THE FERRICHROME RECEPTOR PROTEIN (*tonA*) OF *ESCHERICHIA COLI* IS SYNTHESISED AS A PRECURSOR IN VITRO

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1. Introduction

The importance of iron for bacterial growth is reflected by the number of systems that have been identified for the active transport of iron into *Escherichia coli*. These systems depend upon the solubilization and chelation of ferric-ions from insoluble hydroxy-iron polymers by siderophores. Uptake systems have been identified in *E. coli* for ferrichrome [1], rhodoturulic acid [2], aerobactin [3] and citrate [4] as well as for the endongenous chelator enterochelin [5].

Three components of the ferrichrome transport system have been identified. The initial stage of transport is the binding of ferrichrome to an outer membrane protein, the *tonA* (*fhuA*) gene product. The *tonA* protein is also the receptor for the phages T_1, T_5 and $\phi 80$ and colicin M, and mutants resistant to these agents are also defective in the transport of ferrichrome [1]. Ferrichrome can protect sensitive cells from killing by phage and colicin [1,6,7] which suggests that they share a common binding site with ferrichrome on the receptor. Subsequent transport of the ferric-complex from the outer membrane across the cell envelope requires the *tonB* gene product, an inner membrane protein [8], which is involved in the transport of all the ferric-siderophores and vitamin B_{12} [9]. A third locus *fhuB* has been identified and postulated to have an inner membrane permease function [2]. To study the biogenesis of the *tonA* protein we have used a plasmid clone carrying the *tonA* region and here we describe the identification of the primary translation product of the *tonA* gene in a coupled transcription–translation system. The *tonA* protein synthesized in vitro is ~2000 M_r larger than the mature membrane protein; partial processing of this precursor to the mature form was obtained by the addition of membrane vesicles to the in vitro system.

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2. Materials and methods

The plasmid pL C19-19 was identified from the Clarke and Carbon collection of ColE1–*E. coli* hybrid plasmids [10] as carrying *ponB* and *tonA* [11] (and was obtained from Y. Hirota). *Escherichia coli* K12 strains used were C600 *thr, leu tonA* and KN126 *ilv trp*-am *tyr*-am *sup*D126ts. KN126 *tonA* was obtained by selecting for T_5-resistant mutants. Cultures were grown in M9-glucose medium and labelled with [^{35}S]methionine (Amersham). Dipyridyl was used at a final concentration of 100 μM.

Outer membranes were prepared as sarkosyl-insoluble fractions from total cell envelopes [12]. The acrylamide monomer:dimer ratio was 44:0.3. The in vitro coupled transcription–translation system was based on that in [13] as modified [14]. Partial proteolysis of polypeptides from SDS–polyacrylamide gel slices was as in [15] using *Staphylococcus aureus* V8 protease.

3. Results and discussion

Transformants of C600 (*tonA*) with the plasmid pLC19-19 DNA were sensitive to killing by phages T_1, T_5, $\phi 80$ and colicin M and have therefore gained the *tonA* wild-type allele. Fig.1 shows an SDS–PAGE

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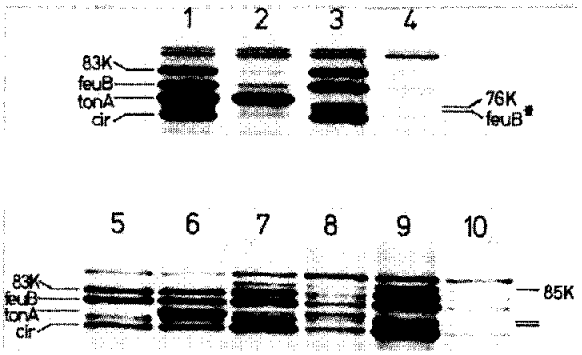

Fig.1. SDS–PAGE of outer membranes from exponentially growing cultures labelled with [^{35}S]methionine in the presence (or absence) of dipyridyl (DP). Only the regions of the gels containing the dipyridyl induced proteins are shown. Slots (1,6) C600 (pLC19-19) plus DP; (2) C600 (pLC19-19) no addition; (3,5) C600 plus DP; (4) C600 no addition; (7) KN126 plus DP; (8) KN126 no addition; (9) KN126 *tonA* plus DP; (10) KN126 *tonA* no addition. See also [18] for identification of proteins 83 000 M_r *feuB* and *cir* induced by iron stress. We also observed additional new bands between *tonA* and *cir*; these could be resolved into two under certain conditions, a 76 000 M_r and a smaller polypeptide which is absent from *feuB* mutants (unpublished), this is probably a degradative product of *feuB*. In addition, an 85 000 polypeptide appeared to be induced in KN126 (slot 9).

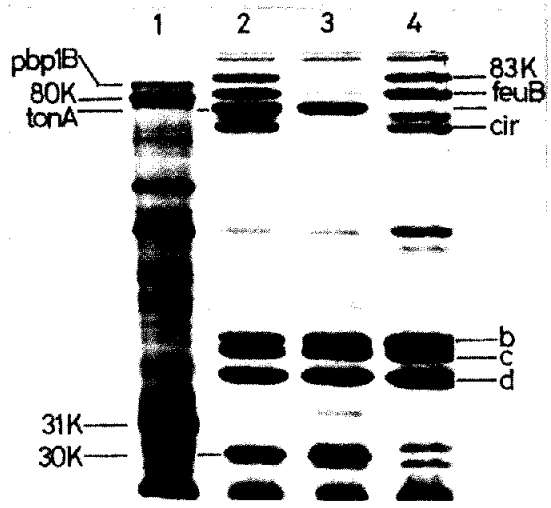

Fig.2. Identification of the *tonA* precursor I: in vitro protein synthesis programmed by pLC19-19 (slot 1) and outer membrane proteins from C600 (pLC19-19) (plus DP slot 2, no addition slot 3) and C600 *tonA* (plus DP slot 4). The positions of the major outer membrane proteins, *b* (*ompF*), *c* (*ompC*) and *d* (*ompA*) are indicated. Other polypeptides indicated are as described in fig.1 or as in the text.

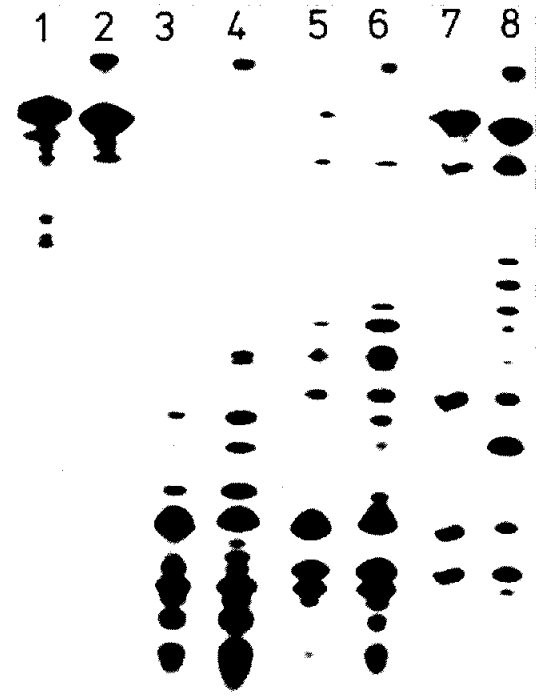

Fig.3. Identification of the *tonA* precursor II: partial proteolysis of *tonA* (slots 2,4,6 and 8) synthesised in vivo and the 80 000 M_r (slots 1,3,5,7) polypeptide synthesised in vitro with *Staphylococcus aureus* V8 protease (slots 1,2, no addition; 3,4, 1 μg protease; 5,6, 0.2 μg; 7,8, 0.04 μg).

analysis of outer membrane proteins from these strains together with a second *tonA* mutant and its parent. Dipyridyl, a non-utilizable iron chelator, was added to each strain (slots 1,3,5–7,9) to induce the synthesis of a number of high relative molecular mass (M_r) proteins, one of which is the ferric-enterochelin receptor (*feuB*). It can be seen that the outer membrane of C600 (pLC19-19) contains, even in the absence of dipyridyl, large amount of a 78 000 M_r protein which migrates at an identical position to the *tonA* protein of strain KN126. The large amount of the *tonA* protein might be expected since the *tonA* gene is present on a multicopy plasmid. Concerning the regulation of *tonA* protein synthesis, increased levels of *tonA* protein were reported [17,18] under conditions of iron stress, although in [6,18] such induction could not be shown. Here, we have shown that *tonA* synthesis is hardly affected by growth of bacteria in the presence of dipyridyl (fig.1, slots 7,8), although *feuB* and a number of other proteins involved in iron uptake are induced. This suggests that the synthesis of *tonA* protein is regulated in a different way to that of *feuB*. Moreover, in C600 (pLC19-19) the *tonA* protein is synthesised in large amounts while

the inducible iron-binding proteins, like *feuB*, are only synthesised at their repressed levels. This is further evidence against a common regulatory system.

The proteins programmed by pLC19-19 DNA in an in vitro transcription–translation system are shown in fig.2 (slot 1). Although there was no protein corresponding to the M_r of the *tonA* protein normally present in outer membranes (slot 2), a major product of 80 000 M_r was synthesised. This polypeptide was shown to be virtually identical to the *tonA* protein by comparing *Staphylococcus aureus* protease digests of this protein with that of *tonA* from the outer membrane of C600 (pLC19-19) (fig.3). Presumably, the 80 000 M_r polypeptide contains a leader polypeptide, required for the insertion of the protein into the membrane, which is cleaved during the assembly process as predicted by the Signal hypothesis [19]. Indeed, the 80 000 M_r polypeptide can be cleaved to produce a 78 000 M_r polypeptide when inverted inner membrane vesicles are included in the in vitro incubation mix (not shown).

Interestingly, plasmid pLC19-19 was found to code for a 30 000 M_r outer membrane protein in vitro, (fig.2, slots 2,3) which also appeared to be synthesized in vitro as a precursor of 31 000 (fig.2, slot 1). The inner membrane protein PBP1B (coded by *ponB*) also coded for by this plasmid did not appear to be synthesized as a precursor (not shown).

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References

[1] Hantke, K. and Braun, V. (1975) FEBS Lett. 49, 301–305.
[2] Kadner, R. J., Heller, K., Coulton, J. W. and Braun, V. (1980) J. Bacteriol. 143, 256–264.
[3] Warner, P. J., Williams, P. H., Bindereif, A. and Neilands, J. B. (1981) Infect. Immun. in press.
[4] Frost, G. E. and Rosenberg, H. (1973) Biochim. Biophys. Acta 330, 90–101.
[5] Rosenberg, H. and Young, I. G. (1974) in: Microbial Iron Metabolism (Neilands, J. B. ed) pp. 67–82, Academic Press, London, New York.
[6] Luckey, M., Wayne, R. and Neilands, J. B. (1975) Biochem. Biophys. Res. Commun. 64, 687–693.
[7] Wayne, R., Frick, K. and Neilands, J. B. (1976) J. Bacteriol. 126, 7–12.
[8] Plastow, G. S. and Holland, I. B. (1979) Biochem. Biophys. Res. Commun. 90, 1007–1014.
[9] Braun, V. and Hantke, K. (1977) in: Microbial Interactions (Reissig, J. L. ed) pp. 99–137, Springer-Verlag, Berlin, New York.
[10] Clarke, L. and Carbon, J. (1976) Cell 9, 91–99.
[11] Takeda, Y., Nishimura, A., Nishimura, Y., Yamada, M., Yasuda, S., Suzuki, H. and Hirota, Y. (1981) Plasmid, in press.
[12] Boyd, A. and Holland, I. B. (1979) Cell 18, 287–296.
[13] Zubay, G. (1973) Annu. Rev. Genet. 7, 267–287.
[14] Collins, J. (1979) Gene 6, 29–42.
[15] Cleveland, D. W., Fischer, S. G., Kirschner, M. W. and Laemmli, U. K. (1977) J. Biol. Chem. 252, 1102–1106.
[16] Pugsley, A. P. and Reeves, P. (1976) Biochem. Biophys. Res. Commun. 70, 846–853.
[17] Pugsley, A. P. and Reeves, P. (1977) Biochem. Biophys. Res. Commun. 74, 903–911.
[18] Braun, V., Hancock, R. E. W., Hantke, K. and Hartman, A. (1976) J. Supramol. Struct. 5, 32–58.
[19] Blobel, G. and Dobberstein, B. (1975) J. Cell Biol. 67, 835–851.