Novel Aerobactin Receptor in *Klebsiella pneumoniae*

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(Received 19 June 1989; revised 11 September 1989; accepted 15 September 1989)

Several *Klebsiella pneumoniae* strains which produced enterochelin but not aerobactin were nevertheless sensitive to cloacin DF13. In contrast, a strain of serotype K1 : O1 which produced both siderophores was cloacin-resistant. Loss by mutation of the O1 but not K1 antigen rendered this strain cloacin-sensitive, indicating that the O1 antigen prevented access of cloacin to the cloacin/aerobactin receptor. Unlike the K1 : O1 strain, the aerobactin-negative strains failed to hybridize in a colony blot assay with an aerobactin receptor gene probe prepared from pColV-K30. However, antisera raised against the 74 kDa pColV-K30 aerobactin receptor cross-reacted with a 76 kDa outer-membrane protein in each *K. pneumoniae* strain. In addition to the 76 kDa protein, the K1 : O1 strain also produced a strongly cross-reacting 74 kDa protein. To determine whether these aerobactin-negative strains could use aerobactin, mutants unable to synthesize siderophores were isolated. Aerobactin promoted the growth of these mutants in iron-deficient media. The evidence presented suggests that some *K. pneumoniae* strains produce an aerobactin iron-uptake system without apparent production of aerobactin and which is probably based on a 76 kDa receptor, the gene for which does not hybridize with aerobactin receptor gene encoded on pColV-K30.

INTRODUCTION

In response to iron deprivation, many Gram-negative bacteria derepress high-affinity iron-sequestering systems based on low-molecular-mass iron-chelating agents (siderophores) and their corresponding outer membrane (OM) receptor proteins (Griffiths, 1987; Neilands, 1981, 1982). Since iron is required for the growth of most pathogenic bacteria, such systems contribute to virulence by enabling the bacterium to sequester transferrin- and lactoferrin-bound iron in body fluids and secretions (Bullen & Griffiths, 1987; Williams, 1988).

In *Escherichia coli*, two complete high-affinity iron-sequestering mechanisms have been described. These are based on enterochelin (enterobactin), a phenolate-type siderophore, and aerobactin, a hydroxamate-type siderophore (Griffiths, 1987; Bagg & Neilands, 1987). The plasmid-encoded ColV-K30 aerobactin-mediated iron-uptake system makes a significant contribution to the virulence of invasive *E. coli* strains (Williams, 1979; Warner *et al.*, 1981) although the selective advantage it confers over strains already producing enterochelin is not fully understood. The superiority of aerobactin over enterochelin in body fluids may, however, reside in its greater efficiency in sequestering transferrin-bound iron in body fluids (Konopka *et al.*, 1982; Williams & Carbonetti, 1986) and in its repeated re-use by the bacterial cell (Braun *et al.*, 1984; Williams & Carbonetti, 1986).

Abbreviations: CAS-agar, chrome azurol S/hexadecyltrimethylammonium bromide agar; EDDA, ethylenediamine-di-(*o*-hydroxyphenylacetic acid); Fe-SSM, iron-depleted simple salts medium; FE + SSM, iron-sufficient simple salts medium; OM, outer membrane; TSB, trypticase soy broth.

The aerobactin iron-uptake systems initially described in *E. coli* were plasmid-encoded (Williams, 1979). However, they are chromosomally encoded in many *E. coli* K1 strains responsible for human neonatal infections (Valvano *et al.*, 1986). Both plasmid- and chromosomally-encoded aerobactin iron-uptake systems have also been described in *Enterobacter*, *Citrobacter*, *Proteus*, *Morganella*, *Serratia*, *Shigella*, *Salmonella* and *Yersinia* (Krone *et al.*, 1985; Waters & Crosa, 1988; Cercenado *et al.*, 1986; Martinez *et al.*, 1987; Payne, 1980; Griffiths *et al.*, 1985; Colonna *et al.*, 1985; Stuart *et al.*, 1986). In *Klebsiella pneumoniae*, the presence of a large conjugative plasmid encoding aerobactin production has been reported to correlate, in part, with the virulence of several K1 and K2 serotype strains (Nassif & Sansonetti, 1986).

The aerobactin gene cluster in pColV-K30 has been reported to consist of an operon containing five genes in the order (*iucABCD iutA*), four of which (*iucABCD*) are involved in aerobactin biosynthesis (Carbonetti & Williams, 1984; Bagg & Neilands, 1987). The fifth gene (*iutA*) encodes a 74 kDa OM protein which functions as the receptor for ferric-aerobactin (Carbonetti & Williams, 1984; Bagg & Neilands, 1987). In some enteroinvasive *E. coli* strains (Griffiths *et al.*, 1985; Marolda *et al.*, 1987), in *Shigella flexneri* (Griffiths *et al.*, 1985) and in *Klebsiella edwardsii* (Krone *et al.*, 1985) the apparent molecular mass of the receptor appears to be 76 kDa whilst that of *Enterobacter cloacae* is 85 kDa (Krone *et al.*, 1985). The aerobactin receptor also functions as the receptor for cloacin DF13 (Van Tiel-Menkveld *et al.*, 1982). This bacteriocin, which is capable of killing susceptible cells, has been used to detect the presence of the ferric-aerobactin receptor in *E. coli*, in *Enterobacter* and in *Klebsiella* (Van Tiel-Menkveld *et al.*, 1982; Krone *et al.*, 1985). However, other bacterial surface structures may impede the access of cloacin to its receptor so that not all aerobactin-producing cells are susceptible (Griffiths, 1987; Carbonetti & Williams, 1985).

In a recent study, we examined the ability of *Klebsiella* species isolated from a variety of infection sites to express high-affinity iron-uptake systems (Williams *et al.*, 1987). In response to iron deprivation, all strains induced between four and six iron-repressible OM proteins in the 70 to 85 kDa range. All of the isolates examined produced enterochelin whilst six strains also made aerobactin (Williams *et al.*, 1987). The present study was prompted by the observation that many of the *Klebsiella* strains which did not produce aerobactin were nevertheless sensitive to cloacin DF13. In contrast, some aerobactin producers were resistant to this bacteriocin. In this paper, we describe the existence of an aerobactin iron-uptake system, probably based on a 76 kDa OM protein, in *Klebsiella pneumoniae* strains which do not produce aerobactin. We also report on an aerobactin-producing, cloacin-resistant, *K. pneumoniae* strain, in which the O-antigen component of lipopolysaccharide appears to be responsible for conferring resistance to cloacin. This strain appears to produce two aerobactin receptors, of 74 and 76 kDa, respectively.

METHODS

Bacterial strains and culture media. The *K. pneumoniae* strains and *E. coli* K12 (pColV-K30) control strain used in this study are listed in Table 1. They were routinely cultured in the iron-depleted simple salts medium (Fe-SSM) described previously (Williams *et al.*, 1984) which contains: glucose 35 mM; NH_4Cl , 25 mM; KCl , 1.5 mM; NaCl , 45.2 mM; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 mM and $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$, 67 mM (pH 7.4). The phosphate buffer was batch-incubated with Chelex 100 ion-exchange resin (Bio-Rad) to remove iron. Fe-SSM contains less than $0.5 \mu\text{M-Fe}^{3+}$ (Lodge *et al.*, 1986). Iron-sufficient simple salts media (Fe + SSM) contained 0.02 mM- FeSO_4 . Cultures were grown at 37 °C with shaking and growth determined by measuring the optical density at 470 nm (OD_{470}).

Siderophore detection and isolation. Culture supernatants were assayed for the production of enterochelin and aerobactin by the chemical assays of Arnow (1937) and Arnold & Viswanatha (1983), respectively. The results were confirmed by bioassay using *E. coli* AN193 (Neilands, 1984) and *E. coli* LG1522 (Carbonetti & Williams, 1985), respectively, as indicator strains. Aerobactin was isolated and purified from the culture supernatants of *E. coli* K12 (pColV-K30) by anion-exchange chromatography on Dowex-1 as described by Gibson & Magrath (1969) followed by solvent extraction with chloroform/phenol (1:1, v/v).

Cloacin susceptibility. A crude preparation of cloacin was prepared by exposing a growing culture of *Ent. cloacae* DF13 (in nutrient broth) to mitomycin C ($0.5 \mu\text{g ml}^{-1}$) followed by overnight incubation at 37 °C with aeration. Cells were removed by centrifugation and the cloacin-containing supernatant sterilized by filtration. *K. pneumoniae* strains were tested for cloacin sensitivity after overnight growth in Fe-SSM by spreading on plates of

the same medium supplemented with 200 μM -ethylenediamine-di-(*o*-hydroxyphenylacetic acid) (EDDA) and solidified by the addition of 1.5% (w/v) agar. Samples (10 μl) of the crude cloacin preparation were added to wells cut in the agar. Cloacin sensitivity was indicated by a cleared zone around the well.

Isolation of mutants. Mutants derived from *K. pneumoniae* strain DL1 lacking either the K1 or O1 or both K1 and O1 antigens were isolated as described previously (Benedi *et al.*, 1989; Tomas *et al.*, 1986, 1988).

Mutants defective in enterochelin iron-uptake were derived from strain DL44 by mutagenesis with diethyl sulphate as described previously (Tomas *et al.*, 1988). Mutants were selected by their failure to produce an orange halo on blue chrome azurol S/hexadecyltrimethylammonium bromide agar (CAS-agar) prepared, as for *E. coli* strains, according to the method of Schwyn & Neilands (1987). The ability of enterochelin-negative mutants to use aerobactin was examined by spreading a lawn of cells on Fe-SSM agar medium containing 200 μM -EDDA; 10 μl aerobactin (20 $\mu\text{g ml}^{-1}$) was added to wells cut in the agar, and following overnight incubation at 37 °C, examined for a halo of growth around the well. The growth kinetics of an enterochelin-negative strain in Fe-SSM, Fe + SSM and Fe-SSM supplemented with 20 $\mu\text{g ml}^{-1}$ aerobactin were determined by incubating cells at 37 °C with aeration and measuring OD₄₇₀.

Detection of aerobactin receptor genes. Each *K. pneumoniae* strain was screened for the presence of genes encoding the aerobactin receptor by using the colony hybridization technique. *E. coli* K12(pColV-K30) was included as a positive control. The aerobactin receptor gene probe was prepared from pEN7, a recombinant plasmid which carries the aerobactin receptor gene of pColV-K30 (Gross *et al.*, 1985). Plasmid DNA was purified by column chromatography on Sephacryl S-1000 (Bywater *et al.*, 1983) and then digested with restriction endonuclease *Bgl*II (Anglian Biotechnology). A 2 kb *iutA* DNA fragment, separated and purified on low-melting-point agarose (Weislander, 1979), was radiolabelled by random priming with [α -³²P]dATP essentially as described by Feinberg & Vogelstein (1984).

Bacterial cells for probing were grown overnight at 37 °C on L-agar. Colonies were transferred to hybridization filters (Colony/Plaque Screen Hybridization Transfer Membrane Discs, Dupont); the cells were lysed, and their DNA was denatured and then bound to the filters by exposure to 254 nm UV light. Pre-hybridization and hybridization of filters was carried out essentially as described by Singh & Jones (1984) except that the hybridization solution contained 200 $\mu\text{g ml}^{-1}$, 2% (w/v) sodium dodecyl sulphate (SDS) and 50% (v/v) formamide (100% de-ionized, Sigma). Hybridization was carried out at 42 °C overnight. The filter discs were then washed once in 2 $\times$ SET/2% (w/v) SDS at room temperature for 15 min and then twice in 0.2 $\times$ SET/2% (w/v) SDS at 65 °C for 15 min (1 $\times$ SET contains 0.15 M-NaCl and 2 mM-EDTA in 30 mM-Tris/HCl, pH 7.4). Discs were exposed to X-ray film overnight at room temperature.

Preparation and electrophoresis of outer membranes. *Klebsiella* and *E. coli* OM's were isolated using 2% (w/v) sodium *N*-lauroyl sarcosinate (Sarkosyl) as described previously (Williams *et al.*, 1984). OM proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously on 10% gels. After electrophoresis, gels were fixed and stained with Coomassie brilliant blue, and photographed or used for immunoblotting.

Preparation of antiserum against the 74 kDa aerobactin receptor protein. The aerobactin receptor protein was isolated from the OM of the *cir*-negative *E. coli* strain B2B 1022(pABN1) by preparative SDS-PAGE essentially as described by Chart & Griffiths (1985) except that membranes were solubilized by heating at 66 °C for 30 min in Tris/HCl buffer (0.06 M, pH 6.8) containing 2% (w/v) SDS, 10% (v/v) glycerol and 0.001% bromophenol blue. The antiserum was raised in a female Dutch rabbit using three injections of receptor protein given subcutaneously in Freund's incomplete adjuvant at intervals of three weeks. Thirteen days after the third injection, the rabbit was bled and serum prepared. Material obtained from three gels was combined for each injection.

Immunoblotting. OM proteins resolved by SDS-PAGE were transferred to nitrocellulose by electrophoretic blotting (Towbin *et al.*, 1979). After transfer, the aerobactin receptor was detected by sequential treatment with: 1% (w/v) skimmed milk to block unbound sites on the nitrocellulose; rabbit antiserum raised against the 74 kDa pColV-K30 aerobactin receptor; and staphylococcal protein A-horse radish peroxidase conjugate (Sigma). Antigenic sites were visualized with a 25 $\mu\text{g ml}^{-1}$ solution of 4-chloronaphthol containing 0.01% H₂O₂. The molecular masses and positions of cross-reacting OM proteins were then confirmed by counter-staining the immunoblots with Ponceau S (0.5%) in 1% (v/v) acetic acid.

RESULTS

Siderophore production and cloacin sensitivity

Five encapsulated *K. pneumoniae* strains of serotypes K1, K21, K22, K44 and K66 were examined for siderophore production and cloacin sensitivity. Enterochelin was detected in the culture supernatants of all strains but only one strain (DL1) produced aerobactin as determined by both colorimetric and cross-feeding assays (Table 1). However, whilst all four aerobactin-

Fig. 1. Growth of *K. pneumoniae* strains DL44 (●) and KN4401 (■) in Fe-SSM, and KN4401 (□) in Fe-SSM supplemented with aerobactin (20 µg ml⁻¹).

Table 1. *Bacterial strains and properties*

Key: E, enterochelin; A, aerobactin; *iutA* probe, hybridization with *iutA* aerobactin receptor gene derived from pColV-K30; +, positive reaction; -, negative reaction; R, resistant; S, sensitive.

<i>K. pneumoniae</i>	Serotype	Origin	Siderophore produced		Cloacin sensitivity	<i>iutA</i> probe
			E	A		
DL1	K1:O1	Blood	+	+	R	+
CAPD	K21:O1	Peritoneum	+	-	S	-
DL37	K22:O1	Blood	+	-	S	-
DL44	K44:O1	Blood	+	-	S	-
C3	K66:O1	Environment	+	-	S	-
KN4401	Derived from DL44		-	-	S	-
KN4402	Derived from DL44		-	-	S	-
<i>E. coli</i> K12 (pColV-K30)			+	+	S	+

negative strains were sensitive to cloacin DF13, the aerobactin producing strain, DL1, was resistant. To determine whether the K1 capsular polysaccharide or O1 LPS antigen presented a barrier to cloacin adsorption, mutants isogenic with DL1 lacking either the K1 or the O1 antigen, or both, were isolated. The K⁺:O⁻ and K⁻:O⁻ mutants were cloacin-sensitive whilst the K⁻:O1 mutant was cloacin-resistant, suggesting that the O rather than the K antigen prevents access of cloacin to the ferric-aerobactin receptor.

Hybridization of the pColV-K30 aerobactin gene probe with K. pneumoniae DNA

The pColV-K30 aerobactin gene prepared from plasmid pEN7 hybridized in a colony blot assay only with the aerobactin-producing *K. pneumoniae* strain DL1 (Table 1). None of the four cloacin-sensitive aerobactin-negative strains gave a positive reaction with the probe.

Fig. 2. (a) SDS-PAGE of the OM proteins of *K. pneumoniae* and *E. coli* K12(pColV-K30) grown in Fe-SSM (lanes A and C–G) and of *K. pneumoniae* DL1 (lane B) grown in Fe + SSM. Lane A, *E. coli*; lane B, DL1; lane C, DL1; lane D, CAPD; lane E, DL37; lane F, DL44; lane G, C3; lane H, molecular mass markers. (b) Immunoblot of the OM proteins of *K. pneumoniae* and *E. coli* K12(pColV-K30) showing the reaction of a polyclonal antiserum raised against the pColV-K30 74 kDa aerobactin receptor. Lanes are as in (a).

Aerobactin utilization by siderophore-negative *K. pneumoniae* mutants

To determine whether the aerobactin-negative, cloacin-sensitive strains could use aerobactin, strain DL44 was selected and mutagenized with diethyl sulphate to obtain mutants unable to produce enterochelin and thus unable to grow in iron-deficient media. Two mutants, KN4401 and KN4402, were selected by their failure to produce an orange halo on CAS-agar plates and by their inability to grow on Fe-SSM agar plates containing 200 μ M-EDDA. Fig. 1 shows the growth kinetics of strain DL44 and the isogenic enterochelin-negative mutant KN4401 in Fe-SSM. Compared with the parent strain DL44, growth of KN4401 in Fe-SSM was markedly reduced but could be restored upon addition of aerobactin to the medium. Aerobactin also promoted the growth of KN4401 on Fe-SSM/EDDA agar. Growth of both KN4401 and DL44 was also stimulated by addition of excess iron to iron-deficient media (data not shown). Thus the cloacin-sensitive *K. pneumoniae* DL44 which failed to hybridize with the pColV-K30 aerobactin receptor gene probe nevertheless possesses a functional aerobactin iron-uptake system.

Immunochemical characterization of the *K. pneumoniae* aerobactin receptor

Fig. 2(a) shows an SDS-PAGE gel of the OM proteins of *K. pneumoniae* grown in Fe-SSM compared with those of *E. coli* K12(pColV-K30) grown in the same medium. In *K. pneumoniae*, up to six proteins in the range 70 to 85 kDa were expressed in Fe-SSM (Fig. 2a). Similar results were also obtained when the organism was grown in Trypticase Soy Broth (TSB) containing

ovotransferrin (1 mg ml⁻¹) (data not shown). Synthesis of these high-molecular-mass proteins was repressed upon addition of excess iron to the medium (Fig. 2*a*, compare lanes B and C). To determine which of these proteins may function as a receptor for aerobactin, proteins separated by SDS-PAGE were transferred to nitrocellulose and probed with a polyclonal antiserum raised against the 74 kDa pColV-K30 aerobactin receptor (Fig. 2*b*). In each *K. pneumoniae* strain, a 76 kDa OM protein was recognized by the antibody. In addition, strain DL1 also produced a strongly reacting 74 kDa protein. Similar results were also obtained with organisms grown in TSB plus ovotransferrin (data not shown).

DISCUSSION

Since the first indications that the aerobactin iron-uptake system was associated with virulence, considerable efforts have been directed towards elucidating its distribution and genetic organization within virulent enteroinvasive, enteropathogenic and enterotoxigenic strains of *E. coli* (Griffiths, 1987; Roberts *et al.*, 1986; Carbonetti *et al.*, 1986; Fernandez-Beros *et al.*, 1988; Marolda *et al.*, 1987). This work has been extended to other enterobacterial pathogens including the Klebsiellae (Nassif & Sansonetti, 1986; Williams *et al.*, 1987). Thus, the aerobactin receptor and biosynthetic genes from pColV-K30 have been cloned into small multi-copy plasmids from which receptor and biosynthesis gene probes have been prepared (Bindereif & Neilands, 1983). These have been used to screen many pathogens for the presence of the aerobactin operon. Alongside these hybridization techniques, cloacin sensitivity has been used to detect synthesis of the aerobactin receptor whilst both chemical and bio-assays for the detection of aerobactin have also been developed.

Using such methodology, the expression of the aerobactin iron-uptake system by *K. pneumoniae* was investigated. Of the five strains examined, only one produced aerobactin and hybridized with the *iutA* gene probe in a colony-blot assay. This K1:O1 serotype strain was, however, resistant to cloacin but became sensitive upon loss, by mutation, of the O1 antigen. However, three of four non-aerobactin producing strains which express K21, K22 and K66 capsular polysaccharides were cloacin-sensitive despite possessing the O1 antigen. Thus the O1 antigen does not appear to block the access of cloacin to its receptor in all serotypes. Furthermore, other K1:O1 serotype strains (Nassif & Sansonetti, 1986) have been reported to be cloacin-sensitive. This may be due to differences in the cell-surface distribution of K and O antigens or perhaps to differences in the O-antigen chain length in the K1:O1 strain. Further work is required to elucidate the precise mechanism by which cloacin is excluded from its receptor in some but not all O1 serotype strains.

Despite the failure of the aerobactin-receptor gene probe to hybridize with the aerobactin-negative strains, their cloacin sensitivity indicated that the cells might possess an aerobactin receptor and be capable of using ferric-aerobactin as an iron source. To determine whether the putative aerobactin receptor was functional, mutants of the aerobactin-negative strain DL44, defective in enterochelin biosynthesis, were isolated. Aerobactin stimulated the growth of these mutants on solid media containing EDDA and in Fe-SSM.

K. pneumoniae therefore appears capable of scavenging ferric-aerobactin without synthesizing the chelator itself, and thus has a potential selective advantage in infection sites where aerobactin produced by other bacteria could be utilized. In the present study, only four aerobactin-negative strains were examined for aerobactin utilization. Further work is required to determine the distribution of *Klebsiella* strains expressing a functional aerobactin receptor without apparent aerobactin production. A high incidence of the aerobactin receptor in the absence of aerobactin production has been observed in *E. coli* strains isolated from children with diarrhoea (Fernandez-Beros *et al.*, 1988). In these strains the receptor was located either on a plasmid or in the chromosome but in contrast to the klebsiellae described here, all cloacin-sensitive strains hybridized with the pColV-K30 aerobactin gene probe (Fernandez-Beros *et al.*, 1988). In another study of some 500 strains of *E. coli* from human extraintestinal infections, Carbonetti *et al.* (1986) reported that around 50% of the strains examined expressed the aerobactin iron-uptake system. Several of these aerobactin-producing strains however, did not hybridize with either the receptor or biosynthesis gene probes.

In a study of the contribution of the aerobactin system to the virulence of *K. pneumoniae*, Nassif & Sansonetti (1986) observed one cloacin-sensitive, aerobactin-negative strain. However, in this strain, the aerobactin receptor gene was located on a small plasmid and hybridized with the pColV-K30 probe. In the present study, using moderately stringent conditions (which would be expected to recognize greater than 75% DNA homology), the probe did not hybridize with the aerobactin-negative *K. pneumoniae* strains. Thus, considerable divergence may exist between this aerobactin receptor gene and that of pColV-K30. To date, examinations of the DNA sequence homology of *E. coli* and *Shigella* aerobactin systems has revealed considerable homology in the biosynthetic and receptor genes, but differences in the physical maps and divergence in the flanking regions (Marolda *et al.*, 1987). In *Enterobacter* however, aerobactin systems which show considerable divergence in the biosynthetic genes and/or the receptor gene when compared with that of pColV-K30 have recently been described (Waters & Crosa, 1988; Crosa *et al.*, 1988).

Despite the lack of hybridization between the pColV-K30 receptor probe and the aerobactin-negative *K. pneumoniae* strains, immunoblotting of OM proteins from *K. pneumoniae* grown in Fe-SSM with a rabbit antibody raised against the purified 74 kDa pColV-K30 aerobactin-receptor revealed the presence of an immunologically cross-reacting, iron-repressible OM protein of 76 kDa. This reaction was however weaker than that of the pColV-K30 protein. Antigenic homology between the aerobactin receptors of *E. coli*, *Ent. cloacae*, *K. edwardsii*, *S. flexneri*, '*A. aerogenes*' and pColV-K30 has also been reported (Krone *et al.*, 1985; Marolda *et al.*, 1987; Waters & Crosa, 1988). The *Ent. cloacae* and *K. edwardsii* aerobactin receptors appear to be immunologically more related to each other than to the pColV-K30-encoded receptor protein (Krone *et al.*, 1985). Thus the 76 kDa protein is probably the aerobactin receptor in these aerobactin-negative strains, although further work is required to confirm its functionality. Interestingly, in addition to the 76 kDa OM receptor, a much more strongly reacting 74 kDa protein was also present in the aerobactin-producing, *iutA*-probe-positive *K. pneumoniae* strain DL1. This strain therefore may possess two aerobactin receptors, one of which is much more closely related to that of pColV-K30.

Thus, of the six iron-repressible proteins observed in the OM of *K. pneumoniae* DL1, three can now tentatively be assigned a function. The 74 and 76 kDa proteins are probably both aerobactin receptors whilst the 81 kDa is the enterochelin receptor (Williams *et al.*, 1987). Since *K. pneumoniae* only produces two siderophores, the remaining three proteins may function as receptors for siderophores produced by other micro-organisms. *E. coli* for example is capable of utilizing fungal siderophores such as ferrichrome and ferrioxamine B (Griffiths, 1987; Neilands, 1982). Exogenously supplied desferrioxamine B has also been reported to be capable of enhancing *Klebsiella* infections (Khimji & Miles, 1978) suggesting that this organism may also possess a ferrioxamine B receptor. Further work is currently under way to characterize more fully the iron-repressible *K. pneumoniae* OM proteins and to determine their genetic localization and organization.

We thank K. Hantke and P. H. Williams for *E. coli* strains and plasmids, I. Ørskov for serotyping the *Klebsiella* strains, T. Baldwin and P. Derbyshire for preparation of DNA probes and W. McPheat for helpful discussions.

REFERENCES

- ARNOLD, L. D. & VISWANATHA, T. (1983). The use of bis(mercaptoacetato-S,O)hydroxoiron(III) complex for the determination of hydroxamates. *Journal of Biochemical and Biophysical Methods* **8**, 307–320.
- ARNOW, L. E. (1937). Colorimetric determination of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. *Journal of Biological Chemistry* **228**, 531–537.
- BAGG, A. & NEILANDS, J. B. (1987). Molecular mechanism of regulation of siderophore-mediated iron assimilation. *Microbiological Reviews* **51**, 509–518.
- BENEDI, V. J., CIURANA, B. & TOMAS, J. M. (1989). Isolation and characterization of *Klebsiella pneumoniae* unencapsulated mutants. *Journal of Clinical Microbiology* **27**, 82–87.
- BINDEREIF, A. & NEILANDS, J. B. (1983). Cloning of the aerobactin-mediated iron assimilation system of plasmid ColV. *Journal of Bacteriology* **153**, 1111–1113.
- BRAUN, V., BRAZEL-FAISST, C. & SCHNEIDER, R. (1984). Growth stimulation of *Escherichia coli* in serum by iron (III) aerobactin. Recycling of aerobactin. *FEMS Microbiology Letters* **21**, 99–103.

- BULLEN, J. J. & GRIFFITHS, E. (1987). *Iron and Infection; Molecular, Physiological and Clinical Aspects*. New York: John Wiley.
- BYWATER, M., BYWATER, R. & HELLMAN, L. (1983). A novel chromatographic procedure for purification of bacterial plasmids. *Analytical Biochemistry* **132**, 219–224.
- CARBONETTI, N. H. & WILLIAMS, P. H. (1984). A cluster of five genes specifying the aerobactin iron uptake system of plasmid ColV-K30. *Infection and Immunity* **46**, 7–12.
- CARBONETTI, N. H. & WILLIAMS, P. H. (1985). Detection of synthesis of the hydroxamate siderophore aerobactin by pathogenic isolates of *Escherichia coli*. In *The Virulence of Escherichia coli, Reviews and Methods*, pp. 419–424. Edited by M. Sussman. New York: Academic Press.
- CARBONETTI, N. H., BOONCHAI, S., PARRY, S. H., VAISANEN-RHEN, V., KORHONEN, T. & WILLIAMS, P. H. (1986). Aerobactin-mediated iron-uptake by *Escherichia coli* isolates from human extra-intestinal infections. *Infection and Immunity* **51**, 966–968.
- CERCENADO, E., BAQUERO, F., DELGADO-IRIBARREN, A. & MARTINEZ, J. L. (1986). Epidemiology of aerobactin production in *Enterobacteriaceae*. *Annals de l'Institut Pasteur/Microbiologie* **137B**, 297–303.
- CHART, H. & GRIFFITHS, E. (1985). Antigenic and molecular homology of the ferric-enterobactin receptor protein of *Escherichia coli*. *Journal of General Microbiology* **131**, 1503–1509.
- COLONNA, B., NICOLETTI, M., VISCA, P., CASALINO, M., VALENTI, P. & MAIMONE, F. (1985). Composite IS1 elements encoding hydroxamate-mediated iron-uptake in F1me plasmids from epidemic *Salmonella* spp. *Journal of Bacteriology* **162**, 307–316.
- CROSA, L. M., WOLF, M. K., ACTIS, L. A., SANDERS-LOEHR, J. & CROSA, J. G. (1988). New aerobactin-mediated iron uptake system in a septicemia-causing strain of *Enterobacter cloacae*. *Journal of Bacteriology* **170**, 5539–5544.
- FEINBERG, A. P. & VOGELSTEIN, B. (1984). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* **137**, 266–267.
- FERNANDEZ-BEROS, M. E., KISSEL, V. & CABELLO, F. C. (1988). Aerobactin-mediated iron-uptake system in intestinal *Escherichia coli*. *FEMS Microbiology Letters* **49**, 397–401.
- GIBSON, F. & MAGRATH, D. I. (1969). The isolation and characterization of a hydroxamic acid (aerobactin) formed by *Aerobacter aerogenes* 62-1. *Biochimica et biophysica acta* **192**, 175–184.
- GRIFFITHS, E. (1987). The iron-uptake systems of pathogenic bacteria. In *Iron and Infection; Molecular, Physiological and Clinical Aspects*, pp. 69–137. Edited by J. J. Bullen & E. Griffiths. New York: John Wiley.
- GRIFFITHS, E., STEVENSON, P., HALE, T. L. & FORMAL, S. B. (1985). Synthesis of aerobactin and a 76,000 dalton iron-regulated outer membrane protein by *Escherichia coli*-K12-*Shigella* hybrids and by enteroinvasive strains of *Escherichia coli*. *Infection and Immunity* **49**, 67–71.
- GROSS, R., ENGELBRECHT, F. & BRAUN, V. (1985). Identification of the genes and their polypeptide products responsible for aerobactin synthesis by pColV plasmids. *Molecular and General Genetics* **201**, 204–212.
- KHIMJI, P. L. & MILES, A. A. (1978). Microbial iron-chelators and their action on *Klebsiella* infections in the skin of guinea-pigs. *British Journal of Experimental Pathology* **59**, 137–147.
- KONOPKA, K., BINDEREIF, A. & NEILANDS, J. B. (1982). Aerobactin-mediated utilization of transferrin iron. *Biochemistry* **21**, 6503–6508.
- KRONE, W. J. A., KONINGSTEIN, G., DE GRAAF, F. K. & OUDEGA, B. (1985). Plasmid-determined cloacin DF13-susceptibility in *Enterobacter cloacae* and *Klebsiella edwardsii*; identification of the cloacin DF13/aerobactin outer membrane receptor proteins. *Antonie van Leeuwenhoek* **51**, 203–218.
- LODGE, J. M. T., WILLIAMS, P. & BROWN, M. R. W. (1986). Influence of growth rate and iron limitation on the expression of outer membrane proteins and enterobactin by *Klebsiella pneumoniae* grown in continuous culture. *Journal of Bacteriology* **165**, 353–356.
- MAROLDA, C. L., VALVANO, M. A., LAWLOR, K. M., PAYNE, S. M. & CROSA, J. H. (1987). Flanking and internal regions of chromosomal genes mediating aerobactin iron uptake systems in enteroinvasive *Escherichia coli* and *Shigella flexneri*. *Journal of General Microbiology* **133**, 2269–2278.
- MARTINEZ, J. L., CERCENADO, E., BAQUERO, F., PEREZ-DIAZ, J. C. & DELGADO-IRIBARREN, A. (1987). Incidence of aerobactin production in Gram-negative hospital isolates. *FEMS Microbiology Letters* **43**, 351–353.
- NASSIF, X. & SANSONETTI, P. J. (1986). Correlation of the virulence of *Klebsiella pneumoniae* K1 and K2 with the presence of a plasmid encoding aerobactin. *Infection and Immunity* **54**, 603–608.
- NEILANDS, J. B. (1981). Microbial iron compounds. *Annual Review of Biochemistry* **50**, 715–731.
- NEILANDS, J. B. (1982). Microbial envelope proteins related to iron. *Annual Review of Microbiology* **36**, 285–309.
- NEILANDS, J. B. (1984). Methodology of siderophores. *Structure and Bonding* **58**, 1–24.
- PAYNE, S. M. (1980). Synthesis and utilization of siderophores by *Shigella flexneri*. *Journal of Bacteriology* **143**, 1420–1424.
- ROBERTS, M., PARTHASARATHY, S., LAM-PO-TANG, M. K. L. & WILLIAMS, P. H. (1986). The aerobactin iron-uptake system in enteropathogenic *Escherichia coli*: evidence for an extinct transposon. *FEMS Microbiology Letters* **37**, 215–219.
- SCHWYN, B. & NEILANDS, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry* **160**, 47–56.
- SINGH, L. & JONES, K. W. (1984). The use of heparin as a simple cost-effective means of controlling background in nucleic acid hybridization procedures. *Nucleic Acids Research* **12**, 5627–5638.
- STUART, S. J., PRPIC, J. K. & ROBINS-BROWNE, R. M. (1986). Production of aerobactin by some species of the genus *Yersinia*. *Journal of Bacteriology* **166**, 1131–1133.
- TOMAS, J. M., BENEDI, V. J., CIURANA, B. & JOFRE, J. (1986). Role of capsule and O-antigen in resistance of *K. pneumoniae* to serum bactericidal activity. *Infection and Immunity* **54**, 85–89.

- TOMAS, J. M., CAMPRUBI, S. & WILLIAMS, P. (1988). Surface exposure of the O-antigen in *Klebsiella pneumoniae* K1:O1 serotype strains. *Microbial Pathogenesis* **5**, 141-147.
- TOWBIN, H., STAHELIN, T. & GORDON, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America* **76**, 4350-4354.
- VALVANO, M. A., SILVER, R. P. & CROSA, J. H. (1986). Occurrence of chromosome- or plasmid-mediated aerobactin iron transport systems and hemolysin production amongst clonal groups of human invasive strains of *Escherichia coli* K1. *Infection and Immunity* **52**, 192-199.
- VAN TIEL-MENKVELD, C. J., MENTJON-VERVUUST, J. K., OUDEGA, B. & DEGRAAF, F. K. (1982). Siderophore production by *Enterobacter cloacae* and a common receptor protein for the uptake of aerobactin and cloacin DF13. *Journal of Bacteriology* **150**, 490-497.
- WARNER, P. J., WILLIAMS, P. H., BINDEREIF, A. & NEILANDS, J. B. (1981). ColV plasmid-specified aerobactin synthesis by invasive strains of *Escherichia coli*. *Infection and Immunity* **33**, 540-545.
- WATERS, V. L. & CROSA, J. H. (1988). Divergence of the aerobactin iron-uptake systems encoded by plasmids pColV-K30 in *Escherichia coli* K12 and pSMN1 in *Aerobacter aerogenes* 62-1. *Journal of Bacteriology* **170**, 5153-5160.
- WEISLANDER, L. (1979). A simple method to recover intact high molecular weight RNA and DNA after electrophoretic separation in low gelling temperature agarose gels. *Analytical Biochemistry* **98**, 305-309.
- WILLIAMS, P. (1988). Role of the cell envelope in bacterial adaptation to growth *in vivo* in infections. *Biochimie* **70**, 987-1011.
- 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.
- WILLIAMS, P., CHART, H., GRIFFITHS, E. & STEVENSON, P. (1987). Expression of high affinity iron uptake systems by clinical isolates of *Klebsiella*. *FEMS Microbiology Letters* **44**, 407-412.
- WILLIAMS, P. H. (1979). Novel iron uptake system specified by ColV plasmids; an important component in the virulence of invasive strains of *Escherichia coli*. *Infection and Immunity* **26**, 925-932.
- WILLIAMS, P. H. & CARBONETTI, N. H. (1986). Iron, siderophores and the pursuit of virulence: independence of the aerobactin and enterochelin iron uptake systems in *Escherichia coli*. *Infection and Immunity* **51**, 942-947.