DETECTION OF A SPECIFIC DNA-CYTOPLASMIC MEMBRANE COMPLEX IN *ESCHERICHIA COLI* BY EQUILIBRIUM DENSITY CENTRIFUGATION ON SUCROSE GRADIENTS

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1. Introduction

There have been many previous reports of the association of *E.coli* DNA with a particulate or "fast sedimenting" fraction containing cell membranes (see review by Klein and Bonhoeffer [1]). The methods used have usually involved the velocity sedimentation of crude cell lysates through sucrose gradients on to a shelf of high density. This technique suffers the disadvantage of the possibility of non-specific trapping of DNA in the particulate fraction and the inability to demonstrate the precise surface structure to which DNA may be bound, and whether in fact the complex really does contain membrane. The recent demonstration by Osborn et al. [2] that the surface layers of *E. coli*, including the outer and inner (cytoplasmic) membranes, can be largely separated by isopycnic centrifugation in a sucrose density gradient, afforded us the opportunity to search for specific DNA-inner membrane complexes under these conditions. In this paper we show that the bulk of the DNA does remain tightly bound to the cell surface during isolation of cell envelopes and then bands with the cytoplasmic but not with the outer membrane when these complexes are centrifuged to equilibrium on a sucrose gradient.

2. Methods

Cell envelope fractions of *E.coli* strain ASH102 [3] were isolated from spheroplasts by osmotic lysis or sonication, purified by two cycles of high speed centrifugation (100,000 *g*) and centrifuged to equilibrium on 30–55% sucrose gradients, as described by Osborn et al. [2].

In DNA labelling experiments cultures were grown for 3–4 generations in supplemented M9 medium [2] containing 1–2 μCi/ml methyl [^{3}H] thymine (Amersham). Gradients were scanned at 256 nm using an Isco model 180, and radioactivity determinations carried out as described previously [4]. Polypeptides present in membrane fractions obtained from the sucrose gradients were analysed by disc-electrophoresis on 10.5% polyacrylamide gels containing sodium dodecyl sulphate (SDS) as described previously [5].

3. Results and discussion

3.1. Identification of the cytoplasmic membrane fractions on sucrose gradients

Strain ASH102 was labelled for several generations by growth in the presence of [2-^{14}C] glycerol [2]. Envelopes were isolated from sonicated spheroplasts [2], purified and centrifuged to equilibrium on a sucrose gradient. The determination of the distribution of labelled glycerol revealed the presence of four major bands (fig.1) containing lipid and corresponding to the 4 bands identified by Osborn et al. [2], H (p = 1.22), M (p = 1.19), L2 (p = 1.16) and L1 (p = 1.14). Fractions L2 and L1 were identified previously by these workers by the presence of cytochromes and dehydrogenases as containing predominantly cytoplasmic membranes, with L2 banding at higher density due to greater contamination with outer membrane; the H-band was iden-

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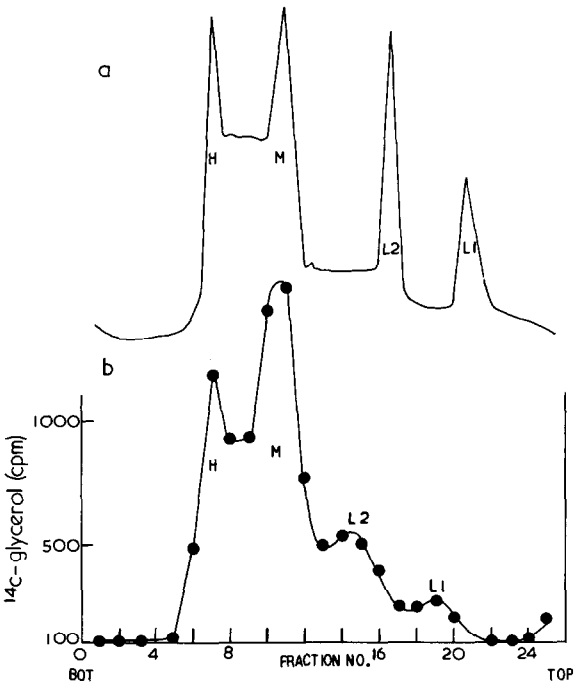

Fig.1. Separation of surface layers by equilibrium centrifugation. Strain ASH102 was grown in the presence of [^{14}C]glycerol for several generations; cells were converted to spheroplasts, lysed by sonication and envelopes isolated. Samples containing 1–2 × 10^{10} cell equivalents were layered on 30–55% (w/w) discontinuous sucrose gradients containing 5 mM EDTA and centrifuged for 16 hr at 150,000 *g* as described by Osborn et al. [2]. Gradients were scanned in an Isco UV-analyser or fractionated and radioactive counts determined. a) Absorbance profile; b) distribution of radioactive glycerol. Density of separated layers H (outer membrane), M, L2 and L1 (inner membrane) as indicated in the text.

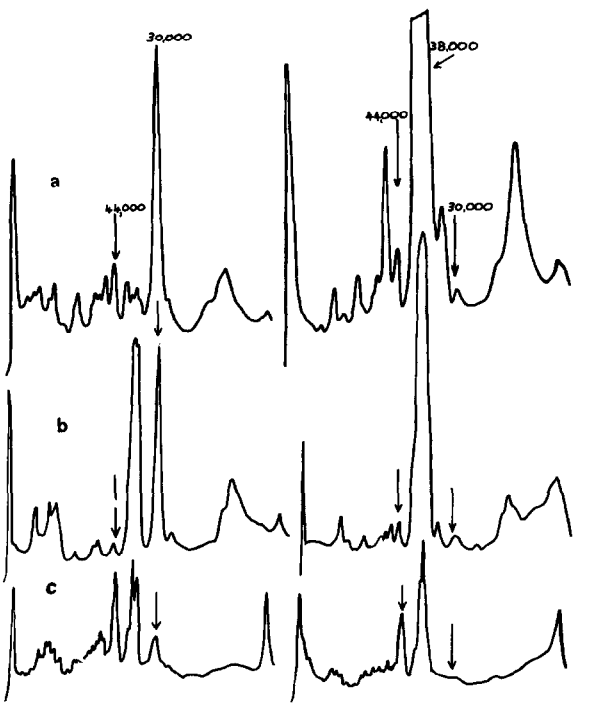

Fig.2. Differences in membrane proteins between outer and inner membrane fractions. Purified envelopes of strain ASH102 were isolated and fractionated on an equilibrium sucrose gradient as in fig.1. Outer membranes (H) and combined L1 and L2 fractions were collected, pelleted and resuspended in 10^{-2} M sodium phosphate buffer containing 1% SDS. Samples were then analysed on 10.5% SDS acrylamide gels as described previously [5]. a) Microdensitometer tracings of Coomassie Blue stained gels of total, unfractionated envelopes; b) outer membrane proteins; c) inner membranes. Left hand tracings, samples heated to 70° for 2 min before electrophoresis; right hand tracings, samples heated to 100° for 2 min prior to analysis. Electrophoresis was from left to right.

tified as the outer membrane by the presence of most of the surface polysaccharide; band M appeared to be a mixture of unseparated membranes. To confirm the distinction of the outer and inner membrane fractions in this study, the protein content of H and of the combined L2 and L1 fractions was analysed on SDS polyacrylamide gels. As shown in fig.2 protein composition was clearly different. Inner membrane fractions were enriched for several minor constituents with apparent molecular weights over 50,000 daltons and contained only small levels of the 30,000 dalton band which constitutes the major polypeptide of the total envelope and of the H or outer membrane fraction. This latter polypeptide band is characterised further by its apparent ability to aggregate to form a major band at the 38–40,000 dalton position when membrane samples are heated to 100° prior to analysis. Localisation of this protein in the outer membrane has now been confirmed by several studies [2, 5–8]. One major band of the cell envelope, apparent molecular weight 44,000 daltons, was greatly enriched in the inner membrane. This major band is only present in mutant strains such as ASH102 used here which show specific tolerance to colicin E2 [5].

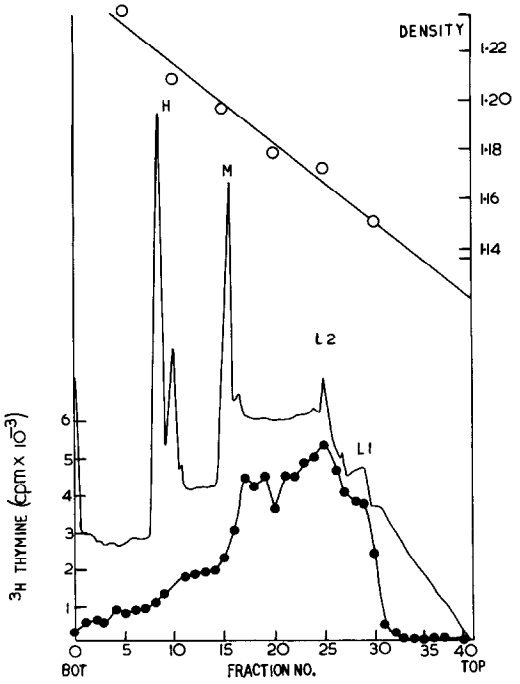

Fig.3. Strains ASH102 was labelled with [^{3}H] thymine, thoroughly washed and spheroplasts prepared and lysed osmotically [2]. Envelopes were isolated and fractionated on a sucrose equilibrium gradient as in fig.1. Gradients were scanned at 256 nm (——) fractionated and the distribution of radioactive thymine determined. (●—●—●): [^{3}H] thymine; (○—○—○) density of sucrose (g/cm^3) determined with a refractometer.

3.2. Association of DNA with the inner membrane fractions L1 and L2

Spheroplasts of cells grown in [^{3}H] thymine for several generations were lysed by osmotic shock [2] to minimise shearing of DNA and the purified membranes centrifuged to equilibrium on a sucrose gradient. The gradient was scanned with an Isco UV analyser and the usual 4 major density bands were obtained. After scanning, the gradient was fractionated and fig.3 shows the distribution of labelled thymine found throughout the gradient. The bulk of labelled DNA was clearly associated with the cytoplasmic membrane fractions L1 and L2 whereas only a small proportion of radioactive counts were found near the H-band and this may be due to Low level contamination of the H-fraction with fragments of inner membrane. Prior incubation of the envelope preparation with DNAase I reduced by threefold the amount of acid precipitable thymine fractionating with the cytoplasmic membrane. The possible significance of the small fraction of DNA which resisted DNAase treatment and still banded with L1 and L2 is currently being investigated.

Treatment of the envelope complex with SDS led to the release of the DNA, whose molecular weight was then determined on a sucrose velocity gradient to be about 8 x 10^7 daltons. Since 10–20% of total spheroplast DNA was present in the envelope fraction we tentatively conclude that the DNA–cytoplasmic membrane complexes detected here may represent up to 8 sites of DNA attachment to the cytoplasmic membrane per cell. We are now attempting to establish whether these sites include the chromosomal origin and if so, how the specific properties of such complexes differ from other possible attachment complexes.

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