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Genetics of Escherichia coli Uropathogenicity: Analysis of the O6 : K15 : H31 Isolate 536*

JÖRG HACKER[1], MANFRED OTT[1], GABRIELE BLUM[1], REINHARD MARRE[2], JÜRGEN HEESEMANN[3], HELMUT TSCHÄPE[4], and WERNER GOEBEL[1]

[1] Institut für Genetik und Mikrobiologie, Universität Würzburg, D-W 8700 Würzburg
[2] Institut für Medizinische Mikrobiologie, Universität Lübeck, D-W 2400 Lübeck
[3] Institut für Hygiene und Mikrobiologie, Universität Würzburg, D-W 8700 Würzburg
[4] BGA, Robert-Koch-Institut, Bereich Wernigerode, D-O 3700 Wernigerode

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Summary

E. coli strain 536 (O6 : K15 : H31) isolated from a case of acute pyelonephritis, expresses S-fimbrial adhesins, P-related fimbriae, common type I fimbriae, and hemolysins. The respective chromosomally encoded determinants were cloned by constructing a genomic library of this strain. Furthermore, the strain produces the iron uptake substance, enterocheline, damages HeLa cells, and behaves in a serum-resistant mode. Genetic analysis of spontaneously arising non-hemolytic variants revealed that some of the virulence genes were physically linked to large unstable DNA regions, termed "pathogenicity islands", which were mapped in the respective positions on the *E. coli* K-12 linkage map. By comparing the wild type strain and mutants in *in vitro* and *in vivo* assays, virulence features have been evaluated. In addition, a regulatory cross talk between adhesin determinants was found for the wild-type isolate. This particular mode of virulence regulation is missing in the mutant strain.

Zusammenfassung

Der *E. coli* Stamm 536 (O6 : K15 : H31), isoliert aus einem Patienten mit akuter Nierenbeckenentzündung, zeichnet sich durch die Ausprägung von S-Fimbrien Adhäsinen, P-verwandten Fimbrien, "common type I" Fimbrien und Hämolysinen aus. Ausgehend von einer Genbank des Stammes 536 konnten die entsprechenden chromosomal kodierten Gene kloniert werden. Weiterhin produziert der Stamm die Eisenaufnahmesubstanz Enterochelin, schädigt HeLa-Zellen und verhält sich Serum-resistent. Genetische Untersuchungen an

* Dedicated to Dr. *I. Ørskov* and to Dr. *F. Ørskov* on the occasion of their 70th birthday.

spontan auftretenden nicht-hämolytischen Varianten zeigten, daß einige der Virulenzgene gekoppelt auf instabilen DNA Regionen vorliegen, die als "Pathogenitätsinseln" bezeichnet werden. Die Lage der Inseln konnte definierten Bereichen auf der *E. coli* K-12 Genomkarte zugeordnet werden. Durch den Vergleich von Wildtyp und Mutanten mittels *in vivo* und *in vitro* Untersuchungen konnten Eigenschaften der Virulenz analysiert werden. Weiterhin wurde eine regulatorische Interaktion von Adhäsindeterminanten bei dem Wildstamm gefunden, welche in den avirulenten Mutanten nicht vorhanden ist.

Introduction

The majority of urinary tract infections (UTI) can be attributed to *Escherichia coli* pathogens causing cystitis or pyelonephritis. *E. coli* UTI isolates exhibit specific virulence properties, enabling colonization of and survival in the urinary tract and leading to severe tissue damage in the case of acute pyelonephritis (9, 21, 28). Fimbrial adhesins mediating binding to host cell surfaces, serum resistance properties, iron uptake substances, and toxin production have been shown to be involved in the infectious process (8, 13, 19). Furthermore, the type of lipopolysaccharide composition (O antigen) and capsule formation (K antigen) are important (1, 13, 17). In the last decade, numerous attempts have been made to study uropathogenicity on a molecular level. Cloning and characterization of virulence determinants from various strains (e.g. J 96, 2980, 20025, IH 3034; 12, 16, 18, 20, 23) belonging to different serotypes provided an insight into the molecular mechanisms of the uropathogenicity of *E. coli*. The present overview of the genetics is dealing with aspects of the virulence of the uropathogenic *E. coli* strain 536.

Isolation and Serotyping of the Uropathogenic Strain E. coli 536

E. coli strain 536 had been isolated from a patient suffering from acute pyelonephritis at the Institut für Hygiene und Mikrobiologie, University of Würzburg in 1980 (2). Serotyping of strain 536 revealed carriage of the O6 lipopolysaccharide antigen, which is frequently found among UTI isolates (3, 31). Furthermore, capsule type K15 and H31 flagellin antigen have been determined (2, 10).

Virulence Features of E. coli Strain 536

As shown by phenotypic characterization, strain 536 exhibits hemolytic properties and mannose-resistant agglutination of human and bovine erythrocytes, as well as mannose-sensitive agglutination of yeast cells (2, 11). Detailed genetic analysis (see below) revealed the existence of two hemolysin (*hly*) determinants, gene clusters encoding the S-fimbrial adhesin (*sfa*), P-related fimbriae (*prf*), and common type I fimbriae (*pil*) (3, 11, 14). Furthermore, the ability to survive in non-immune serum could be observed (7). The strain produces the iron uptake substance enterocheline (*Rabsch* and *Reissbroth*, personal communication) but does not produce aerobactin. In addition, strain 536 produces cytotoxic effects in HeLa cells (*Heesemann*, unpublished) which might be due to the expression of the cytotoxic necrotizing factor (*cnf*; 4, 5). A schematic diagram of the virulence features of *E. coli* strain 536 is given in Fig. 1.

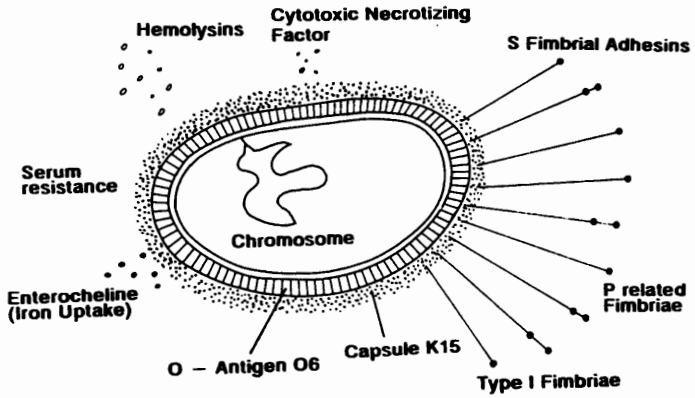

Fig. 1. Schematic representation of *E. coli* strain 536.

Isolation of Non-pathogenic Mutants

Non-pathogenic variants of the wild type strain were isolated by screening single colonies on blood agar plates for loss of the hemolytic property. Such mutants occurred at a frequency of approximately 10^{-3} (14). Besides mutants, generated *in vitro*, non-hemolytic variants could also be isolated following infection of the rat urinary tract (mutant 536-22; 6). Further phenotypic analysis revealed that the loss of the hemolytic phenotype is accompanied by a reduced mannose-resistant and mannose-sensitive agglutination activity, the loss of fimbria formation and of toxicity for HeLa cells. The mutants were also serum-sensitive (7, 11, 14, *Blum* et al., in preparation). The isolated mutants had been very helpful for evaluating the virulence properties of the wild type strain in *in vivo* and *in vitro* models, and led to the identification of particularly unstable DNA regions, termed "pathogenicity islands" (14), which comprise physically linked virulence genes (6) and also influence the expression of pathogenicity determinants still present on the chromosome (see below).

In vivo and in vitro Virulence Assays

To test the influence of the pathogenicity factors mentioned above on the virulence of the strains, the wild type isolate 536 and the mutant 536-21 were analysed in various *in vitro* and *in vivo* assays (see Table 1). It can be seen that adhesion to uroepithelial cells and serum resistance were markedly reduced in the mutant strain as compared to the wild type isolate. Furthermore, the generation of leukotrienes and histamine produced by human granulocytes and rat mast cells following incubation with strain 536 dropped considerably when using mutants 536-21 (15, 29). In addition, the mutants had also lost their *in vivo* virulence demonstrated by the use of different animal assays. As shown in different studies (7, 8, 19), the introduction of cloned determinants coding for hemolysin and/or adhesins into mutant 536-21 increased the virulence capacity of the transformants. Thus, it could be demonstrated that the expression of cloned adhesins contributed to colonization of the strains while

Table 1. *In vivo* and *in vitro* assays for determination of virulence properties of strain 536 (wild type) and mutant 536–21

virulence assay	Wild-type 536	Mutant 536–21	Ref.
Adhesion (bacteria/cell)	6.2	1.9	19
Serum resistance (% survival/3 h)	250	0.1	7
Lethality (mouse) (LD_{100})	7×10^7	1×10^9	7, 8
Inflammation (leukotriene C_4/pg)	25	0	15, 29
Nephrovirulence (rat) (renal counts/log)	4.8	2.7	19
Lethality (rat) (% lethality/48 h)	31	0	7

hemolysin production revealed an effect on mouse lethality and inflammatory response. Following transformation with different plasmids encoding virulence factors and subsequent chromosomal integration of the plasmid DNA, strains were isolated which exhibited an *in vivo* virulence comparable to that of the wild-type strain (19).

Cloning and Mapping of Virulence-associated Genes

The *E. coli* strain 536 does not harbour plasmids, consequently genetic attempts to analyse its virulence properties started with the construction of a genomic library of this strain, and of one of the non-hemolytic mutant 536-21 generated *in vitro* (2, 14). The cosmid vector pHC 79 was used for random cloning of 35–40 kB (kilobases) stretches of chromosomal DNA in *E. coli* K-12 HB 101. Various recombinant cosmids were identified according to the phenotypes of hemolysis and agglutination in the mannose-sensitive and mannose-resistant manners (Table 2). The S-fimbrial adhesin and Type 1 fimbriae were cloned on cosmids pANN 801 and pGB 30, respectively.

Table 2. Characterization of cosmids derived from a genomic library of *E. coli* strain 536

Strain/cosmid	Sfa	Pil	Prf	HlyI	HlyII
536 WT (wild-type)	+	+	+	+	+
HB101 (pANN 801)	+	−	−	−	−
HB101 (pGB 30)	−	+	−	−	−
HB101 (pANN 10–21)	−	−	+	−	−
HB101 (pCos 473	−	−	+	−	+
HB101 (pCos 10)	−	−	−	+	−
HB101 (pHC 79)	−	−	−	−	−

Sfa, S-fimbrial adhesin; Pil, type 1 fimbriae; Prf, P-related fimbriae; Hly I, II, hemolysin I, II.

Furthermore cosmids conferring hemolytic properties to the *E. coli* K-12 host strain were identified (pCos 10; pCos 473). Using a DNA probe specific for P fimbriae derived from the *pap* determinant of strain J96 (18), it could be shown that cosmids pANN 10–21 and pCos 473 carried sequences highly related to P fimbriae (6). Subcloning of this particular region confirmed the expression of fimbriae, which were identified electron-microscopically. Agglutination with human erythrocytes or Gal-Gal coated latex beads specific for P fimbriae, however, has not been observed (6, *Vetter* et al., in preparation). Thus, the corresponding determinant was termed *prf* (P-related fimbriae; 6).

The data elaborated by cosmid analysis revealed a physical linkage of the determinants coding for one hemolysin (*hly*II) and P-related fimbriae (*prf*). The *hly*I gene cluster was not linked to hlyII and *prf*. By analysing cosmids from a genomic library of the mutant strain 536-21, it has become evident, that there were large chromosomal deletions in the genome of this strain which comprises *hly*I, *hly*II and *prf*, which could be mapped by chromosome walking (14). Two distinct unstable DNA regions carrying the above mentioned determinants were identified. Preliminary data speak in favour of the location of the genes for cytotoxic necrotizing factor (*cnf*) in one of these regions.

The gene clusters for *sfa* and *pil* were still present on the mutant genome, but they were poorly expressed (Table 3). By constructing Hfr strains of the *E. coli* wild type isolate 536, the location of the unstable regions could be mapped according to the *E. coli* K-12 linkage map at 55 min (region I) and 97 min (region II). Additionally, the *sfa* and *pil* determinants were identified at postions 91 min and 98 min, respectively. The data have been summarized in Fig. 2.

Table 3. Genetic and phenotypic characterization of wild-type strain 536 and mutants

Strain	Origin	Virulence determinants				
		sfa	*pil*	*prf*	*hly*I	*hly*II
536	uropathogenic wild-type	present expressed	present expressed	present expressed	present expressed	present expressed
536–21	laboratory mutant	present repressed	present repressed	deleted	deleted	deleted
536–22	mutant generated *in vivo*	present repressed	present repressed	deleted	deleted	deleted

Analysis of Mutant Strains

To yield further insight into the physical structure of the "pathogenicity islands" of strain 536, the genomes of the wild type isolate and of mutant 536-21 were analysed by pulsed field gel electrophoresis. In Fig. 3 (A), it can be seen that the genomic *Xba*I profiles of the strains differ, as a result of the loss of the unstable DNA regions. Using DNA probes derived from the cloned virulence genes in Southern hybridizations (Fig. 3 B–F), it could be demonstrated that two fragments of 370 kb and 50 kb in size reacted

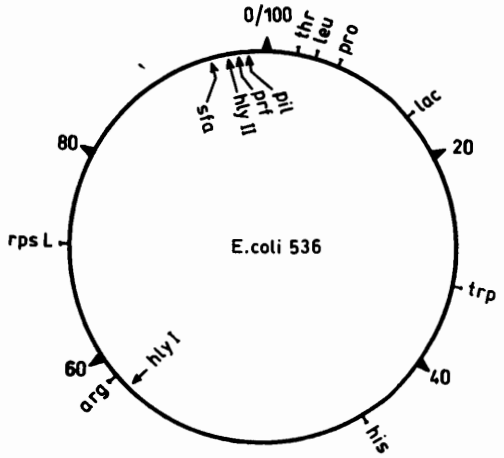

Fig. 2. Chromosomal map positions of virulence determinants of *E. coli* strain 536.

with the *hly* probe in the wild type strain, while the *prf* probe hybridized only in the 370 kb fragment. No hybridization occurred in the mutant genome. Using an *sfa*-specific probe, a 470 kb *Xba*I fragment could be detected in both strains, whereas the wild type reacted with a *pil* probe in a 320 kb fragment and the mutant, in a 500 kb fragment. To prove that the fragments of 370 kb and 320 kb were adjacent in the wild type, and that the loss of the *hly* region II resulted in a 500 kb fragment, hybridization was carried out by using a DNA probe comprising the fusion point of this region which had been derived from the genomic library of mutant 536-21. It can be seen that the DNA probe termed HD1 (14) hybridized in the respective fragments. In this way, the size of the deleted region II could be calculated to be 190 kb (Fig. 4), while the size of the unstable region I had been determined before by *Knapp* et al. by chromosome walking, which was 70 kb (14).

The deletion events comprise the *hly,* and *prf* determinants, while the *sfa* and *pil* gene clusters are still present on the chromosome of the mutant. This was confirmed by using DNA probes spanning the entire coding regions of these determinants (11). Phenotypic analysis, however, revealed a reduced expression of type 1 fimbriae and S-fimbrial adhesins (7, 11, 14; cf. Table 3). To evaluate the differences in expression of *sfa* more precisely, *sfa–lacZ* translational fusions were generated in wild type and mutant variants which had a Tn*5* insertion in the original *lac* gene (27). Determination of LacZ expression in these strains, reflecting the degree of Sfa production, revealed a more than 10-fold decrease of the LacZ values for the mutant as compared to the wild type (Table 4).

These data strongly suggest a *trans* regulatory effect of a gene product encoded by the unstable DNA regions on the expression of the *sfa* gene. Anaylsis of mutants which had lost either of the two regions revealed that the pathogenicity island II was responsible for this effect (14). Since in this region, the *prf* determinant is located (6), and *sfa* and P fimbriae determinants share a high degree of homology according to their *trans* acting regulatory cistrons (25, 26), it might be concluded, that a positive cross-talk between *prf* and *sfa* exists.

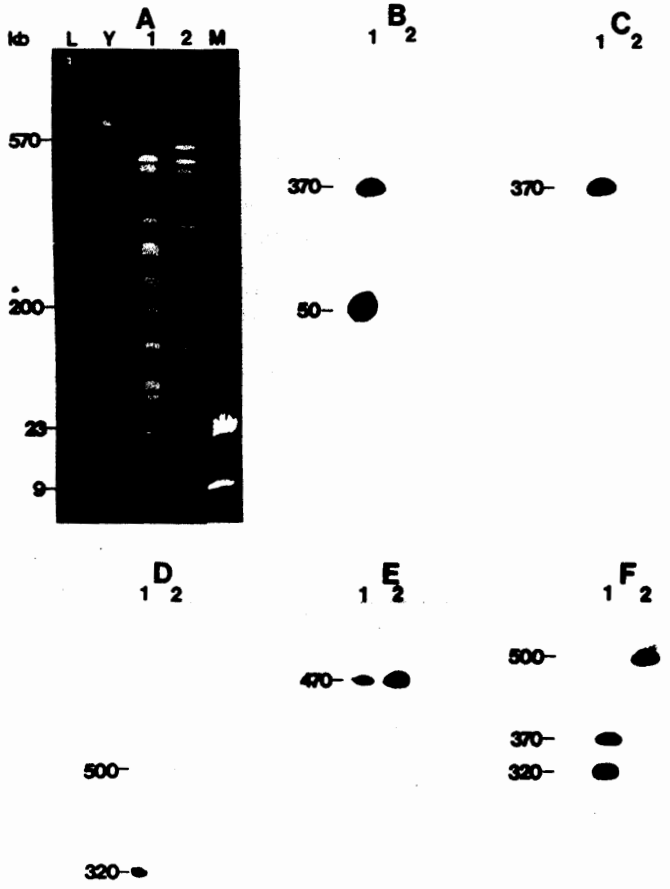

Fig. 3. Genomic *Xba*I pattern of *E. coli* strain 536 wild type (lane 1) and mutant 536-21 (lane 2) obtained by pulsed field gel electrophoresis (A) and Southern hybridization using *hly* (B), *pap* (C), *pil* (D), and *sfa* (E) specific fragments and the cloned junction fragment HD 1 of *hly* region II (F, see Fig. 4) as DNA probes. Yeast chromosomes (Y), lambda concatemers (L), and *Hind*III cleaved lambda DNA (M) were used as DNA size markers.

Unstable DNA regions as observed for strain 536 seem to be a general phenomenon, as in O18 : K1 strains and in the O4 : K6 isolate J96, DNA regions similar to the pathogenicity island II have been detected which also carry physically linked virulence determinants (6).

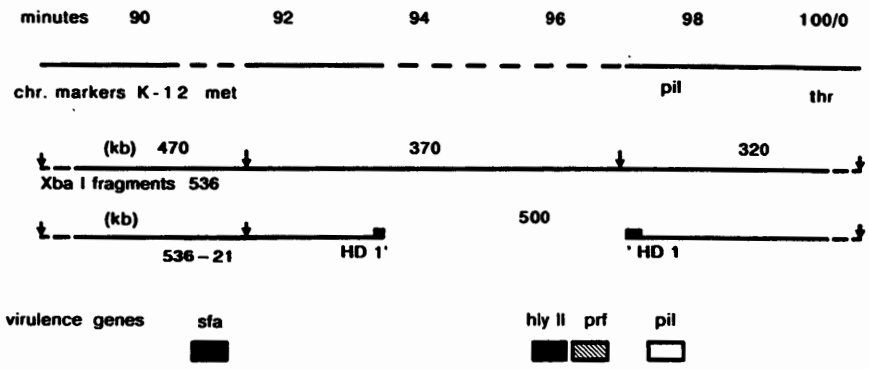

Fig. 4. Map of the *hly*II region in strains 536 and 536-21 obtained by *Xba*I cleavage. The locations of gene clusters are given for *pil, prf, hly*II, and *sfa*. In the upper part, the *E. coli* K-12 linkage map is given.

Table 4. Determination of Sfa expression in *E. coli* 536 wild-type (WT) and mutant 536–21 via *sfa–lacZ* translational gene fusions

Strain	LacZ units
536 WT	5321[1]
536–21	5516[1]
536 WT (*lacZ*::Tn*5*)	14[1]
536–21 (*lacZ*::Tn*5*)	12[1]
536 WT (*sfa–lacZ*)	603
536–21 (*sfa–lacZ*)	52

[1] Induced with IPTG.

Conclusion

Cloning of virulence genes from the chromosome of uropathogenic *E. coli* isolates enabled molecular studies of the properties determining pathogenicity (13). Furthermore, using cloned fragments as DNA probes in Southern hybridization, the determinants could be mapped in the genome, as well as the occurrence of virulence genes among various isolates could be analysed for epidemiological purposes (3, 22, 23). There is a dynamic situation in the chromosome of extraintestinal *E. coli* isolates, as some virulence factors are located on unstable DNA regions (6, 14) which also influence the expression of other virulence genes on the chromosome (11, 14). One might speculate about the biological advantages of such deletions. A resulting smaller genome favours a decrease of the generation time of the bacteria which can be useful at certain stages during the infectious process, and stable negative phenotypes due to the loss of the genes may be helpful for circumvention of the immune response. Furthermore, the origin of the virulence-associated chromosomal islands might be of particular interest. Recently, *Zagaglia* et al. (30) reported on the integration of virulence plasmids into the

chromosome of enteroinvasive *E. coli* and *Shigella flexneri*. Maybe these UTI *E. coli* "pathogenicity islands" originate from the integration of hemolysin plasmids frequently found among isolates from animal sources (13). A lot of questions arise with regard to the regulatory events in the expression of virulence factors *in vitro* and *in vivo*. It is known that virulence factors are not expressed constitutively (27). Rather, they are controlled by environmental parameters, leading to different levels of expression (24). Additionally, there might be a cross-talk between different gene clusters. Future studies with strains harbouring gene fusions of virulence determinants with reporter genes will be useful for evaluating coordinate virulence gene expression which is a crucial feature of uropathogenic *E. coli*.

To study these various aspects of uropathogenicity, the genetically well characterized *E. coli* strain 536 represents a useful model.

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Professor Dr. *Jörg Hacker,* Institut für Genetik und Mikrobiologie, Universität Würzburg, Röntgenring 11, D-W 8700 Würzburg