Comparative Biochemistry of the Cell Envelopes of *Photobacterium leiognathi* and *Escherichia coli*

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(Received 28 September 1982)

Photobacterium leiognathi closely resembles *Escherichia coli* with respect to cell lysis by lysozyme, and the fractionation of outer and cytoplasmic membranes. The two organisms differ in their phospholipid contents and, more significantly, in outer membrane protein compositions.

INTRODUCTION

Photobacterium leiognathi (*P. mandapamensis*) is a luminescent marine bacterium found as the specific symbiont in the luminous organ of Leiognathid and Apogonid fish, as a marine intestinal commensal or free-living in sea water (Reichelt & Baumann, 1973; Nealson & Hastings, 1979). The genus shares many properties with the *Enterobacteriaceae* (Baumann & Baumann, 1977). In particular, electron microscopy of thin sections of this organism reveals a cell envelope which is morphologically very similar to terrestrial Gram-negative bacteria, with the characteristic membrane, cell wall, periplasmic space and outer membrane (Reichelt & Baumann, 1973). It would not be surprising if closer analysis of the cell envelope of *P. leiognathi* revealed considerable differences from typical Gram-negative bacteria, since the marine organism must cope with a constant high osmotic pressure. At the same time, there is some evidence for greater membrane permeability in this genus. We have observed that *P. leiognathi* has a relatively high sensitivity to a variety of antibiotics and chemical mutagens. In addition, the periplasmic β -lactamase of *Photobacterium* species does not generally display the 'crypticity' typical of this enzyme in most other Gram-negative bacteria (Richmond and Sykes, 1973), and usually taken as a measure of outer membrane impermeability to β -lactam antibiotics (K. Smith & S. Lamb, unpublished results). We have accordingly analysed various aspects of the biochemistry of the cell envelope of *P. leiognathi* with particular emphasis on the composition of the outer membrane, and using *Escherichia coli* for comparative purposes.

METHODS

Growth of bacteria. The maintenance and growth of *Escherichia coli* ML308 225 have been described previously (Allen & Scott, 1979). *Photobacterium leiognathi* strain K-721 (Nealson & Hastings, 1977) was obtained from the Roche Research Institute of Marine Pharmacology in Sydney, and was grown in liquid cultures using minimal medium (Reichelt & Baumann, 1973) without CaCl_2 and supplemented with 0.2% (w/v) yeast extract. This bacterial strain was maintained on plates made by adding 1.2% (w/v) agar to the liquid medium.

Membrane preparation. Membranes from *E. coli* ML308 225 in the late-exponential growth phase were prepared and fractionated by sucrose density gradient centrifugation as described previously (Allen & Scott, 1979). A 500 ml culture of *P. leiognathi* in the late-exponential growth phase was harvested by centrifugation, resuspended in 20 ml deionized water and mixed with 2 mg egg white lysozyme (Sigma) in 20 ml 30 mM-Tris/HCl buffer pH 7.5. Following incubation at 37 °C for 15 min, the mixture was centrifuged for 3×10^4 g-min to remove intact cells and then for 2×10^6 g-min. The resulting precipitate was resuspended in 2.0 ml 10 mM-HEPES buffer pH 7.2 and the membranes were fractionated by sucrose density centrifugation as for *E. coli* ML308 225. Pooled fractions (6 ml) from these gradients were extracted with 50 ml chloroform/methanol (3:1, v/v) for 60 min at room temperature to

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extract phospholipids. The aqueous residues were dialysed against deionized water, freeze-dried and analysed qualitatively for proteins by electrophoresis on 10% (w/v) SDS-polyacrylamide gels (Laemmli, 1970).

Bacterial extractions. Outer membrane proteins were also extracted from both species of bacteria using the method described previously (Allen & Scott, 1979) and were analysed by SDS gel electrophoresis (Laemmli, 1970). Phospholipids and lipopolysaccharides (LPS) were extracted from both species of bacteria and analysed as previously described (Guan & Scott, 1980), with one exception, as follows: the liquid phase for GLC of fatty acid methyl esters was Silar 9 C P (Allied Science Laboratories Inc., State College, Pa., U.S.A.) on Chromosorb G in a 1.2 m $\times$ 6 mm column, and the temperature programme was 180 °C for 2 min after sample injection, followed by heating at 8 °C min⁻¹ to 240 °C. This regime improved the separation between palmitic and hexadecenoic fatty acid methyl esters.

Analysis of intact phospholipids was carried out on total phospholipid extracts only, using TLC on 20 $\times$ 20 cm silica gel plates with chloroform/methanol/acetic acid/water (85:15:10:3, by vol.) as the solvent. Standard phospholipids were purchased from Sigma. Phospholipids were detected by exposure to iodine vapour.

LPS binding to bacteria. This was measured indirectly, in terms of its effect on the serum bactericidal reaction of *E. coli* ML308 225. Bactericidal assays were carried out as described previously, with and without *P. leiognathi* LPS at a final concentration of 150 μ g ml⁻¹.

RESULTS AND DISCUSSION

In an isotonic salts suspension, *P. leiognathi* was not lysed by lysozyme. When resuspended in a hypotonic buffer (30 mM-Tris/HCl pH 7.5), the organism was stable but was very readily lysed by lysozyme. In this respect, it resembles *E. coli*, in which an osmotic shock is necessary for lysozyme to penetrate the outer membrane before it can hydrolyse the peptidoglycan cell wall (Witholt *et al.*, 1976). Following low speed centrifugation of the *P. leiognathi* lysate, the supernatant was centrifuged at high speed and the resulting precipitate was fractionated by sucrose density gradient centrifugation. As can be seen in Fig. 1, the elution profile of the gradient is almost identical to fractionation of *E. coli* membranes under the same circumstances. The two protein peaks in the *E. coli* elution profile correspond to the outer and cytoplasmic membranes (Schnaitman, 1970; Allen & Scott, 1979). On this basis, fractions 4–6 were pooled as the outer membranes and fractions 10–12 were pooled as the inner membranes for each gradient elution.

Following extraction with chloroform/methanol, the pooled outer membrane fractions were concentrated and analysed for membrane proteins by SDS gel electrophoresis. These results are presented in Fig. 2. The profile for the *P. leiognathi* outer membrane shows a single major protein with a similar mobility to the characteristic major outer membrane protein complex of *E. coli*. The remaining proteins are few and present in much smaller quantities. This represents a marked contrast to the *E. coli* outer membrane protein profile, which is more complex. The electrophoretic technique used in these experiments (Laemmli, 1970) resolves the major outer membrane protein complex more effectively than that used previously (Bragg & Hou, 1972; Allen & Scott, 1979).

Extraction of a crude particulate fraction from *E. coli* with SDS buffer after a first extraction with a Triton X-100 buffer gives an outer membrane protein preparation which is similar to that obtained from outer membranes purified by sucrose density gradient centrifugation (Schnaitman, 1973; Allen & Scott, 1979). Similar results are apparent from Fig. 2 in the case of *E. coli*, but the profile for the *P. leiognathi* extract is much more complex than the corresponding profile derived from purified outer membranes. Thus the differential solubility technique which is so convenient for isolating outer membrane proteins from *E. coli* is not suitable for use with *P. leiognathi*.

The chromatographic purification of LPS from *P. leiognathi* was successfully carried out by the same methods as reported for *E. coli* ML308 225 (Allen & Scott, 1980). A similar total lipid content (measured as fatty acid methyl esters) of 11% was found. When added to a mixture of *E. coli* ML308 225 and normal human serum, the LPS from *P. leiognathi* caused a 50% inhibition of the complement-dependent bactericidal reaction. We have shown previously that this inhibition is due to LPS binding to the bacterial surface, and can also be observed with *E. coli* K12 (Allen & Scott, 1980, 1981). The binding of LPS to *Salmonella typhimurium* outer membranes has also been reported (Jones & Osborn, 1977). The common feature of LPS which can interact in this

Fig. 1. Sucrose density gradient fractionation of membranes from *E. coli* (○) and *P. leiognathi* (●). Bacterial protoplasts were lysed by dilution into cold deionized water and particulate fractions were sedimented by centrifugation for 2000000 *g*-min. These fractions were layered on to a 35 ml discontinuous sucrose gradient (Schnaitman, 1970) and centrifuged for 77000000 *g*-min. Fractions (2 ml each) were collected from the bottom of the tubes and the absorbance at 280 nm was used as a measure of protein concentration.

Fig. 2. Electrophoresis on SDS-polyacrylamide gels of outer membrane proteins from *E. coli* and *P. leiognathi*. Outer membrane proteins were extracted from purified outer membranes, or from the particulate fraction of bacterial lysates as described in the text. Polyacrylamide gels (10%, w/v) containing SDS were prepared, electrophoresed and stained (Laemmli, 1970), and scanned with an ISCO model 1310 densitometer. The origin of the gel is on the left in each case. (a) Extract of *E. coli* outer membrane. (b) Extract of *P. leiognathi* outer membrane. (c) SDS extract of *E. coli* particulate fraction. (d) SDS extract of *P. leiognathi* particulate fraction.

way with intact bacterial cells is an incomplete outer polysaccharide chain and it has been suggested that this feature minimizes steric interference between the added and the intrinsic LPS (Jones & Osborn, 1977; Allen & Scott, 1981). The present result with *P. leiognathi* LPS suggests that, in this respect, it is similar to the incomplete LPS molecules produced by 'rough' *Enterobacteriaceae*.

Phospholipid analysis by TLC was qualitative, but phosphatidyl ethanolamine was clearly the predominant phospholipid from both species of bacteria. Phosphatidyl glycerol, phosphatidyl serine and cardiolipin were also detected in smaller quantities in each case. These results are typical of Gram-negative bacteria (Cronan, 1978).

Samples of the total phospholipid and of both membranes from *P. leiognathi* were analysed for fatty acid methyl esters and the results were as follows. The total lipid comprised 26% palmitic, 57% hexadecenoic and 17% octadecenoic acids. In contrast, *E. coli* comprises 32%, 34% and 25% of these fatty acids respectively, as well as 2% myristic and 7% methylene hexadecenoic acids (Guan & Scott, 1980). The latter fatty acids were not detected in *P. leiognathi*. The outer membrane of *P. leiognathi* contained 43% palmitic, 47% hexadecenoic and 9% octadecenoic acids. The cytoplasmic membrane contained 31% palmitic, 50% hexadecenoic and 19% octadecenoic acids. The discrepancy between the total lipid and membrane lipid compositions is at variance with the observed results in *E. coli* (Koplow & Goldfine, 1974).

In conclusion, the features of the cell envelope of *P. leiognathi* which have been elucidated in this study are almost entirely typical of terrestrial Gram-negative bacteria, the main exception being the rather simple outer membrane protein composition. There is no evidence at this level of analysis for unusual membrane properties which might account for the ability of the organism to withstand high osmotic pressures. Further information on this ability will probably come from an examination of the dynamic properties of the cytoplasmic membrane. However, the apparent predominance of a single protein species in the outer membrane can possibly be reconciled with the permeability properties of the membrane. Many of the different outer membrane proteins in *E. coli* function in the specific uptake of nutrients from the medium (Di Rienzo *et al.*, 1978). In the apparent absence of a multiplicity of such proteins, it is possible that the single major protein in the outer membrane of *P. leiognathi* forms a single 'pore' of rather broad specificity. This hypothesis would account for the low permeability barrier presented to a variety of small molecules by the outer membrane of this organism.

We wish to thank Mrs R. J. Allen for technical assistance, Miss S. Lamb for helpful discussions, and Mrs M. Smith and Mrs S. Treseder for secretarial assistance.

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