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IDENTIFICATION OF AN *ESCHERICHIA COLI* INNER MEMBRANE POLYPEPTIDE SPECIFIED BY A λ-*tonB* TRANSDUCING BACTERIOPHAGE

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SUMMARY: Analysis by polyacrylamide gel electrophoresis of the proteins coded by a λ*ton*B transducing phage, after infection of UV-irradiated bacteria, revealed the presence of at least 7 new polypeptides. Three of these were identified as proteins of the *trp* operon whilst three others were deleted by a spontaneous mutation in the *ton*B region carried by the phage. A single polypeptide, molecular weight 40,000 was absent from a phage carrying a proflavine induced mutation in *ton*B. We conclude that this protein, which was localised in the inner membrane by sarkosyl fractionation of the envelope, is the *tonB* product.

The uptake of chelated iron complexes (1,2) and vitamin B_{12} (3), the killing action of bacteriophages T_1, φ80 (4) and of the group B colicins (5), in E.coli all require a functional *ton*B gene product. The initial event in the transport of the 'substrates' is their energy-independent binding to a variety of outer membrane receptors (6). Subsequent uptake into the cytoplasm requires energy generated by the electron transport chain or Ca, Mg ATPase in the cytoplasmic membrane (7, 8) and the *ton*B gene product. Bypassing the outer membrane step of some of these processes, e.g. for T_1 (9) or for ferric-enterochelin (10), has been shown to eliminate the requirement for *ton*B, and has led to the conclusion that the role of the *ton*B gene product is to couple the outer membrane components of the transport

Abbreviations: SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; kd, kilodaltons.

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systems with those of the energized inner membrane (9, 10, 11). Treatments that disrupt the normal envelope structure such as filtration of a culture (12) or treatment with a specific penicillin, mecillinam (13) seem to interfere with the action of *ton*B product, consistent with its location in the envelope. However, comparative analysis of the envelope fractions of wild type bacteria and of *ton*B deletion mutants by SDS-PAGE have previously failed to identify the *ton*B product. We therefore decided to identify and find the cellular location of the *ton*B gene product to help to elucidate its function.

MATERIALS AND METHODS

Media and Phage: Phage stocks were prepared and assayed as described by Moir and Brammar (14) except that phage were dialysed against lambda buffer (6 mM Tris, 10^{-2} M, $MgSO_4$, 0.005% gelatin pH 7.2), before infection of irradiated bacteria.

Phage λ_{269} (*trp*ED$^+$ *ton*B$^+$) [att-C_{III}]$^\nabla$ was derived from $\lambda_h att^{\phi 80} C_I^+$ parent and obtained from Dr N. Murray. Lysogens of transducing phages were obtained by selecting for *trp*$^+$ or *ton*B$^+$ transductants of *trp* or *ton*B strains (*ton*B transductants were selected by their ability to grow in the absence of citrate on M9 glucose medium (15)).

Isolation of *tonB* mutants: Spontaneous mutants were isolated from a λ_{269} lysogen of a strain carrying a chromosomal *ton*B, *trp*ABC deletion (*trp*A9; 16) by adding phage ϕ80vir (10^{10} pfu) colicins B and V (0.2 ml of a UV induced lysate of a *colV*, *colB*$^+$ strain) and λC_{I26} (10^{10} pfu) for 15 min at room temperature before spreading on nutrient agar plates (Oxoid No.2 nutrient broth 25 g, Oxoid agar No.3 14.5 g/litre). Surviving colonies were checked for immunity to λ and screened for a requirement for citrate (10 mM) for growth on M9 glucose agar, indicative of *ton*B mutants (15).

Proflavine was also used to mutagenize λ_{269} according to the method of De Mars (17). Mutants were isolated by infecting a *trp*ED, *ton*B host with dilutions of the mutagenized phage, superinfecting with ϕ80vir (10^8 pfu) in the presence of colicin V and plating on M9 glucose agar containing citrate.

Phages were then isolated from the lysogens by UV-induction (18) and the presence of *trp*E and D and the absence of a functional *ton*B were confirmed by transduction tests. Phage λ_{402} is a spontaneous *ton*B deletion mutant and λ_{403} a proflavine induced *ton*B mutant.

Phage infection of UV-irradiated cells: The method was based on that of Hendrix (19). Bacteria of a λ*ind*⁻ lysogen of strain 159 (20) were irradiated with UV light (12,000 ergs mm^{-2}), infected with transducing phages (MOI = 5) and the proteins synthesised after infection labelled with ^{35}S - methionine (Amersham Radiochemicals, U.K.; specific activity > 100,000 μCi/mmol).

Preparation of cell fractions for gel electrophoresis: Whole cell lysates were prepared by washing the labelled samples in envelope buffer (21), resuspending the pellet in 100 μl SDS-sample buffer (0.062 M Tris HCl pH 6.8, 20% w/v glycerol 4% w/v SDS, 5% w/v mercaptoethanol) and boiling for 5 min. The combined cytoplasmic plus periplasmic fraction was recovered from the envelope supernatant by acetone precipitation with 4 volumes of acetone at -20°C. Membranes were prepared and SDS-PAGE performed as described previously (12, 21).

RESULTS

Two new proteins were synthesised and therefore labelled with ^{35}S -methionine after infection of irradiated cells of a λ*ind*⁻ lysogen of the *E.coli* strain 159 with wild type phage λ (result not shown). These were the phage C_I repressor (26kd) and rex (29kd) gene products. In contrast, infection with the specialized transducing phage λ_{269} carrying *ton*B and genes of the *trp* operon, resulted in the production of several other polypeptides as shown by SDS-PAGE analysis of whole cell lysates (Fig.1). Three of these polypeptides were produced in greatly increased amounts in a derivative of strain 159 lacking the repressor of the tryptophan operon (Fig.1, lanes 6 and 7). The apparent molecular weight of two of these polypeptides, 58kd and 52kd agree with the known molecular weights of the trpD and E proteins respectively (14). The third, molecular weight 35,000, is presumably a 'trpC-fusion' polypeptide generated by an internal deletion during formation of λ_{269}. In addition to the 'trp' proteins, four other polypeptides were detected in varying amounts in different experiments

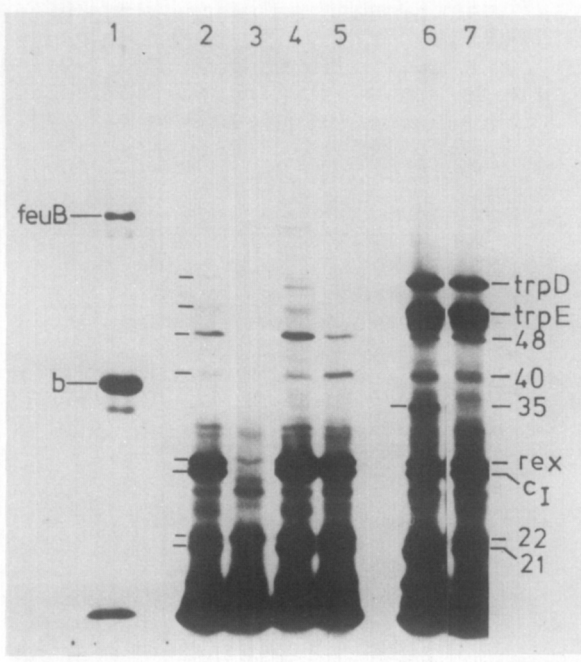

Fig. 1. Gel fluorograph of whole cell lysates from infection of irradiated host cells. The concentration of acrylamide was 11% and electrophoresis was from top to bottom in all the figures. An outer membrane fraction from E. coli B/r (lane 1) was included as additional molecular weight markers (feuB protein is 81kd and b, the major porin is 36.5kd). Lanes 2-5 trpR$^+$ host, infected with λ_{402} (2), uninfected control (3), λ_{403} (4), λ_{269} (5). Lanes 6 and 7 trpR$^-$ host infected with λ_{269} (6), λ_{402} (7).

in lysates of λ_{269} infected cells. These had apparent molecular weights of 48, 40, 22 and 21 kd.

Evidence relating to the functional role of the *ton*B product predicts that it would have an envelope location and to help identify the presence of a possible *ton*B polypeptide, cell fractionation of the infected cells was carried out. The results obtained, shown in Fig.2, revealed trace amounts of the trp proteins, the 48kd and the C_I repressor polypeptides in the inner membrane fraction whilst the 40kd polypeptide and the λrex product (presumed to be located in the inner membrane (22)) were greatly enriched in this fraction. The 22kd polypeptide was also

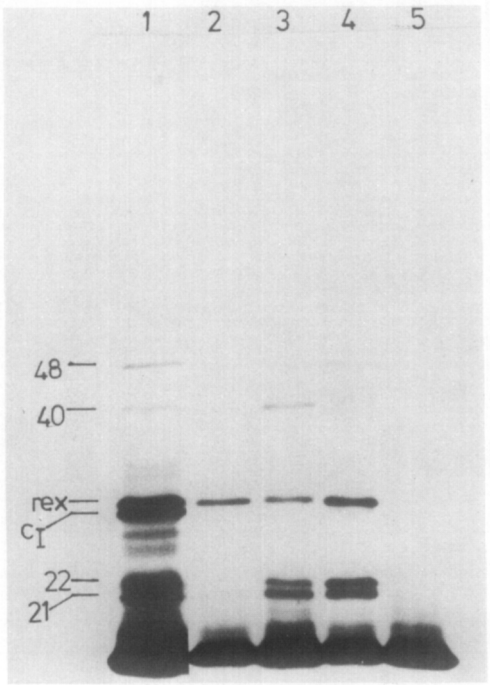

Fig. 2. Gel fluorograph of the cytoplasmic membrane fractions from infections of a *trpR*$^+$ host. A whole cell lysate infected with λ_{269} is included for comparison (lane 1). Lane 2. λ_{402}, 3. λ_{269}, 4. λ_{403}, 5. uninfected control.

found in the inner membrane fraction whilst the 21kd polypeptide was apparently located in both the outer and inner membranes (Fig. 2, 3).

The 40kd polypeptide was produced in low amounts relative to the C_I and rex proteins, and in some experiments the presence of this protein was obscured when whole cell lysates were analysed by a protein of similar molecular weight present in the uninfected control. Analysis of the isolated inner membrane fraction however, removed this ambiguity and facilitated the ready identification of this protein in infected cells.

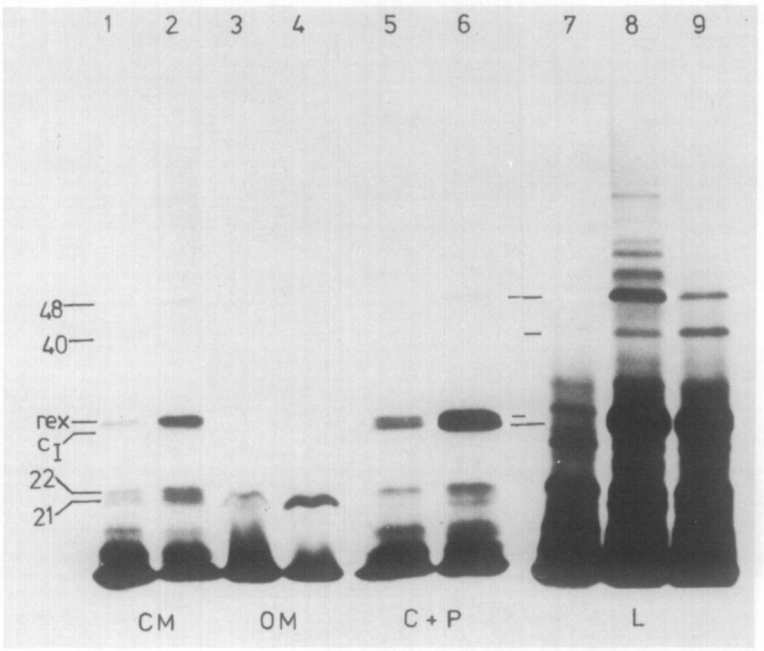

Fig. 3. Cell fractionation of a trpR$^+$ irradiated host after infection. CM - cytoplasmic membrane, OM - outer membrane, C + P - cytoplasm plus periplasm, L - whole cell lysates. Lanes 1, 3, 5 and 9 λ_{269}, 2, 4, 6 and 8 λ_{403}, 7 uninfected control.

In a further attempt to identify the *ton*B gene product amongst the proteins determined by λ_{269}, mutations of the *ton*B gene were obtained in this phage by selecting for the *ton*B phenotype (see Methods) in λ_{269} lysogens in which the chromosomal *ton*B region had been deleted. Infection of irradiated bacteria with one such phage, λ_{402}, carrying a spontaneous mutation to *ton*B$^-$, failed to produce the 40, 35 (the trp-fusion protein), 22 and the 21kd polyeptides indicating that this phage carried a deletion in the *ton*B region (Fig. 2, lane 2). Similar results were obtained with a second spontaneous *ton*B$^-$ mutant. However, λ_{403} (a proflavine induced *ton*B mutant which was also revertable by proflavine) only failed to produce the 40kd inner membrane polypeptide (Fig. 2, lane 4, and Fig. 3) and we conclude that this is probably the *ton*B product.

DISCUSSION

The results described above suggest that the *ton*B gene product is associated with the inner membrane of *E.coli*. The level of synthesis observed in the UV-irradiated system indicates that the protein may be a minor component of the envelope *in vivo*, which would explain the previous failure to identify the product. To unambiguously identify the *ton*B product we initially sought to isolate an amber mutation using a host with a temperature sensitive suppressor tRNA: however, we have so far failed to isolate such a mutant. As an alternative we anticipated that a frame shift mutation induced by proflavine in *ton*B should produce a radical change in the molecular weight of the corresponding polypeptide, thereby facilitating its identification. Although we have not excluded the possibility that the mutation in λ_{403} may be polar and that the 40kd polypeptide may be coded by a gene promoter distal in the same operon as *ton*B, the loss of the 40kd polypeptide in this mutant, together with its location in the cell envelope, provides strong evidence that this is indeed the *ton*B product. This view is further strengthened by calculations which indicate that the polypeptides made from the transducing fragment in λ_{269} account for more than 90% of the coding capacity of the fragment. Moreover, in a recent paper, Postle and Reznikoff (23) reported some unpublished data that the *ton*B product identified by them had a molecular weight of 36kd very similar to that found in this study.

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