PROTEIN *d*, AN IRON-TRANSPORT PROTEIN INDUCED BY FILTRATION OF CULTURES OF *ESCHERICHIA COLI*

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1. Introduction

We have previously described a 76 000 dalton membrane protein of *E. coli* B/r, whose synthesis is apparently restricted to a brief period during the cell cycle [1]. We also reported that a membrane protein of identical molecular weight can be induced by thymine starvation [2]. Gudas, James and Pardee [3] have also described a similar protein, designated protein D, apparently synthesised during a brief period during the cell-cycle of *E. coli* B/r. In addition Gudas et al. reported that bursts of protein *d* synthesis occurred following re-addition of amino acids to a starved culture and after removal of nalidixic acid from a treated culture. On the basis of this body of data Gudas et al. proposed that protein *d* has a role in control of DNA replication.

Recently our attention was drawn to several outer membrane proteins of similar molecular weight to protein D which are present at high levels in *tonB* mutants [4,5] and in wild-type strains under conditions of limiting iron concentration [6,7]. In this paper we present evidence which clearly indicates that the initially designated 76 000 dalton protein (protein *d*) is in fact identical with the product of the *feuB* locus, a binding protein essential for enterochelinmediated iron-transport [8]. Furthermore we present evidence that the synthesis of this and a related group of outer membrane proteins is induced by merely filtering a bacterial culture and resuspending the cells in identical fresh medium. Much of the evidence pertaining to a role for protein *d* in DNA replication is thus invalidated.

2. Materials and methods

2.1. *Strains, growth conditions and filtration of bacteria*

E. coli B/r LEB16 has been described previously by Meacock and Pritchard [9] and by Churchward and Holland [1]. *E. coli* K12 strains AB2847 and VR42/B9 were kindly supplied by Dr V. Braun. Strain VR42/B9 is a *feuB cir* strain, defective in ferric enterochelin uptake. Strain AB2847, the parent strain, is *aroB thi tsx* λ^r.

E. coli B/r LEB16 was grown in proline–alanine minimal medium supplemented with thymine, 20 μg/ml, as described previously [1]. The K12 strains were grown in M9-glucose medium supplemented as follows: tyrosine 40 μg/ml, tryptophan 20 μg/ml, phenylalanine, 40 μg/ml, shikimic acid 40 μg/ml, vitamin B1 2 μg/ml.

Removal of medium from growing cultures was achieved by filtration through a membrane filter (Sartorius, 0.45 μm pore size) previously washed with fresh, warm resuspension medium, followed by washing and resuspension of bacteria in fresh, warm medium.

2.2. *Pulse-labelling*

Cultures were pulse-labelled by removal of 1.0 ml culture into 1.0 ml warm medium containing either 1 μCi L-[U-^{14}C]leucine (Amersham 311 mCi/mmol) or 8.5 μCi L-[^{35}S]methionine (Amersham 290 Ci/mmol). After 4 min the pulse was terminated by addition of chloramphenicol (final concentration 300 μg/ml) and either leucine or methionine (final concentration 2 mg/ml).

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2.3. *Envelope preparation*

The basic procedure was as described by Churchward and Holland [1] except that 10 mM $MgSO_4$ was omitted from the buffer. To prepare an outer membrane fraction, washed envelope pellets were resuspended in 0.2 ml 0.5% w/v Sarkosyl NL97, incubated at room temperature for 30 min and the outer membrane material pelleted by centrifuging at 70 000–100 000 × *g* for 2 h.

2.4. *Polyacrylamide gel electrophoresis*

SDS–PAGE was performed either as described previously by Churchward and Holland [1] using an acrylamide monomer : dimer ratio of 37.5 to 1 or by the modified method described by Hancock et al. [10] using a monomer : dimer ratio of 147 to 1 which gives better resultion, particularly of these polypeptides in the 70–90 K range.

2.5. *Autoradiography and microdensitometry*

Gels were prepared for fluorography as described previously [1] except that the PPO-impregnated gels were dried on a Bio-Rad Gel Slab Dryer.

Autoradiography was as before [1] except that Kodak RPH X-ray film was used. The resulting autoradiograms were canned using a Joyce-Loebl microdensitometer. The area under a peak was used as a measure of the rate of synthesis of that particular polypeptide. Ratios of peak areas were thus used as a measure of relative rates of synthesis. The particular peaks used are indicated in the text.

3. Results and discussion

When cultures of *E. coli* B/r LEB16 growing in medium containing thymine are filtered and resuspended in fresh medium lacking thymine the induced synthesis of a 76 000 dalton membrane protein (protein *d*) can be demonstrated by SDS–PAGE using the basic Laemmli procedure [2].

As shown in figs 1 and 2 the synthesis of certain outer membrane proteins is still nevertheless induced when the LEB16 strain is filtered and resuspended under conditions where thymine is present throughout. The induction pattern is in fact more complex than that obtained previously, due to increased resolution afforded by the use of the detergent Sarkosyl to prepare an outer membrane fraction, and the use of the modified gel system in this study [10].

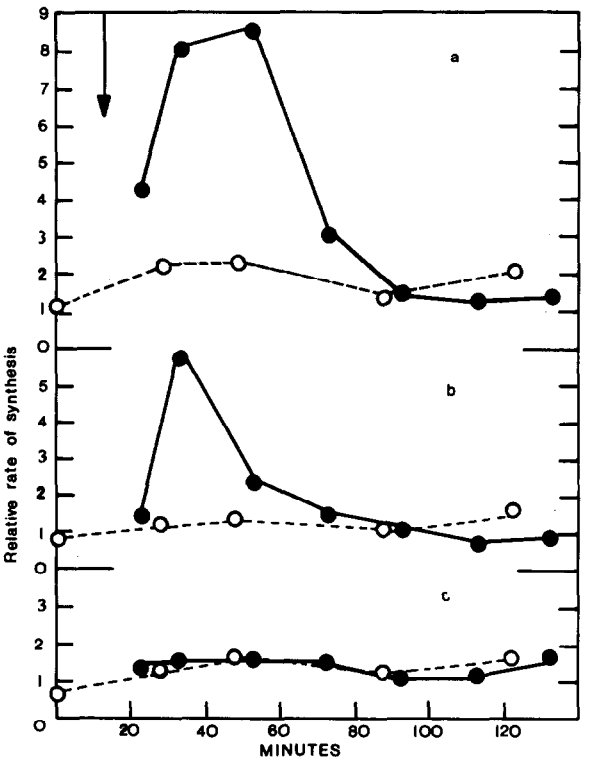

Fig.1. Relative rates of synthesis of outer membrane proteins. An exponentially growing culture of *E. coli* B/r was pulse-labelled with [^{14}C]leucine. Half of the culture was filtered at the time indicated by the arrow and resuspended in fresh medium, the other half was left untreated. Samples of both cultures were then pulse-labelled at intervals. Outer membranes were prepared and run on an 11% acrylamide gel. The resulting autoradiograph (not shown) was scanned using a Joyce-Loebl microdensitometer. The data shown are rates of synthesis relative to a non-inducible polypeptide in the 58 000 dalton band (see fig.2). (a) Relative rate of synthesis of the 81 000 dalton polypeptide. Similar results although not shown are obtained for the 74 000 dalton polypeptide. (b) Relative rate of synthesis of the 79 000 dalton polypeptide. (c) Relative rate of synthesis of the 90 000 dalton polypeptide, a non-inducible protein. Open circles denote untreated culture; filled circles denote filtered culture.

Three outer membrane proteins are seen to be induced, the largest (81 000 daltons*) being identical

*Relative mobilities in the modified gel system differ from these previously obtained with the basic Laemmli procedure: for simplicity therefore we have used molecular weight assignments designated by Hancock et al. [10] for these polypeptides.

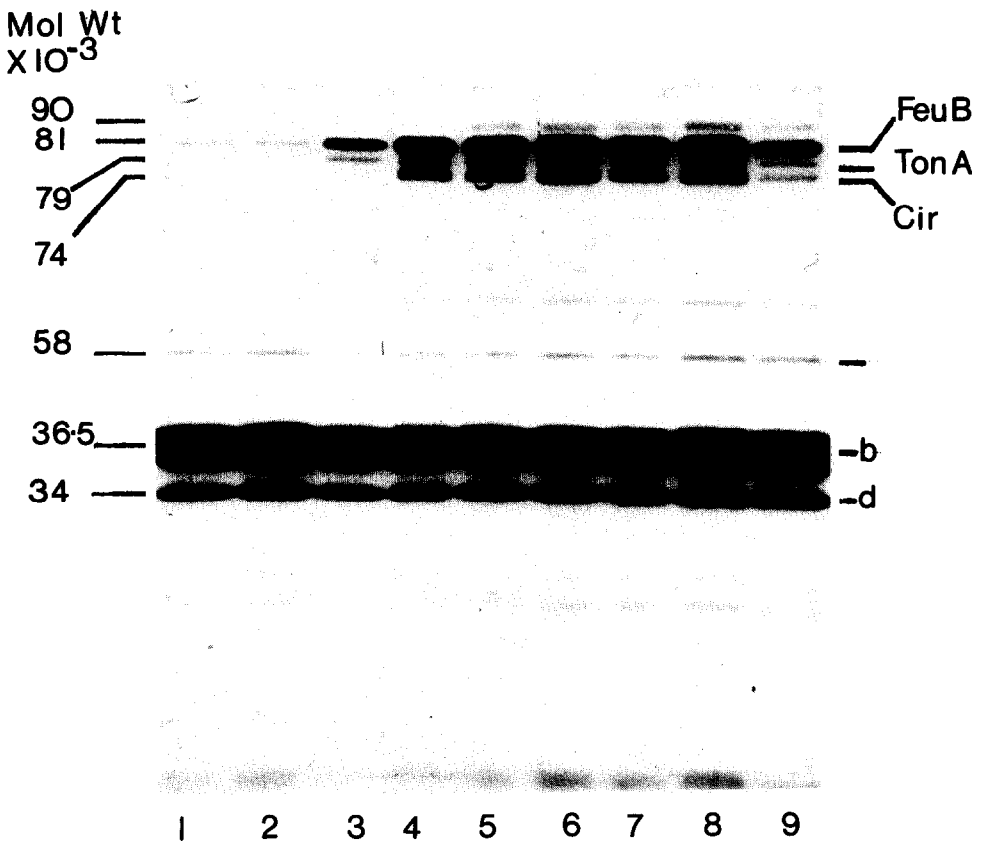

Fig.2. Autoradiograph of 10% slab-gel profiles of outer membranes. Samples of an exponentially growing culture of *E. coli* B/r were pulse-labelled with [^{14}C]leucine. The cells were filtered and resuspended in warm conditioned medium obtained by filtration of an exponential culture. Half of this suspension was then pulse-labelled at intervals. The remainder was filtered again and finally resuspended in fresh warm medium before pulse-labelling. Outer membranes were prepared and subjected to electrophoresis. Slots (1) and (2), 20 min and 10 min before filtration. (3) and (4) 10 min and 20 min after filtration and resuspension in conditioned medium. (5) 25 min in fresh medium. (6) 30 min in conditioned medium. (7) 35 min in fresh medium. (8) 40 min in conditoned medium. (9) 40 min in fresh medium.

to the 76 000 dalton polypeptide as observed with the original Laemmli gel system. Similar results are also obtained when a thymine independent revertant of LEB16 is filtered and re-incubated. Inhibition of DNA synthesis by withdrawal of thymine is not therefore required to promote induction as previously suggested [2]. Moreover, under these conditions induction is only transient and after about 60 min the rate of synthesis returns to the control level. We wish to emphasise here that these effects of filtration are quite specific since no disturbance of the normal rate of synthesis of cytoplasmic protein, bulk envelope protein or the majority of individual outer membrane proteins (e.g., the 90 K protein, fig.1) is detected.

Attempts were made to further identify the filtration-induced proteins by analysis of *E. coli* B/r and K12 strains AB2847 and its derivatives VR42/B9; the latter is a *cir, feuB* double mutant, which, as shown in fig.3, lacks two specific iron-binding proteins, 74 000 and 81 000 daltons respectively [10]. The data in fig.3 shows that in contrast to the B/r strain filtration of the double mutant fails to induce the synthesis of any polypeptides with these molecular weights. However, an 83 000 dalton polypeptide is induced and this appears to be identical to another iron-binding protein described by Hancock and Braun

[7]. This polypeptide, which fails to separate from the *feuB* protein using the basic Laemmli gel system, has not so far been observed in *E. coli* B/r under any conditions. A further filtration induced protein, in both *E. coli* B/r and strain VR42/B9 is identical to the *tonA* gene product, a polypeptide absent from the outer membrane of some phage T1 resistant B/r and K12 strains ([6,7] and our unpublished data).

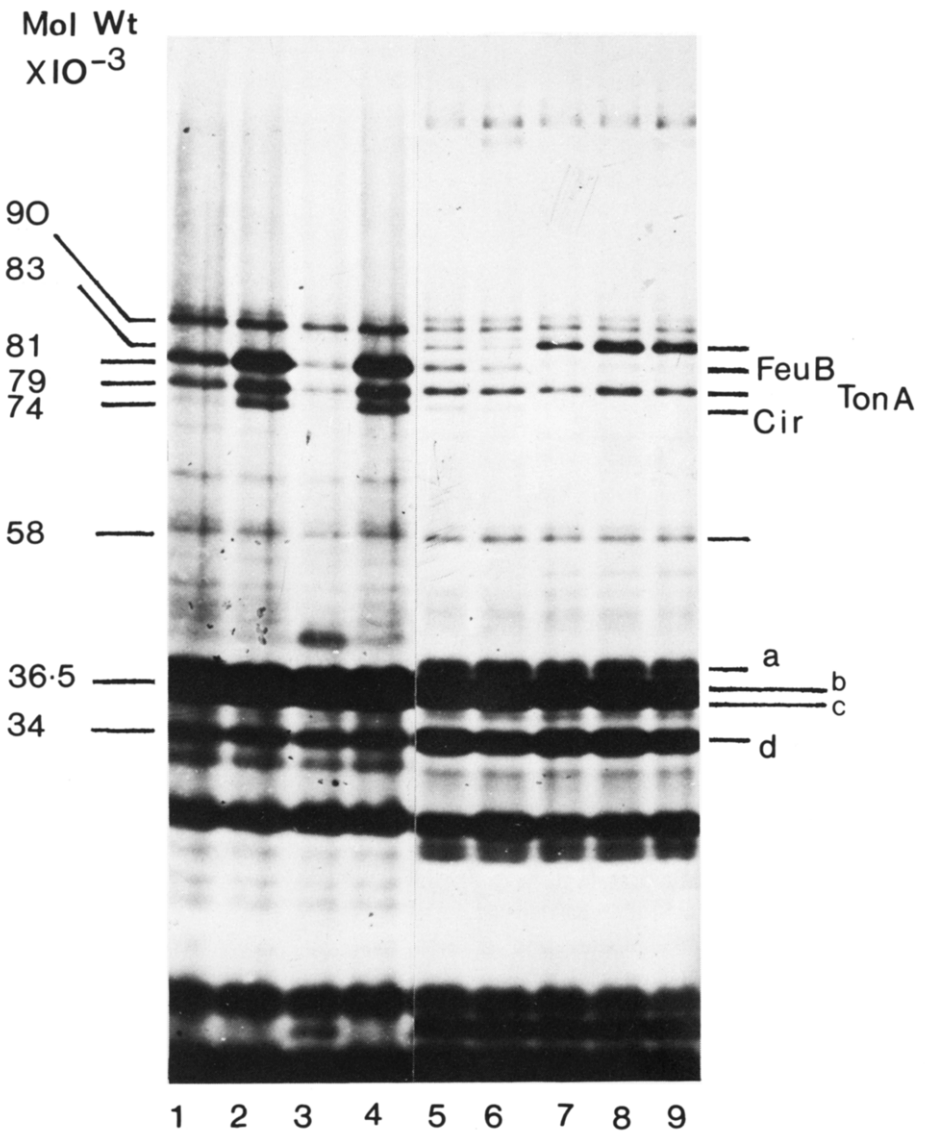

Fig.3. Autoradiography showing induction of iron-transport proteins in *E. coli* B/r and in a *feuB, cir* mutant of *E. coli* K12. Strains were filtered and resuspended in the usual way and then pulse labelled with [^{35}S]methionine at intervals. In the case of *E. coli* B/r half of the resuspended culture was immediately supplemented with $FeCl_3$ (final concentration, 100 μM). Outer membranes were isolated and analysed on an 11% acrylamide slab-gel [10]. Slots (1–4) *E. coli* B/r. (1) Labelled before filtration. (2) Filtered and labelled after 20 min. (3) Filtered + $FeCl_3$. After 20 min or after 30 min. (4) Minus $FeCl_3$, after 30 min. Slots (5), (6) strain AB2847 unfiltered. (7) Strain VR42/B9 labelled before filtration. (8) Labelled 20 min after filtration. (9) Labelled 30 min after filtration. Approximately equal numbers of radioactive counts were applied to each slot. Bands (a,b,c,d) designated according to ref. [13].

Examination of fig.3 also indicates that the extent of induction of the iron-binding proteins in *E. coli* VR42/B9 is much less than that obtained with the B/r strain. In fact, some *E. coli* K12 strains have been found to be poorly inducible by filtration whilst others behave like *E. coli* B/r. The basis for this effect is not known but may relate to other factors affecting the integrity of the outer membrane.

To test the hypothesis that filtration-induced synthesis of these outer membrane proteins is a response to low intracellular levels of iron, a filtered culture of LEB16 was resuspended in fresh medium containing 100 μM $FeCl_3$. This high level of iron should provide sufficient intracellular iron, via the postulated passive iron-uptake system [11], to relieve any iron-deficit. The data in fig.3 clearly show that the induction of the three outer membrane proteins in LEB16 was largely supressed by the presence of 100 μM $FeCl_3$. In contrast, the presence of 100 μM $FeCl_3$ had no apparent effect on the overall growth of the culture or on any other outer membrane proteins.

Attempts to suppress the induction of these proteins by resuspension of the filtered cells in conditioned medium prepared either by filtration (fig.2) or centrifugation (unpublished data) have so far failed and it thus appears that induction of synthesis may be due to filtration per se rather than to resuspension in fresh medium lacking enterochelin, ferrichrome, or any other iron-chelating compounds normally present in an undisturbed culture.

In order to clarify further the relationship between these iron-binding polypeptides and the protein *d* described by Gudas et al. we note that studies carried out in this laboratory, involving amino acid starvation of *E. coli* K12 strains (involving filtration of cells) or temperature-shift experiments with a *dnaA* mutant also lead to the induction of two polypeptides identical in molecular weight to the *feuB* and *cir* proteins [12].

The results presented above, which clearly identify the previously designated 76 000 dalton polypeptide or protein D as the *feuB* gene product, have important implications for previous studies of this protein. These studies indicated that this protein is only synthesised during a brief period of the cell-cycle of *E. coli* B/r [1,3] and that this might be related to its possible role in control of DNA synthesis [3]. In our own studies [1] the generation of synchronous cultures did involve prior filtration of the bacteria and therefore the apparent periodicity of protein *d* synthesis under these conditions should now be re-investigated. Similarly, much of the published data [3] on the role of protein *d* in DNA replication was based upon experiments involving filtration or procedures likely to promote envelope damage (e.g., mecillinam treatment [14]) much of this data must now be considered invalid.

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