The *Yersinia* high-pathogenicity island and iron-uptake systems in clinical isolates of *Escherichia coli*

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The ability to acquire iron is crucial for bacteria during an infection. The capacity of 35 strains of *Escherichia coli*, isolated from clinical specimens, to use various strategies to obtain iron was analysed. The isolates employed several iron-uptake mechanisms, including production of enterobactin (86 %) and aerobactin (71 %). The majority of the isolates also excreted yersiniabactin, which is encoded by the *Yersinia* high-pathogenicity island (HPI). However, PCR analysis of the *Yersinia* HPI revealed diversity in its genetic organization. Use of human transferrin (91 %), lactoferrin (94 %), haemoglobin (80 %) and haemoglobin–haptoglobin complex (63 %) as the sole source of iron was common among *E. coli* isolates. Multiple iron-uptake systems may be of benefit to bacteria during an infection.

INTRODUCTION

Under conditions of iron stress, the vast majority of micro-organisms induce high-affinity iron-transport systems to overcome the low availability of this essential element. In mammalian hosts, it is sequestered by iron-withholding proteins, such as transferrin in serum and cerebrospinal fluid or lactoferrin in cerebrospinal fluid, tears, milk and secretions of the respiratory, gastrointestinal and genital tracts. Most intracellular iron is bound by haem or stored in ferritin and haemosiderin (Weinberg, 1978; Payne, 1988). A typical high-affinity iron-uptake system consists of a low-molecular-mass, Fe(III)-specific ligand (termed a siderophore), its cognate membrane receptor and several proteins involved in transport of the ferri-siderophore across the cell wall (Griffiths *et al.*, 1988; Martinez *et al.*, 1990).

Escherichia coli has evolved several mechanisms to acquire iron, including the production of siderophores such as enterobactin and aerobactin. Some pathogenic strains produce cell-bound or secreted haemolysins that release haemoglobin from erythrocytes, making it available for haemoglobin proteases (Payne, 1988; Otto *et al.*, 1998). Moreover, *E. coli* strains are capable of utilizing ferric dicitrate and iron that are bound to exogenous hydroxamate siderophores such as coprogen, rhodotorulic acid, ferri-chrome and ferrioxamine B (Payne, 1988; Ratledge & Dover, 2000).

Recent studies have revealed that some pathogenic *E. coli* isolates carry genes that encode yersiniabactin-mediated iron-uptake systems, clustered in a pathogenicity island

(Schubert *et al.*, 1998; Bach *et al.*, 2000). This pathogenicity island was originally found in *Yersinia* spp. isolates and was named a 'high-pathogenicity island' (HPI), as its presence is correlated with virulence. The *Yersinia* HPI displays features typical of a pathogenicity island: (i) it is a large fragment of the chromosome (36–43 kb, depending on the species); (ii) it carries genes essential for virulence; (iii) it is located in the vicinity of a tRNA gene; (iv) it contains insertion sequences and an integrase gene; and (v) it differs in G+C content from the rest of the chromosome (Carniel, 2001).

So far, iron-acquisition studies in *E. coli* have not simultaneously investigated all mechanisms that can be used by strains of this species; therefore, the aim of the present work was to perform an extensive study of iron-uptake mechanisms used by clinical *E. coli* isolates, with focus on occurrence and genetic organization of the *Yersinia* HPI.

METHODS

Bacterial strains. Thirty-five *E. coli* strains were isolated from clinical specimens and identified using ID 32 GN strips in the ATB Expression system (bioMérieux). The strains were stored at –75 °C in heart infusion broth (Difco) that contained 50 % (v/v) glycerol. Origins of the strains are shown in Table 1.

Siderophore production. Bacterial ability to produce siderophores was checked initially by using chrome azurol S (CAS) assay solution (Schwyn & Neilands, 1987). Production of enterobactin and aerobactin was detected by performing cross-feeding assays, which tested the ability of bacteria to promote growth of indicator strains grown under iron starvation. Enterobactin was detected by *Salmonella typhimurium* TA 2700, which is defective in the biosynthesis of this siderophore but retains the receptor for the iron–enterobactin complex. Aerobactin production was detected by *E. coli* LG 1522, a strain that is deficient in the biosynthesis of this siderophore but has an intact receptor for ferri-

Abbreviations: CAS, chrome azurol S; HPI, high-pathogenicity island.

Table 1. Haemolytic activity, siderophore production and presence of *Yersinia* HPI in clinical strains of *E. coli*

Strain	Source	Haemolytic activity	Siderophore production			Size of PCR product (bp)		
			Enterobactin	Aerobactin	Yersiniabactin	<i>irp1</i>	<i>irp2</i>	<i>fyuA</i>
RK 3	Urine	+	+	+	+	240	280	780
RK 6	Urine	+	+	+	+	—	280	780
RK 13	Urine	+	+	+	+	240	280	750
RK 14	Urine	—	+	+	+	240	280	780
RK 15	Urine	—	+	+	+	240	280	780
RK 16	Urine	—	+	+	+	—	—	780
RK 17	Urine	+	+	—	+	240	280	780
RK 18	Urine	+	+	—	+	240	280	750
RK 20	Urine	—	+	+	+	—	280	—
RK 21	Urine	+	+	+	+	240	280	780
RK 22	Urine	+	+	—	+	240	280	750
RK 23	Urine	+	+	—	+	240	280	780
RK 24	Urine	+	+	—	+	240	280	780
RK 26	Urine	—	+	—	+	—	280	780
RK 27	Urine	+	+	—	+	240	280	—
RK 30	Urine	—	+	—	—	—	—	—
RK 36	Urine	—	+	+	+	240	280	780
RK 37	Urine	—	+	+	+	—	280	780
RK 4	Blood	+	+	+	+	240	280	780
RK 5	Blood	—	+	—	—	—	—	—
RK 7	Blood	—	—	+	—	—	—	—
RK 10	Blood	—	—	+	—	—	—	—
RK 12	Blood	+	+	+	+	240	280	750
RK 33	Cervical canal	—	+	+	+	240	280	780
RK 34	Cervical canal	—	+	+	+	240	280	780
RK 35	Cervical canal	+	+	—	+	240	280	—
RK 19	Semen	+	+	+	+	240	280	780
RK 28	Semen	—	—	+	+	240	280	780
RK 29	Semen	+	+	+	+	—	280	780
RK 11	Conjunctiva	—	+	+	+	—	280	780
RK 25	Conjunctiva	—	—	+	+	—	—	780
RK 9	Wound	+	+	+	+	240	—	780
RK 31	Foreskin abscess	+	+	+	+	240	280	780
RK 32	Wound	—	+	+	+	240	280	780
RK 8	Cerebrospinal fluid	—	—	+	—	—	—	—

aerobactin (Reissbrodt & Rabsch, 1988). Yersiniabactin detection was done with *Yersinia enterocolitica* 5030, a strain that uses exogenous yersiniabactin, and *Y. enterocolitica* 5092, a negative-control strain that neither produces nor utilizes yersiniabactin (Haag *et al.*, 1993). *Aureobacterium* (formerly *Arthrobacter*) *flavescens* JG-9 was used for the detection of hydroxamate siderophores other than aerobactin (Reissbrodt & Rabsch, 1988).

Haemolysin production assay. Aliquots (10 µl) of bacterial suspension were transferred to holes (4 mm in diameter) punched in human blood agar plates. The plates were incubated for 24 h at 37 °C and checked for haemolysis (Beecher & Wong, 1994).

Utilization of human iron sources. Iron-deficient LB agar plates were prepared by adding ethylenediamine-di-(*o*-hydroxyphenylacetic acid)

(EDDHA) at a concentration sufficient to inhibit bacterial growth. Overnight cultures were inoculated into molten Luria agar at a density of 10⁴ cells ml⁻¹. Human haemoglobin, haemoglobin-haptoglobin complex, transferrin and lactoferrin (Sigma) were dialysed to remove contamination, incubated with FeCl₃ to obtain 50 % iron saturation and sterilized through a 0.22 µm filter (Staags & Perry, 1991). Sterile paper discs were impregnated with these iron-binding proteins (0.01 µmol per disc) and placed onto the inoculated agar plates, which were then incubated for 48 h at 37 °C and examined for zones of growth around the discs (Massad *et al.*, 1991).

Detection of *Yersinia* HPI genes. HPI genes in clinical strains of *E. coli* were examined by using a PCR-based method. Bacterial DNA was isolated by using a QIAamp DNA Mini kit (Qiagen). Recombinant *Taq* polymerase and other PCR reagents were purchased from MBI

Fermentas. Primers were synthesized by Genset Oligos; their sequences were published by Karch *et al.* (1999). PCR amplifications were performed in a 50 µl volume with 5 µl 10× PCR buffer with NH₄(SO₄)₂, 0.6 µM each primer, 200 µM dNTP mix, 2.5 mM MgCl₂, 2 U *Taq* polymerase and 1 µg genomic DNA. Amplification involved an initial denaturation step (94 °C, 5 min) followed by 30 cycles of denaturation (94 °C, 1 min), annealing (Karch *et al.*, 1999) and extension (72 °C, 1 min), with a final extension step (72 °C, 8 min). PCR products were separated in 1.5 or 2 % agarose gel. All experiments were performed in duplicate.

RESULTS AND DISCUSSION

Production of enterobactin and aerobactin in clinical *E. coli* isolates

CAS assay solution contains a dye that is blue when chelated with iron and turns to orange when a stronger chelator, such as a siderophore, removes the iron. All strains gave positive results in the assay, indicating production of iron-chelating compounds. The type of siderophore produced by the strains was determined in bioassays that examined the capability of each strain to induce the growth of indicator strains that are deficient in iron-uptake mechanisms. As the growth of the aerobactin indicator strain *E. coli* LG 1522 can also be stimulated by rhodotorulic acid (Reissbrodt & Rabsch, 1988), we performed an additional bioassay with *A. flavescens* JG-9. All strains gave negative results in this test, which excluded the production of rhodotorulic acid.

Results of the cross-feeding assays are shown in Table 1. Twenty of 35 *E. coli* isolates produced both enterobactin and aerobactin. Ten strains produced enterobactin only; aerobactin alone was secreted by five isolates. All uropathogenic *E. coli* isolates produced enterobactin, either alone or in combination with aerobactin.

Haemolytic activity

Seventeen (49 %) of 35 *E. coli* isolates showed β-haemolysis on human blood agar plates (Table 1). Haemolysin-producing strains were isolated from all sources except for conjunctiva and cerebrospinal fluid.

Iron utilization studies

A measure of the effectiveness of iron-acquisition systems is the capacity of bacterial strains to use iron-binding proteins as the sole iron source. We demonstrated the ability of *E. coli* strains to grow in the presence of human haemoglobin, haemoglobin–haptoglobin complex, transferrin and lactoferrin (Table 2). The ability to use transferrin and lactoferrin was prevalent (91 and 94 % of strains, respectively). All haemolytic strains expressed growth in the presence of haemoglobin or the haemoglobin–haptoglobin complex. Overall, the proportions of isolates that were capable of using haemoglobin and haemoglobin–haptoglobin were 80 and 63 %, respectively. Utilization of haemoglobin, transferrin and lactoferrin has also been reported previously (Law *et al.*, 1992; Dall'Agnol & Martinez, 1999). *E. coli* was considered to be incapable of using the haemoglobin–haptoglobin

Table 2. Human iron sources utilized by *E. coli* isolates

Strain	Haemoglobin	Haemoglobin–haptoglobin	Transferrin	Lactoferrin
RK 3	+	+	+	+
RK 6	+	+	+	+
RK 13	+	+	+	+
RK 14	+	+	+	+
RK 15	+	+	+	+
RK 16	–	–	+	+
RK 17	+	–	+	+
RK 18	+	–	+	+
RK 20	+	+	+	+
RK 21	+	+	+	+
RK 22	+	+	+	+
RK 23	+	+	+	+
RK 24	+	+	+	+
RK 26	–	–	+	+
RK 27	+	+	+	+
RK 30	–	–	+	+
RK 36	+	+	+	+
RK 37	+	+	+	+
RK 4	+	+	+	+
RK 5	+	+	+	–
RK 7	+	–	+	–
RK 10	–	–	+	+
RK 12	+	+	+	+
RK 33	–	–	+	+
RK 34	+	+	+	+
RK 35	+	+	+	+
RK 19	+	–	+	+
RK 28	+	–	+	+
RK 29	+	+	+	+
RK 11	–	–	+	+
RK 25	–	–	–	+
RK 9	+	+	+	+
RK 31	+	+	+	+
RK 32	+	–	+	–
RK 8	+	+	–	+

complex (Eaton *et al.*, 1982; Helms *et al.*, 1984); however, the uropathogenic strain CFT073, which possesses multiple iron-uptake systems, can use this complex (Torres *et al.*, 2001).

Screening for *Yersinia* HPI in *E. coli* strains

The presence of the *Yersinia* HPI in genomes of *E. coli* isolates was detected by a PCR-based method that used primers specific for the *Yersinia pestis* genes *irp1* and *irp2* (yersinia-bactin biosynthesis genes) and *fyuA*, also named *psn*, which encodes the ferri-yersiniabactin receptor (Karch *et al.*, 1999). All three HPI genes were present in 54 % of *E. coli* strains. Overall, *irp1* and *irp2* were present in 63 and 77 % of *E. coli*

strains, respectively, and *fyuA* was detected in 77 % of isolates. Five strains (14 %) were completely negative for the presence of *Yersinia* HPI genes (Table 1).

The sizes of PCR products generally tallied with expected values, although four isolates (RK 12, RK 13, RK 18 and RK 22) demonstrated a 750 bp product of *fyuA* that was shorter than the corresponding fragment in *Y. pestis*, which is 780 bp long (Karch *et al.*, 1999).

HPI organization in *E. coli* isolates

Nine *E. coli* strains were chosen for detailed analysis of the organization of the *Yersinia* HPI. The following genotypes were investigated: *irp1*⁺*irp2*⁺*fyuA*⁺ (isolates RK 17, RK 28 and RK 33), *irp1*⁺*irp2*⁺*fyuA*-750 bp (RK 18 and RK 22), *irp1*⁺*irp2*⁺*fyuA*⁻ (RK 27 and RK 35) and *irp1*⁻*irp2*⁺*fyuA*⁺ (RK 26 and RK 37). Strains were subjected to a set of PCR amplifications with primers complementary to single HPI genes as well as to regions that contained fragments of consecutive genes, which allowed us to determine the order of the genes. PCR products were obtained from the vast majority of investigated HPI regions and their sizes corresponded to *Y. pestis* homologues (Fig. 1). Analysis revealed diversity of the HPI in *E. coli* clinical isolates. Only one of nine scrutinized isolates, namely RK 33, possessed the whole set of *Yersinia* HPI genes. The other isolates failed in amplification of one or more of the following regions: *ybtS*, *ybtQ/ybtA*, *irp1*, *ybtT/fyuA* and/or *fyuA*. In clinical *E. coli* isolates, the HPI was located in the vicinity of *asnT* (asparagine-specific tRNA gene). Amplification of the *asnU/int* and *asnV/int* regions gave no product. Insertion of the HPI in *Yersinia pseudotuberculosis* can occur in three tRNA loci: *asnT*, *asnU* and *asnV*

(Buchrieser *et al.*, 1998), whereas in *E. coli* it occurs mainly in the *asnT* gene (Schubert *et al.*, 1998, 2000; Karch *et al.*, 1999), although it is also possible in other locations (Clermont *et al.*, 2001).

A shorter-than-expected PCR product of *fyuA* in some strains (Table 1) suggests partial deletion of the gene, which may affect the function of its product, the ferri-yersiniabactin receptor. A truncated *fyuA* gene has also been demonstrated in certain pathogenic *E. coli* isolates (Schubert *et al.*, 1998). Deletions have also been shown for another HPI gene, *int*, which encodes an integrase (Karch *et al.*, 1999; Gophna *et al.*, 2001; Girardeau *et al.*, 2003). As integrase genes can be involved in the mobility of pathogenicity islands (Hacker *et al.*, 1997; Hensel *et al.*, 1997), partial deletion of *int* may result in a non-functional integrase and stabilization of the HPI in the chromosome of *E. coli* isolates (Karch *et al.*, 1999). A second possibility is that these strains have acquired the HPI by a non-integrase-based transfer event (Gophna *et al.*, 2001). Amplification with primers designed for the IS100 sequence gave no product in strains RK 28 and RK 37. In other isolates, this PCR resulted in a 950 bp product, whereas the corresponding region in *Y. pestis* is 100 bp. Moreover, in some cases, we did not obtain a PCR product for a particular gene, despite obtaining amplicons of regions that link it with adjacent genes.

Production of yersiniabactin

The presence of *Yersinia* HPI genes in the chromosome of *E. coli* strains, detected by PCR amplification, does not confirm that the yersiniabactin-mediated iron-uptake system is functional. Expression of *fyuA*, *irp1* and *irp2* in *E. coli* has

Fig. 1. Genetic organization of *Yersinia* HPI in nine clinical *E. coli* isolates. Genes are represented by black bars. White indicates chromosome fragments that are negative for PCR amplification.

been demonstrated recently to be iron-regulated, which is typical of siderophore-mediated iron-acquisition systems in bacteria (Schubert *et al.*, 1998; Karch *et al.*, 1999; Bach *et al.*, 2000). However, in some *fyuA*-positive isolates, the gene was not expressed (Karch *et al.*, 1999). It has been demonstrated that supernatants of HPI-positive *E. coli* strains grown under iron-deficient conditions enhance expression of *fyuA* in *Y. enterocolitica* strain WA-CS *irp1* :: Kan^R (pCJG3.3N), which harbours an *fyuA*–*gfp* reporter gene fusion (Schubert *et al.*, 2000). Provided that extracellular yersiniabactin positively regulates the expression of FyuA outer-membrane yersiniabactin receptor (Pelludat *et al.*, 1998), this would indicate yersiniabactin production in *E. coli*.

In this study, all *E. coli* isolates were examined for production of yersiniabactin by using a bioassay with *Y. enterocolitica* 5030. Thirty of 35 isolates stimulated growth of the indicator strain in iron-poor medium (Table 1); therefore, we considered them to be HPI-positive. Moreover, none of them promoted growth of *Y. enterocolitica* 5092, indicating that they did indeed produce yersiniabactin. This suggests that the lack of PCR product for regions involved in yersiniabactin biosynthesis, i.e. *ybtS*, *irp1* and *ybtT/fyuA*, in certain strains was probably caused by minor alterations of *E. coli* target sequences, which did not affect production of the siderophore.

Despite the lack of PCR product of some HPI marker genes in several isolates, we demonstrated yersiniabactin production in these strains. This suggests that detection of the HPI based on PCR amplification of one or even two genes alone may not be sufficient to show its presence in *E. coli* isolates. Thus, an appropriate bioassay or several PCRs should be carried out.

Our results showed that the vast majority of pathogenic isolates of *E. coli* possessed at least two iron-uptake systems. This feature may contribute to higher pathogenicity and facilitate colonization of the host organism. Whilst enterobactin- and aerobactin-mediated iron-uptake mechanisms have been studied thoroughly, the role of yersiniabactin in *E. coli* virulence is unclear. Both enterobactin and aerobactin have been shown to be prevalent in *E. coli* (Martinez *et al.*, 1987; Reissbrodt & Rabsch, 1988; Montgomerie *et al.*, 1994). Production of haemolysins is less frequent (Johnson *et al.*, 1988; Aumont *et al.*, 1989). As enterobactin is bound by albumin and IgA in human serum (Moore & Earhart, 1981), its function as a virulence factor is doubtful. The role of aerobactin in pathogenicity has been supported by epidemiological evidence (Valvano & Crosa, 1984). Bearing in mind that enterobactin is inactivated in serum and that some isolates do not produce aerobactin, an additional siderophore (i.e. yersiniabactin) that has a higher affinity for ferric iron than aerobactin (Perry *et al.*, 1999) would be of benefit to bacteria. Recently, Schubert *et al.* (2002), by using a mouse infection model, have shown that yersiniabactin contributes to virulence in extraintestinal pathogenic *E. coli* strains that lack both aerobactin and haemolysin. Multiple siderophore systems may function in different environments within the host, or at different stages during the course of an

infection (Torres *et al.*, 2001). The role of yersiniabactin in the virulence of *E. coli* needs further investigation. Moreover, demonstration of the coexistence of yersiniabactin- and aerobactin- or enterobactin-mediated iron-uptake systems in *E. coli* clinical isolates contributes to re-evaluation of the function of aerobactin and enterobactin in *E. coli* virulence, as yersiniabactin has not yet been taken into consideration when making such an evaluation.

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