

## Deletions of chromosomal regions coding for fimbriae and hemolysins occur *in vitro* and *in vivo* in various extraintestinal *Escherichia coli* isolates

Jörg Hacker,<sup>1</sup> Larisa Bender,<sup>1</sup> Manfred Ott,<sup>1</sup> Jochen Wingender,<sup>1</sup>  
Björn Lund,<sup>2</sup> Reinhard Marre<sup>3</sup> and Werner Goebel<sup>1</sup>

<sup>1</sup>Institut für Genetik und Mikrobiologie, University of Würzburg, Röntgenring 11, D-8700 Würzburg, F.R.G., <sup>2</sup>Department of Microbiology, University of Umea, S-901 87 Umea, Sweden, and <sup>3</sup>Institut für Medizinische Mikrobiologie, University of Lübeck, Ratzeburger Allee 160, D-2400 Lübeck, F.R.G.

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Fimbrial adhesins and hemolysins contribute to pathogenicity of extraintestinal *Escherichia coli* isolates causing urinary tract infections (UTI), sepsis and new born meningitis (NBM). Using gene cloning techniques and pulse field electrophoresis in combination with Southern hybridizations it was demonstrated that the genetic determinants coding for P and 'P-related' fimbrial adhesins and hemolysins are closely linked on the chromosomes of different pathogenic *E. coli* wild-type isolates. For two UTI strains, 536 (O6:K15) and J96 (O4:K6), a co-deletion of the linked gene clusters coding for hemolysin and fimbriae was observed. The deleted DNA regions which also comprise flanking DNA sequences were termed 'pathogenicity DNA islands'. Such 'pathogenicity DNA islands' were also detected in the genome of O18:K1 isolates of OMP type 6 but were absent on the chromosomes of O18:K1 strains of OMP type 9. A mutant strain, 536-22 was selected from rat kidneys after intraurethral infection of animals with the wild-type parental strain 536. This particular isolate also shows deletions of 'pathogenicity islands' leading to a non-pathogenic phenotype. It is therefore concluded that excisions of 'pathogenicity islands' from chromosomes of pathogenic *E. coli* strains are not restricted to the laboratory but also occur *in vivo*. The generation of deletions may represent a general mechanism of bacterial virulence modulation.

**Key words:** P-fimbriae; hemolysin; genomic deletions; extraintestinal *E. coli*; virulence modulation.

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### Introduction

*Escherichia coli* strains may be the causative agents of intestinal and extraintestinal infections. Urinary tract infections (UTI), sepsis and new born meningitis (NBM) are often found to be associated with *E. coli*. Certain virulence factors, i.e. surface antigens (O- and K-antigen), iron-binding substances, hemolysins (Hly) and adhesins contribute to the pathogenicity of extraintestinal *E. coli*.<sup>1-3</sup> Hemolysins, which are able to

damage red blood cells and also stimulate the release of inflammatory substances from granulocytes, are found predominantly among UTI isolates.<sup>4,5</sup>

The binding of virulent *E. coli* to host tissues is mediated by complex fimbrial adhesin structures which are subdivided into several classes according to their receptor specificity.<sup>6</sup> The majority of fimbrial adhesins consist of major and minor subunits and can be detected by agglutination of erythrocytes or yeast cells.<sup>6-8</sup> P fimbrial adhesins (also termed Pap, pili associated with pyelonephritis), which bind to *Ga*- $\beta$ -1-4-*Ga*/residues are produced by the majority of UTI strains. S fimbrial adhesins (Sfa) which are more often associated with NBM isolates, interact with sialyl- $\beta$ -2-3-galactoside receptor structures.<sup>1,9,10</sup> P-related sequences (Prs) recognize *Ga*/Nac-1-3-*Ga*/Nac-containing receptors. S-related fimbriae and F1C fimbriae which are devoid of hemagglutinating activity have also been described.<sup>11-13</sup>

Cloning and genetic characterization of hemolysin and fimbrial adhesin determinants has yielded insight into the molecular structure of these pathogenicity gene clusters,<sup>5,13</sup> but limited information exists on the distribution of these gene clusters in the bacterial genome. Furthermore the genetic basis of the variability of the virulence properties of pathogenic *E. coli* strains *in vitro* and *in vivo* is unclear. In this study we present data on the linkage of hemolysin and fimbrial gene clusters in the genomes of different *E. coli* strains. Furthermore we describe co-deletions of pathogenicity determinants from chromosomes which occur *in vitro* and *in vivo*, suggesting that such processes may represent a special type of virulence modulation of wild-type strains.

## Results

### *Isolation of wild-type strains and mutants*

The wild-type strains listed in Table 1 were isolated from cases of urinary tract infections (strains 536, J96), new born meningitis (strains RS218, IH3034; IH3036) and from human feces (strain RS226). The O:K-serotypes of the strains are described elsewhere.<sup>14-17</sup> The mutants 536-21, 536-27, 536-29 and J96-M1 were isolated from blood agar cultures of their parental strains 536 and J96 as described.<sup>14</sup> The mutants were selected by screening for altered hemolysin phenotype. In contrast to the laboratory generated mutants, strain 536-22 was isolated from rat kidney after intraurethral infection of animals by the wild-type isolate 536. As shown in Table 2, 10<sup>4</sup> renal isolates were screened for hemolysin production at 1, 7 and 14 days after infection. One out of 3  $\times$  10<sup>4</sup> bacteria screened was non-hemolytic.

### *Hemolysin and fimbrial adhesin production of wild-type strains and mutants*

As shown in Table 1 the strains J-96, RS218, RS226 and 536 were hemolytic. It was demonstrated previously<sup>14,18</sup> that strain 536 carries two hemolysin determinants, termed *hly*I and *hly*II. Mutant strains 536-21, 536-22, 536-27 and 536-29 as well as the O18:K1 isolates IH3034 and IH3036 were non-hemolytic. Mutant strain J96-M1 produced a reduced amount of hemolysin compared to the wild-type strain J96.

For characterization of fimbrial adhesins, receptor-specific hemagglutination was carried out and fimbrial preparations of strains were analysed by SDS-PAGE (Table 1 and Fig. 1). The wild-type strain 536 [Fig. 1(B), lane 1] produced two fimbrial subunits of 22 kDa and 16 kDa, the latter represents the S major subunit protein.<sup>19</sup> The 22 kDa protein is referred to as the Prf ('P related' fimbriae) subunit<sup>18,20</sup> (see below). Mutants 536-21 and 536-22 [Fig. 1(B), lanes 2, 3] and also mutants 536-27 and 536-29 did not produce any fimbrial adhesins (Table 1). The wild type strain J96 and the mutant J96-M1 produced the P- (Pap-) specific adhesins. J96 also showed Prs-specific hemagglutination while J96-M1 was Prs-negative (Table 1). Both strains

**Table 3** Recombinant DNAs

Recombinant plasmid/ cosmid	Characteristics <sup>a</sup>	Reference
pANN 19B2 cosmid <sup>b</sup>	Ap <sup>r</sup> , <i>hlyII</i> , <sup>c</sup> <i>prf</i> , Hly <sup>+</sup> <sup>d</sup>	This study
pANN 6 A4-2 cosmid <sup>b</sup>	Ap <sup>r</sup> , <i>hlyII</i> , <sup>c</sup> <i>prf</i>	This study
pANN 15 B4 cosmid <sup>b</sup>	Ap <sup>r</sup> , <i>hlyII</i> , <sup>c</sup> <i>prf</i>	This study
pANN 10-21 cosmid <sup>b</sup>	Ap <sup>r</sup> , <i>hlyII</i> , <sup>c</sup> <i>prf</i>	14
pANN 8-63 cosmid <sup>b</sup>	Ap <sup>r</sup> , <i>hlyII</i> <sup>c</sup>	14
pANN 4-73 cosmid <sup>b</sup>	Ap <sup>r</sup> , <i>hlyII</i> , <sup>c</sup> <i>prf</i> , Hly <sup>+</sup> <sup>d</sup>	14
pANN 215 plasmid <sup>c</sup>	Cm <sup>r</sup> , $\Delta$ <i>hly A</i> , $\Delta$ <i>hly B</i>	47
pRHU 845 plasmid	Tc <sup>r</sup> , <i>pap</i>	11

<sup>a</sup> Ap<sup>r</sup>, ampicillin resistance; Cm<sup>r</sup>, chloramphenicol resistance; Tc<sup>r</sup>, tetracycline resistance; *hly*, hemolysin gene cluster; *prf*, P-related fimbriae gene cluster; *pap*, pili associated with pyelonephritis gene cluster.

<sup>b</sup> Cosmids which are based on pHC79 vector were derived from a gene library of strain 536 (see Knapp *et al.*<sup>14</sup>).

<sup>c</sup> *hlyII* designates cloned DNA fragments from the hemolysin region II of strain 536 (see Fig. 2).

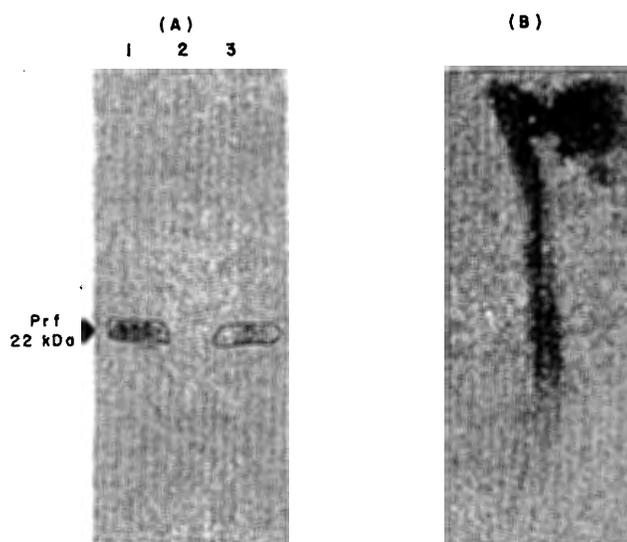
<sup>d</sup> Hly<sup>+</sup>, hemolysin production.

<sup>e</sup> *hly* specific DNA cloned from the plasmid pHly 152.

#### Linkage of hemolysin and fimbrial determinants on the chromosomes of extraintestinal *E. coli* isolates

The *prf*-positive clones indicated in Table 3 were further tested for hemolysin production. It was demonstrated that the clones HB101 (pANN 4-73) and HB101 (pANN 19B2) were hemolytic (see Table 3). Genetic analysis of the *prf*-specific cosmid DNAs (Table 3) revealed that the *prf* gene cluster is very closely linked to a hemolysin determinant. Restriction analysis showed that *prf* is located next to the *hlyII*-determinant of strain 536. A genetic map of the chromosomal region comprising the gene clusters *prf* and *hlyII* of strain 536 is given in Fig. 2.

In order to confirm the genetic linkage of the *hly* and *prf* determinants of strain 536



**Fig. 3.** (A) Western blot analysis using the anti-Prf-22 kDa antiserum (see text). The following *E. coli* fimbriae preparations are shown: lane 1, HB101 (pANN19B2); lane 2, HB101 (pHC 79 cosmid vector); lane 3, 536. Equal amounts of fimbriae preparations were applied to the SDS-PAGE. The 22 kDa Prf protein is indicated. (B) Electron-microscopic examination of isolated fimbriae from clone HB101 (pANN 19B2) approximately  $\times 40\,000$ .

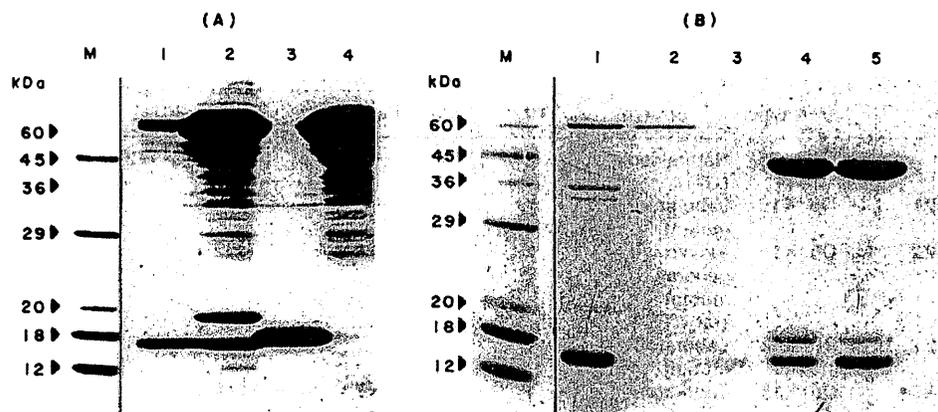


Fig. 1. SDS-PAGE analysis of fimbriae isolated from the following *E. coli* strains. (A) Lane 1, RS218; lane 2, RS226; lane 3, IH3034; lane 4, IH3036; (B) lane 1, 536; lane 2, 536-21; lane 3, 536-22; lane 4, J96; lane 5, J96-M1. Equal amounts of each preparation were applied, containing approximately 1–20  $\mu$ g of protein, as determined by the method of Bradford.<sup>46</sup> Protein markers are separated in lanes M.

the *pap* homologous DNA segment a cosmid library of strain 536<sup>14</sup> was screened by DNA-DNA colony hybridization with a *pap*-specific gene probe (Fig. 2, probe b; cf. Fig. 4 and Table 3). The cosmids pANN19B2, pANN10-21, pANN4-73, pANN6A4-2 and pANN15B2 were identified which contained *pap* homologous DNA fragments (Table 3, see Fig. 2). The cosmid clones did not express P-specific binding properties but fimbrial structures could be detected by electron microscopy as shown for HB101 (pANN19B2) in Fig. 3(B). Antiserum was prepared from rabbits against the 22 kDa fimbrial subunit proteins which were isolated from the 536 wild-type strain. Western blot analysis with the anti-22 kDa antiserum revealed the presence of fimbrial subunits of 22 kDa on the cosmid clones HB101 (pANN19B2) [Fig. 3(A), lane 1] HB101 (pANN 10-21) and HB101 (pANN 4-73) (data not shown) and on wild-type strain 536 [Fig. 3(A), lane 3]. From these data it became clear that the 22 kDa fimbriae of strain 536 are encoded by *pap* homologous sequences. Therefore the genetic locus was termed *prf* ('P-related fimbriae').

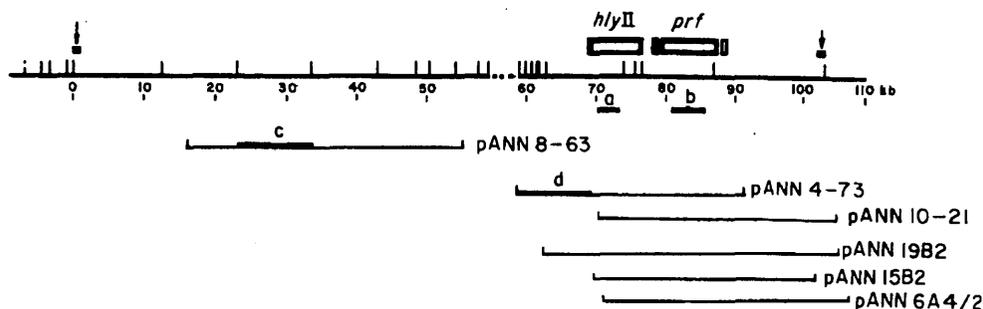


Fig. 2. Organization of the *hlyII* region of strain 536. The vertical bars represent *Hind*III restriction recognition sites. The genes for hemolysin (*hly*) and P related fimbriae (*prf*) are indicated as boxes with strong lines, where the borders and the extent of the *prf* determinant are not known precisely. The DNA probes used for Southern blot hybridizations are indicated below (a, b, c, d) as solid bars. The positions of cosmid DNAs relevant for this study are shown. The borders of the *hlyII* region are marked as solid rectangles with an arrow above (cf. Knapp *et al.*),<sup>14</sup> where the region not cloned is indicated as interrupted line. DNA probes a and b were isolated from plasmids pANN 215 which contains DNA from plasmid pHly152<sup>47</sup> and pRHU845,<sup>11</sup> respectively.

**Table 1** Phenotypic characterization of strains and mutants

Strain	O:K type	Source <sup>a</sup>	Hly <sup>b</sup>	Adhesins <sup>c</sup>				Fimbrial subunits (kDa)	Reference
				Sfa	Pap	Prs	Prf		
536	O6:K15	Natural isolate (UTI)	+	+	-	-	+	16 22	14
536-21	O6:K15	Laboratory mutant	-	-	-	-	-		14
536-22	O6:K15	<i>In vivo</i> generated mutant (rat UTI) <sup>d</sup>	-	-	-	-	-		This study
536-27	O6:K15	Laboratory mutant	-	-	-	-	-		This study
536-29	O6:K15	Laboratory mutant	-	-	-	-	-		This study
J96	O4:K6	Natural isolate (UTI)	+	-	+	+	-	18 19	1, 21
J96-M1	O4:K6	Laboratory mutant	(+) <sup>e</sup>	-	+	-	-	18 19	This study
RS218	O18:K1	Natural isolate (NBM)	+	+	-	-	-	17	5, 16
RS226	O18:K1	Natural isolate (FEC)	+	+	-	-	+	17 20	5, 16
IH3034	O18:K1	Natural isolate (NBM)	-	+	-	-	-	18	1, 16
IH3036	O18:K1	Natural isolate (NBM)	-	-	-	-	-		1, 16
HB101	K-12	Laboratory strain	-	-	-	-	-		35

<sup>a</sup>UTI, urinary tract infection; NBM, new born meningitis; FEC, fecal isolate.

<sup>b</sup>Hly, hemolysin production.

<sup>c</sup>Adhesins were determined as described in Materials and methods; Sfa, S fimbrial adhesin; Pap, pili associated with pyelonephritis; Prs, P-related sequence; Prf, P-related fimbriae.

<sup>d</sup>Strain 536-22 was isolated from a rat kidney after urethral infection with strain 536.<sup>2</sup>

<sup>e</sup>Reduced hemolysin production in comparison to the wild type strain J96, confirmed by a liquid assay.<sup>4</sup>

produced fimbrial subunits of 18 kDa and 19 kDa [Fig. 1(B), lanes 4, 5]; the 18 kDa protein subunit can be referred to the Pap and Prs fimbrial subunits of serotype F13.<sup>11</sup> The O18:K1 isolates, RS218, RS226 and IH3034 [Fig. 1(A), lanes 1-3; Table 1] produced S fimbriae as indicated by S-specific hemagglutination and by the occurrence of SfaA subunit proteins of 17-18 kDa.<sup>16</sup> In the case of strain RS226 an additional protein band of 20 kDa was detected. Strain IH3036 did not produce any demonstrable fimbrial adhesins [Fig. 1(A), lane 4; Table 1].

#### *Analysis of P-related fimbriae of strain 536*

It was demonstrated by Southern hybridizations (see below) that a chromosomal DNA region of strain 536 is homologous to P- (*pap*-) specific sequences. In order to analyse

**Table 2** Nephropathogenicity of strain 536 to rats and isolation of a non-hemolytic mutant

Days after infection	cfu/g kidney	Number of Hly <sup>-</sup> variants/ number of renal isolates tested
1	1.3 × 10 <sup>5</sup>	0/10 <sup>4</sup>
7	7.0 × 10 <sup>4</sup>	1/10 <sup>4</sup>
14	9.0 × 10 <sup>2</sup>	0/10 <sup>4</sup>

and to analyse the association of pathogenicity gene clusters of other strains, orthogonal field alternation gel electrophoresis (OFAGE) studies of chromosomal DNAs of the isolates listed in Table 1 were performed (see Fig. 4). The DNAs were digested with *Xba*I which cleaves *E. coli* chromosomal DNA very rarely and hybridized with *hly* as well as with P fimbriae-specific gene probes (probes a, b in Fig. 2) the latter of which is homologous to *pap*, *prs* and *prf* gene clusters. Wild-type strain 536 exhibited two *hly* specific *Xba*I fragments of 370 kb and 50 kb in size [Fig. 4(A), lane 7] reflecting the two *hly* determinants of this strain.<sup>14</sup> The P-fimbriae probe b only hybridized to the 370 kb fragment [Fig. 4(B), lane 7]. This indicates the presence of the gene clusters *hly*II and *prf* on the 370 kb *Xba*I fragment. In the case of the O18:K1 Hly<sup>+</sup> strains [Fig. 4(A), (B), lanes 3, 4] a 390 kb (strain RS226) and a 480 kb (strain RS218) *Xba*I fragment was recognized by both probes. *Xba*I cleaved genomic DNA of wild-type strain J96 shows strong hybridization signals of a fragment of 330 kb using the *hly* and *pap* gene probes [Fig. 4(A), (B), lane 5]. We suggest that the hybridization signal represents a double band (see below). The identical patterns after hybridization of the chromosomal DNAs with *hly* as well as with the P-fimbriae probe indicate a linkage of the two gene clusters on chromosomes of the wild-type strains 536, RS226, RS218 and J96.

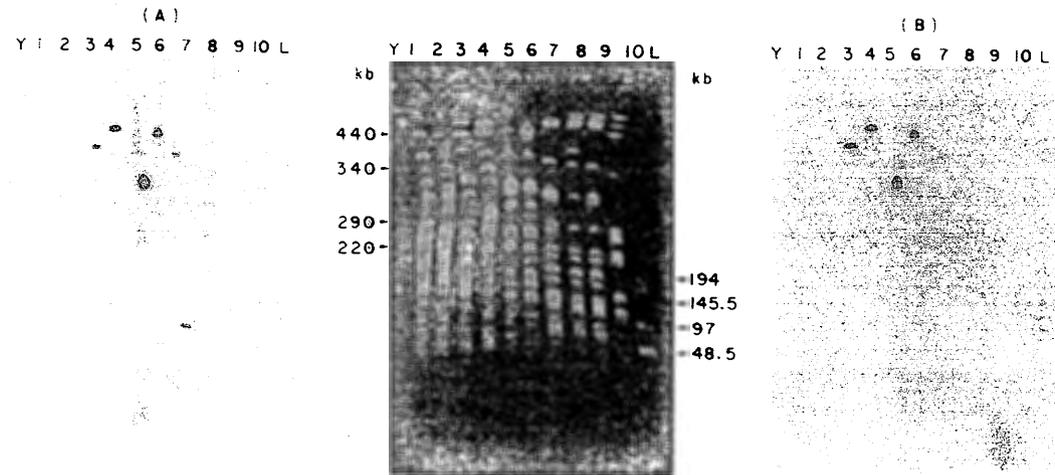
#### *Co-deletion of hemolysin and fimbrial determinants*

To analyse the genetic basis for the altered hemolysin and fimbrial phenotypes of the mutants and variants, chromosomal DNAs were extracted and hybridized with *hly*- and *pap*-specific gene probes. As shown in Fig. 4, DNA isolated from strains 536-21, 536-22, IH3034 and IH3036 did not exhibit any signals after hybridization with probes a and b [cf. Fig. 2, Fig. 4(A), (B), lanes 1, 2, 8, 9] which argues that *hly*- and P-specific sequences are absent from the chromosomes of the corresponding strains.

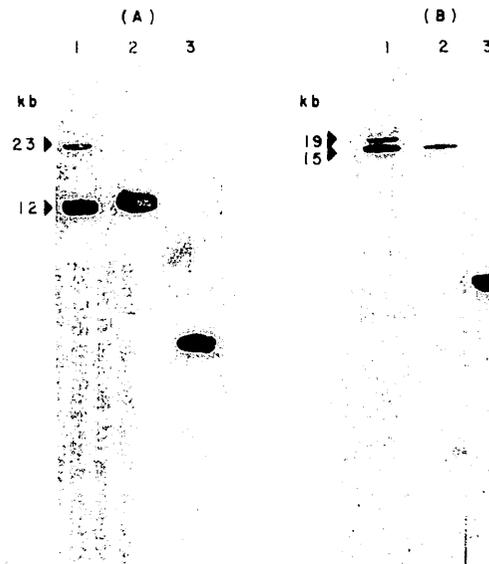
Strain J96-M1 shows an enlarged *Xba*I fragment of 440 kb recognized by probes a and b compared to the parental strain J96 [Fig. 4(A), (B), lane 6]. In order to determine the genetic processes leading to the altered phenotype of strain J96-M1 compared to J96, the chromosomal DNAs of both strains were further cleaved with the enzymes *Sal*I and *Eco*RI and then hybridized with probes a and b (Fig. 5). As *Sal*I does not cleave inside the *hly* determinants<sup>5</sup> thus clearly one of the two *hly* gene clusters of strain J96 [Fig. 5(A), lane 1] is deleted in strain J96-M1 [Fig. 5(A), lane 2] leading to the altered hemolysin phenotype of this mutant. It is also demonstrated that the *prs* determinant coding for the sheep erythrocyte hemagglutinating Prs fimbriae of strain J96 [Fig. 5(B), lane 1] is missing in the genome of strain J96-M1 as the 19 kb *prs*-specific *Eco*RI fragment is lost [Fig. 5(B), lane 2] (cf. Lund *et al.*).<sup>11</sup> These data suggest that a co-deletion of the linked hemolysin and fimbrial determinants from the chromosomes of mutants 536-21, 536-22 and J96-M1 occurred. It is also evident that the O18:K1 strains IH3034 and IH3036 differ from the isolates RS218 and RS226 by the absence of *hly*- and P-fimbriae-specific sequences.

#### *Southern hybridization with hlyII and prf flanking gene probes of strain 536*

To determine whether the chromosomal deletions observed also comprise flanking sequences of the hemolysin and fimbrial determinants, *Hind*III-cleaved chromosomal DNAs of the strains indicated in Table 1 were hybridized against DNA fragments located in the vicinity of the *hly*II and *prf* gene clusters of strain 536 (Figs 2 and 6). Using probe d (an 8 kb *Eco*RI/*Bam*HI fragment of cosmid pANN4-73) located very close to the *hly*II determinant of strain 536 (see Fig. 2) it can be seen that this sequence is not present on the genomes of the Hly-negative strains 536-21, 536-22, IH3034, IH3036 and on the chromosome of strain J96-M1 [Fig. 6(A), lanes 3, 4, 7,

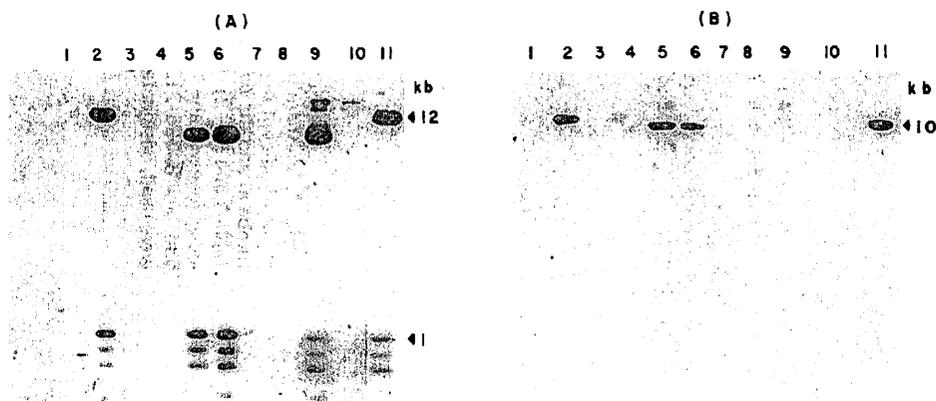


**Fig. 4.** Southern hybridization of *Xba*I cleaved chromosomal DNAs using a *hly* probe (A) comprising the cloned *Hind*III fragment of plasmid pHly 152, containing *hlyA*- and *hlyB*-specific DNA sequences (cf. Noegel *et al.*)<sup>47</sup> (probe a) and the *pap*-specific *Hind*III fragment of 4.4 kB size (cf. Lund *et al.*)<sup>11</sup> (B) from plasmid pRHU 845 (cf. Table 3 and Fig. 2). In the middle the OFAGE separated *Xba*I fragments of the genomic DNAs are visible. Strains are as follows: lane 1, IH3034; lane 2, IH3036; lane 3, RS226; lane 4, RS218; lane 5, J96; lane 6, J96-M1; lane 7, 536; lane 8, 536-21; lane 9, 536-22; lane 10, HB101. As size markers yeast chromosomes and lambda-oligomers were used, designated 'Y' and 'L', respectively. DNA sizes are indicated.



**Fig. 5.** Southern blot analyses using the *hly*-(A) and *pap*-(B) specific gene probes (cf. Figs 2, 4, Table 3 and see text). Genomic DNAs of strains J96 (lane 1) and J96-M1 (lane 2) were cleaved with *Sal*I, blot (A) and *Eco*RI, blot (B). Sizes of hybridizing fragments are indicated (cf. text). In lanes 3 are the plasmid DNAs pANN 215×*Hind*III (A) source of *hly* probe; pRHU 845×*Hind*III (B) source of *pap* probe.

8, 10]. This DNA region was detected, however, on the chromosomes of the hemolysin-positive isolates 536, RS218, RS226 and J96 [Fig. 6(A), lanes 2, 5, 6, 9]. Southern hybridization with probe c (a 10 kb *Hind*III fragment of cosmid pANN8-63; Fig. 2) which is located about 30 kb upstream of *hly*II<sup>14</sup> shows homology to the genomes of the Hly<sup>+</sup> strains [Fig. 6(B), lanes 2, 5, 6] with the exception of strain J96 [Fig. 6(B), lane 9] whereas the DNAs of the Hly negative strains and mutant J96-M1 did not hybridize [Fig. 6(B), lanes 3, 4, 7, 8, 10]. The non-pathogenic *E. coli* K-12 strain HB101 shares no common sequences to the used DNA probes a-d [Fig. 4(A), (B), lane 10, and Fig. 6(A), (B), lane 1], underlining the specificity of the whole DNA region for pathogenic *E. coli* strains.



**Fig. 6.** Southern blot analyses of *Hind*III cleaved chromosomal DNA using probes d and c from cosmids pANN 4-73, blot (A) and pANN 8-63, blot (B), respectively (Fig. 2 and see text). The strains are as follows: lane 1, HB101; lane 2, 536; lane 3, 536-21; lane 4, 536-22; lane 5, RS218; lane 6, RS226; lane 7, IH3034; lane 8, IH3036; lane 9, J96; lane 10, J96-M1. In lanes 11 the respective cosmid DNAs cleaved with *Hind*III, from which the DNA probes were isolated, are applied as control. DNA sizes are indicated.

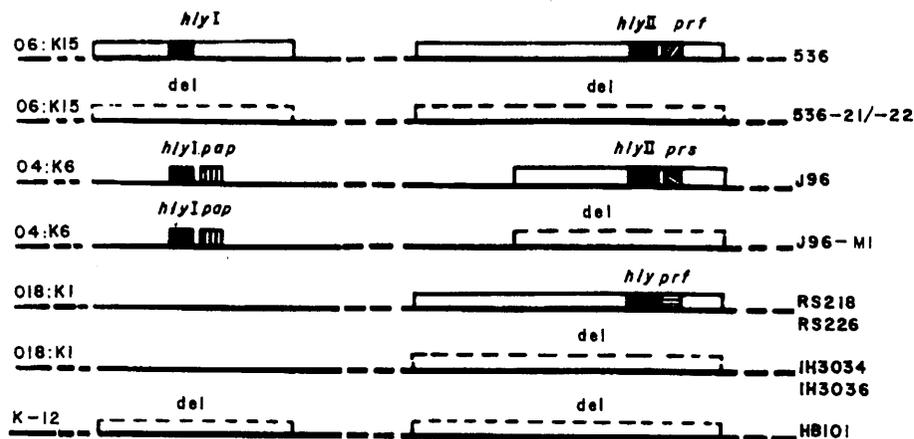


Fig. 7. Genomic DNA island structures of various extraintestinal *E. coli* isolates and mutants. On the left the serotype, and on the right the designation of strains is indicated. The *hly*, *pap*, *prs* and *prf* determinants are marked as boxes with different styling (for abbreviations see Table 1, cf. text). Missing chromosomal DNA is indicated by hatched boxes, whereas the corresponding island structures are shown as boxes with closed lines.

#### Discussion and conclusions

In this study we present data on the genetic linkage of a hemolysin (*hly*) determinant and the genes coding for P-related fimbriae (*prf*) on the chromosome of the wild-type *E. coli* strain 536. DNA segments which carry more than one pathogenicity gene cluster in close association have been previously termed as 'virulence gene blocks'.<sup>13,21,22</sup> Whereas virulence gene blocks of *E. coli* uro-virulence determinants have been described by several authors,<sup>21-25</sup> deletion events involving more than one pathogenicity gene cluster are demonstrated here for the first time. As shown in Fig. 7 the gene clusters of strain 536 coding for P related fimbriae and hemolysin are completely lost in the mutant strains 536-21 and 536-22. The two virulence gene clusters are deleted together with additional sequences indicated by the use of gene probes c and d which are located more than 30 kb apart (Fig. 2). These data are in agreement with previous observations<sup>14,20</sup> showing that large regions which comprise the two hemolysin determinants (*hlyI*, *hlyII*) of strain 536 may delete from the chromosome. These regions were termed as 'pathogenicity DNA islands'.

The presence of virulence gene blocks as part of pathogenicity DNA islands and their excision from the chromosomes of pathogenic isolates is not restricted to strain 536. As shown here mutant strain J96-M1 was isolated from the wild-type strain J96 which carries two hemolysin determinants which are associated with the adhesin gene clusters *pap* and *prs*, respectively.<sup>11,21</sup> As indicated in Fig. 7 strain J96-M1 has lost one hemolysin gene cluster together with the adhesin determinant *prs*. While the OFAGE analysis (Fig. 4) did not provide clear-cut evidence for excision of *hly* and *prs* sequences, the DNA hybridization experiments shown in Fig. 5 demonstrate a codeletion of the two determinants, a fact which corresponds well to the phenotypic characterization of strains J96 and J96-M1 (see Table 1).

In this respect the variants of serotype O18:K1 are of special interest. It was described previously<sup>15,17</sup> that the Hly-positive O18:K1 isolates RS218 and RS226 belong to one particular clonal group characterized by the outer membrane pattern (OMP) 6, while the Hly-negative isolates IH3034 and IH3036 are members of the clonal group OMP 9. Our data show that the isolates RS226 and RS218 carry

pathogenicity islands comprising the *hly* determinant and a DNA segment which is homologous to P-specific gene clusters (Fig. 7). It is suggested that the fimbriae consisting of 20 kDa subunits produced by strain RS226 are encoded by these P-fimbriae-homologous DNA sequences (see Fig. 1). In contrast to the OMP 6 isolates the strains IH3034 and IH3036 do not carry these pathogenicity DNA islands. It is speculated that the strains IH3034 and IH3036 may be 'deletion mutants' of the strains RS226 and RS218 which may have lost the pathogenicity islands.

Analogous physical linkages of pathogenicity genes have also been reported for *Bordetella pertussis*.<sup>26</sup> In addition, different enterobacteria such as *Yersinia*, *Shigella* and intestinal *E. coli* strains bear plasmids which carry more than one specific pathogenicity gene cluster.<sup>27-29</sup> Deletion events leading to a loss of virulence expression have also been described for *Haemophilus influenzae* capsule antigen b.<sup>30</sup>

We also demonstrate that deletion events comprising the virulence determinants *hly* and *prf* of *E. coli* strain 536 occurred *in vitro* and *in vivo*. Mutant 536-22 was recovered from the rat urinary tract after infection with the wild-type parental strain 536. One might speculate over the sense of losing such large DNA regions, containing important virulence genes. In evolution an elaborate genetic mechanism has been established, selecting for regions on the chromosome which are dispensable for the bacterial survival either at a certain stage of infection or in a special environment. For example it can be advantageous for bacteria not to express certain immunogenic surface structures like fimbriae to aid in avoidance of host responses. Also repressing the hemolytic phenotype could be of benefit in this respect. Clinical reports<sup>31</sup> have shown, that patients obstructed or suffering from diabetes are more susceptible to UTI *E. coli* strains not exhibiting virulence phenotypes. This indicates a natural selection of *E. coli* strains with deleted virulence regions living in an appropriate environment.

From a molecular point of view, several modes of modulation of bacterial virulence have been elucidated in the last years,<sup>32</sup> where the action of *trans*-regulatory factors is a predominant theme. In this report we present a mechanism controlling coordinate virulence gene expression which, interestingly, is in marked contrast to the commonly known modes because of its irreversibility. The absence of these dynamic pathogenicity islands in the genome of the *E. coli* K-12 laboratory strain (Fig. 7) underlines the specificity of these processes for certain pathogenic isolates. Long range genome analysis should yield more insight into the organization and distribution of pathogenicity DNA islands, which could be helpful for epidemiological studies.

## Materials and methods

**Bacterial strains, plasmids and cosmids.** The strains used are listed in Table 1. Plasmids and cosmids are listed in Table 3. As recipient strain the K-12 isolate HB101 was used.<sup>35</sup>

**Media, chemicals and enzymes.** Bacteria were grown in Luria Bertani (LB) broth. Radiochemicals were purchased from Amersham-Buchler, F.R.G. Antibiotics were a gift from Bayer, F.R.G. All other chemicals were obtained from Merck, F.R.G. Restriction enzymes were purchased from Boehringer, F.R.G.

**Isolation of chromosomal and plasmid DNA.** Chromosomal DNA was isolated as described by Knapp *et al.*<sup>14</sup> Plasmid and cosmid DNA was isolated by the clear lysate method and purified over CsCl gradients.<sup>34,35</sup> DNA fragments were eluted from agarose gels by the freeze squeeze method.<sup>36</sup>

**Cleavage of DNA with restriction enzymes and electrophoresis.** DNA was treated with the appropriate restriction enzymes under respective conditions and electrophoresed in 1% agarose gels.<sup>35</sup>

**Gene probes and radioactive labelling.** The gene probes used are listed in Fig. 2. After elution from agarose gels the DNA fragments were labelled by the method of Feinberg and Vogelstein<sup>37</sup> with a random priming kit purchased from Boehringer, F.R.G.

**Colony hybridization.** For identification of cosmid clones from a gene library, colony dot hybridization was performed, as described by Maniatis *et al.*<sup>35</sup>

**Southern hybridization.** The transfer of DNA from agarose gels to nitrocellulose paper and the washing and autoradiography of the filters were performed as described earlier.<sup>35,38</sup> The filters were hybridized in 50% Formamid for 24 h at 42°C. Stringent conditions were used for the washing procedure. For reusing Southern blot filters, they were incubated for 10 min in 1 mM EDTA pH 7.5 at 100°C. Total removal of the DNA probe was controlled by autoradiography of the nitrocellulose filter for at least 3 days. Then the filter could be used for a second hybridization.

**Orthogonal field alternation gel electrophoresis (OFAGE).** Chromosomal DNA for OFAGE analysis was isolated as described by Grothues and Tümmler.<sup>39</sup> Agarose blocks containing chromosomal DNA were equilibrated in restriction enzyme buffer for 3 h on ice and cleaved in fresh buffer with 30 units restriction enzyme for 3 h at the appropriate incubation temperature. OFAGE was performed with a Consort equipment (Consort, F.R.G.) using 1% agarose gels in 0.25×TBE buffer. Constant voltage of 250 V for 72 h was applied. The electric fields were arranged in a 120° angle alternating every 20 s.<sup>40</sup>

**Isolation of fimbriae.** Fimbriae were isolated from *E. coli* strains as described earlier.<sup>41</sup> Bacteria used for fimbriae preparation were grown on solid media.

**Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of fimbrial protein subunits.** After disintegration of fimbriae by boiling for 5 min in 10 mM Tris hydrochloride (pH 7.8) containing 4% sodium dodecyl sulfate, 0.01 ml of mercaptoethanol per ml, 0.2 ml of glycerol per ml, and 0.002% bromphenol blue, the fimbrial samples were run on slab gels as described previously.<sup>42,43</sup>

**Preparation of antisera and Western blot analysis.** Western blotting was performed by the method of Towbin *et al.*<sup>44</sup> Antisera used were prepared from rabbits with purified fimbrial protein eluted from SDS–PAGE gels.<sup>41</sup> The preimmune serum did not react with any fimbriae isolated.

**Determination of adhesins.** Pap-specific haemagglutination was performed with human P1-erythrocytes. Prs agglutination was carried out with sheep erythrocytes as described.<sup>11</sup> Sfa agglutination was confirmed by using neuraminidase treated bovine red blood cells described by Hacker *et al.*<sup>45</sup>

**Hemolysin production.** Erythrocyte lysis was detected on meat agar plates containing washed human erythrocytes or in a liquid assay.<sup>4</sup>

**Electron microscopy.** Fimbriae preparations were directly applied to Formvar-coated copper grids. After soaking in 1% phosphotungstic acid (pH 6.4) for 30 s. The samples were examined under a Zeiss-10 A transmission electron microscope.<sup>45</sup>

**Rat pyelonephritis model.** Female Wistar rats (strain Han-WIST; initial weight, 200 g) were anesthetized with phenobarbital (Nembutal; Abbott Laboratories), at a dose of 25 mg/kg of body weight administered intraperitoneally. *Escherichia coli* strain 536 was grown overnight on blood agar and suspended in phosphate buffer (0.1 M; pH 7.0) to give a final concentration of  $5 \times 10^7$  cfu/ml. Of this suspension 1.5 ml was injected via the urethra into the bladder of the rats. Rats were killed after 1, 7 and 14 days, and the kidneys were removed. Viable counts of the *E. coli* were made from kidney homogenate on MacConkey agar and blood agar plates.<sup>2</sup> Non-hemolytic variants were furthermore selected on blood agar plates and tested for the presence of the O6 and K15 antigen, as well as for streptomycin resistance. Variant, designated *E. coli* 536-22, was further characterized.

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