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Iron-Suppressible Production of Hydroxamate by *Escherichia coli* Isolates

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A total of 476 strains of *Escherichia coli* isolated from humans, pigs, cattle, poultry, potable water, or effluent were examined for iron-suppressible ability to produce hydroxamate. Isolates able to produce such material (Hyd$^+$ isolates) are presumed to be able to carry out hydroxamate-dependent transport of iron. The percentages of Hyd$^+$ isolates found among *E. coli* isolated from the feces of breast-fed babies (71%), adults (46%), milk-fed calves (32%), or poultry (28%) were significantly greater ($P < 0.01$) than the percentages isolated from potable water and effluent (6%) or from the feces of suckling piglets (6%), weaned pigs (6%), or weaned cattle (4%). The percentages of Hyd$^+$ isolates found among *E. coli* associated with diarrhea in humans (51%), weaned pigs (7%) or calves (25%) were not significantly different ($P > 0.1$) from those found among strains isolated from corresponding nondiarrheic hosts. Many of the *E. coli* isolated from cases of *E. coli* bacteremia in humans and poultry were Hyd$^+$ (64% and 83%, respectively). We conclude that ability to carry out hydroxamate-mediated transport of iron is widely distributed among natural isolates of *E. coli* but that the distribution of Hyd$^+$ *E. coli* is not random. *E. coli* isolated from sources where levels of available iron might be expected to be low tend to be Hyd$^+$. It seems that a link may exist between prevalence of Hyd$^+$ *E. coli* and active host-defense based on restricted availability of iron.

Like other living organisms, *Escherichia coli* cells require iron for growth. Continuous supplies of this nutrient must be obtained from the growth milieu, where the metal is commonly present either in the form of very insoluble colloidal hydroxides (28) or bound to organic ligands (5). To obtain iron under such conditions, bacteria have evolved a number of transport systems (22), important components of which are low-molecular-weight iron-binding compounds termed siderophores.

In *E. coli*, three distinct chromosomally determined systems for transporting iron have been identified (13, 16, 35). The enterochelin-dependent system appears to be widespread and may be the normal means by which iron is taken up under conditions of iron-deficiency (23). Under such conditions, *E. coli* cells synthesise and secrete enterochelin, a cyclic trimer of 2,3-dihydroxy-*N*-benzoyl-L-serine (DBS), into the growth milieu. Here, a stable iron-siderophore complex (ferric enterochelin) is formed, and this is thought to be transported into the bacterial cytoplasm. Iron is released for cellular metabolism after enzymatic hydrolysis of the ligand to DBS (16). In contrast with the enterochelin-dependent system, the other two high-affinity systems involve ligands not supplied by the *E. coli* cells. Thus, transport mediated by citrate or ferrichrome occurs only when the required ligand is present in the growth medium (11, 13).

We have recently reported the existence in certain *E. coli* of an additional hydroxamate-based iron-transport system, which is at least sometimes plasmid determined (29). What is presumably an identical system has been described independently by Williams (33). Plasmids controlling such hydroxamate-dependent transport of iron were originally detected in ColV$^+$ strains of *E. coli*, but it soon became obvious that some noncolicinogenic *E. coli* are also able to produce hydroxamate (see below).

The aim of the survey reported here was to determine the distribution of *E. coli* able to carry out hydroxamate-dependent iron transport. The results obtained support the view that the frequency with which such organisms are found in a particular situation is related to severity of bacterial iron deprivation.

A brief account of this work has been presented elsewhere (S. J. Stuart and R. K. J. Luke, Abstr. Annu. Meet. Aust. Soc. Microbiol. 1981, C6, p54).

MATERIALS AND METHODS

Bacteria. Apart from 20 isolates supplied by R. Bishop of the Royal Children's Hospital, Melbourne, Australia (RCH) (14 from 6- to 12-month-old infants

and 6 from adults) and a further 18 isolates (from adults) supplied by P. Cavanagh of the Fairfield Infectious Diseases Hospital, Melbourne (FIDH), strains from nondiarrheic patients were isolated at La Trobe University, Bundoora, Australia. Samples of freshly voided feces were collected from breast-fed babies (2 to 10 days old) in Melbourne maternity hospitals; from rectal swabs from suckling piglets (1 to 4 weeks old); from weaned pigs and poultry in the Bendigo district of Victoria; from milk-fed calves (1 to 3 weeks old) in the Warragul district of Victoria; and from weaned cattle in the Dandenong district of Victoria. Samples were spread directly onto eosin methylene blue agar (Oxoid Ltd.). Five isolated colonies which exhibited a greenish metallic sheen were selected from each plate and further purified on MacConkey agar (Oxoid no. 3). Bacteria were presumptively identified as *E. coli* if they produced indole and fermented lactose at 44°C.

E. coli isolated from the feces of diarrheic infants were obtained from RCH (11 strains) or from FIDH (9 strains), whereas those from the feces of diarrheic adults (19 strains) were obtained from FIDH. *E. coli* isolated from the feces or small intestines of scouring pigs (60 strains) or calves (48 strains) were obtained from J. Craven of the Attwood Veterinary Research Laboratory, Melbourne. In all instances, the *E. coli* isolated from diarrheic feces were obtained from cases characterized by a heavy, predominant growth of the organism on primary isolation plates.

A total of 50 *E. coli* isolates from blood cultures of humans with septicemia (bacteremic *E. coli*) were obtained from R. Stoner of the Institute of Medical and Veterinary Science, Adelaide, Australia (35 isolates), and from V. Petrocheilou of the Bristol Royal Infirmary, Bristol, England (15 isolates). Isolates from *E. coli* bacteremia of chickens were supplied by H. Williams-Smith of the Houghton Poultry Research Station, Houghton, England (6 isolates), and from P. Coloe of the Veterinary Research Institute, Melbourne (17 isolates).

A total of 48 "nonanimal" isolates of *E. coli*, derived from potable water (30 isolates) and effluent (18 isolates), were supplied by A. McNeill of the State Rivers and Water Supply Commission of Victoria, Australia.

E. coli K-12 strain AN194 (16) was used as an indicator in tests for colicin production. *E. coli* strain MW was obtained from H. Williams-Smith (27), and *E. coli* strains B188 (ColV$^-$), B188 (ColV-B188), and B188 (ColV I-K94) were obtained from H. Williams-Smith (25, 26) via K. G. Hardy.

Media and chemicals. Stock cultures of survey isolates were stored in nutrient broth (Oxoid no. 2 plus 3 g of Oxoid yeast extract per liter) containing 6% (vol/vol) dimethyl sulfoxide at −70°C, and working cultures were maintained on nutrient agar slants. The mineral salts mixture used in minimal medium was the medium 56 described by Monod et al. (21), except that ferrous sulfate was omitted and 100 μM 2,2'-dipyridyl was included to reduce further the availability of iron. Low-iron minimal medium containing 0.7 μM iron was prepared as described previously (29). Glucose (30 mM) was used as the carbon source. When required, 1% (wt/vol) casein hydrolysate (Oxoid) was added to minimal medium to promote growth of auxotrophic *E. coli*. Ajax agar (12 g/liter) was used to prepare solid media.

All chemicals used in the preparation of buffers and media were of analytical grade.

Growth of cells and preparation of culture supernatant fluids. Survey strains were grown at 37°C with orbital shaking (200 rpm) in 100-ml Erlenmeyer flasks containing 10 ml of medium. Cultures (48 h) were centrifuged at 12,000 × *g* for 20 min and the resulting clear supernatant fluid tested for the presence of hydroxamate.

Detection of strains able to produce colicin. Colicin production was detected by using a double-layer technique described previously (18).

Assay for hydroxamate production. The method used to detect hydroxamate in culture supernatant fluids was that described by Csáky (9), except that modified hydrolysis conditions were used (12).

Statistical tests. The significance of differences in the percentages of strains producing hydroxamate material was determined by using the chi-square test described by Clarke (7).

RESULTS

Detection of hydroxamate-dependent systems for iron-transport. Organisms to be tested for ability to produce hydroxamate were grown initially in minimal medium containing 2,2'-dipyridyl. Those found to produce Csáky assay-reactive material were then inoculated into flasks of two minimal media, one containing approximately 0.7 μM Fe and the other containing approximately 50 μM Fe. Where the presence of 50 μM Fe led to a more than 90% decrease in the production of detectable Csáky assay-reactive material, in comparison with that in the low-iron treatment, the (Hyd$^+$) isolate was presumed to possess an hydroxamate-dependent system for iron transport. It was found that the levels of bound hydroxylamine nitrogen in supernatant fluids obtained from low-iron treatments were generally greater than 10^{-4} M, whereas the levels detected in the high-iron treatments were generally less than 5×10^{-6} M.

Distribution of Hyd$^+$ strains among natural isolates of *E. coli*. The distribution of Hyd$^+$ strains among 476 *E. coli* isolates derived from different sources is shown in Table 1. Where more than one isolate from a particular fecal specimen was examined, only the result of the first isolate tested was included in the results shown. The proportions of Hyd$^+$ *E. coli* isolated from the feces of apparently healthy suckling-piglets, weaned pigs, or weaned cattle were not significantly different ($P > 0.1$) from the proportion of Hyd$^+$ organisms isolated from nonanimal sources. However, in comparison with strains isolated from potable water and effluent, greater proportions of isolates from the feces of apparently healthy humans, poultry, and calves were Hyd$^+$ ($P < 0.001$). The proportions of Hyd$^+$ isolates among *E. coli* isolated from diarrheic feces of humans, weaned pigs, and calves were not significantly different ($P > 0.1$) from the

TABLE 1. Distribution of Hyd+ *E. coli* among natural isolates

Source of *E. coli*	No. of isolates	No. (%) of Hyd+ isolates[a]
Nonanimal		
Potable water or effluent	48	3 (6)
Humans		
Feces from breast-fed babies	7	5 (71)
Infants	14	3 (21)
Adults	24	11 (46)
Infants[b]	20	8 (40)
Adults[b]	19	12 (63)
Blood	50	32 (64)
Swine		
Feces from suckling piglets	18	1 (6)
Weaned pigs	32	2 (6)
Weaned pigs[b]	60	4 (7)
Cattle		
Feces from milk-fed calves	22	7 (32)
Weaned cattle	51	2 (4)
Calves[b]	48	12 (25)
Poultry		
Feces from laying hens	40	11 (28)
Chicken blood	23	19 (83)

[a] Iron-suppressible production of hydroxamate material was detected as described in the text. Results obtained with isolates from a particular animal type were pooled since no statistically significant differences were observed among results obtained with samples making up a group.
[b] Diarrheic feces.

percentages of Hyd+ isolates among strains isolated from the feces of the respective nondiarrheic hosts. Significantly greater proportions of Hyd+ isolates were detected among *E. coli* isolated from humans or chickens with bacteremia than were detected among nonanimal isolates ($P < 0.001$). However, the proportion of Hyd+ isolates among *E. coli* implicated in bacteremia of humans was not significantly different ($P > 0.1$) from the proportion of Hyd+ organisms detected among isolates from human feces. Interestingly, a significant difference ($P < 0.05$) was observed between the proportion of Hyd+ among Col+ (70%) and Hyd+ among Col− (57%) *E. coli* isolated from human bacteremia. The ColV+ strain MW, previously shown (27) to be invasive, was found to be Hyd+.

Recovery of *E. coli* and incidence of Hyd+ isolates. *E. coli* was not detected in a number of nondiarrheic fecal samples obtained from humans, calves, or poultry (Table 2, group 1). In contrast, nondiarrheic fecal specimens from piglets, weaned pigs, and weaned cattle were all found to harbor *E. coli* (Table 2, group 2). An inverse relationship was observed between the frequencies with which *E. coli* was detected in feces of a particular animal type and the prevalence of Hyd+ isolates among the *E. coli* present in these specimens. Among *E. coli*-containing fecal samples from group 1 animals, the proportion of fecal samples harboring Hyd+ isolates was significantly greater than was that among *E. coli*-containing fecal samples from group 2 animals ($P < 0.001$). Among all *E. coli* isolated from those fecal samples which were obtained from

TABLE 2. Recovery of *E. coli* from nondiarrheic fecal samples and presence of Hyd+ isolates

Source of fecal sample	No. of fecal samples	No. (%) of fecal samples harboring *E. coli*	No. (%) of *E. coli*-containing fecal samples harboring Hyd+ isolates[a]	Proportion (%) of Hyd+ isolates in fecal samples harboring Hyd+ *E. coli*[b]
Group 1				
Breast-fed babies	29	7 (24)	5 (71)	25/25 (100)
Milk-fed calves	26	22 (85)	9 (41)	21/37[c] (57)
Adult humans	23	18 (78)	8 (44)	—[d]
Poultry	51	40 (78)	12[c] (30)	50/53[c] (94)
Group 2				
Suckling piglets	18	18 (100)	4 (22)	9/20[c] (45)
Weaned pigs	32	32 (100)	5 (16)	13/24[c] (54)
Weaned cattle	51	51 (100)	4 (8)	12/20[c] (60)

[a] In all instances where fecal samples contained *E. coli*, except where samples were obtained from adult humans, five isolates were purified and examined for iron-suppressible production of Csáky assay-reactive material as described in the text.
[b] The proportion of Hyd+ strains among all *E. coli* isolated from fecal samples harboring Hyd+ organisms.
[c] *E. coli* isolates from some fecal samples did not grow in the liquid media used for testing production of hydroxamate.
[d] —, Only one *E. coli* strain was isolated from each fecal sample.

group 1 animals and which contained Hyd^+ *E. coli*, the proportion of Hyd^+ isolates was significantly greater ($P < 0.001$) than was the proportion detected among *E. coli* isolated from similar fecal specimens obtained from group 2 animals.

Hydroxamate production and ability to colonize the gastrointestinal tract. The results outlined above provide a basis for proposing (see below) that Hyd^+ *E. coli* may possess enhanced ability to survive in the gut. Since many but not all $ColV^+$ *E. coli* are also Hyd^+, and since something is known about the abilities of certain $ColV^+$ *E. coli* to survive in the human intestinal tract (25, 26), two previously described ColV plasmids were tested for the presence of the Hyd determinant. A ColV I-K94 transconjugant of a $ColV^-$ derivative of strain B188 known to lack enhanced colonizing ability (26) failed to produce detectable hydroxamate. On the other hand, a ColV-B188 transconjugant of the $ColV^-$ strain B188, which has enhanced colonizing ability compared with that of the $ColV^-$ form (25), was found to be Hyd^+.

DISCUSSION

It is now widely accepted that deprivation of iron ("nutritional immunity") contributes to defense against bacterial infection. Iron in mammalian hosts is largely unavailable to bacteria since the bulk of the metal is stored in tissue and erythrocytes and the levels of free ionic iron in fluids and secretions are kept low by partially saturated iron-binding proteins (36). Both transferrin, which is the most abundant iron-binding protein in blood and lymph, and lactoferrin, which is commonly found in milk and other body secretions, have very high affinities for iron (1). It is generally assumed that *E. coli* cells which are able to multiply rapidly in serum or milk must possess high-affinity transport systems with which they can wrest iron from host proteins (3, 32). Two major types of microbial siderophores are recognized; phenolic compounds which contain at least one 2,3-dihydroxybenzoyl group, and compounds which contain at least one hydroxamate group (5).

The Csáky assay, which has commonly been used to detect hydroxamate siderophores, is based on the selective oxidation of bound hydroxylamine nitrogen. However, nonhydroxamate substances such as oxime-containing compounds may also produce a positive Csáky reaction. In previous studies, this assay has been used to detect well-characterized hydroxamate siderophores produced by selected microorganisms (6, 12, 19), and under such conditions there was little likelihood that false-positive responses would be obtained as a result of the wider specificity of the assay. We, however, have used the Csáky assay to detect material produced by a heterogeneous collection of *E. coli* isolates, and this warrants some comment.

During examination of isolates for ability to produce hydroxamate, production of Csáky assay-reactive material has always been found to be iron suppressible. Iron-deficient media have been prepared by two methods, and similar results have been obtained whether the bulk of iron has actually been removed from the media used or whether an iron-chelator has been included to decrease availability of iron. We therefore consider that, although our approach would not distinguish between different types of hydroxamate siderophore which may be produced by *E. coli*, iron-suppressible production of Csáky assay-reactive material is an accurate indicator of capacity to carry out hydroxamate-dependent iron transport. This assumption has been verified in the case of strain RJ80 which carries the plasmid pRJ1000 (29). Moreover, purified Csáky assay-reactive material produced by this strain has characteristics (data not shown) similar to those of aerobactin (12), and two other *E. coli* ColV plasmids have been shown to carry determinants for aerobactin biosynthesis (2, 31).

Where invading bacteria are subject to iron stress because of the presence of partially saturated iron-binding proteins, one might expect Hyd^+ strains to enjoy a selective advantage over their Hyd^- counterparts. The low proportion of Hyd^+ strains among the isolates from potable water and effluent is consistent with our view that monitoring of the Hyd^+ phenotype can provide a useful indicator of the availability of iron for microbial growth in a particular situation.

Our finding that bacteremia isolates tend to be Hyd^+ is consistent not only with this view, but also with the finding of Williams and Warner (34) that mutations which affect plasmid-controlled iron transport can eliminate enhanced virulence conferred by the unmutated plasmid. Furthermore, the finding that *E. coli* strain MW is Hyd^+ and the observations of Smith and Huggins (27) are consistent with the view that ability to produce hydroxamate is important in facilitating growth of low inocula under iron-deficient conditions. Smith and Huggins (27) observed that only when a high inoculum was used was a $ColV^-$ form of strain MW as virulent as its $ColV^+$ counterpart when inoculated into chicken muscle.

Our observation that *E. coli* isolated from the feces of breast-fed babies tended to be Hyd^+ is consistent with the view that iron present in breast milk is not readily available. A similar trend was evident among *E. coli* isolated from feces of calves fed fresh bovine milk, but, in striking contrast, *E. coli* isolated from the feces

of suckling piglets was rarely found to produce hydroxamate. Iron-dependent bacteriostasis induced by human milk has been demonstrated previously both in vitro and in vivo in neonate intestines (4, 30), and, moreover, it is known that the milks from different mammalian species vary in composition. Significantly, an increasing level of unsaturated iron-binding capacity that was associated with the lactoferrin present in the three milks (15, 17, 24) corresponded with increasing proportions of Hyd$^+$ isolates among the fecal *E. coli*.

It is of interest that very few Hyd$^+$ isolates were detected in the feces of weaned pigs or weaned cattle, but that greater proportions of isolates from the feces of adult humans and poultry were Hyd$^+$. It would seem that iron in the intestinal tracts of weaned pigs and weaned cattle must be more readily available to *E. coli* cells or that the *E. coli* isolated from these sources possess an as yet unrecognized iron acquisition system. It is generally recognized that, in adult humans, populations of *E. coli* are transient, and occasionally such bacteria cannot be detected at all (8, 10). It has been suggested (10) that host-controlled systems inhibit the growth of *E. coli* in human intestinal tracts, and in the light of our results it is tempting to speculate that iron deprivation may contribute to such control. Lactoferrin is, in fact, secreted into the intestine (20), and it is thought that very little free ionic iron is present in the digest passing through the human intestinal tract (14). The inverse relationship between the rate of recovery of *E. coli* from fecal samples and incidence of the Hyd$^+$ phenotype (Table 2) further supports such speculation. Smith and Huggins (25, 26) have demonstrated that, unlike the ColV plasmid present in strain B188 (ColV-B188), the ColV I-K94 plasmid does not confer increased capacity to survive in the human intestine. Smith and Huggins suggested that derepression, with or without some other genetic difference between the two plasmids, might account for the different patterns of survival. Our finding that ColV-B188 confers ability to produce hydroxamate, whereas ColV I-K94 does not, strongly supports the view that iron deprivation may restrict growth of *E. coli* in human intestines.

In conclusion, ability to carry out hydroxamate-dependent iron-transport appears to be a capacity which is widely distributed among natural isolates of *E. coli*. The apparent relationship between presence of Hyd$^+$ organisms and the severity of host-produced iron stress at the site of isolation has already prompted a reassessment of the role of bacterial iron transport systems in the establishment of infections. In our view, the enterochelin-dependent system for iron transport may not always be able to supply sufficient iron to *E. coli* cells growing in animal fluids. We consider that hydroxamate-mediated iron transport operating either independently or in concert with enterochelin-dependent transport may facilitate acquisition of iron in vivo and that Hyd$^+$ cells may thus have a selective advantage over Hyd$^-$ cells in certain in vivo situations.

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