Incidence of the Aerobactin Iron Uptake System Among \textit{Escherichia coli} Isolates From Infections of Farm Animals

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(Received 2 July 1986; revised 5 November 1986)

To assess the importance of aerobactin-mediated iron uptake as a bacterial virulence determinant in animal infections, a total of 576 strains of \textit{Escherichia coli} isolated from cattle, chickens, sheep and pigs were screened by colony hybridization to determine the presence of the aerobactin genetic determinants, and by a bioassay to detect aerobactin secretion in iron-limited conditions. Results obtained by the two complementary methods correlated well. The incidence of the aerobactin system was very high among septicaemia isolates, particularly those from cattle and chickens, an observation that strongly suggests an important role for this mechanism of iron assimilation in pathogenesis. On the other hand, the incidence of the aerobactin system among mastitis strains was not significantly higher than among faecal isolates from healthy animals. No classical enterotoxigenic \textit{E. coli} strains tested carried the aerobactin genetic determinants. Although most strains that produced aerobactin were also able to make colicin \textit{V}, the fact that the two characteristics existed separately in a significant minority of isolates suggested that colicin testing alone could not be reliably used to determine the presence of the aerobactin system.

INTRODUCTION

Iron is an essential trace element for aerobic bacterial growth. However, because ferric iron is virtually insoluble at physiological pH levels, a variety of mechanisms have evolved in bacteria for the solubilization and active uptake of iron. Enterobacteria secrete a catechol iron-chelating agent (siderophore) called enterochelin, which sequesters ferric iron in the medium and transports it by specific inner and outer membrane components into bacterial cells (Rosenberg & Young, 1974). Intracellular release of iron involves enzymic degradation of the siderophore molecule (Langman et al., 1972).

Some strains of \textit{Escherichia coli} also synthesize the hydroxamate siderophore aerobactin (Braun, 1981; Warner et al., 1982), and a cognate outer membrane receptor protein with which ferric-aerobactin formed in the external medium interacts (Bindereif et al., 1982; Grewal et al., 1982). Although the binding constant of ferric-aerobactin is several orders of magnitude less than that of ferric-enterochelin (Neilands, 1981), the ability to secrete the hydroxamate siderophore apparently confers a significant selective advantage on bacteria growing in iron-limited environments such as the fluids and tissues of an infected animal body where iron is complexed with lactoferrin and transferrin (Williams, 1979; Williams & Warner, 1980).

Several properties of aerobactin may contribute to its greater effectiveness as a siderophore compared with enterochelin. For example, aerobactin is repeatedly reusable (Braun et al., 1984)

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and can therefore support bacterial growth in conditions of iron stress at very low concentrations (Williams & Carbonetti, 1986). Moreover, aerobactin-acquired iron appears to be efficiently channelled wherever it is required for cellular growth rather than simply released into an intracellular pool (Williams & Carbonetti, 1986).

The aerobactin system was first found associated with plasmids that specify colicin V (ColV plasmids), which were particularly prevalent among E. coli strains isolated from human and animal bacteraemia (Smith, 1974; Smith & Huggins, 1976). Furthermore, in a recent extensive survey that involved direct screening for the aerobactin system, its incidence was significantly higher among isolates from various human extraintestinal infections than among faecal isolates from healthy individuals (Carbonetti et al., 1986). Indeed, in a group of strains isolated from human pyelonephritis, the frequency of the aerobactin system was as high as that of P-fimbriae, and higher than that of haemolysin, both of which are recognized as virulence-enhancing factors in uropathogenic E. coli (Brooks et al., 1980; Svanborg-Edén et al., 1983; Waalwijk et al., 1983).

To assess the significance of aerobactin as a bacterial virulence determinant in animal infections we have used the same direct approaches to screen a large collection of E. coli isolates from various types of infection of farm animals. Strains were tested in a bioassay for the ability to secrete aerobactin in iron-limited conditions, as well as by colony hybridization for the presence of the aerobactin genetic determinants. In addition, we have examined the association between colicin V and the aerobactin system in these strains.

## METHODS

### Clinical isolates
A total of 576 strains of E. coli isolated from various infections of cattle, sheep, chickens and pigs, and from the faeces of healthy animals, were tested. Some strains were isolated specifically for this survey; others were acquired from the culture collections of Dr J. E. T. Jones (Royal Veterinary College, London, UK) and Dr H. Williams Smith (Houghton Poultry Research Station, Huntingdon, Cambridgeshire, UK). The strains from clinical cases of bovine mastitis were obtained through 23 Veterinary Investigation Laboratories by liaising with the National Mastitis Survey organized by the Central Veterinary Laboratory, Weybridge, UK.

### Aerobactin screening
A bioassay for aerobactin developed in our laboratory (Carbonetti & Williams, 1985) uses as an indicator an E. coli K12 strain, designated LG1522, which is unable to utilize ferric-enterochelin (fepA) and which harbours a Col-V-K30 mutant plasmid that is defective in aerobactin biosynthesis (iuc), but which specifies the outer membrane ferric-aerobactin receptor protein (iur^+). Thus, a lawn of strain LG1522 on M9 minimal agar (Roberts et al., 1983) containing the iron-chelating agent a,a'-dipyridyl (200 μM), will form visible growth only if cross-fed by any aerobactin-producing strains point-inoculated (up to 12 per plate) onto the lawn. The bioassay can be modified to detect aerobactin in solutions placed into small wells cut in a seeded agar plate.

Direct detection of the aerobactin genetic determinants by colony hybridization has been described previously (Carbonetti et al., 1986). The aerobactin system was cloned as a 16 kb HindIII fragment of plasmid ColV-K30 (Bindereif & Neilands, 1983). The recombinant plasmid, designated pABN1 (Fig. 1a), was shown by transposon mutagenesis and deletion mapping to contain a cluster of five genes, of which four mediate siderophore biosynthesis, and the fifth specifies the receptor protein (Carbonetti & Williams, 1984). A 2 kb AvaI fragment derived from within the biosynthesis region was used as the probe for colony hybridization screening (Fig. 1a). Laboratory strains known to lack the aerobactin system were included on each filter as negative controls.

### Detection of radiolabelled aerobactin
Aerobactin synthesized from precursor L-[U-14C]lysine monohydrochloride (Amersham; specific activity 348 mCi mmol−1, 129 GBq mmol−1) was detected by electrophoresis as described by Ford et al. (1986), or by chromatography on Whatman 3MM paper, using an ascending system with n-butanol/acetic acid/water (60:15:25, by vol.) for a period of 16 h. When appropriate, 100 μg unlabelled aerobactin, purified from culture supernates of Aerobacter aerogenes 62-1 as described by Gibson & Magrath (1969), was added to samples as an internal standard. Radiolabelled compounds were detected by autoradiography and the aerobactin standard was visualized by spraying the paper with 1:25% (w/v) FeCl3 in 1 N-HCl.

### Colicin V bioassays
Clinical isolates were tested for colicinogenicity on nutrient agar plates using E. coli K12 strains sensitive to all colicins as indicators (Smith & Huggins, 1976). Derivatives carrying the ColV plasmid were then used to identify colicin V on the basis of specific immunity to bactericidal activity. Note that the V colicins, like E and I colicins, may comprise more than one immunity type (Davies et al., 1981), so that the bioassay described here identifies only colicins of the same immunity group.

### Cloning of the colicin V gene and preparation of a hybridization probe
Plasmid ColV-K30 DNA was purified from cleared Triton X-100 lysates of a plasmid-containing strain by caesium chloride equilibrium density gradient ultracentrifugation in the presence of ethidium bromide (Radloff et al., 1967). Purified plasmid DNA was cleaved with HindIII, ligated to pACYC184 vector plasmid DNA (Chang & Cohen, 1978) cut with the same enzyme, and
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Fig. 1. Physical maps of recombinant plasmids pABN1 (a) and pLG180 (b) used to derive hybridization probes. Thin lines represent DNA fragments of plasmid ColV-K30, and thick lines indicate vectors (pPlac for pABN1; pACYC184 for pLG180). Cleavage sites for restriction endonucleases EcoRI (E), HindIII (H) and SalI (S) are shown, as are the two AatI (A) sites in pABN1 relevant to probe derivation. Stalks on the map of pLG180 indicate sites of transposon Tn1000 insertion; open symbols represent plasmid derivatives in which colicin V production was unaffected and filled symbols indicate insertions resulting in loss of colicin activity.

used to transform E. coli K12 strain RB308 (Carbonetti & Williams, 1984); selected chloramphenicol-resistant transformants were tested in bioassays for their ability to produce colicin V. Plasmid DNA purified from colicinogenic transformants consisted of an approximately 17 kb fragment of ColV-K30 DNA in the 4 kb vector. Restriction analysis of this plasmid (designated pLG180) with a number of enzymes generated the map shown in Fig. 1(b). Transposon mutagenesis with Tn1000 (Guyer, 1978) was used as previously described (Carbonetti & Williams, 1984) to generate colicin V-deficient mutants of pLG180; the sites of transposon insertion in six of these were mapped by restriction analysis to a 1.5 kb region of the plasmid. Ten plasmids in which transposon insertion did not affect colicin activity were also mapped. A 4 kb EcoRI fragment spanning the region defined by the colicin-defective mutants was isolated for use as a probe for colony hybridization screening of clinical isolates (Fig. 1b). In control experiments this fragment hybridized to DNA of ColV strains, but not to that of plasmid-cured derivatives.

Colony hybridization. The strains to be tested were grown overnight on nitrocellulose filters placed on the surface of nutrient agar plates. Bacteria were lysed in situ by exposure to alkali, and the released DNA was immobilized by baking the filter at 80 °C for 2 h (Grunstein & Hogness, 1975). Baked filters were scrubbed gently with hot water to remove cell debris. Restriction fragments for use as probes were isolated from low-melting-temperature agarose gels and labelled with 32P by the method of Feinberg & Vogelstein (1984). After hybridization, filters were washed in 0.1 x SSC (1 x SSC is 0.15 M-NaCl, 0.015 M-sodium citrate) as described previously (Carbonetti et al., 1986).

RESULTS AND DISCUSSION

Incidence of the aerobactin system among clinical isolates

Table 1 shows the results of screening 570 E. coli isolates from various animal sources for the presence of the aerobactin system. The frequency of positive strains was significantly higher among strains isolated from septicaemia, particularly of cattle and chickens, than among normal faecal isolates from healthy animals (P < 0.05). The data are in general agreement with reported surveys of human isolates of E. coli in which the incidence of aerobactin was greater in strains isolated from extraintestinal infections than among control faecal isolates (Montgomerie et al., 1984; Carbonetti et al., 1986). However, the incidence of aerobactin production among human faecal isolates (42 and 34%, respectively, in the two studies cited) was much higher than in the control isolates from farm animals reported here (2–14%), which enhances the
significance of the very high frequency of aerobactin production by bovine and chicken septicaemic strains.

There was no evidence of a role for aerobactin in the virulence of E. coli strains associated with mastitis. The incidence of the aerobactin system among such strains was marginally greater than that of faecal strains, but the difference was not significant at the 5% level. It has been suggested that coliform mastitis is not a specific but an opportunistic infection; strains isolated from cases of mastitis belong to a wide range of O-serogroups, and no specific mastitis virulence determinants have been identified (Wray et al., 1984; Linton & Robinson, 1984). However, part of the host animal's response to infection of the mammary gland is an increase in the concentration of lactoferrin in the milk to complex available iron (Harmon et al., 1976; Bishop et al., 1976), and so an efficient iron chelator could in theory be a candidate virulence factor in mastitis strains. Our survey indicates, however, that aerobactin is probably not involved.

Furthermore aerobactin is clearly not a virulence factor in cases of enterotoxic diarrhoea, since all the classical ETEC strains (producing LT or ST) tested for the presence of the aerobactin system were negative. However, the majority of bovine O26 strains were able to synthesize aerobactin whether they were isolated from the heart blood of septicaemic calves, or were faecal isolates from calves with diarrhoea. Four out of the six O26 bovine intestinal isolates also produced the Shiga-like enterotoxin (verotoxin) and in this respect were similar to human EPEC O26 strains (M. A. Linggood, unpublished data). It has previously been reported (Williams & Roberts, 1985; Roberts et al., 1986) that several human O26 and other EPEC strains possess the aerobactin system. Its role in the virulence of these strains may be to acquire iron complexed with lactoferrin in the mother's milk (Bullen et al., 1972; Williams & Roberts, 1985). Moreover, EPEC strains, unlike ETEC, characteristically adhere very intimately to the intestinal epithelium (Rothbaum et al., 1982). The presence of lactoferrin-rich secretions at the mucosal surface, therefore, may create a microenvironment in which the activity of the aerobactin iron uptake system might be selectively advantageous for bacterial colonization (Roberts et al., 1986). The same considerations may apply to the pathogenesis of the O26 strains isolated from calf scours.

Comparison of screening methods

In the data shown in Table 1, 139 strains (24%) were positive in both tests for aerobactin, and 415 (73%) were negative by both screening methods. Despite this close correlation, however, discrepant results for a significant number of isolates (16, approximately 3% of the total) serve to emphasize the complementary nature of the two procedures, and the desirability of doing both tests if possible. Thirteen strains (five each from bovine and chicken septicaemia, and one each from sheep septicaemia, bovine mastitis and chicken faeces) were positive by colony hybridization, but negative in bioassays done as described in Methods. The aerobactin system in these strains may be mutated, or fortuitously maintained in a repressed state during inoculum growth on the bioassay plates. More interesting, perhaps, are the three isolates (one each from

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**Table 1. Presence of the aerobactin system in E. coli isolates from animals**

The number of strains positive in either screening test is given as a fraction of the total number in each diagnostic group, and as a percentage (in parentheses) where appropriate.

<table>
<thead>
<tr>
<th>Diagnostic group</th>
<th>Origin of isolate:</th>
<th>Frequency of occurrence</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Cattle</td>
<td>Chickens</td>
</tr>
<tr>
<td>Septicaemia</td>
<td>46/54 (85%)</td>
<td>54/61 (89%)</td>
</tr>
<tr>
<td>Mastitis</td>
<td>18/148 (12%)</td>
<td>3/12 (25%)</td>
</tr>
<tr>
<td>Enterotoxic diarrhoea</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>Faecal (healthy animals)</td>
<td>3/73 (4%)</td>
<td>3/27 (11%)</td>
</tr>
</tbody>
</table>

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**Notes:**
- Significance of the very high frequency of aerobactin production by bovine and chicken septicaemic strains.
- There was no evidence of a role for aerobactin in the virulence of E. coli strains associated with mastitis.
- Aerobactin was marginally greater among mastitis strains compared to faecal strains, but not significant at the 5% level.
- Coliform mastitis is an opportunistic infection with a wide range of O-serogroups.
- Increased lactoferrin concentration in milk complexes available iron, possibly creating an environment for aerobactin activity.
- Discrepant results for 3% of the total suggest complementary nature of tests, with 16 isolates positive by one method and negative by the other.
- Three isolates were positive by hybridization but negative in bioassays, possibly due to mutation or repressed state.

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**Further Reading:**
- Wray et al., 1984
- Linton & Robinson, 1984
- Harmon et al., 1976
- Bishop et al., 1976
- Bullen et al., 1972
- Williams & Roberts, 1985
- Roberts et al., 1986
- Rothbaum et al., 1982
- Williams & Roberts, 1985
- Bullen et al., 1972
- Williams & Roberts, 1985
- Rothbaum et al., 1982
- Roberts et al., 1986
- Williams & Roberts, 1985
- Rothbaum et al., 1982
- Roberts et al., 1986
- Williams & Roberts, 1985
- Rothbaum et al., 1982
- Roberts et al., 1986
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bovine, sheep and pig mastitis) that were positive in the bioassay, but apparently negative by hybridization done as described in Methods.

Nature of the siderophore secreted by hybridization-negative strains

In subsequent studies with the three strains that gave a negative result by colony hybridization, isolated DNA was resolved on agarose gels, blotted to nitrocellulose (Southern, 1975), and probed with the AvaI restriction fragment. This approach confirmed a lack of significant homology, even at much lower stringency (up to $1 \times SSC$ in the final wash), so ruling out the possibility that capsular material or other cell debris might reduce the efficiency of colony hybridization. The suggestion that these three strains, and similar isolates from human extraintestinal infections identified in a previous survey (Carbonetti et al., 1986), make a different siderophore has also been ruled out. Biochemical studies indicated that when these bacteria were fed with radioactive lysine (a precursor of aerobactin), they synthesized a labelled compound whose chromatographic and electrophoretic properties were identical with those of authentic aerobactin added as an internal standard.

Electrophoresis at pH 7.0 also revealed the synthesis at variable levels of an uncharged compound, probably N$^a$-acetyl-N$^o$-hydroxylysine (Neilands, 1983), by these and other aerobactin-producing clinical isolates. However, when radiolabelled components of extracts to which no unlabelled aerobactin had been added were resolved by preparative paper electrophoresis and eluted in distilled water, only material with the known mobility of aerobactin was found subsequently to co-chromatograph with standard aerobactin, and also to support the growth of strain LG1522 in a bioassay. The nature of the aerobactin system in these strains and its possible significance in the pathogenesis of mastitis and other extraintestinal infections are currently being investigated.

Association of aerobactin and colicin V among clinical isolates of E. coli

First reports of the aerobactin system in E. coli clearly indicated its physical association, in terms of genetic linkage on the same plasmid, with the ability to synthesize colicin V (Williams, 1979; Stuart et al., 1980). To investigate the correlation between aerobactin and colicin V production in detail, we first used a bioassay to screen isolates of animal origin to determine if they secreted the colicin, and then determined whether they contained nucleotide sequences homologous with a DNA fragment carrying the colicin V genetic determinant. Clinical isolates that caused the killing of a ColV$^-$ indicator strain, but not of its ColV$^+$ derivative, were considered able to synthesize colicin V of the same immunity type, and, as expected, these strains gave a positive result in colony hybridization screening. On the other hand, clinical isolates that caused the killing of both bioassay indicator strains clearly produced a colicin of another immunity type, but it was not possible to determine, without a much more extensive and elaborate bioassay system, if they also made homoimmune colicin V. Hybridization provides a rapid and reliable method for identifying the ability of multiply colicinogenic strains to produce colicin V, regardless of immunity type; for instance, out of 32 isolates of this type from animal septicaemia or mastitis, 24 contained DNA sequences homologous with the colicin V gene probe. Positive hybridization was also observed, however, for 14 out of 53 septicaemia and mastitis strains that were apparently non-colicinogenic on the basis of the bioassay. Apparent masking of colicin V activity in bioassays performed on nutrient agar has been reported previously, and it is possible that detection of colicinogenicity in these clinical isolates would require the use of more refined media such as diagnostic sensitivity test agar (Davies et al., 1981). It is also possible, of course, that the hybridization probe contains sequences apart from the colicin gene that are common to other related plasmids present in this group of strains.

Table 2 shows the association between the colicin V and aerobactin systems in representative samples of 80 animal septicaemia isolates and 45 mastitis strains. Clearly the majority of strains were able to synthesize either both products (61%) or neither (27%), but a few of the aerobactin-producing strains were unable also to make colicin V, and a small number of ColV$^+$ isolates did not also carry the aerobactin genes. Thus although there was very close correlation between the two characters among the isolates tested, it is clear that merely testing for colicinogenicity is
Table 2. Association between colicine V and aerobactin among septicaemia and mastitis isolates of E. coli

Aerobactin production and colicinogenicity were each determined by both bioassay and colony hybridization with the appropriate probe.

<table>
<thead>
<tr>
<th>Diagnostic group</th>
<th>No. Col V⁺</th>
<th>No. Col V⁻</th>
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<tbody>
<tr>
<td>Septicaemia (n = 80)</td>
<td>61</td>
<td>6</td>
</tr>
<tr>
<td>Mastitis (n = 45)</td>
<td>15</td>
<td>3</td>
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</table>

likely to lead to substantial error in assessing the incidence of the aerobactin system. None of the O26 isolates made colicine V.

Hybridization analysis of isolated DNA resolved on an agarose gel and blotted onto nitrocellulose (Southern, 1975) indicated the presence of sequences homologous with the aerobactin probe associated with large plasmids in some of the ColV⁻ strains (data not shown). Moreover, in 14 randomly chosen strains that produced both aerobactin and colicine V, treatment with SDS in conditions known to cause plasmid curing (Smith, 1974) led, as expected, to coincident loss of both phenotypes. Also hybridization analysis indicated the presence of sequences homologous with both the aerobactin and colicine V probes associated with a single large plasmid in these strains (data not shown).

It was recently shown that the aerobactin genetic determinants may be flanked by insertion sequences, and may therefore comprise a transposable element (Bindereif & Neilands, 1985; Colonna et al., 1985; Perez-Casal & Crosa, 1984; Roberts et al., 1986), so that there is no a priori reason why the two phenotypic characters should not exist independently. Indeed, among the human isolates reported previously (Carbonetti et al., 1986), fewer than 40% of aerobactin-producing strains synthesized a colicine of any kind (M. Roberts, unpublished observations). Furthermore, there is evidence that the aerobactin gene complex may be located on the bacterial chromosome or on other types of plasmid (Valvano & Crosa, 1984; Bindereif & Neilands, 1985; Valvano et al., 1986; Roberts et al., 1986). We confirm here that the association between colicine V and aerobactin production is not absolute, and we stress that the importance of aerobactin as a virulence determinant can be assessed only by screening strains directly for the presence and expression of the aerobactin gene cluster, and cannot be deduced from data based simply on screening for colicine V.

We are grateful to Nicholas Carbonetti and David Reynolds for help with several aspects of this work. We thank Sally Clarke for mapping the cloned colicine V genetic determinant, and Carolyn Wight for carrying out biochemical analyses of aerobactin as part of their undergraduate research projects. M. R. acknowledges receipt of a Science and Engineering Council CASE Studentship. Part of this work was supported by a project grant awarded by the Wellcome Trust to P. H. W. and Dr R. A. Cooper, Department of Biochemistry, University of Leicester.

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