrane-enriched fraction of *E. coli* was separated at a similar density of 1.16, whereas the outer membrane-enriched fraction had a higher density (1.22) (ref. 23). The membrane fraction from *M. fulvus* contains constituants and activities of the electron transport chain characteristic for the cytoplasmic membrane. There is, however, some lipopolysaccharide present (about 7% of the membrane dry weight) which could not be removed by salt or EDTA. This lipopolysaccharide could also not be separated from the membrane in solubilization and reconstitution experiments with sodium dodecyl sulfate (H. Kleinig, M. Boll and H. Falk, in preparation). There are three interpretations possible for this phenomenon. First, the lipopolysaccharide present in the fraction is bound by hydrophobic bonds to the cytoplasmic membrane. Second, outer and inner layer of the envelope from *M. fulvus* are very similar with respect to their basic constituants, which results in an identical boyant density. Third, the membranes are hybridized membranes as described recently for *Escherichia coli* membranes[35]. Which of these interpretations is correct can not be decided at the moment. There are, however, general assumptions that the envelope of the gliding myxobacteria may be different from that of the eubacteria[5]. Vesicular membranes and short fragments can be found in ultra-thin sections (Fig. 1) as well as in negatively stained preparations of the membrane fraction from *M. fulvus*. The relative frequency of the fragments depends on the duration of

sonication and is not correlated to the lipopolysaccharide content. Extensively sonicated preparations exhibit large proportion of fragments. It seems therefore that both forms, vesicles and fragments, are not principally different.

M. fulvus as well as other myxobacteria (unpublished results) are interesting exceptions of the statement that Gram-negative bacteria contain ubiquinones, Gram-positive bacteria contain menaquinones, and enterobacteria contain both[33]. Only menaquinones are present in the Gram-negative myxobacteria.

The carotenoids and carotenoid glucosides of *M. fulvus* are located within the membrane fraction described. In spite of the bright red color of these membranes, the concentration of the pigments is surprisingly low when compared with other minor membrane constituants such as menaquinone. About twenty different carotenoid glycosides have been described as yet from bacteria and blue green algae (for review see ref. 34). Their function and biosynthesis, however, is generally not understood. The enzymatically active membrane fraction from *M. fulvus* may serve as a tool for studying these questions.

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