Antigenic and Molecular Homology of the Ferric Enterobactin Receptor Protein of *Escherichia coli*

By HENRIK CHART* AND ELWYN GRIFFITHS

National Institute for Biological Standards and Control, Holly Hill, London NW3 6RB, UK

(Received 13 November 1984)

The ferric enterobactin receptor protein (81 kDal) of *Escherichia coli* O111 was purified by preparative sodium dodecyl sulphate-polyacrylamide gel electrophoresis and used to raise polyclonal antiserum in rabbits. This antiserum was used in conjunction with the immunoblot technique to examine the degree of antigenic homology of the ferric enterobactin receptor protein among 17 pathogenic and laboratory strains of *E. coli*. Both the molecular weight and the antigenic properties of the enterobactin receptor were highly conserved. However, the laboratory strain C and a pathogenic enteroinvasive strain, *E. coli* O164, were unusual in not producing the 81 kDal protein. The antiserum also recognized an 81 kDal protein from iron-restricted *Salmonella typhimurium* and an 83 kDal protein from iron-restricted *Klebsiella pneumoniae*.

INTRODUCTION

The availability of iron in the tissues of the mammalian host is severely restricted. Most of the iron is found intracellularly as ferritin, haemosiderin or haem and that which is extracellular in body fluids is attached to the iron-binding glycoproteins transferrin and lactoferrin. Since these proteins have a high association constant for iron and are normally only partly saturated, they ensure that little free iron is available to invading micro-organisms. Consequently, pathogenic bacteria have evolved high-affinity uptake systems which can successfully compete with these iron-binding glycoproteins for essential ferric ions (Finkelstein *et al.*, 1983; Griffiths, 1983). When *Escherichia coli* is placed in an iron-limited environment, several outer membrane proteins are derepressed and at least one high-affinity iron-chelating agent (siderophore) is synthesized (Neilands, 1982). One of the induced proteins, the 81 kDal product of the *fep* gene, is the receptor for ferric iron complexed with the siderophore enterobactin. Enterobactin-mediated iron uptake is of particular interest in the study of bacterial pathogenicity, since both the chelator and its outer membrane receptor are produced by *E. coli* growing *in vivo* during infection (Griffiths & Humphreys, 1980; Griffiths *et al.*, 1983).

Recent results from this laboratory have shown that normal human adult sera contain naturally occurring IgG antibodies which react with the outer membrane proteins of *E. coli* (Griffiths, 1983; Griffiths *et al.*, 1985). Interestingly, the major antigenic components recognized by these antibodies were the ompA proteins and the iron-regulated outer membrane proteins, including the enterobactin receptor. The possibility that antibodies to the enterobactin receptor might interfere with iron uptake and inhibit bacterial growth is currently being investigated, as is the degree of antigenic homology of the 81 kDal protein in different strains of *E. coli*. In the present study we isolated the ferric enterobactin receptor protein from *E. coli* O111 by preparative polyacrylamide gel electrophoresis and used antiserum raised to this preparation to assess the antigenic homology of the 81 kDal protein of 17 strains of *E. coli*. Our results suggest that the molecular weight and antigenic properties of the enterobactin receptor protein are highly conserved and that this correlation extends to other members of the *Enterobacteriaceae*.

Table 1. *Strains of E. coli*

Strain	Designation	Source
1. K12	Laboratory strain	This laboratory
2. W3110	Laboratory strain	C. Yanofsky, Stanford University, Calif., USA
3. W	Laboratory strain	} M. Buck, Biochemistry Dept, University of California, Berkeley, USA
4. C	Laboratory strain; ATCC 3121	
5. MRE 600	Laboratory strain	This laboratory
6. O128 K67 H2	Enteropathogenic	J. Dolby, Clinical Research Centre, Harrow, UK
7. O111 K58 H2	Enteropathogenic	This laboratory
8. O15	Enteropathogenic	J. Dolby
9. O78 K80	Extraintestinal; ColV ⁺	} H. Williams Smith, Houghton Research Station, Huntingdon, UK
10. O18 K1 H7	Extraintestinal; ColV ⁺	
11. O2 H1	Extraintestinal	This laboratory
12. O128 H49	Enterotoxigenic; ST ⁻ LT ⁺	} Central Public Health Laboratory, Colindale, London, UK
13. O159 H20	Enterotoxigenic; ST ⁺ LT ⁻	
14. O78 H11 K2	Enterotoxigenic; ST ⁺ LT ⁺ CFA/I ⁺	} This laboratory
15. O149 K91 H10	Enterotoxigenic	
16. O164 H ⁻	Enteroinvasive	} Central Public Health Laboratory, Colindale, London, UK
17. O124 H30	Enteroinvasive	

METHODS

Bacteria. Strains of *E. coli* used in this study are listed in Table 1. Isolates of *Salmonella typhimurium* LT2 and *Klebsiella pneumoniae* were obtained from stocks held at The National Institute for Medical Research, Mill Hill, London, UK.

Bacterial surface antigens. Lipopolysaccharide (LPS) prepared from *E. coli* O111 K58 H2 by the methods of Fitzgerald & Rogers (1980) was kindly donated by Dr H. J. Rogers.

Media and culture conditions. Strains were stored at -70°C in brain-heart infusion broth (Difco) containing 10% (v/v) glycerol. Iron-restricted growth was achieved by growing the bacteria in trypticase soy broth (BBL) containing either ovotransferrin as described previously (Griffiths & Humphreys, 1978), or 200 μM - α,α' -dipyridyl (Sigma). Iron-replete organisms were grown in trypticase soy broth alone.

Isolation of outer membranes. Bacterial outer membranes were isolated as described by Chart & Trust (1983) with the exception that the cells were disrupted by sonication (150 W, 5 min, 0°C). The protease inhibitor benzamidine (Sigma) was used at a concentration of 10 mM (Hollifield *et al.*, 1978). ^{125}I -labelled proteins were obtained by radio-iodinating membrane proteins exposed on the surface of intact *E. coli* O18 as described by Griffiths *et al.* (1983).

Electrophoresis. Outer membrane protein samples were prepared for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Chart & Trust (1983). Protein bands were detected with Coomassie Brilliant Blue R (Chart & Trust, 1983) or by the silver staining method of Wray *et al.* (1981). LPS was detected in gels by the procedure of Tsai & Frasch (1982).

Immunoblotting. SDS-PAGE profiles were transferred by electroblotting to nitrocellulose papers and reacted with sera as described by Stevenson & Griffiths (1985). Rabbit antibody was detected by using ^{125}I -labelled goat anti-rabbit antiserum (Miles Laboratories).

Rabbit antiserum. Antiserum was raised in a female Dutch rabbit. Isolated receptor protein (30 μg) was injected subcutaneously in Freund's complete adjuvant at multiple sites. A second injection in incomplete adjuvant was administered intramuscularly 3 weeks later. After 10 d, the rabbit was bled from the marginal ear vein and the serum was separated after clot formation.

RESULTS

Purification of the 81 kDal protein

E. coli O111 was chosen as a source of ferric enterobactin receptor protein since this strain produced particularly large amounts of the protein under iron-restricted conditions. For protein isolation, outer membranes were prepared from *E. coli* O111 grown in 1 litre volumes of trypticase soy broth containing ovotransferrin. Since the protease inhibitor benzamidine retards proteolytic degradation of the 81 kDal protein of *E. coli* K12 (Hollifield *et al.*, 1978) it was added to all reagents used in the protein isolation procedure.

Preparative gel electrophoresis was used to isolate the 81 kDal protein. Samples (1 mg) of outer membrane protein preparation were applied to a single well extending the whole width of the gel. After electrophoresis, gels were stained briefly in Coomassie Blue until the 81 kDal band was visible (5 min). The entire band was then cut out and macerated by passage through a 10 ml plastic syringe. Protein was eluted from the macerate with 125 mM-Tris/HCl pH 6.8 containing 0.5% (w/v) SDS (2 d, 4 °C) and precipitated from the eluate with acetone/triethylamine/glacial acetic acid (90:5:5, by vol.) at -20 °C. The precipitates were collected by centrifugation (4000 g, 20 min, 4 °C) in conical glass tubes.

SDS-PAGE of the protein preparations showed only a single 81 kDal band after staining with Coomassie Blue; a trace of an additional 74 kDal protein was also visible on silver staining (results not shown). Staining for LPS by the method of Tsai & Frasch (1982) failed to detect O-polysaccharide in the gel profiles. This protein preparation was used to raise antibodies in rabbits.

Specificity of antibodies to the 81 kDal protein

The growth of *E. coli* O111 in broth containing the iron chelator α, α' -dipyridyl resulted in the expression of large amounts of the 81 kDal protein. Since this chelator was more economical and convenient to use than ovotransferrin it was used to bring about the expression of the iron-regulated outer membrane proteins in the strains tested. The SDS-PAGE profiles of outer membrane proteins isolated from different strains of *E. coli* grown in the presence of α, α' -dipyridyl are shown in Fig. 1(a). By using the 81 kDal protein band of strain K12 as a reference (lane 1), it can be seen that all strains except C and O164 have an 81 kDal band (arrowed). As noted previously, however (Griffiths *et al.*, 1983), there was considerable variation between strains with respect to the number and quantity of iron-regulated proteins expressed.

The electrophoresed proteins were transferred from gels onto nitrocellulose paper and reacted with the antiserum raised to the purified 81 kDal protein. Fig. 1(b) shows that an 81 kDal band (arrowed) was recognized in all strains except C and O164. In addition, the antiserum produced a weak reaction with an antigen (arrowed) having a relative mobility equivalent to a protein of 24 kDal. This antigen was present in all strains tested, as well as in iron-replete *E. coli* O111 (results not shown). In order to show that the antiserum was reacting with the 81 kDal protein, the outer membrane proteins of *E. coli* O18 were extrinsically labelled with ^{125}I and the labelled preparation used as markers in SDS-PAGE. An unlabelled outer membrane protein preparation from iron-restricted *E. coli* O111 was electrophoresed in a gel adjacent to the ^{125}I -labelled proteins and the separated proteins were transferred onto a nitrocellulose sheet. This was cut into two between the lanes, using pinking shears to produce a serrated edge, and the paper carrying the unlabelled proteins was reacted in the usual way with the antiserum raised to the 81 kDal protein of *E. coli* O111. Both the immunoblot and the nitrocellulose paper carrying the separated ^{125}I -labelled proteins were finally placed onto X-ray film and aligned using the serrated edges. In this way an outer-membrane protein profile was produced on the film alongside and aligned with the antiserum blot. The results showed that the antiserum was indeed recognizing the 81 kDal protein (Fig. 2a). This antiserum failed to detect an 81 kDal band in the outer membrane protein profile of iron replete *E. coli* O111 (not shown).

Two bacterial species other than *E. coli* were also examined to ascertain the degree of antigenic conservation of the ferric-enterobactin receptor protein. The results in Fig. 2(b) clearly show that the anti-receptor antibodies recognized an 81 kDal band in the outer membrane protein profile of *S. typhimurium* and an 83 kDal band in that from *K. pneumoniae*. These were the only bands detected.

Interestingly, the antiserum also reacted with what appeared to be LPS in *E. coli* O111, O18 and to a lesser extent W3110 (Fig. 1b). To investigate this observation, the antiserum was reacted with purified LPS from homologous *E. coli* O111. An enzyme-linked immunosorbent assay demonstrated that the anti-receptor antiserum did indeed contain antibodies to LPS. The SDS-PAGE profile of LPS from *E. coli* O111, stained for LPS by the silver staining technique of Tsai & Frasch (1982) (Fig. 3a), closely resembled the outer membrane components recognized

Fig. 1 (a) SDS-PAGE profiles of outer membrane proteins (30 µg per lane) isolated from various strains of *E. coli* cultured in the presence of α,α' -dipyridyl. The arrow ($\blacktriangleright$) indicates the position of the ferric enterobactin receptor protein (81 kDal). Lane numbers refer to the strains listed in Table 1. Only the region of the gel containing the iron-regulated outer membrane proteins is shown. (b) Autoradiogram of an immunoblot showing antibodies raised to purified ferric enterobactin receptor protein of *E. coli* O111 reacting with an 81 kDal protein ($\blacktriangleright$) in the outer membrane protein profile of all *E. coli* strains tested, except *E. coli* C (lane 4) and *E. coli* O164 (lane 16) (15 µg protein per lane; 20 µl antiserum per lane). The white arrow ($\triangleleft$) indicates an antigen with an apparent molecular mass of 24 kDal recognized by the antiserum in all strains examined. Antibodies in the antiserum also reacted with the LPS of *E. coli* W3110 (lane 2), *E. coli* O111 (lane 7) and *E. coli* O18 (lane 10) (see Fig. 3).

Fig. 2(a) Immunoblot of the separated iron-regulated outer membrane proteins of *E. coli* O111 (15 μ g per lane) reacted with the antiserum (20 μ l per lane) raised to the 81 kDal protein of *E. coli* O111; in this experiment antibodies to LPS were absorbed by addition of homologous LPS (lane 2) (see also Fig. 3b). Lane 1 carried 125 I-labelled outer membrane proteins of iron-restricted *E. coli* O18. The radiolabelled proteins were electrophoresed at the same time as and transferred to the nitrocellulose paper along with the unlabelled proteins run in lane 2. The immunoblot and the nitrocellulose paper carrying the 125 I-labelled proteins were aligned as described in the text, before being placed on the X-ray film. Only the region of the gel containing the iron-regulated outer membrane proteins is shown.

(b) Immunoblot of the iron-regulated outer membrane proteins (15 μ g per lane) isolated from *K. pneumoniae* and *S. typhimurium* reacted with the antiserum (20 μ l per lane) raised to the 81 kDal protein; the bacteria were grown in the presence of α, α' -dipyridyl. The antibodies recognized an 83 kDal protein in *K. pneumoniae* (lane 1) and an 81 kDal protein in *S. typhimurium* (lane 2). Only the region of the gel containing the iron-regulated outer membrane proteins is shown.

Fig. 3(a) SDS-PAGE profile of LPS from *E. coli* O111. Only the higher molecular weight region of the gel is shown. (b) Immunoblot of the outer membrane proteins isolated from *E. coli* O111 cultured in the presence of α, α' -dipyridyl and reacted with antibodies to the ferric enterobactin receptor protein. Lane 1 was reacted with antiserum absorbed with purified LPS from *E. coli* O111; no reaction with LPS can be seen. In contrast, lane 2 shows that the nonabsorbed serum recognized both the 81 kDal receptor protein and LPS. (See also Fig. 1b, lane 7). Only the higher molecular weight region of the gel is shown.

by the antiserum in the above three strains. Adding purified *E. coli* O111 LPS to the anti-receptor antiserum absorbed the anti-LPS antibodies, leaving the remaining antibodies to react with the enterobactin receptor (Fig. 3b) and the antigen with an apparent molecular mass of 24 kDal (results not shown).

DISCUSSION

The results reported in this paper show that both the molecular weight and at least some antigenic properties of the ferric-enterobactin receptor protein are conserved within the strains of *E. coli* examined. Antigenic similarities between the receptor proteins of several *E. coli* strains would help to explain why normal human sera from a range of sources contained antibodies to the 81 kDal protein of the test strains (Griffiths, 1983; Griffiths *et al.*, 1985). The degree to which antibodies reacting with the iron-regulated outer membrane proteins, and specifically with the ferric enterobactin receptor, might be involved in host defence is unknown.

Preparative gel electrophoresis proved to be a successful way of isolating the 81 kDal protein. However, the presence in the antiserum of antibodies reacting with LPS suggests that there was an extremely low level of contaminating LPS present in the purified protein used for immunization, even though silver staining of SDS-PAGE gels failed to detect it. Examination of LPS profiles in SDS-PAGE gels showed that the 81 kDal protein and some of the high molecular weight LPS co-migrate.

The recognition by the antiserum of an additional antigen with a molecular mass equivalent to a protein of 24 kDal indicated that antibodies to the 81 kDal protein were either cross-reacting with common epitopes on another outer membrane component or that this material was a proteolytic breakdown product of the 81 kDal protein. The latter seems unlikely since this antigen was present in strains which did not have a ferric enterobactin receptor protein (*E. coli* C and O164) and also in iron-replete *E. coli* O111. Furthermore, it was not present in the profile of *S. typhimurium* which contained an 81 kDal protein.

Antibodies to the ferric enterobactin receptor of *E. coli* O111 also cross-reacted with the iron-regulated outer membrane proteins of two other bacterial species. The recognition of an 81 kDal band in the outer membrane protein profile of iron-restricted *S. typhimurium* was not surprising since this organism can both synthesize and utilize enterobactin (Neilands, 1982). However, the reaction of the anti-receptor antibodies with an 83 kDal protein from iron-restricted *K. pneumoniae* was unexpected. Williams *et al.* (1984) reported that under iron restriction, *K. aerogenes* produced an 83 kDal protein but not an 81 kDal protein, although this organism could utilize ferric enterobactin as an iron source. From the reaction that we observed, it would seem that in *K. pneumoniae* and *K. aerogenes* the enterobactin receptor protein has a molecular mass of 83 kDal and not 81 kDal.

We thank Pauline Stevenson for helpful advice and discussion, and the World Health Organization and Medical Research Council for financial support.

REFERENCES

- CHART, H. & TRUST, T. J. (1983). Acquisition of iron by *Aeromonas salmonicida*. *Journal of Bacteriology* **156**, 758–764.
- FINKELSTEIN, R. A., SCIORTINO, C. V. & MCINTOSH, M. A. (1983). Role of iron in microbe–host interactions. *Reviews of Infectious Diseases* **5**, S759–777.
- FITZGERALD, S. P. & ROGERS, H. J. (1980). Bacteriostatic effect of serum: role of antibody to lipopolysaccharide. *Infection and Immunity* **27**, 302–308.
- GRIFFITHS, E. (1983). Adaptation and multiplication of bacteria in host tissues. *Philosophical Transactions of the Royal Society* **B303**, 85–96.
- GRIFFITHS, E. & HUMPHREYS, J. (1978). Alterations in tRNAs containing 2-methylthio-*N*⁶-(Δ^2 -isopen-tenyl)-adenosine during growth of enteropathogenic *Escherichia coli* in the presence of iron binding proteins. *European Journal of Biochemistry* **82**, 503–513.
- GRIFFITHS, E. & HUMPHREYS, J. (1980). Isolation of enterochelin from the peritoneal washings of guinea-pigs lethally infected with *Escherichia coli*. *Infection and Immunity* **28**, 286–289.
- GRIFFITHS, E., STEVENSON, P. & JOYCE, P. (1983). Pathogenic *Escherichia coli* express new outer membrane proteins when growing *in vivo*. *FEMS Microbiology Letters* **16**, 95–99.
- GRIFFITHS, E., STEVENSON, P., THORPE, R. & CHART, H. (1985). Naturally occurring antibodies in human sera that react with the iron regulated outer membrane proteins of *Escherichia coli*. *Infection and Immunity* **47**, 808–813.
- HOLLIFIELD, W. C., FISS, E. H. & NEILANDS, J. B. (1978). Modification of a ferric enterobactin receptor protein from the outer membrane of *Escherichia coli*. *Biochemical and Biophysical Research Communications* **83**, 739–746.
- NEILANDS, J. B. (1982). Microbial envelope proteins related to iron. *Annual Review of Microbiology* **36**, 285–309.
- STEVENSON, P. & GRIFFITHS, E. (1985). Detection of antibodies and antigens by immunoblotting. In *The Virulence of Escherichia coli*. Edited by M. Sussman (Society for General Microbiology Special Publication no. 13). London: Academic Press.

- TSAI, C. M. & FRASCH, C. E. (1982). A sensitive silver stain for detecting lipopolysaccharide in polyacrylamide gels. *Analytical Biochemistry* **119**, 115–119.
- WILLIAMS, P., BROWN, M. R. W. & LAMBERT, P. A. (1984). Effect of iron deprivation on the production of siderophores and outer membrane proteins in *Klebsiella aerogenes*. *Journal of General Microbiology* **130**, 2357–2365.
- WRAY, W., BOULIKAS, T., WRAY, P. & HANCOCK, A. (1981). Silver staining of proteins in polyacrylamide gels. *Analytical Biochemistry* **118**, 197–203.