AN INDUCIBLE OUTER MEMBRANE PROTEIN INVOLVED IN AEROBACTIN-MEDIATED IRON TRANSPORT BY ColV STRAINS OF *ESCHERICHIA COLI*

K. K. GREWAL, P. J. WARNER* and P. H. WILLIAMS†
Department of Genetics, University of Leicester, Leicester, LE1 7RH, England

Received 8 February 1982

1. Introduction

Iron is an essential trace element for microbial growth. One of the factors affecting the virulence of an invasive bacterial pathogen is its ability to sequester sufficient iron for growth from complexes with iron-binding proteins such as transferrin in the serum of infected animals [1]. *Escherichia coli* secretes the high affinity catechol siderophore enterobactin (enterochelin [2]) into its environment, and ferric-enterobactin is subsequently actively transported back across the bacterial membranes [3]. A significant proportion of bacteraemic isolates of *E. coli* utilise an additional independent iron uptake system which is plasmid specified [4], and which involves the hydroxamate siderophore aerobactin [5–7]. Genetic experiments have indicated that the uptake of ferric-aerobactin by these strains requires both chromosomal genes (including *tonB*) and plasmid gene functions [8]. We report here the identification of an outer membrane protein, specified by a ColV plasmid, whose synthesis is regulated by the availability of Fe^{3+}.

The outer membrane of *E. coli* may contain a number of minor protein species of high relative molecular mass (M_r) whose synthesis is clearly induced in conditions of iron deprivation. They include the products of the *fep* gene ([3], M_r 81 000) and the *fecA* gene ([9], M_r 80 000) which are involved in the uptake of ferric-enterobactin and ferric-citrate, respectively. Increased levels of the product of the *tonA* (*fhuA*) gene ([10], M_r 78 000) have also been reported in conditions of iron stress [11]. However, there is evidence that results are complicated by the presence of a degradation product of the Fep protein at a similar position in polyacrylamide gels [12]. A protein (M_r 83 000) which has not yet been defined genetically [3], and the *cir* gene product ([13], M_r 74 000), the receptor for colicins V and I, are also induced during iron starvation, but neither has so far been implicated in any known iron uptake system. The plasmid-specified outer membrane protein described here has, at least under the conditions used for analysis up to now, the same apparent molecular mass as the Cir protein, and is therefore revealed only in *cir* mutants. Strains carrying mutant ColV plasmids rendering them unable to transport aerobactin-complexed iron into the cell are shown to lack the plasmid specific 74 000 M_r (74 kM_r) outer membrane protein.

2. Materials and methods

Strains of *E. coli* K12 used in this work are described in table 1. Strain LG1419, which was isolated following *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis [8], carries a revertable (point) mutation in the ColV-K30 plasmid. Since it could not be ruled out that mutants of this type might show normal assembly of inactive protein into the membrane, a phenotypically similar mutant was also derived by transposition mutagenesis; insertion of a transposon will be expected to result in failure to synthesise a product. ColV-K30 was transferred by conjugation at 30°C to strain UB281(pMR5) which harbours a temperature-sensitive mutant of RP1 [14]. The colicinogenic derivative was grown at 42°C and used as the donor in conjugation with AN1937; selection was

* Present address: Department of Microbiology, National Institute for Research in Dairying, Shinfield, Reading, England

† To whom correspondence should be addressed

Published by Elsevier Biomedical Press
00145793/82/0000–0000/$02.75

Table 1
Bacterial strains

Designation	Characteristics[a]
AN1937	*ara entA lac leu mtl proC rpsL supE thi tonA trpE xyl* [4]
LG1315	Derivative of AN1937 carrying plasmid ColV-K30 Iu^+ [4]
LG1419	Derivative of AN1937, *nalA*, carrying plasmid ColV-K30*iut* [8]
UB281(pMR5)	*pro met nalA*, carrying plasmid pMR5 (Ap^r [Tn*801*] Km^r Tc^r) [14]
LG1514	Derivative of AN1937, *nalA*, carrying plasmid ColV-K30::Tn*801 iut*
LG1562	Derivative of AN1937, *cir* (spontaneous)
LG1565	Derivative of LG1562 carrying plasmid ColV-K30 Iu^+ from LG1315
LG1564	Derivative of LG1562 carrying plasmid ColV-K30*iut* from LG1419
LG1563	Derivative of LG1562 carrying plasmid ColV-K30::Tn*801 iut* from LG1514

[a] Plasmid phenotypes: Ap^r ampicillin resistance, Km^r kanamycin resistance, Tc^r tetracycline resistance, Iu^+ iron uptake proficient, *iut* defective in transport of iron

made for conjugal transfer of ampicillin resistance, which will occur only if there has been transposition of Tn*801* (Ap^r) from the RP1 mutant to ColV-K30. Transconjugants which synthesised colicin V were tested for their ability to grow in iron-limiting conditions; one derivative which failed to do so was designated LG1514. Both LG1419 and LG1514 over-produce aerobactin, and are therefore assumed to be defective in some aspect of the transport of iron into the cell [8].

Growth media and culture conditions, and the procedure for detecting the uptake of radioactive iron into non-growing bacteria have been described in [4,8].

Outer membranes were prepared by the rapid isolation technique in [15]. Briefly, this involved conversion of cells to spheroplasts by treatment with lysozyme–EDTA, and lysis with Triton X-100. The outer membrane fraction was recovered by centrifugation (40 000 × *g*), washed repeatedly and analysed on 13% sodium dodecyl sulphate (SDS)–polyacrylamide gels (monomer/dimer ratio, 44:0.3) with 7% stacking gels.

3. Results

Strain AN1937 is defective in enterobactin biosynthesis (*entA*) and cannot therefore actively take up iron from the external medium. After a shift from iron-rich nutrient broth, in which growth is supported by non-specific entry of Fe^{3+} into the cell, to minimal medium relatively low in iron (2 μM), the strain continues to grow for several generations due to the presence of intracellular iron pools. As these are depleted, the onset of iron starvation causes induction of the characteristic outer membranes over 74 000–83 000 M_r (fig.1a). Strain LG1562 is a *cir* derivative of AN1937, resistant simultaneously to colicins V and I. The mutation results in failure of the 74 000 M_r Cir protein to assemble in the outer membrane (it should be noted that not all *cir* mutants are of this type), although induction of synthesis of the other high molecular mass proteins in conditions of iron stress occurs normally (fig.1b).

Wild-type plasmid ColV-K30 was transferred by conjugation into *cir* mutant LG1562. The transconjugant, designated LG1565, sequesters iron from the growth medium by the plasmid-specified aerobactin system. When iron is freely available, synthesis of the outer membrane proteins involved in iron transport is

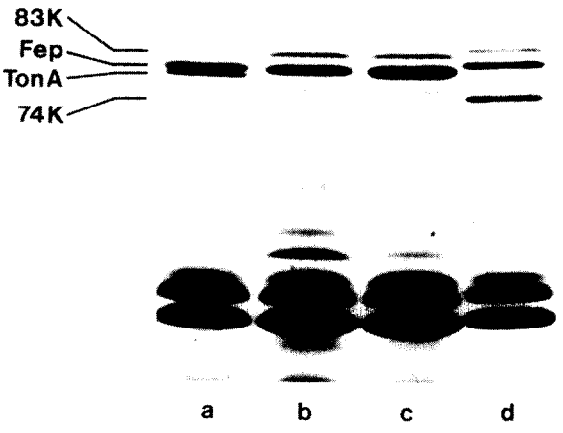

Fig.1. Identification of a plasmid-specified 74 000 M_r (74 K) outer membrane protein. Outer membrane preparations of the following strains grown as described in the text were analysed by SDS–polyacrylamide gel electrophoresis: (a) AN1937 (*entA* Cir^+); (b) LG1562 (*cir*); (c) LG1565 (*cir* ColV-K30 Iu^+); (d) LG1565 in the presence of transferrin. The 83 K protein (M_r 83 000) has not yet been assigned a function. Fep (M_r 81 000) is the receptor for ferric-enterobactin [3]. Cir (M_r 74 000) is the receptor for colicins V and I [13]. It is not clear whether the TonA protein (M_r 78 000), the receptor for ferric-ferrichrome [10], is inducible under the conditions of this experiment; the bands indicated may comprise significant levels of a degradation product of the Fep protein [12].

repressed (fig.1c), but effective reduction of the level of available iron by addition of excess transferrin (6 μM) to the growth medium markedly induces the synthesis of Fep and the other high M_r proteins. In addition, a novel plasmid specific protein with app. M_r 74 000 is also induced (fig.1d). This protein is masked in Cir$^+$ strains such as AN1937.

Evidence for the involvement of this inducible protein (74 kM_r) in iron transport comes from analysis of outer membranes of derivatives of LG1562 carrying *iut* mutant plasmids. One such plasmid, having a point mutation, has been described in [8]. A similar mutant, derived by insertion of transposon Tn*801*, was isolated as in section 2. Strains harbouring either plasmid secrete aerobactin, but are nevertheless unable to transport radioactive iron into the cell (table 2). Furthermore, derivatives of LG1562 harbouring either plasmid (designated LG1563 and LG1564, see table 1) do not exhibit significant levels of the plasmid-specified 74 kM_r protein in the outer membrane when grown to iron starvation in minimal medium, conditions which effectively promote induction of synthesis of the other characteristic outer membrane proteins.

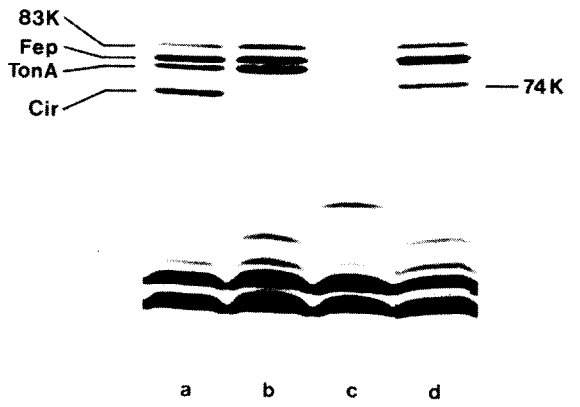

Fig.2. Absence of 74 kM_r protein from outer membranes of strains carrying *iut* mutant plasmids. Outer membrane preparations of the following strains grown as described in the text were analysed by SDS–polyacrylamide gel electrophoresis: (a) LG1562 (*cir*); (b) LG1564 (*cir* ColV-K30 *iut*); (c) LG1563 (*cir* ColV-K30::Tn*801 iut*); (d) LG1565 (*cir* ColV-K30 Iu$^+$) in the presence of transferrin. See fig.1 for identification of protein bands.

4. Discussion

We report here the identification of a 74 000 M_r protein (74 kM_r) in the outer membrane of *E. coli* strains carrying plasmid ColV-K30 grown in the presence of transferrin. The observation that a particular protein is inducible by conditions of iron stress is not, of course, proof of its involvement in iron uptake. Direct evidence for the proposal that the protein described here has a role in the uptake of ferric-aerobactin is provided by the finding that mutants defective in the uptake of ^{55}Fe lack the 74 kM_r protein in the outer membrane, and we conclude therefore that this protein represents at least part of the ferric-aerobactin receptor.

A similar conclusion has been reached by an alternative approach. Thus, in *Enterobacter cloacae*, which secretes both catechol and hydroxamate siderophores, the outer membrane receptor for ferric-aerobactin is also the receptor for cloacin DF13, a bacteriocin produced by certain strains of this organism [16]. Furthermore, the presence of ColV plasmids in *E. coli* strains (normally insensitive to cloacin DF13) renders them susceptible to killing by this agent [16,17]. The observation that aerobactin competitively inhibits the lethal effect of cloacin DF13, and the finding that cloacin resistant mutants of *E. coli* harbouring a ColV plasmid lack a plasmid-specified 74 000 M_r outer membrane protein [17], also strongly suggest that the 74 kM_r protein participates in ferric-aerobactin uptake. As would be predicted, we find that strains carrying *iut* mutant plasmids are resistant to cloacin DF13.

Table 2
Uptake of radioactive iron by strain AN1937 (*entA*) and plasmid carrying derivatives[a]

Bacterial strain	Plasmid	^{55}Fe retained on filters (cpm)
AN1937	–	41
LG1315	ColV-K30 Iu$^+$	18 841
LG1419	ColV-K30*iut*	337
LG1514	ColV-K30::Tn*801 iut*	25

[a] Bacteria growing exponentially in appropriately supplemented iron-depleted medium (<0.4 μM) were harvested by centrifugation, washed and resuspended to 5 × 10^8 cells/ml in similar medium lacking L-tryptophan but containing 100 μM sodium nitrilotriacetate. Carrier-free $^{55}FeCl_3$ (1 μCi/ml) was added, and suspensions were incubated with aeration by shaking for 30 min at 37°C. Cells from 10 ml of each suspension were washed on membrane filters with medium containing 100 mM sodium citrate. Filters were air-dried and the level of ^{55}Fe label retained was counted in a Packard model 3255 liquid scintillation counter

Acknowledgements

We are grateful to Martyn Robinson for strain UB281(pMR5), to Barry Holland for helpful discussions, and to Maureen Burns for excellent technical assistance. This work was supported by project grant G979/461/C from the Medical Research Council.

References

[1] Weinberg, E. D. (1978) Microbiol. Rev. 42, 45–66.
[2] O'Brien, I. G. and Gibson, F. (1970) Biochim. Biophys. Acta 215, 393–402.
[3] Hancock, R. E. W., Hantke, K. and Braun, V. (1976) J. Bacteriol. 127, 1370–1375.
[4] Williams, P. H. (1979) Infect. Immun. 26, 925–932.
[5] Stuart, S. J., Greenwood, K. T. and Luke, R. K. J. (1980) J. Bacteriol. 143, 35–42.
[6] Braun, V. (1981) FEMS Microbiol. Lett. 11, 225–228.
[7] Warner, P. J., Williams, P. H., Bindereif, A. and Neilands, J. B. (1981) Infect. Immun. 33, 540–545.
[8] Williams, P. H. and Warner, P. J. (1980) Infect. Immun. 29, 411–416.
[9] Wagegg, W. and Braun, V. (1981) J. Bacteriol. 145, 156–163.
[10] Hantke, K. and Braun, V. (1975) FEBS Lett. 49, 301–305.
[11] Pugsley, A. P. and Reeves, P. (1977) Biochem. Biophys. Res. Commun. 74, 903–911.
[12] Plastow, G. S., Pratt, J. M. and Holland, I. B. (1981) FEBS Lett. 131, 262–264.
[13] Hancock, R. E. W. and Braun, V. (1976) FEBS Lett. 65, 208–210.
[14] Robinson, M. K., Bennett, P. M., Falkow, S. and Dodd, H. M. (1980) Plasmid 3, 343–347.
[15] Hantke, K. (1981) Mol. Gen. Genet. 182, 288–292.
[16] Van Tiel-Menkveld, G. J., Oudega, B., Kempers, O. and De Graaf, F. K. (1981) FEMS Microbiol. Lett. 12, 373–380.
[17] Bindereif, A., Braun, V. and Hantke, K. (1981) personal communication.