Iron-regulated Outer-membrane Proteins of *Escherichia coli* Strains Associated with Enteric or Extraintestinal Diseases of Man and Animals

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The SDS-PAGE patterns of the iron-regulated outer-membrane proteins from 70 strains of *Escherichia coli* isolated from various human and animal infections were analysed and the nature of the siderophores produced was examined. Iron-regulated 81 kDa and 74 kDa protein bands seen in SDS-PAGE gels were characterized further by immunoblotting using anti-81 kDa and anti-74 kDa (Cir) sera. The results showed considerable differences between the patterns of the iron-regulated outer-membrane proteins exhibited by the different strains. Nevertheless, three distinct and characteristic profiles, based on the most prominent bands expressed, could be identified, although not all strains produced patterns which matched with one of these. These results suggest the possibility of using the pattern of iron-regulated outer-membrane proteins expressed, as well as siderophores produced, as a new set of markers to characterize groups of pathogenic *E. coli*.

INTRODUCTION

Many different pathogenic strains of *Escherichia coli* have been identified and these are known to be responsible for a variety of human and animal infections (Sussman, 1985). Properties reportedly associated with the virulence of these various pathogens include the possession of certain O and K antigens and their ability to produce specific adhesive factors, toxins or haemolysins. However, it is now known that the possession of a single trait is not sufficient for virulence and there is evidence that the pathogenicity of *E. coli* is a consequence of certain independent factors occurring together. Considerable attention has therefore been given to the analysis of various bacterial properties and to the grouping of isolates having similar characteristics (Stenderup & Ørskov, 1983; Achtman *et al.*, 1983, 1986; Korhonen *et al.*, 1985; Vaisanen-Rhen *et al.*, 1984; Achtman & Pluschke, 1986; Selander *et al.*, 1986; Valvano *et al.*, 1986). Although some of these studies have revealed clonal groups of *E. coli*, no unique relationships between such clones and virulence or different clinical disease specificities have been identified (Achtman & Pluschke, 1986). This has led to the suggestion that pathogenicity can be explained by the differential association of particular, and often unknown, bacterial properties with the individual clonal groupings (Achtman & Pluschke, 1986).

In most studies to date, the properties of pathogenic *E. coli* have been examined in organisms grown in laboratory media and such characteristics may not correlate with the virulence of the strains. When bacterial pathogens multiply *in vivo* during infection they can be phenotypically quite different from the same organisms grown in laboratory media (Griffiths, 1983, 1987). Analysis of the properties of organisms grown *in vivo* should therefore add to our understanding of bacterial pathogenicity. One important environmental factor leading to this difference in phenotype is the availability of iron; the level of freely available iron in the healthy mammalian

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host is severely restricted. Many pathogens produce high-affinity iron-uptake systems when growing in iron-restricted environments both *in vitro* and *in vivo* during infection (Griffiths, 1987) and there is increasing evidence that these behave as virulence determinants. An essential feature of several of these systems is the synthesis and secretion of siderophores, together with the production of new outer-membrane proteins, some of which are receptors for ferric-siderophores and are essential for iron uptake.

Under iron-restricted growth conditions, pathogenic *E. coli* produce either the phenolic siderophore enterobactin alone, or they produce enterobactin plus the hydroxamate siderophore aerobactin (Williams, 1979; Braun, 1981; Griffiths, 1987). Generally, aerobactin production is associated with strains which cause extraintestinal infections, the necessary genes being located either on a plasmid or in the chromosome (Stuart *et al.*, 1982; Montgomerie *et al.*, 1984; Griffiths *et al.*, 1985*b*; Carbonetti *et al.*, 1986; Marolda *et al.*, 1987). During iron restriction, *E. coli* also derepresses the synthesis of several outer-membrane proteins with molecular masses in the range 70–83 kDa. Some of these have been shown to be receptors for ferric siderophores, for example the 81 kDa ferric-enterobactin receptor, whereas others, such as the 74 kDa Cir protein, have no known function (Neilands, 1982). Previously, we reported a variation in the quantities of iron-regulated proteins produced by different *E. coli* strains, as well as differences in the SDS-PAGE patterns obtained from the very few pathogenic strains examined (Griffiths *et al.*, 1983, 1985*a*; Chart & Griffiths, 1985). In the study reported here, we examined these differences further by analysing SDS-PAGE patterns of iron-regulated outer-membrane proteins from 70 strains of *E. coli* isolated from various human and animal infections.

METHODS

Bacterial strains and growth conditions. The *E. coli* strains used in this study are listed in Table 1. Strains were stored at -70°C in brain/heart infusion broth (Difco) containing glycerol (10%, v/v) and grown in trypticase soy broth (BBL Microbiology Systems); ferric dicitrate ($25\text{ }\mu\text{M}$) was added when producing iron-replete organisms in order to ensure a plentiful supply of iron. Expression of iron-regulated outer-membrane proteins was induced by growing bacteria in trypticase soy broth (37°C , 6 h) containing the iron-chelator Desferal (0.5 mM, Ciba-Geigy). Bacteria were also grown in the Tris-buffered medium of Simon & Tessman (1963) supplemented with sodium succinate (10 g l^{-1} , pH 6.8). The rationale for using a Tris/succinate medium is discussed by Braun (1981) and Neilands (1982).

Electrophoresis of outer-membrane proteins. Outer membranes were prepared using Triton X-100 as described by Griffiths *et al.* (1983) or using sodium lauryl sarcosinate (Filip *et al.*, 1973); similar outer-membrane protein profiles were obtained by both procedures. Where indicated in the text, the protease inhibitors phenylmethylsulphonyl fluoride (PMSF) (10 mM) or benzamidine (10 mM) were added to the buffer medium prior to sonication.

Table 1. *Strains of E. coli isolated from animal and human sources*

Strain designation*	Serotype	Source†	Membrane pattern (MP)‡	Siderophore production§
<i>Human</i>				
1. O111 ^a	O111:K58:H2	EPEC	—	ent
2. SC48 ^b	O15	NHF	—	ent
3. 13 ^b	O128:K67:H2	EPEC	—	ent
4. 10407 ^c	O78:K2:H11	ETEC	—	ent
5. E16970/O ^c	O25:H ⁻	ETEC	—	ent
6. E25079/O ^c	O6:H16	ETEC	—	ent
7. A112 ^d	O1:K1	NHF	5	ent
8. A194 ^d	O1:K1	NHF	5	ent
9. A25 ^d	O1:K1	UTI	9	ent/aero
10. A266 ^d	O1:K1	UTI	9	ent/aero
11. A265 ^d	O1:K1	UTI	9	ent/aero
12. A100 ^d	O1:K1	UTI	9	ent/aero
13. A190 ^d	O1:K1	UTI	9	ent/aero
14. A174 ^d	O18:K1	UTI	6	ent
15. A155 ^d	O18:K1	UTI	6	ent
16. A294 ^d	O18:K1	UTI	6	ent
17. A292 ^d	O18:K1	UTI	6	ent
18. A300 ^d	O18:K1	UTI	6	ent

19. A105 ^d	O18:K1	UTI	9	ent/aero
20. A106 ^d	O18:K1	UTI	9	ent/aero
21. A53 ^d	O18:K1	NBM	9	ent/aero
22. A54 ^d	O18:K1	NBM	9	ent/aero
23. A58 ^d	O18:K1	NBM	9	ent/aero
24. A59 ^d	O18:K1	NBM	9	ent/aero
25. A60 ^d	O18:K1	NBM	9	ent/aero
26. 780401 ^e	O18:K1	NBM	—	ent/aero
27. H247 ^f	O18:K1	NBM	—	ent/aero
28. 536 ^g	O6	UTI	—	ent
29. 477 ^g	O18	UTI	—	ent/aero
30. 764 ^g	O18	UTI	—	ent/aero
31. 275 ^g	O75	UTI	—	ent/aero
32. 341 ^g	O75	UTI	—	ent
33. 367 ^g	O4	UTI	—	ent

Animal

34. B44 ^f	O9:K30:K99	Bovine E	—	ent
35. B85 ^f	O9:K35:K99	Bovine E	—	ent
36. B79 ^f	O101:K32:K99	Bovine E	—	ent
37. B41 ^f	O101:K30:K99	Bovine E	—	ent
38. S13 ^f	O8:K85:K99	Ovine E	—	ent
39. A82 ^h	O8:K85:K99	Ovine E	—	ent
40. P18 ^f	O8:K87:K88ab	Porcine E	—	ent
41. P8 ^f	O138:K81	Porcine O	—	ent
42. P14 ^f	O147:K89:K88ac	Porcine E	—	ent
43. P115 ^f	O149:K91:K88ac	Porcine E	—	ent
44. P3 ^f	O139:K82	Porcine O	—	ent
45. Abbotstown ^h	O149:K91:K88ac	Porcine E	—	ent
46. G7 ^h	O8:K87:K88ab	Porcine E	—	ent
47. B184 ^f	O78:K80	Bovine B/S	—	ent/aero
48. B190 ^f	O78:K80	Bovine B/S	—	ent/aero
49. B179 ^f	O78:K80	Bovine B/S	—	ent/aero
50. B197 ^f	O78:K80	Bovine B/S	—	ent/aero
51. B188 ^f	O78:K80	Bovine B/S	—	ent/aero
52. X136/85 ^h	O78:K80	Bovine B/S	—	ent/aero
53. RVC95 ^h	O26:K60	Bovine B/S	—	ent
54. S3 ^f	O78:K80	Ovine B/S	—	ent
55. S1 ^f	O78:K80	Ovine B/S	—	ent
56. S2 ^f	O78:K80	Ovine B/S	—	ent/aero
57. RVC330 ^h	O78:K80	Ovine B/S	—	ent/aero
58. S5 ^h	O15:K	Ovine B/S	—	ent
59. F1 ^h	O1:K1	Chicken B/S	—	ent/aero
60. P21 ^f	O141:K85	Porcine O	—	ent
61. P25 ^f	O141:K85	Porcine O	—	ent
62. P26 ^f	O141:K85	Porcine O	—	ent
63. P27 ^f	O141:K85	Porcine O	—	ent
64. P28 ^f	O141:K85	Porcine O	—	ent
65. P58 ^f	O141:K85	Porcine O	—	ent
66. P60 ^f	O141:K85	Porcine O	—	ent
67. P61 ^f	O141:K85	Porcine O	—	ent
68. P62 ^f	O141:K85	Porcine O	—	ent
69. P63 ^f	O141:K85	Porcine O	—	ent
70. P23 ^f	O141:K85	Porcine O	—	ent

* The sequential numbers used here are for ready reference in the text and figures. The original strain designations are those from the source laboratories. Source laboratories are indicated by superscript letters: *a*, This laboratory; *b*, J. Dolby, Clinical Research Centre, Harrow, UK; *c*, S. M. Scotland, Central Public Health Laboratory, Colindale, London, UK; *d*, M. Achtman, Max-Planck Institute, Berlin, West Germany; *e*, H. C. Zanen, University of Amsterdam, The Netherlands; *f*, H. Williams Smith, Houghton Research Station, Huntingdon, UK; *g*, J. Hacker, University of Würzburg, W. Germany; *h*, J. A. Morris, Central Veterinary Laboratory, Weybridge, Surrey, UK.

† B/S, bacteraemia/septicaemia; E, enteropathogen; EPEC, enteropathogenic; ETEC, enterotoxigenic; NBM, new-born meningitis; NHF, normal human faeces; O, oedema disease; UTI, urinary tract infection.

‡ Major outer-membrane protein classification; see Achtman *et al.* (1983).

§ ent, produces enterobactin; aero, produces aerobactin.

|| Requires niacin for growth.

Benzamidine retards proteolytic degradation of the 81 kDa protein of *E. coli* K12 (Hollifield *et al.*, 1978). Outer-membrane proteins were analysed by SDS-PAGE using the extended electrophoresis times described by Griffiths *et al.* (1985a). The iron-regulated 81 kDa and 74 kDa outer-membrane proteins of *E. coli* O111 (Chart & Griffiths, 1985) were used as molecular mass markers. After electrophoresis, the gels were either stained with Coomassie Blue or used for immunoblotting. Immunoblotting was done essentially as described by Stevenson & Griffiths (1985) with antiserum raised in rabbits with the 81 kDa or the 74 kDa (Cir) protein of *E. coli* O111 :K58 :H2; the reaction with the second antibody, goat anti-rabbit immunoglobulin G (Miles Laboratories), was carried out for 24 h. The 81 kDa and 74 kDa proteins of *E. coli* O111 were purified by preparative SDS-PAGE as described by Chart & Griffiths (1985).

Assay for siderophore production. Enterobactin was assayed using the Arnow test for the detection of phenolic compounds (Arnow, 1937). The hydroxamate siderophore aerobactin was assayed using the ferric perchlorate test (Atkin *et al.*, 1970).

Haemolysin production. This was assessed by growing strains on trypticase soy agar plates containing 5% (v/v) washed horse or sheep erythrocytes. Cultures were examined for zones of haemolysis following incubation at 37 °C for 24 h.

RESULTS

Analysis of iron-regulated outer-membrane proteins from E. coli strains of human origin

Strains of *E. coli* representing the various groups pathogenic for humans, as well as isolates from normal human faeces, were grown under iron-restricted conditions in order to examine the expression of iron-regulated outer-membrane proteins. The strains studied produced new outer-membrane proteins with molecular masses in the region of 70–83 kDa during growth in the presence of the iron chelator Desferal (Fig. 1). A comparison of a number of strains showed that when the organisms were grown under iron-replete conditions these proteins were either not expressed or were expressed at an extremely low level (for example see Fig. 2a), as has been shown previously with other strains (Griffiths *et al.*, 1983, 1985a, b; Neilands, 1982). Figs 1 and 2(a) also show striking differences in the protein pattern produced on SDS-PAGE by the various *E. coli* strains. The identity of the 81 kDa and 74 kDa (Cir) proteins was confirmed by immunoblotting using a polyclonal anti-81 kDa serum (Chart & Griffiths, 1985) and an anti-74 kDa (Cir) serum (Griffiths *et al.*, 1985b); representative strains exhibiting both of these proteins are shown in Fig. 2(b).

Although there were quantitative and qualitative differences in the iron-regulated proteins expressed by the various isolates, three characteristic patterns could be consistently distinguished (Fig. 2a). The protein profiles produced by the enterotoxigenic, enteropathogenic and commensal strains of *E. coli* studied are shown in Fig. 1, lanes 1–8. The SDS-PAGE profiles in lanes 1–6 are similar to each other in that the predominant iron-regulated proteins expressed are the 81 kDa and 74 kDa (Cir) proteins, the identity of which was confirmed by immunoblotting; traces of a 78 kDa and an 83 kDa protein can also be seen in some instances, and strain 2 has an additional iron-regulated protein of about 71 kDa. The characteristic profile expressed by the majority of these strains is typified by that of *E. coli* O25 :H⁻ (Table 1, strain 5; Fig. 2a, b, lane 1).

The most prominent iron-regulated proteins seen in profiles 7–13 in Fig. 1 are those with molecular masses of approximately 70 kDa and 74 kDa, together with a slightly weaker band of about 71 kDa. This type of protein profile is typified by that of *E. coli* O1 :K1 (Table 1, strain 12, Fig. 2a, b, lane 2). All of the strains whose profiles are seen in lanes 7–13 of Fig. 1 belong to serotype O1 :K1, and all, except those in lanes 7 and 8, were isolated from urinary tract infections. All except one of these strains produced small amounts of an 81 kDa protein, although this protein band was not always easily seen. The presence of an 81 kDa protein, which is the receptor for ferric-enterobactin, was confirmed by immunoblotting (Fig. 3) using the polyclonal anti-81 kDa serum. However, this antiserum failed to detect an 81 kDa protein in the outer-membrane protein profile of *E. coli* O1 :K1 strain A25 (Table 1, Figs 1, and 3, strain 9). The antiserum raised with the 74 kDa (Cir) protein of *E. coli* O111 as antigen reacted with the 74 kDa protein produced by *E. coli* strains 9–13 but failed to recognize the protein of approximately 74 kDa in strains 7 and 8 (Table 1, Fig. 3), which means that these two strains

Fig. 1. SDS-PAGE profiles of outer-membrane proteins (30 µg per lane) isolated from various strains of *E. coli* of human origin grown in trypticase soy broth containing Desferal (0.5 mM). Lane numbers refer to the strains listed in Table 1. Electrophoresis was carried out for an extended period (see Methods). The positions of the iron-regulated 81 kDa and 74 kDa proteins are indicated by arrows.

Fig. 2. (a) Lanes 1–3 show representative SDS-PAGE profiles consistently distinguished amongst the iron-regulated outer-membrane proteins; only the relevant portion of the gel is shown. Lanes 4, 5 and 6 show the same portions of the gels after SDS-PAGE of the outer-membrane proteins from the same organisms grown under iron-replete conditions. Lanes 1 and 4, *E. coli* O25 : H⁻ (Table 1, strain 5); lanes 2 and 5, *E. coli* O1 : K1 (Table 1, strain 12); lanes 3 and 6, *E. coli* O18 : K1 (Table 1, strain 24). Each lane carried 25 µg protein. (b) Immunoblot of the iron-regulated outer-membrane proteins (20 µg per lane) of: lane 1, *E. coli* O25 : H⁻ (Table 1, strain 5); lane 2, *E. coli* O1 : K1 (Table 1, strain 12); lane 3, *E. coli* O18 : K1 (Table 1, strain 24) reacted with a mixture of the anti-81 kDa serum (20 µl per lane) and the anti-74 kDa serum (20 µl per lane). Only the region of the gel containing the iron-regulated outer-membrane proteins is shown. The positions of the 81 kDa and 74 kDa proteins are indicated by arrows.

cannot be considered as having an iron-regulated outer-membrane protein profile like that shown in Fig. 2(a), lane 2. It should be noted that strains 7 and 8 also have a different major outer-membrane protein pattern from strains 9–13 (Table 1, MP5 and MP9).

The SDS-PAGE profiles of proteins expressed under iron restriction by *E. coli* strains 14–27 (Fig. 1, lanes 14–27) show four to six prominent bands, with molecular masses in the range 68–83 kDa, and they are quite distinct from those of strains 1–13; Fig. 2(a), lane 3, shows an example of this type of profile. *E. coli* strains 14–27 all belong to the O18 : K1 serogroup and were isolated from urinary tract infections (strains 14–20) or from newborn infants with meningitis (strains 21–27). Each produced an 81 kDa and a 74 kDa (Cir) protein during iron-restricted growth, as confirmed by immunoblotting. Most, but not all, of these strains expressed a 68 kDa protein (Fig. 1, compare lanes 18 and 19), but there is some doubt whether this protein is regulated by iron since varying quantities can be seen under iron-replete growth conditions. Also, a doublet can be seen in the region of the 78 kDa band in several strains. Interestingly, *E. coli* strains showing a doublet 78 kDa band belong to clonal type O18 : K1 MP9 (Achtman *et al.*, 1983); clonal type O18 : K1 MP6 strains appear to have a single band in this region. The possibility that protein degradation during the isolation of the membrane proteins might have been responsible for producing this type of profile was tested by including protease inhibitors in the reagents used. Degradation of the 81 kDa protein in *E. coli* K12 has been reported by Hollifield *et al.* (1978) and this proteolysis was inhibited by benzamidine. However, the presence of the protease inhibitors benzamidine or PMSF in the reagents used for preparation of outer-membrane proteins had no effect on the production of the multiple-band pattern of iron-regulated proteins observed here.

E. coli strains numbered 28, 32 and 33 (Table 1) exhibited iron-regulated outer-membrane proteins similar to those of strains 14–27 (data not shown). These organisms were again isolates from cases of urinary tract infections but they belonged to different serogroups (O6, O75 and O4). They did not produce the 68 kDa protein seen in the SDS-PAGE profile of most of the *E. coli* O18 : K1 strains. Two *E. coli* O18 strains which were K1 negative (Table 1, strains 29 and 30) exhibited a iron-regulated protein pattern identical to those produced by strains 9–13, as did another isolate of *E. coli* O75 (Table 1, strain 31; data not shown). All of these strains produced an 81 kDa and a 74 kDa (Cir) protein as identified by immunoblotting.

Analysis of iron-regulated outer-membrane proteins from E. coli strains of animal origin

Fig. 4 shows that the majority of animal strains examined expressed outer-membrane proteins with molecular masses in the region of 74–83 kDa when grown in broth containing Desferal. The iron-regulated outer-membrane proteins of *E. coli* O111 (Fig. 4, lane 1) were used as molecular mass markers. As before, a comparison of a number of strains showed that these proteins were not expressed when the organisms were grown under iron-replete conditions (data not shown). Although some variation between the pattern of proteins produced by different strains can be seen, the striking differences in the SDS-PAGE profiles produced by the various human isolates were not observed (compare Figs 1 and 2a with Fig. 4). Many strains, viz. 34–37, 40, 42, 58, 59, as well as a group of 10 isolates of *E. coli* serotype O141 : K85 (Table 1, strains 60–69; data not shown), produced an iron-regulated outer-membrane profile like that exhibited by the O25 : H⁻ strain 5 (Fig. 2a, lane 1); these produced both the 81 kDa and 74 kDa (Cir) proteins.

In other instances, although there was clearly variation between isolates, no well-defined patterns could be discerned. For example, two *E. coli* O8 : K85 : K99 strains (Fig. 4, lanes 38 and 39) seemed not to express a 74 kDa protein and the anti-74 kDa (Cir) serum failed to react with a band in this region on immunoblotting (data not shown). Again, two isolates of *E. coli* O149 : K91 : K88ac, as well as an O26 : K60 strain, showed a doublet protein band at approximately 74 kDa (Fig. 4, lanes 43, 45 and 53), and two of the ten *E. coli* O78 : K80 isolates produced a doublet band in the region of the 78 kDa protein (Fig. 4, lanes 48 and 56). Proteins of 74 kDa from *E. coli* O78 isolates 48, 50, 52, 56 and 57 failed to react with the anti-74 kDa serum. All other strains which produced a 74 kDa band reacted with this antiserum. One isolate of *E. coli* O141 : K85 (Table 1, strain 70) did not show a 74 kDa protein on SDS-PAGE and no protein band in this region reacted with the anti-74 kDa serum on immunoblotting (data not shown).

Fig. 3. Immunoblot of the iron-regulated outer-membrane proteins (35 µg per lane) of seven isolates of *E. coli* O1 : K1 reacted with a mixture of the anti-81 kDa serum (20 µl per lane) and the anti-74 kDa serum (20 µl per lane). Lane numbers refer to the strains listed in Table 1. Lane 9 shows no reaction with the anti-81 kDa serum and lanes 7 and 8 show no reaction with the anti-74 kDa serum. Only the relevant portion of the immunoblot is shown.

Fig. 4. SDS-PAGE profiles of outer-membrane proteins (30 µg per lane) isolated from *E. coli* strains of animal origin. The bacteria were grown in trypticase soy broth containing Desferal (0.5 mM). Lane numbers refer to the strains listed in Table 1. Electrophoresis was carried out for an extended period (see Methods).

The anti-81 kDa serum reacted with the 81 kDa protein of every animal *E. coli* isolate tested.

Two iron-regulated outer-membrane protein profiles of *E. coli* from animal sources were distinctly different from the rest. These are the profiles produced by strains 41 and 44 (Fig. 4). Interestingly, these two patterns belong to *E. coli* O138:K81 and O139:K82, respectively, both of which were isolated from cases of porcine oedema disease (Smith *et al.*, 1983). The pattern produced by these two organisms is not unlike that produced by the human *E. coli* O18:K1 strains (Fig. 2*a*, lane 3). However, not all strains causing oedema disease in pigs produced this particular pattern of iron-regulated outer-membrane proteins. Ten isolates of *E. coli* O141:K85 obtained from cases of porcine oedema disease (Table 1, strains 60–69) produced a profile identical to that seen in Fig. 2(*a*), lane 1 (data not shown).

Production of siderophores and haemolysin

The various strains of *E. coli* were grown in the Tris-buffered medium of Simon & Tesson (1963) containing sodium succinate, and culture supernatants were analysed for phenolic and hydroxamate siderophores. All strains grew well in this medium with the exception of strain 2 and strains 14–18 (Table 1), which required niacin (0.3 g l^{-1}) for growth. The results in Table 1 show that all the enteric pathogens and commensal isolates of *E. coli* examined, whether from human or animal sources, produced only the phenolic siderophore enterobactin; this was also the case with isolates 14–18 and 28, 32 and 33 from human urinary tract infections, isolates 53, 55 and 58 from animal septicaemias, and isolates 60–70 from cases of porcine oedema disease. Aerobactin was produced only by human strains 9–13, 19–27 and 29–31, and by animal isolates 47–52, 56, 57 and 59 (Table 1); all were isolates from extraintestinal infections, either urinary tract infections, septicaemias or meningitis.

Isolates 14–18 and 28–33 (Table 1) were haemolytic, as were the strains causing enteric disease in sheep and pigs (Table 1, numbers 38, 39 and 40–46).

DISCUSSION

This paper presents for the first time, an analysis of the iron-regulated outer-membrane proteins from a large number of pathogenic *E. coli* strains isolated from various sources. Although considerable qualitative and quantitative differences were seen in the SDS-PAGE profiles exhibited by the different strains, three distinct and characteristic patterns, based on the most prominent bands expressed, could be identified (Fig. 2*a*). However, not all strains produced profiles which matched precisely with one of these. The use of the anti-81 kDa and anti-74 kDa (Cir) sera provided further characterization of the 81 kDa and 74 kDa proteins seen. Among the isolates examined here, only those giving rise to the protein patterns seen in lanes 2 or 3 of Fig. 2(*a*) seem to be associated with extraintestinal infections in man. These two patterns were not observed in the SDS-PAGE profiles of proteins from *E. coli* isolated from cases of human enteric diseases, nor in *E. coli* which caused septicaemia or enteric diseases of animals. However, two animal isolates, *E. coli* O138:K81 and O139:K82, both of which are strains associated with oedema disease in pigs (Smith *et al.*, 1983), did express an iron-regulated outer-membrane protein profile which was similar, but not identical, to that shown in Fig. 2(*a*), lane 3. The role in bacterial iron metabolism, and therefore possibly in virulence, of many of the iron-regulated membrane proteins described in the present report is unknown; nor is it known whether the genetic information for their synthesis is encoded on plasmids or in the chromosome. The loss of the ColV plasmid encoding the aerobactin iron-uptake system from one strain of *E. coli* O18:K1 had no effect on the visible pattern of iron-regulated membrane proteins it expressed (Griffiths *et al.*, 1985*a*).

Clearly, the different patterns of iron-regulated outer-membrane proteins expressed by *E. coli* are not restricted to one particular serogroup. However, one interesting variation amongst an established group of *E. coli* was noticed and this appeared to be related to established clonal types. *E. coli* strains exhibiting the characteristic iron-regulated outer-membrane protein pattern seen in Fig. 2(*a*), lane 3, and belonging to clonal type O18:K1 MP9 (Table 1), showed a doublet protein band at 78 kDa, whereas strains belonging to clonal type O18:K1 MP6 (Table 1) produced only a single band in this position. It is also interesting that of two *E. coli* O6 isolates

examined, one, a human enterotoxigenic strain (Table 1, strain 6), produced a protein profile like that in Fig. 2(a), lane 1, whereas the other, an isolate from a case of human urinary tract infection (Table 1, strain 28), gave a protein pattern similar to that seen in Fig. 2(a), lane 3. Again, all five *E. coli* O1 : K1 isolates from human urinary tract infections (Table 1, strains 9–13) exhibited iron-regulated outer-membrane protein profiles like that represented in Fig. 2(a), lane 2, but the one O1 : K1 strain of animal origin, from chicken septicaemia (Table 1, strain 59) showed an iron-regulated protein profile like that in Fig. 2(a) lane 1. One human isolate of *E. coli* O1 : K1 (Table 1, strain 9) appeared not to produce an 81 kDa protein, the receptor for ferric enterobactin, although it did synthesize this chelator as well as aerobactin. Not only was there no 81 kDa protein visible on SDS-PAGE of membrane proteins from this strain, but no reaction was observed on immunoblotting with the anti-81 kDa serum. This antiserum reacts with all other 81 kDa proteins found so far (Chart & Griffiths, 1985; this paper), as well as with an iron-regulated 81 kDa protein in 17 clinical isolates of *Klebsiella pneumoniae* (Williams *et al.*, 1987) and an 81 kDa protein from *Salmonella typhimurium* (Chart & Griffiths, 1985). It is unlikely, therefore, that this antiserum failed to recognize the enterobactin receptor, if present. *E. coli* strain 9 (Table 1) is therefore unusual in that it is the only one of more than 70 clinical isolates examined which failed to produce the 81 kDa protein. Less is known about the specificity of the antiserum raised to the 74 kDa (Cir) protein of *E. coli* O111. The fact that it failed to react on immunoblotting with a number of 74 kDa proteins indicates either that there is antigenic variation amongst these proteins, or that the 74 kDa protein seen on SDS-PAGE is not the 74 kDa (Cir) protein. It is already known that the plasmid-encoded aerobactin receptor protein is a 74 kDa protein (Bindereif *et al.*, 1982; Grewal *et al.*, 1982). Work is in progress to characterize further the 74 kDa proteins exhibited by the various strains.

Our data on the nature of the siderophore or siderophores produced by these various isolates are in general agreement with previous reports (Stuart *et al.*, 1982; Montgomerie *et al.*, 1984; Carbonetti *et al.*, 1986; Linggood *et al.*, 1987). All of the strains in our study produced the iron chelator enterobactin but only those strains causing extraintestinal infections produced the additional hydroxamate-type chelator, aerobactin. However, not all strains isolated from extraintestinal infections were aerobactin producers. Five *E. coli* O18 : K1 strains isolated from human urinary tract infections failed to produce aerobactin; these organisms exhibited the major outer-membrane protein group 6 (MP6) (Achtman *et al.*, 1983), required niacin for growth in the Tris/succinate medium and produced only enterobactin during iron restriction (Table 1, strains 14–18). Interestingly, Valvano *et al.* (1986) have recently reported that of 12 *E. coli* O18 : K1 strains exhibiting outer-membrane protein pattern MP6 examined by them none contained a functional aerobactin iron-uptake system. Two of these *E. coli* O18 : K1 MP9 strains (Valvano *et al.*, 1986) did however produce aerobactin, a result which is consistent with our data on seven *E. coli* O18 : K1 MP9 strains (Table 1). In agreement with Achtman *et al.* (1983) and Valvano *et al.* (1986) we also found that *E. coli* O18 : K1 MP6 strains were haemolytic. Another group of isolates in our study, again from human urinary tract infections, strains 28–33 (Table 1), produced haemolysins which lysed both horse and sheep erythrocytes. Three of these strains (two O18 and one O75) also produced aerobactin. However, four strains isolated from cases of bovine or ovine septicaemia did not produce aerobactin nor were they haemolytic (Table 1, strains 53, 54, 55 and 58). These strains may have lost plasmid-determined aerobactin genes during laboratory subculture. However, Linggood *et al.* (1987) have identified a number of animal isolates which were negative for aerobactin production although positive by colony hybridization for the aerobactin genetic determinants; they suggest that such strains may be mutated.

In conclusion, our results show the distribution of a new set of markers, the iron-regulated outer-membrane proteins, together with siderophore production, amongst previously established clones and other pathogenic strains of *E. coli* of unknown clonal types. Such characteristics should be taken into consideration when attempts are made to group pathogenic *E. coli* according to parameters which may be important for virulence. We believe that analysis of organisms grown in an iron-restricted environment, a condition known to resemble more closely that usually found in host tissues, will add considerably to the study of pathogenic *E. coli*,

and other pathogens, and may give a clearer insight into bacterial characteristics associated with virulence and with different disease specificities. Such an analysis has already proved useful in studying the properties of *Shigella flexneri* and enteroinvasive *E. coli* (Griffiths *et al.*, 1985b; Marolda *et al.*, 1987; Nassif *et al.*, 1987; Lawlor *et al.*, 1987).

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