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Pathogenic diversity of *Escherichia coli* and the emergence of ‘exotic’ islands in the gene stream

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Abstract – *Escherichia coli* is a highly adaptive bacterial species that is both a member of the commensal intestinal flora and a versatile pathogen associated with numerous types of intestinal and systemic infections in humans and other animals. The spectrum of diseases caused by *E. coli* is due to the acquisition of specific virulence genes harbored on plasmids, bacteriophages, or within distinct DNA segments termed pathogenicity islands (PAIs) that are absent from the genomes of commensal *E. coli* strains. PAIs are likely to have been transferred horizontally and may have integrated into the *E. coli* chromosome through bacteriophage or plasmid integration or transposition. The contribution of intergenic inheritance to the adaptation and evolution of *E. coli*, types of PAIs associated with different groups of pathogenic *E. coli* and approaches to identify unique sequence islands (USIs), some of which might confer pathogenicity, in *E. coli* and other bacteria are presented. © Inra/Elsevier, Paris.

***Escherichia coli* / pathogenicity island / horizontal gene transfer / adaptation**

Résumé – Diversité du pouvoir pathogène d'*Escherichia coli* et fluidité du génome bactérien : acquisition d'ADN « exotiques » organisés en îlots au sein du chromosome. *Escherichia coli* est une espèce bactérienne ayant une grande faculté d'adaptation. Elle fait partie de la flore intestinale commensale mais c'est également un pathogène versatile responsable d'infections systémiques et intestinales chez l'homme et les animaux. La grande diversité des maladies provoquées par *E. coli* est due à l'acquisition de gènes de virulence spécifiques se trouvant sur des plasmides et des bactériophages ou à l'intérieur de régions chromosomiques appelées îlots de pathogénicité (PAIs). Ceux-ci sont absents du génome des souches d'*E. coli* commensales. L'acquisition des PAIs est probablement la conséquence d'un transfert horizontal d'ADN étranger au génome des *E. coli* lors de l'intégration dans le chromosome de bactériophages, de plasmides ou de transposons. Nous présenterons dans cette revue la contribution de ces transferts d'ADN à l'adaptation et à l'évolution de *E. coli*, les types de PAIs associés aux différents groupes de *E. coli* pathogènes ainsi que les approches permettant l'identification des îlots constitués de séquences uniques (USIs) et dont une partie pourrait conférer le pouvoir pathogène chez *E. coli* et chez d'autres bactéries. © Inra/Elsevier, Paris.

***Escherichia coli* / îlot de pathogénicité / transfert horizontal de gène / adaptation**

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1. INTRODUCTION

In addition to being a common member of the intestinal microflora of humans and other animals, particular subsets of *Escherichia coli* strains have adapted the capacity to cause a variety of intestinal and systemic infections. Different types of pathogenic *E. coli* exhibit host and tissue specificity as well as age specificity for particular hosts [48, 66, 162]. The diarrheagenic *E. coli* pathogens include: enterotoxinogenic *E. coli* (ETEC) associated with travellers' diarrhea and porcine post-weaning diarrhea; enteropathogenic *E. coli* (EPEC) that cause watery diarrhea in children and animals, enterohemorrhagic *E. coli* (EHEC) associated with hemorrhagic colitis and hemolytic uremic syndrome, enteroaggregative *E. coli* (EAEC) associated with persistent diarrhea, and enteroinvasive *E. coli* (EIEC) and *Shigella* that cause invasive intestinal infections, watery diarrhea and dysentery in humans and other primates [75, 120, 122]. *E. coli* associated with extra-intestinal diseases include uropathogenic *E. coli* (UPEC) that cause urinary tract infections in humans, dogs and cats [13, 41, 61, 175], *E. coli* associated with neonatal meningitis [174], and *E. coli* associated with septicemia in humans [24, 153, 174] and other animals [4, 46, 71,

111, 176]. Other *E. coli* pathogens are associated with host-specific diseases such as airsacculitis and cellulitis in poultry caused by avian pathogenic *E. coli* (APEC), diarrhea in rabbits caused by rabbit enteropathogenic *E. coli* (REPEC) [2, 37] and *E. coli* that cause edema disease in pigs [176].

The schism that separates commensal *E. coli* strains from pathogenic *E. coli* and the diversity of diseases they cause has largely been attributed to the acquisition of different sets of virulence genes that permit *E. coli* pathogens to gain entry to, colonize, and promote the development of specific pathological changes in host niches that are inaccessible or inhospitable to commensal *E. coli* [52]. Genes contributing to the virulence of pathogenic *E. coli*, as well as other bacterial pathogens, have been acquired by inheritance of specific plasmids or phages and through the integration of large blocks of DNA, termed pathogenicity islands (PAIs), into their chromosomes [52, 63, 70, 101]. In this review, we summarize some of the aspects of PAIs and discuss the mechanisms by which PAIs may have been acquired. PAIs that have been identified and characterized in pathogenic *E. coli* are reviewed, and finally some of the approaches that may be useful for identifying and characterizing PAIs are presented.

2. EXOTIC ISLANDS IN THE GENE STREAM CONTRIBUTE TO BACTERIAL EVOLUTION

PAIs have been described as large and sometimes unstable DNA regions that are present in pathogenic variants of a bacterial species, but are absent from strains of the same species that exhibit low or no virulence [63, 70, 101]. These DNA islands are 'exotic' in the sense that genes encoded by these sequences often possess a nucleotide composition and codon usage that is atypical to that of the bacterial genome within which they presently reside. Differences in G+C content and codon usage suggest that these DNA regions were most likely acquired at some time in the past from foreign species that may now be extinct, through lateral transmission. This rationale is based on the idea that, when first acquired, DNA from intergenic sources with differing G+C contents will demonstrate the greatest level of sequence divergence from the recipient into which it has integrated. Over-time random genetic drift should ameliorate the third codon (P_3) to a base composition that is more like that of the surrounding average G+C content of the host [6, 99].

Incorporation of foreign DNA into bacterial genomes is not restricted to PAIs, and it is likely that horizontal gene transfer is a major force in the evolution of bacterial genomes such as *E. coli* or *Salmonella enterica* [98, 100]. Bacterial populations of *E. coli* or *Salmonella* have been shown to be clonal based on multilocus enzyme electrophoresis (MEE) [154, 171]. Sequencing of housekeeping genes encoding common required functions shared by all strains within a group demonstrate limited DNA transfer among clones [25, 121, 164]. In contrast, genes involved in clone adaptation and diversification show a higher degree of intergenic acquisition and include *rfb* gene clusters [9, 141, 159, 168] as well as PAI-encoded genes [63, 70, 101].

Analysis of the complete genome sequence of the *E. coli* K-12 strain MG1655

[16] has allowed Lawrence and Ochman [100] to estimate that at least 17.6 % of the open reading frames (ORFs), 755 of 4 288 ORFs totalling 547.8 kb, in the *E. coli* K-12 genome were acquired through horizontal gene transfer. The distribution of these horizontally acquired segments in *E. coli* K-12 [100], as well as PAIs in pathogenic *E. coli* (table 1) and other bacteria [63, 70, 101] are often associated with tRNA gene loci. In addition, horizontally acquired regions of *E. coli* K-12 are also frequently associated with the presence of insertional sequence (IS) elements. Similarly, PAIs from different bacterial species are frequently associated with IS-related, or plasmid-encoded sequences. These findings suggest that the mechanisms for horizontal transfer of PAIs as well as the acquisition of metabolic or niche adaptive genes by commensal *E. coli* strains are shared.

The association of tRNA genes implicates temperate bacteriophages as the mediators of horizontal gene transfers. The integration sites of a number of bacteriophages, such as P4 and related coliphages, are within or adjacent to tRNA genes [31, 142]. Horizontal acquisition through tRNA gene homing bacteriophages is a plausible means of facilitating intergenic recombination since tRNA sites are highly conserved across species. The association of different PAIs with tRNA genes and the presence of phage- or plasmid-related sequences bordering or within PAIs supports the likelihood that these virulence-associated sequences may have been acquired from bacteriophages or integrative plasmids that carried bacteriophage integrase or attachment sites. IS sequences may also mediate acquisition or loss of PAIs, either directly by transposition events in the case of functional mobile elements such as the *IS100* flanked unstable *pgm* locus of *Yersinia pestis* [54, 55, 134]. The presence of non-functional remnants of IS sequences or repetitive sequences, however, may also contribute to integration, subsequent recombination or deletion of horizontally acquired DNA.

The genomes of natural *E. coli* isolates based on physical mapping vary by as much as 1 Mb, with chromosome sizes ranging from 4.5 to 5.5 Mb in length [12]. Clinical *E. coli* isolates possess the largest genome sizes, whereas *E. coli* K-12 is situated at the lower end of the genome sizes with 4.63 Mb. This further supports the likelihood that the continued success and adaptation of *E. coli* to exploit specialized niches involves gene capture from external sources. There are therefore numerous and diverse additions to pathogenic *E. coli* strains compared to the adaptive changes that *E. coli* K-12 has acquired.

3. *E. COLI* PATHOGENICITY ISLANDS

Various virulence-associated genes have integrated into the genome of *E. coli*, and have endowed this organism with a capacity to successfully adapt to a pathogenic lifestyle in different host tissues and species and cause a spectrum of different disease syndromes. In its broadest definition horizontally acquired segments of DNA that contribute to the pathogenicity of *E. coli* and closely related *Shigella* spp. could also include virulence plasmids and temperate phages, as well as large blocks of DNA containing virulence genes that have integrated into the chromosome. The acquisition of plasmids and temperate phages harboring genes that encode toxins, adhesins, or other virulence factors most definitely contributes to the virulence of certain pathogenic *E. coli* and *Shigella* spp. [7, 8, 120, 127]. Strictly speaking, these genetic elements are not, however, considered to be PAIs and are not included in the following sections. A summary of some of the characteristics of the PAIs described below are presented in *table 1*.

3.1. Pathogenicity islands associated with uropathogenic *E. coli*

E. coli strains are the most frequent cause of urinary tract infections (UTIs) in children

and adults. Uropathogenic *E. coli* (UPEC) comprise a distinct subset of *E. coli* strains that belong to certain O serogroups and possess virulence determinants which are less frequently associated with *E. coli* isolated from the fecal flora. These virulence determinants include P and S/FIC fimbriae, aerobactin, hemolysin, cytotoxic necrotizing factor 1 (CNF1), and certain capsular (K) antigens [41, 86]. UPEC-associated virulence genes are generally encoded on the chromosome and are often clustered together on discrete blocks of DNA [51, 79, 103]. These distinct virulence gene blocks that could spontaneously delete from the chromosome of UPEC strains 536 and J96 were first termed 'pathogenicity DNA islands' by Hacker et al. [69], and later shortened to 'pathogenicity islands' [68]. The PAIs of three different UPEC strains (536, J96 and CFT073) have been characterized. There are diverse differences in the five PAIs characterized from these three strains including sizes of the PAIs, different flanking direct repeat sequences, association with different tRNA genes and location on the *E. coli* K-12 map (*table 1*). Interestingly, none of the PAIs from strains J96 and CFT073 share any common sequences except for the *hly* and *pap* genes [65, 163]. The fact that *hly* and *pap* sequences occur independently in the genomes of some pathogenic *E. coli* [44, 58, 86, 109] also supports the likelihood that various PAIs associated with UPEC strains have emerged independently and have undergone significantly different gene additions or deletions over time.

3.1.1. PAI-1 and PAI-2 of UPEC strain 536

Uropathogenic *E. coli* strain 536 (O6:K15:H31) contains two chromosomal copies of *hly* gene clusters encoding hemolysin, produces S fimbriae, P-related fimbriae (Prf) and type 1 fimbriae and is resistant to serum [69, 92]. Spontaneous hemolysin-negative deletion mutations from strain 536 occur at a high rate (10^{-3} to 10^{-4})

Table 1. Summary of the characteristics of the *Escherichia coli* pathogenicity islands (PAIs).

<i>E. coli</i> pathotype	PAI name	Genes/ attributes	PAI size (kb)	Location (min)/ tRNA gene	G+C ^a content (%)	Deletion rate	References
Uropathogenic							
Strain 536	PAI-1	<i>hly</i>	70	82.6/ <i>selC</i>	41	10 ⁻⁴ – 10 ⁻⁵	[19, 67]
	PAI-2	<i>hly, prf</i>	190	96.9/ <i>leuX</i>	41	10 ⁻⁴ – 10 ⁻⁵	[19, 67]
Strain J96	PAI-4	<i>hly, pap</i>	>170	67.0/ <i>pheV</i>	41	stable	[163]
	PAI-5	<i>hly, prs, cnf1</i>	110	94.0/ <i>pheU</i>	41	10 ⁻⁵	[20, 163]
Strain CFT703	PAI-6	<i>hly, pap</i>	58	26.7/63.5/ <i>metV</i>	42.9	stable	[65, 88]
Attaching effacing							
EPEC-strain E2348/69	LEE (PAI-3)	<i>eae, esp, esc</i>	35.6	82.6/ <i>selC</i>	38.4	stable	[48, 115]
EHEC-strain EDL933	LEE	<i>eae, esp, esc</i> P4-like prophage	43.4	82.6/ <i>selC</i>	38.4	stable	[130]
EHEC2 clonal group	LEE	<i>eae, esp, esc</i>	~35?	94.0/ <i>PheU</i>	?	stable	[157]
<i>Shigella</i> and enteroinvasive							
<i>S. flexneri</i> 2a	?	contact hemolysin, antibiotic resistance	99	22-24/ <i>serT, serX?</i>	?	unstable	[137]
<i>Shigella</i> /EIEC	<i>she</i> PAI	<i>she, sigA, set1ab</i>	~51	20-24/?	50.2	10 ⁻⁵ – 10 ⁻⁶	[138]
	black hole	more toxin activity by loss of <i>cadA</i>	-190 ^b	92-94.3/ <i>pheU</i>	N.A. ^c	N.A.	[112]
Various							
Various pathotypes	group II/III capsule islands	<i>kps</i> gene cluster	~20	67.0/ <i>pheV</i>	33-42	stable	[34, 150]
Various pathotypes ^d	<i>Yersinia</i> HPI	<i>irp2, fyuA</i> yersiniabactin	~45 ^e	?/ <i>asn</i> tRNA gene	56-59	some deletions	[140, 152]

^a G+C content is often determined from only part of the PAI that has been sequenced. Average *E. coli* K-12 G+C content is 50.8% [16].

^b 190-kb deletion compared to *E. coli* K-12 in *S. flexneri* 2a and similar deletions in other *Shigella* spp. and enteroinvasive *E. coli*.

^c N.A. = not applicable.

^d Most commonly associated with enteroaggregative *E. coli* and *E. coli* blood culture isolates.

^e Size based on data from *Yersinia* spp. but actual size of PAIs in *E. coli* have not been determined.

in vitro [67] and were also observed in isolates from the urinary tract of infected rats [68]. Loss of the hemolysin phenotype was associated with the loss of large DNA regions flanking the *hly* clusters [67, 93]. Genomic analysis and sequencing demonstrated that these *hly* gene clusters are

located on two distinct PAIs that could excise from the chromosome of strain 536 [93]. Loss of these PAIs resulted in avirulence of the strain, sensitivity to serum, and also affected the expression of S and type I fimbrial adhesin production, although these fimbrial gene clusters were not asso-

ciated with the PAIs and were not excised from the chromosome of strain 536 PAI deletion mutants [69, 93].

The two PAIs of *E. coli* 536 are termed PAI-1 and PAI-2. PAI-1 is 70 kb in size, contains a *hly* gene cluster (*hly* I) and is located at 82.6 min on the *E. coli* K-12 map [19, 93], whereas PAI-2 is 190 kb in size, contains a second *hly* gene cluster (*hly* II), a P-related fimbriae encoding the *prf* gene cluster, and is located at 96.9 min on the *E. coli* K-12 map [19]. PAI-1 and PAI-2 are flanked, respectively, by 16- and 18-bp direct repeats that share the motif 'TTCGA', and these repeats are part of tRNA encoding genes. PAI-1 is flanked by the *selC* selenocysteinyl tRNA (tRNA^{Sec}) gene [21], whereas PAI-2 is flanked by *leuX*, the leucanyl tRNA (tRNA₅^{Leu}) [95] encoding gene. Excision of either PAI leaves only one direct repeat and results in disruption and abolition of transcription of *selC* or *leuX* [19].

Loss of the PAIs from strain 536 does indeed result in a loss of virulence; however, it appears that much of the reduction in virulence is not directly associated with loss of the *hly*, *prf* or other as yet unknown genes located on these unstable DNA regions. Rather, decreased virulence of strain 536 PAI deletion mutants is primarily due to the disruption of the tRNA gene *leuX* upon excision of PAI-2, resulting in alterations in global gene transcription [145]. Absence of tRNA^{Sec} due to disruption of *selC* affected the metabolism of a strain 536 PAI-1 deletion mutant and rendered the strain incapable of anaerobic growth owing to an incapacity to produce formate dehydrogenases (which contain selenocysteine) and an inability to utilize selenium, but did not affect the virulence of the strain [145]. In contrast, absence of tRNA₅^{Leu}, caused by excision of PAI-2 had no effect on anaerobic growth, but rendered the strain serum sensitive, dramatically affected production of enterobactin-mediated iron acquisition, type I and S fimbriae, and flagella, and greatly reduced the virulence of this strain.

Inhibition of type 1 fimbrial production in strain 536 PAI-2 mutants is clearly dependent on *leuX* [146]. The gene encoding the FimB recombinase that mediates a switch to phase-on expression for type 1 fimbriae in strain 536 contains five tRNA₅^{Leu}-specific TTG codons and its expression is suppressed entirely in a *leuX*-negative background, however, altering the five TTG codons to CTG in *fimB* renders *fim* expression independent of *leuX* [146]. It is likely that other *leuX*-dependent characteristics such as enterobactin and flagellar production and motility are also due to a dependence for a substantial amount of TTG codons for expression of these products. Expression of *leuX* also contributes to the survival of strain 536 in mouse bladder mucus [40] and colonization and nephropathogenicity in rats [161].

In addition to the effects of tRNA gene excision and the loss of virulence factors on the PAIs themselves, another PAI excision-associated phenomenon has been observed. Loss of PAI-2 by strain 536 greatly reduces the transcription of *sfa* genes that encode the S fimbriae [69, 93]. However, decreased *sfa* expression is not dependent on *leuX*, but is attributed to the absence of the *prf*-encoded regulatory genes *prfI* and *prfB* [118]. PrfI and PrfB are highly similar to the *sfaB* and *sfaC* gene products that mediate regulation of expression of S fimbriae, and PrfI and PrfB enhance expression of S fimbriae in strain 536 by cross-talk in trans [118]. Overall, the spontaneous loss of PAIs from strain 536, the excision of tRNA genes, and alterations in the metabolism and global regulation of virulence genes are inextricably linked. Although Hacker et al. have also demonstrated spontaneous loss of PAIs in other UPEC, namely PAI-5 of strain J96 [68], it remains to be demonstrated if spontaneous loss of other PAIs associated with *E. coli* from UTI also occur and whether such events are also mediated by tRNA gene associated direct repeats that disrupt a tRNA gene upon excision.

3.1.2. PAI-4 and PAI-5 of UPEC strain J96

UPEC strain J96 (O4:K6) is a prototype *E. coli* strain from which various extra-intestinal *E. coli* virulence factors have been cloned and characterized [77, 125, 170]. Strain J96 possesses two copies of gene clusters encoding P (*pap*-encoded) and P-related (*prs*-encoded) fimbriae, produces F1C and type 1 fimbriae, contains two *hly* gene clusters encoding hemolysin and produces cytotoxic necrotizing factor type 1 (CNF1). All of these virulence-associated genes are located on the chromosome. Some of the virulence factors on strain J96 and other UPEC strains are linked to each other [51, 68, 74, 78, 79, 103]. Blum et al. [20] demonstrated that the virulence-associated genes encoding Prs fimbriae, hemolysin and CNF1 on strain J96 were contained within a 610-kb *Sfi*I fragment and are part of a PAI that has the capacity to spontaneously delete from the chromosome [68]. This PAI is now termed *E. coli* PAI-5, whereas another region containing the *pap* and *hly* gene clusters is termed PAI-4 [70, 163].

PAI-4 and PAI-5 have been further characterized by analysis of overlapping cosmid clones, hybridization to the *E. coli* K-12 Kohara ordered phage library [94], and sequencing of internal and flanking regions [163]. PAI-5 spans 110 kb and is inserted at 94 min on the *E. coli* K-12 map. The precise junction points of PAI-5 contained nearly perfect 135-bp direct repeats with an intact *PheU* (*PheR*) gene at the right junction and a 25-bp segment of the 3' end of *PheU* at the left junction. The *pheU* gene encodes phenylalanyl tRNA^{*pheU*} and is located at 94 min on the *E. coli* K-12 map [16]. The size of PAI-4 was only partially determined and spans over 170 kb. The right junction of PAI-4 was within 250 bp of another phenylalanyl tRNA gene, *pheV*, located at 67 min on the *E. coli* K-12 map and also exhibited sequences similar to the *kps* (group II and III capsular polysaccharide) genes. Group II and group III capsule-encoding genes are

also inserted near *pheV* in certain pathogenic *E. coli* strains [34, 150] (see below).

Random sequencing of cosmid subclones containing sequences unique to PAI-4 or PAI-5 demonstrated similarities to genes encoding adhesins and other virulence determinants from diverse pathogens including *Streptococcus parasanguis*, *Proteus mirabilis*, *Serratia marcescens*, and enterotoxinogenic *E. coli* as well as similarities to IS elements (*IS100*, *IS630*, *IS911*), R1 plasmid genes, and P4 and P2 phage genes. Aside from the *hly* and *pap/prs* gene clusters few common sequences internal to PAI-4 and PAI-5 were observed, except for sequences related to phage P4 located at the borders of the two islands. Although the PAI-4 and PAI-5 insertion sites are associated with phenylalanyl tRNA-encoding genes, differences in the sequences present within and flanking these PAIs suggest that they were acquired and have evolved independently. Although Hacker et al. have isolated spontaneous PAI-5 deletion mutants of strain J96 [20, 68], other groups have observed that the PAIs of strain J96 are stable (E. Oswald, pers. comm.).

Aside from the *pap*- and *hly*-related gene clusters, it is currently unknown whether the PAI-4 and PAI-5 sequences related to virulence genes from diverse sources play a role in UTI pathogenesis or whether they are associated with other UPEC or pathogenic *E. coli* strains associated with human or animal infections. Certain *E. coli* associated with diarrhea and septicemia of cattle and pigs produce CNF1 and also possess *hly* and *pap* gene clusters [46, 108]. The genes encoding CNF1, hemolysin and P-related adhesins are clustered together on PAIs in these porcine and bovine *E. coli* strains and fall into two distinct groups: i) PAIs that are inserted near *PheU* as is the case for PAI-5 of strain J96; and ii) strains that contain PAI-5-type islands that are inserted elsewhere in the chromosome (Herauld et al., unpublished results). Whether these PAIs contribute to intestinal or extra-intestinal infections in pigs is currently being

investigated (Oswald and Fairbrother, pers. comm.).

3.1.3. The PAI of UPEC strain CFT073 (PAI-6)

The UPEC strain CFT073 was isolated from the blood and urine of a woman with acute pyelonephritis. This strain is Hly⁺, Pap⁺, Foc⁺ and Fim⁺ and is known to contain two functional *pap* gene clusters [88]. One of these *pap* gene clusters lies within a 58-kb PAI (that we have named PAI-6) and was initially identified and characterized by ordered overlapping cosmid clones and sequencing of flanking DNA that was also common to *E. coli* K-12 [88]. The location of PAI-6 within the genome of CFT073 is less clear compared to most other described PAIs. Cosmid sequencing determined that the left flanking region starts 7 bp downstream of *dadX* (26.7 min), whereas the right end terminates within an uncharacterized *E. coli* gene (f447) (GenBank accession no. ECAC000365) at a site that is 75 bp downstream of the *metV* tRNA gene (63.5 min). The authors confirmed that these *E. coli* K-12 sequences flank PAI-6 and suggested that CFT073 may have undergone chromosomal rearrangements relative to the K-12 linkage map. However, it remains to be determined if strain CFT073 has incurred major chromosomal inversions centered around PAI-6 or whether additional DNA sequences homologous to those located at 26.7 or 63.5 min of *E. coli* K-12 have inserted adjacent to either side of PAI-6. The junctions on either side of the PAI are bordered by 9-bp direct repeats and may have mediated the integration of PAI-6 into the chromosome; however, PAI-6 appears to be quite stable [88]. Regions of PAI-6 were used as probes and hybridized to 80 % of the DNA from strains associated with pyelonephritis or cystitis, whereas only 22 % of the DNA from fecal strains on average hybridized with these probes [65, 88].

Much of a 61-kb region encompassing the 586-kb PAI-6 has been sequenced and

contains 44 ORFs [65]. In addition to the *pap* and *hly* gene cluster, there are two notable regions showing homologies to other sequences. The left-hand region is highly similar to a 6.3-kb segment of DNA from enteropathogenic *E. coli* (EPEC) strain E2348/69 (accession no. U85771) (pers. obs.). This PAI-6 region homologous to that of the EPEC strain contains *E. coli* K-12-related genes located at 26.8 min adjacent to *dadX* as well as three ORFs absent from *E. coli* K-12 that may be involved in iron transport. The second notable homology is an ORF (R4) at the right end of the PAI that is homologous to an iron siderophore receptor from *Bordetella bronchiseptica*. In addition, there are ORFs similar to transposase genes, IS sequences or plasmid-encoded genes located before the *pap* gene cluster and in between the *pap* and *hly* gene clusters. There is also a stretch of 8 kb within PAI-6 that is similar to six ORFs from *E. coli* K-12 that are located at 59.8 min. The G+C content of PAI-6, excluding sequences with homology to *E. coli* K-12 DNA, is 42.9 % and is significantly different from that of the *E. coli* K-12 genome (50.8 %) [16]. Similarly, the G+C mol % of the PAIs from strains 536 and J96 have also been estimated to be 41 % [70] supporting the likelihood that sequences within these UPEC PAIs have been acquired by lateral gene transfer from organisms with a lower average G+C content.

3.2. Locus of enterocyte effacement (LEE) (PAI-3)

Certain diarrheagenic *E. coli* and other pathogenic enterobacteria adhere intimately to and remodel the actin cytoskeleton of host cells, leading to the formation of what are called attaching and effacing (A/E) lesions [117, 158] (for recent reviews see this volume of *Veterinary Research* (30 (1999)) and [42, 56, 89, 90, 120]). Enterobacterial pathogens known to cause these lesions are widespread among different ani-

mal species and include enteropathogenic *E. coli* (EPEC) from humans [120], swine [73, 180], cattle [33, 57], dogs [5, 47] and rabbits [1, 2, 37], enterohemorrhagic *E. coli* (EHEC) in humans and cattle [22, 120]; certain strains of *Hafnia alvei* [144] and the murine pathogen *Citrobacter rodentium* (formerly *C. freundii* biotype 4280) [60, 151].

Initial studies demonstrated that a gene termed *eae* (for *E. coli* attaching and effacing) [84], was required for the development of A/E lesions by EPEC strains, but that alone did not induce A/E lesions when transferred to *E. coli* K-12. The *eae* gene was found to be located on the chromosome [85] and similar sequences were present in other A/E-producing *E. coli* and enterobacteria [11, 59, 85, 151, 178].

McDaniel et al. [115] demonstrated that the A/E capacity of EPEC strain E2348/69 (O127:H6) was encoded by a large ~35-kb region termed the LEE (for locus of enterocyte effacement). Hybridization of segments of the LEE demonstrated that similar genetic loci were present in other A/E pathogens, but were absent from non-A/E pathogenic *E. coli*, *E. coli* K-12 and other bacterial pathogens. The LEE, when cloned into *E. coli* K-12 recipients, endowed these strains with a capacity to induce A/E lesions on cultured epithelial cells [114], demonstrating that this unique DNA region can autonomously mediate the characteristic trait of attaching and effacing *E. coli*.

The LEE of strain E2348/69 is inserted 16 bp downstream of the tRNA^{Sec} gene *selC* located at 82.6 min on the *E. coli* chromosome [115], the exact site of insertion of PAI-1 from uropathogenic *E. coli* strain 536 [19]. Interestingly, *selC* is also the site of insertion of the *E. coli* P4-like retronphage Φ R73 [80, 160]. Although the LEE insertion site was first characterized as *selC* in a few EPEC and EHEC strains [115], in many cases the LEE has inserted at locations other than *selC* [62, 157, 173]. The LEE was shown to be generally inserted at sites other

than *selC* in bovine EPEC and EHEC strains [62] and is not the site of insertion in rabbit enteropathogenic *E. coli* (REPEC) [102] and EHEC strains of serogroup O103 (E. Oswald, pers. comm.).

Weiler et al. [173] found that the insertion site of the LEE is dependent on the evolutionary lineage of the strains. Two groups of related strains called EPEC1 and EHEC1, belonging to serotypes O127:H6, O55:H6, O55:H7 and O157:H7, were inserted at *selC*, whereas a second cluster of EPEC and EHEC strains (EPEC2 and EHEC2) belonging to serogroups O111, O26, O45 and O128 had LEE that were inserted elsewhere on the chromosome. A second integration site for the LEE has been determined for some strains belonging to the EHEC2 lineage [157]. Certain EHEC2 strains of serogroups O111 or O26 were shown to contain LEE insertions within the tRNA gene *pheU* (*pheR*), located at 94 min on the *E. coli* K-12 map, which is also the insertion site of PAI-5 of UPEC strain J96 [163]. It is likely that the LEE has inserted within more than just these two sites as *E. coli* and other enterobacteria that induce A/E lesions comprise a diverse evolutionary group. As an example, a strain of serotype O111ab:H25 that belongs to a lineage other than the EPEC/EHEC groups 1 and 2 [172] contains a LEE that is inserted elsewhere than the *selC* or *pheU* sites [157]. Despite the fact that the LEE appears to have inserted at multiple times and within multiple sites during the evolution of enterobacteria that induce A/E lesions, and in contrast to some other *E. coli* PAIs, the LEE seems to be stable.

The complete sequence of the LEE from strain E2348/69 encompasses 35 624 bp [50] and has an average G+C content of 38.4 %, well below the *E. coli* K-12 chromosome value of 50.8 % [16], supporting the likelihood that the LEE, as with many other PAIs, originated from a bacterial species with a sufficiently different G+C content. Analysis of the sequence predicts 41 ORFs within the LEE island that comprise at

least three major functional regions from left to right: i) a large region encoding a type III secretion apparatus; ii) a central region containing the intimin adhesin encoding *eae* [59], and *tir* (translocated intimin receptor) genes [91]; and iii) a region encoding secreted Esp (*E. coli* secreted proteins) gene products required for signal transduction in host cells. Numerous gene names have also been changed to follow a more standardized nomenclature of type III secretion-encoding genes [23, 177]. Uncharacterized ORFs include a H-NS related regulatory protein (Orf1), situated at the start of the first operon encoding type III secretion genes. HN-S proteins are global regulators that bind to curved DNA regions that are often AT-rich, and it is compelling to speculate that Orf1 may regulate expression of functional genes within the AT-rich LEE. Three ORFs encode proteins that bear similarities to proteins on virulence plasmids of *Shigella* and *Salmonella* spp. Sixteen remaining ORFs exhibit no relevant homologies to current database entries. Orfd4 has recently been characterized and named *espF* [116]. EspF is a proline-rich secreted protein that is exported by the type III secretion apparatus. Non-coding sequences on the strain E2348/69 LEE include previously described remnants of an IS600 transposase-related sequence at the extreme right end [43], a large enterobacterial repeat intergenic consensus (ERIC) sequence at the left end of the LEE, as well as 1 570 bp of deleted DNA adjacent to *selC* that is present in *E. coli* K-12 but absent from sequences flanking the LEE in strain E2348/69 [115]. The absence of these K-12 flanking sequences suggests that the LEE may have inserted in the past through a mechanism involving IS sequences, but that such mobile insertion elements were perhaps excised and truncated owing to recombination or deletion events.

The LEE from EHEC strain EDL933 (O157:H7) has recently been published and compared to the E2348/69 LEE [130]. The EDL933 LEE which is inserted at the *selC*

locus is 43 359 bp in size. This increased size as compared to the E2348/69 LEE is due to a 7.5-kb putative P4 family prophage designated 933L present at the left end of the EDL933 LEE. The integration site for bacteriophage P4 is known to be a tRNA gene [132], and the P4-like retrorhage Φ R73 inserts within the *selC* tRNA gene. [80, 160]. The LEE of three related EHEC1 strains (one O55:H7 and two O157:H7 serovars) described by Wieler et al. [173] also contained prophage sequences, whereas these sequences were absent from E2348/69 and two clonally related O55:H6 EPEC1 strains. Aside from the presence of the prophage 933L, the LEEs of E2348/69 and EDL933 are quite homologous. Both LEE sequences contain 41 conserved ORFs and an average nucleotide identity of 93.9%. Interestingly, individual genes exhibiting lower identity scores and the most non-synonymous base changes included the genes encoding proteins that interact directly with the host (*eae*, *tir*, *espA*, *espB*, *espD*), whereas most of the other genes were highly homologous. Differences between proteins in the EPEC and EHEC LEEs may reflect some of the discrepancies that have been observed in the mechanisms underlying pathogenic adaptation of EPEC and EHEC strains and host specificity [89, 120].

3.3. Sugar islands: group II and III polysaccharide capsule-encoding gene clusters

E. coli that cause extra-intestinal disease in humans and animals are often encapsulated [82, 83]. All known *E. coli* capsules (K antigens) are encoded on the chromosome and are divided into three major groups, based on differences in chemical composition [128]. Genes encoding group I capsules are located near the *rfb* and *his* loci at 45 min on the *E. coli* map and may be produced in varying amounts by most *E. coli* strains [148]. In contrast, groups II and III capsules are encoded by gene clusters of

about 20 kb in size situated near *serA* [126, 167], and are absent from *E. coli* K-12. Certain group II (e.g. K1, K4, K5) and group III (e.g. K54, K10) K antigens are associated with invasive *E. coli* strains causing meningitis, sepsis or urinary tract infections in humans or animals. Some *E. coli* group II or III capsules are also structurally related to capsules of other invasive pathogens such as *Neisseria meningitidis* and *Haemophilus influenzae* [83, 119]. Furthermore, group II or III capsules have been shown to be required for enhanced virulence and invasiveness of extra-intestinal *E. coli* strains [17, 149].

Sequencing of regions flanking the K5 and K1 (group II)- and K54 (group III)-encoding gene clusters has pinpointed the insertion sites to 150 bp 3' of tRNA *pheV* [34, 150] at 67.0 min of the *E. coli* chromosome. The insertion region of PAI-4 from uropathogenic *E. coli* strain J96 is also located at 67.0 in the region of *pheV* (see above) [163]. Recently, it has been shown that the genes specifying synthesis of the group III K54 capsule are inserted within a group II-type gene cluster and were probably acquired through IS110-mediated horizontal gene transfer [150]. Furthermore, group II and III capsular antigen genes have a low G+C content (33–42%) compared to the *E. coli* K-12 chromosome, again suggesting interspecific gene transfer.

Group II and III capsule-encoding gene clusters produce over 80 different polysaccharide surface structural antigens [83]. It is likely that ancestral capsule gene clusters were acquired by horizontal gene transfer near the *pheV* locus and have since evolved by recombination and additional horizontal gene transfer events that have conserved the flanking genes required for proper transport and assembly of these bacterial surface structures. This has resulted in an array of antigenically diverse capsular types among different *E. coli* strains. As these chromosomally encoded group II and III capsular polysaccharide-encoding gene clusters may also contribute to the virulence of pathogenic

E. coli, they can in certain cases also be considered as pathogenicity islands.

3.4. Unique DNA regions specific to *E. coli* causing meningitis or sepsis

Pathogenic *E. coli* that cause meningitis and sepsis in humans belong to a limited group of strains that frequently possess certain virulence traits. In particular, the K1 capsule and certain O serogroups as well as other virulence factors including S/F1C and P fimbrial adhesins, hemolysin, the aerobactin siderophore system and Ibe10 (invasion of brain endothelium) protein are associated with *E. coli* causing neonatal sepsis or meningitis [14, 15, 24, 96, 156].

Bloch et al. have devised a means of determining chromosome physical distance discrepancies between *E. coli* strains, and used this approach to chart an extensive physical map of the genome of the *E. coli* strain RS218 (O18:K1:H7), isolated from neonatal meningitis [18, 147]. This comparative *E. coli* genome analysis system involves the use of engineered Tn10-based mobile markers containing rare-cutting restriction endonuclease (RCP) sites [106, 107] and parallel analysis of *E. coli* RS218 and *E. coli* K-12 restriction fragment sizes by pulsed field gel electrophoresis. IS10 markers in *E. coli* K-12 can be transferred to pathogenic *E. coli* or other strains of interest by bacteriophage P1-mediated transduction, and the sizes of restriction fragments between *E. coli* K-12 and wild-type *E. coli* bearing the RCP cassettes can be determined and compared.

By comparative genome analysis of strain RS218 and *E. coli* K-12 MG1655, it is estimated that the genome of RS218 is 11% greater than MG1655 and contains 12 additional regions of greater than 20 kb that are absent from *E. coli* MG1655 [147]. The *kps* gene cluster encoding the K1 capsule, the *rfb* genes involved in O antigen synthesis, the *sfa* gene cluster encoding the S fimbriae, the *ibe10* gene involved in invasion of the

brain endothelial cell barrier [76], and the temperate λ phage-encoded *bor* gene [7, 8] are now known to be encoded within some of the additional 'loops' or 'bulges' in strain RS218 [17]. The importance of the unique region containing the K1 capsule has been clearly demonstrated in strain RS218 [17]. P1 transduction-mediated construction of a RS218-K-12 chimera bearing a replacement of the unique region encompassing the K1 capsule resulted in lack of invasiveness in newborn rats as compared to the wild-type RS218 strain. In addition, both the chimera and RS218 strain grew equally well in rich or minimal medium indicating that lack of invasiveness was not due to non-specific alterations to the physiology of the replacement mutant. Whether some of the other regions present in strain RS218 and other invasive *E. coli* strains should be termed pathogenicity islands is a question that requires further elucidation. It is probable that some of these greater than 20-kb regions were acquired by horizontal gene transfer; however, further studies will be needed to determine whether these regions contribute to the pathogenesis of meningitis and septicemia.

3.5. Unique regions of avian pathogenic *E. coli*

Avian pathogenic *E. coli* (APEC) comprise a limited set of *E. coli* strains, particularly of serogroups O78, O2 and O1, which are associated with respiratory disease (airsacculitis), septicemia and other pathologies in poultry [39, 64]. Virulence factors known to be associated with APEC include aerobactin production, resistance to serum and the production of adhesins [38, 44, 45, 97, 113, 135]. The mechanisms by which APEC cause poultry disease are largely unknown.

To determine the genetic basis for the virulence of APEC, Brown and Curtiss [26] utilized a subtractive DNA hybridization technique to identify regions in the genome

of the APEC strain χ 7122 (O78:H7:K80) that are not present in *E. coli* K-12. By using this approach, 12 unique sequence islands (USIs) from strain χ 7122 were identified and were mapped by hybridization to the *E. coli* K-12 ordered Kohara phage library [94]. Four of these unique regions in proximity to 0, 6.1, 45 and 66.6 min were replaced by using sequential P1 phage transduction with lysates from *E. coli* K-12 strains bearing different antibiotic resistance markers flanking either side of the unique regions. Two of these regions, the 45.0-min region, encoding the O78 *rfb* gene cluster, and the 0.0-min region, encoding as yet uncharacterized genes were necessary for virulence of strain χ 7122 in an intra-air sac infection model. Replacement of other χ 7122 USIs with *E. coli* K-12 DNA, determination of whether any of these regions are required for virulence, and determination of the sizes and further characterization of these unique regions are currently underway in our laboratory.

3.6. Pathogenicity islands present in *Yersinia* spp. and certain *E. coli* pathotypes

Highly pathogenic strains of *Yersinia* spp. comprise *Y. pestis* (the plague bacillus), *Y. pseudotuberculosis* and *Y. enterocolitica* biotype 1B. In addition to the presence of virulence plasmids, high-pathogenicity strains contain chromosomal determinants on 'high-pathogenicity islands' (HPI) that are absent from low- or non-pathogenic *Yersinia* spp. *Yersinia* HPIs contain genes involved in iron uptake and storage and are required for virulence in mice [28, 55]. The HPI from *Y. pestis*, also known as the pigmentation (*pgm*) locus [54], spans 102 kb and contains three known virulence-associated factors: i) a hemin storage (*hms*) locus that gives a pigmented phenotype to colonies grown on agar plates containing Congo red [131]; ii) *irp1* and *irp2* [105] and *ybtT* and *ybtE* [10] genes associated with

yersiniabactin production; and iii) the *fyuA* (*psn*) gene that encodes the yersiniabactin receptor and also acts as a receptor for the bacteriocin pesticin [72]. The *Y. pestis* HPI is flanked by IS100 sequences [134] and can frequently delete from the chromosome [54, 55]. Loss of the HPI results in loss of pigmentation and reduced virulence in subcutaneously infected mice [81].

Highly pathogenic *Y. pseudotuberculosis* and *Y. enterocolitica* 1B are pigmentation-negative and contain truncated HPIs. These smaller HPIs span 45 kb and contain the yersiniabactin production locus – *irp1*, *irp2*, *ybt* homologues (*irp4* and *irp5*) [129] and *fyuA* genes – but lack the *hms* gene and IS100 flanking sequences [29, 140]. HPIs in *Y. pseudotuberculosis* and *Y. enterocolitica* are more stable than the larger *Y. pestis* HPI, although spontaneous deletion of either *fyuA* alone or both *fyuA* and *irp2* can occur [29, 134]. The HPI of *Y. enterocolitica* 1B strain Ye 8081 has been further characterized and compared with other *Yersinia* HPI [29]. In addition to the *irp-fyuA* gene cluster, a gene encoding tRNA-Asn, almost identical to the *asnT* gene of *E. coli*, and single copies of four other repeat sequence (RS) elements that include IS1328 and IS1400 were identified. The HPI was conserved for the most part among other *Y. enterocolitica* strains except for the 15-kb stretch that contains three of the RS elements located downstream of *fyuA*. It is hypothesized that the *asn* tRNA gene and/or RS sequences on the outskirts of the HPI may play some role in the instability of the island leading to excision of part or all of the HPI in certain strains [29, 139].

Two lineages of the *Yersinia* HPI have been established based on DNA sequence comparisons: a *Y. enterocolitica* 1B group and a *Y. Pestis/Y. pseudotuberculosis* group [140]. Interestingly, four pesticin-sensitive *E. coli* strains were shown to contain functional *Yersinia irp2* and *fyuA* genes related to the *Y. pestis* lineage. A more extensive investigation has demonstrated that certain pathogenic *E. coli* from humans contain the

irp-fyuA HPI of *Yersinia* spp. [152]. HPI sequences were frequent in enteroaggregative *E. coli* (EA/EC) (93 %) and blood culture isolates (80 %), were less frequent in enteroinvasive *E. coli* (EIEC) (27 %), enteropathogenic *E. coli* (EPEC) (5 %) and enterotoxinogenic *E. coli* (ETEC) (5 %), and were absent from enterohemorrhagic *E. coli* (EHEC), *Shigella* and *Salmonella* strains. Production of *irp2*- and *fyuA*-encoded proteins was also demonstrated in HPI-containing *E. coli*. The *hms* locus of *Y. pestis* was absent from all HPI-positive *E. coli*, although the *irp2* and *fyuA* sequences from *E. coli* were closer to the *Y. pestis* lineage of the HPI and were also associated with the *asn* tRNA gene. However, insertion location of HPI sequences within the *E. coli* genome has not been determined. As with the *Yersinia* spp. some *E. coli* strains (3 %) containing *irp2* genes exhibited *fyuA* deletions. The *fyuA-irp* genes have a G+C content of 56–59 % which is higher than that of the overall G+C content of *Yersinia* (46–50 mol %) or *E. coli* (50.8 %). Hence, it is likely that the HPI pathogenicity island associated with an *asn* tRNA gene has disseminated from an organism with a higher G+C content to *Yersinia* spp. and to certain pathogenic *E. coli* strains either directly or sequentially. Clearly the HPI-encoded sequences contribute to the virulence of *Yersinia* species. Whether HPI-encoded proteins contribute to the virulence of pathogenic *E. coli*, particularly EA/EC or septicemia-causing *E. coli* from humans remains to be determined. At present, the presence of HPI-associated gene clusters in pathogenic *E. coli* or commensal *E. coli* strains from animals has yet to be investigated.

3.7. Pathogenicity islands in *Shigella*

Shigella spp. are associated with invasive intestinal disease, watery diarrhea and dysentery in humans and other primates and comprise four different groups – *S. dysen-*

teriae, *S. flexneri*, *S. boydii* and *S. sonnei* – all of which can be considered as clones of *E. coli* [123, 136]. *Shigella* spp. invade the colonic epithelium, and elaborate one or more secretory enterotoxins, phenotypes that are encoded by both plasmid and chromosomal loci [127]. In addition, to virulence plasmid-encoded genes, PAIs have been identified on the chromosome of *Shigella flexneri* 2a [137, 138]. A 99-kb deletion from the chromosome of *Shigella flexneri* 2a strain YSH6000 was identified and localized between the *Shigella ompA* and *pyrC* gene homologues situated on the *NotI* fragment D of the *Shigella* physical map [124] (22–24 min on *E. coli* K-12) [137]. Loss of this 99-kb fragment resulted in a reduction in contact hemolysin activity and rendered the strain sensitive to ampicillin, chloramphenicol, streptomycin and tetracycline.

The 99-kb *S. flexneri* 2a deletion mutant was used to identify a second *Shigella flexneri* PAI with a capacity to spontaneously delete from the chromosome by using an ‘island probing’ counterselection method [138]. A *tetAR* cassette, encoding tetracycline resistance was inserted within the *she* gene of the Tet^S 99-kb deletion mutant by homologous recombination. The *she* gene encodes the ShMu protein, a member of the IgA protease-like autotransporters, and also encompasses the *set1a* and *set1b* genes encoding the *Shigella* enterotoxin 1 (ShET1) [53, 138]. Tet^S revertants were selected for by growing cells on plates containing fusaric acid [110], and were obtained at a frequency of 10⁻⁵ to 10⁻⁶. Restriction digests migrated on pulsed-field gels demonstrated that these Tet^S revertants had lost a region of ~51 kb from their genome that was located on *NotI* fragment D of the *Shigella flexneri* physical map [124]. A total of 25 kb flanking either side of *she* was sequenced and found to contain a second IgA-protease autotransporter-related gene designated *sigA*, complete copies of IS2 and IS600 and an IS629 sequence that was disrupted by an *E. coli* chromosomal group II

intron-like sequence. Flanking regions from this strain were specific to *she*⁺ *Shigella* strains, indicating that the *she* PAI is specific to a limited group of *Shigella* strains. These findings correlate with the fact that ShET1 is almost exclusively associated with *S. flexneri* 2a strains [122].

3.8. Sometimes less is more: ‘black holes’ in *Shigella* and enteroinvasive *E. coli*

Unlike most pathogenic or commensal *E. coli* strains, enteroinvasive *E. coli* (EIEC) and *Shigella* spp. are typically unable to ferment lactose and also lack the *cadA* gene that encodes lysine decarboxylase (LDC). Recently, Maurelli et al. [112] identified large genomic gaps they termed ‘black holes’ in the region containing *cadA*, in *Shigella* spp. and EIEC. To investigate whether lack of LDC production contributes to the pathogenicity of *Shigella flexneri* 2a, Maurelli et al. [112] initially attempted to introduce *cadA* into *S. flexneri* by P1 phage transduction with an *E. coli* K-12 lysate. No LDC⁺ transductants were obtained, however, suggesting that *S. flexneri* may have a large deletion compared to *E. coli* K-12 that precludes legitimate recombination in the region encompassing *cadA*. Alternatively, a plasmid bearing a copy of *cadA* was transformed into *S. flexneri* rendering it LDC⁺. LDC activity had no effect on the capacity of *S. flexneri* to invade and lyse cells in culture; however, *cadA*⁺ *S. flexneri* exhibited little enterotoxin activity and was attenuated in virulence. The specific inhibitor of ShET1 and ShET2 enterotoxin activity was shown to be cadaverine, a metabolic product of *cadA*-mediated lysine decarboxylation.

Physical enzyme restriction mapping demonstrated that the *S. flexneri* 2a strain has a large deletion of ~190 kb compared to *E. coli* K-12 in the region surrounding *cadA* (93.9 min on *E. coli* K-12), and large gaps in *Shigella* spp. and EIEC were deter-

mined using hybridization probes spanning the *cadA* region from *lexA* (91.7 min) to *vacB* (95.0 min.). These findings illustrate that genomic deletions or 'black holes' and loss of a metabolic gene present in non-pathogenic *E. coli* as well as the acquisition of virulence genes encoded on plasmids, phages or PAIs can contribute to the adaptation of *E. coli* (*Shigella* spp.) to a specific pathogenic lifestyle. The search for other 'black hole' genomic deletions by comparative physical mapping and analysis of biochemical or physiological differences between closely related pathogenic and non-pathogenic counterparts or related pathogens that exhibit tropisms for different host species merits further investigation.

4. CONCLUSION – DISCOVERING AND EXPLORING UNCHARTED ISLANDS

Throughout the descriptions of PAIs presented above various approaches were taken to identify and characterize different *E. coli* PAIs. Methods to detect the loss of virulence-associated genes and to first identify PAIs included the detection of the spontaneous loss of the hemolysin phenotype in UPEC strains 536 and J96 phenotypes [67, 68], as well as the island-probing method that uses counterselection for tetracycline-sensitive revertants following insertion of a *TetAR* cassette into a specific gene on the chromosome [138]. A number of other counterselectable markers that may be adapted for screening potentially unstable PAIs has recently been presented by Reyrat et al. [143].

Screening methods such as island probing combined with physical genome analysis by pulsed-field electrophoresis may be very beneficial for initially detecting and sizing unstable regions that are potentially PAIs and in analyzing their mechanisms of spontaneous deletion. The determination of the role that newly identified USIs may contribute to virulence without knowing the

equivalent *E. coli* K-12 DNA sequences bordering them are, however, difficult to assess. Spontaneous deletions in strains, as demonstrated by the studies of Ritter et al. [145, 146], may involve the disruption of tRNA genes that affect global regulation. For this reason, the replacement of USIs by the corresponding flanking *E. coli* K-12 DNA by P1 phage-mediated transduction [17, 26] or other methods to delete large and defined chromosomal regions [87, 165] may be more suitable for determining whether USIs contribute to the pathogenicity of particular *E. coli* strains.

The availability of the complete *E. coli* K-12 genome [16] as well as refined methods for genetic manipulation of *E. coli* and analysis of genomes by physical mapping and pulsed-field electrophoresis are a tremendous advantage for characterizing and determining the location of PAIs or black holes in *E. coli* and *Shigella* [18, 112, 138, 147]. Other recent methods that select for specifically unique sequences or genes required for survival or virulence in the host may also lead to the discovery of new USIs or black holes that may contribute to the pathogenicity of *E. coli* or other bacterial pathogens. Examples of such technologies include subtractive DNA hybridization [26, 49] and subtractive RNA hybridization [133, 166], signature tagging [32, 155], in vivo expression technology [35, 36, 104, 169], representational difference analysis [27, 30], and mRNA differential display [3, 179]. Furthermore, DNA sequencing of PAIs [50, 65, 163] and computer-assisted sequence comparisons will provide insights into the mechanisms through which PAIs may have been acquired as well as the possible functions of PAI-encoded proteins. The use of infection studies using animal models or cell and tissue cultures should provide an indication of the roles such PAI-encoded genes may play in pathogenesis. However, as there may be a number of homologous or heterologous loci encoding for common virulence attributes within the genome of particular pathogenic *E. coli* strains, dele-

tion of individual USIs that may contribute cumulatively to the pathogenic capacity of a strain may not dramatically alter the virulence of a strain. Determining whether sequences encoded by USIs are common to particular pathogenic strains should also provide an indication as to whether these DNA sequence regions may promote the pathogenicity of these strains, and will provide insight into what gene products may be potentially useful for the development of protective vaccines against these pathogens.

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