Moreover, haemoglobin proteases (Payne, 1988; Otto) degrade haemoglobin from erythrocytes, making it available for enterobactin and aerobactin. Some pathogenic strains produce extracellular iron-uptake systems, clustered in a pathogenicity island. Isolates carry genes that encode yersiniabactin-mediated iron-uptake systems, 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 serous 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-
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 10^5 cells ml^{-1}. Human haemoglobin, haemoglobin–haptoglobin complex, transferrin and lactoferrin (Sigma) were dialysed to remove contamination, incubated with FeCl_3 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 mol 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.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>Haemolytic activity</th>
<th>Siderophore production</th>
<th>Size of PCR product (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Enterobactin</td>
<td>Aerobactin</td>
<td>Yersiniabactin</td>
</tr>
<tr>
<td>RK 3</td>
<td>Urine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RK 6</td>
<td>Urine</td>
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<td>+</td>
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<tr>
<td>RK 13</td>
<td>Urine</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>RK 14</td>
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<td>+</td>
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<td>RK 15</td>
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<td>RK 16</td>
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<td>+</td>
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<td>RK 21</td>
<td>Urine</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RK 22</td>
<td>Urine</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>RK 23</td>
<td>Urine</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>RK 24</td>
<td>Urine</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>RK 26</td>
<td>Urine</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>RK 27</td>
<td>Urine</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
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<td>Urine</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>RK 36</td>
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<td>+</td>
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<tr>
<td>RK 37</td>
<td>Urine</td>
<td>–</td>
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<td>+</td>
</tr>
<tr>
<td>RK 4</td>
<td>Blood</td>
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<td>RK 34</td>
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<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
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<td>Cervical canal</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>RK 19</td>
<td>Semen</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RK 28</td>
<td>Semen</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>RK 29</td>
<td>Semen</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RK 11</td>
<td>Conjunctiva</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RK 25</td>
<td>Conjunctiva</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>RK 9</td>
<td>Wound</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RK 31</td>
<td>Foreskin abscess</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RK 32</td>
<td>Wound</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>RK 8</td>
<td>Cerebrospinal fluid</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>
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 (Reishbrodt & 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). Haemolyisin-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 human haemoglobin, haemoglobin–haptoglobin 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 isolates.
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).

**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:

- irp1⁰⁻irp2⁰⁻fyuA⁰ (isolates RK 17, RK 28 and RK 33),
- irp1⁰⁻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
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 trp1:: Kan^R (pIC33.AN), which harbours an fyuA-gfp reporter gene fusion (Schubert et al., 2000). Provided that extracellular yersiniabactin positively regulates the expression of FyuA outer-membrane yersinia-bactin 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 ybtTffyA, 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 (Martínez et al., 1987; Reissbrodt & Rabsch, 1988; Montgomerie et al., 1994). Production of haemolysin, aerobactin and enterobactin by strains of Escherichia coli causing bacteraemia in cancer patients, and their resistance to human serum. Res Microbiol 140, 21–26.


Acknowledgements

We are grateful to Dr Rolf Reissbrod, Professor Klaus Handke and Professor Shelley M. Payne for kindly providing indicator strains. This work was partially supported by grant 6 P04C 100 20 from the State Committee for Scientific Research to K. K.

References


Iron-uptake systems in E. coli

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