tDNA locus polymorphism and ecto-chromosomal DNA insertion hot-spots are related to the phylogenetic group of *Escherichia coli* strains

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tRNA-encoding genes (tDNA) are known hot-spots for the integration of ecto-chromosomal DNA (ECDNA) including genomic islands. However, only a few loci are currently known to be targeted by such insertions in *Escherichia coli*. A PCR-based screening of tDNA integrity was therefore performed on a collection of *E. coli* strains in order to identify tDNA loci that are most frequently intact and those that are preferred ECDNA insertion sites. It was shown that only a subset of tDNAs were hot-spots for ECDNA insertions, and that the majority of loci were never targeted by such insertions. Polycistronic tDNAs, highly transcribed tDNAs or tDNAs encoding tRNAs recognizing frequently used codons were generally not targeted by ECDNA insertions. Most interestingly, strains of different ECOR groups showed different patterns of tDNA loci polymorphism. More subtle differences were also observed between strains of different pathotypes.

INTRODUCTION

Escherichia coli is a very diverse species including both commensal and pathogenic strains. Pathogenic *E. coli* strains have been classified into intestinal or extra-intestinal pathogens according to their phenotypes and origin (Kaper *et al.*, 2004). Among these extraintestinal pathogens are strains responsible for diseases in humans, such as uropathogenic *E. coli* (UPEC) or newborn meningitis *E. coli* (NMEC) strains, but also in avian species, such as avian pathogenic *E. coli* (APEC) strains (Ron, 2006).

The diversity of the *E. coli* species has been studied in great detail and six major phylogenetic groups (called ECOR groups A, B1, B2, C, D and E) have been identified (Escobar-Paramo *et al.*, 2004; Lecointre *et al.*, 1998; Ochman &

Selander, 1984; Selander *et al.*, 1987). This diversity of *E. coli* is closely related to the plasticity of the *E. coli* genome, whereby a strain can acquire or lose a specific function through horizontal gene transfers or mutational events (Hacker *et al.*, 2003). From genome sequence data one can define a 'core' gene pool containing the genetic information required for essential cellular functions and a 'flexible' gene pool made of ecto-chromosomal DNA (ECDNA) and conferring upon the bacteria more specific traits necessary to adapt to certain niches (Dobrindt *et al.*, 2004; Hayashi *et al.*, 2001; Welch *et al.*, 2002). ECDNA regions, such as genomic islands, may confer various new properties including pathogenicity, antibiotic resistance and fitness traits (Dobrindt *et al.*, 2004; Dozois & Curtiss, 1999) and are frequently found downstream of tDNA loci (DNA encoding tRNA – for a list of tDNA-associated genomic islands in *E. coli* strains see Supplementary Table S1, available with the online version of this paper).

Among the numerous tDNAs present in the genome of *E. coli* only a few are currently known to be targeted by ECDNA insertions (see Table S1) (Blattner *et al.*, 1997; Hayashi *et al.*, 2001). One can therefore ask whether all tDNA loci are potential targets for ECDNA insertions. This study reports a PCR-based screening of the integrity of a subset of tDNA loci in the genome of a collection of extraintestinal

Abbreviations: APEC, avian pathogenic *E. coli*; EAEC, enteroaggregative *E. coli*; ECDNA, ecto-chromosomal DNA; EHEC, enterohaemorrhagic *E. coli*; EIEC, enteroinvasive *E. coli*; ETEC, enterotoxigenic *E. coli*; EPEC, enteropathogenic *E. coli*; ExPEC, extra-intestinal pathogenic *E. coli* of human origin; IPEC, intestinal pathogenic *E. coli*; MFCA, multiple factorial analysis of correspondences; NMEC, newborn meningitis *E. coli*; NP, non-pathogenic *E. coli*; UPEC, uropathogenic *E. coli*.

Three supplementary tables and two supplementary figures are available with the online version of this paper.

pathogenic *E. coli* strains of avian (APEC) or human (ExPEC) origin as well as on a subset of intestinal pathogenic *E. coli* (IPEC) or faecal isolates.

METHODS

Strains and growth conditions. Strains used in this study are described in Supplementary Table S2. To get information on potential ECDNA regions present in specific isolates an emphasis was placed on APEC and, to a lesser extent, on ExPEC strains. IPEC and faecal isolates were also included in order to unravel any very significant difference from other isolates. A significant number of strains from each of the four major phylogenetic groups are present in our subset. APEC strains were collected in France, Spain and Belgium between 1991 and 1999 (EU contract FAIR6 98-4093) (Stordeur *et al.*, 2002). They were isolated from birds harbouring colibacillosis lesions and were confirmed to be pathogenic using the day-old chick model (Dho & Lafont, 1984). All 22 APEC strains were epidemiologically unrelated (Stordeur *et al.*, 2002). The ExPEC and IPEC isolates analysed in this study as well as faecal isolates and strains from the ECOR collection (*E. coli* reference collection) were from the strain collection of the Institut für Molekulare Infektionsbiologie, Universität Würzburg, and have been described before (Boyd & Hartl, 1998; Dobrindt *et al.*, 2001; Ochman & Selander, 1984). IPEC isolates included in this study belonged to different pathotypes, with one enteropathogenic (EPEC), seven shiga-toxin producing *E. coli* including the EHEC strain EDL933, one enterotoxigenic (ETEC), one enteroaggregative (EAEC) and one enteroinvasive (EIEC) strain. Bacteria were grown in Luria–Bertani (LB) broth at 37 °C with agitation unless otherwise stated.

Sequence analysis. Sequences of the entire genome of *E. coli* K-12 strain MG1655, the uropathogenic strain CFT073 and the enterohaemorrhagic strains Sakai and EDL933 (referred to in the text as MG1655, CFT073, Sakai and EDL933 respectively) were downloaded from the NCBI server (<http://www.ncbi.nlm.nih.gov/>) and analysed using the VectorNTI software (Invitrogen).

Chromosomal DNA extraction. Chromosomal DNA was purified from 1 ml overnight culture using the QIAamp DNA mini kit (Qiagen). Chromosome quality was checked by 1 % agarose gel electrophoresis.

Primers and PCR conditions used for tDNA screening. A subset of tDNA loci likely to be associated with ECDNA was selected out of the 85 tDNAs located in the *E. coli* core genome (except for *ileY* all the other 85 tDNA loci of strain MG1655 were also present in the genome of strains CFT073, Sakai and EDL933). Because of their stability and because their disruption might adversely affect the growth of strains or their adaptation to changing physiological conditions, tDNAs located inside rDNA (DNA corresponding to rRNA) operons were not studied further (Anton *et al.*, 1998; Asai *et al.*, 1999; Condon *et al.*, 1995). Similarly, insertion inside polycistronic tDNA was considered unlikely since it might disturb the processing of the transcribed RNA into tRNA (King *et al.*, 1986; Li & Deutscher, 1996, 2002; Morl & Marchfelder, 2001). For this reason, when dealing with polycistronic tDNAs, only the last tDNA was selected.

Primer pairs used in this study, and PCR conditions, are listed in Supplementary Table S3. Primers were designed so that their sequences were present in the genome of all four strains (MG1655, CFT073, EDL933 and Sakai). In all cases, one of the primers was located just 5' of the tDNA analysed. The second primer was located downstream of the tDNA analysed in a region common to all four strains. *pheV* was the only exception since the design of the second primer would then have required amplification of DNA fragments larger than

30 kb in the four sequenced strains and presumably in a significant number of the other tested strains. This situation has also been described by Ou *et al.* (2006). Primer pheV2 was therefore designed so that its sequence is present in strains MG1655 and CFT073 but not in strains EDL933 and Sakai. Because the order of the *asnUVW* tDNAs is not identical in the four strains (*asnWUV* in strains MG1655, EDL933 and Sakai, *asnVUW* in strain CFT073) one set of primers was designed to encompass the entire *asnUVW* region and it was considered as a single locus in the rest of the study.

PCR reactions (50 µl) were performed using an Applied Biosystems 9700 PCR apparatus and were set up in 1 × buffer (ThermoPol buffer; New England Biolabs) using 1 U *Taq* DNA polymerase (New England Biolabs), 200 µM of each dNTP, 0.8 µM of each primer and 100 ng of chromosomal DNA. Cycling conditions were as follows: 1 cycle of 5 min at 95 °C, 30 cycles of 30 s at 95 °C, 30 s at 52 °C and 1 min per kb at 72 °C, and a final extension of 5 min at 72 °C. PCR products were separated on 1 % agarose gels for 1 h at 10 V per cm of gel.

For amplification of long PCR fragments, the LA PCR kit Ver2.1 (Takara) was used and cycling conditions were modified according to the manufacturer's instructions (see Table S3 for details).

Scoring scheme. An integrity score was calculated for each locus. A value of 0 was given for a negative PCR result and 1 for the presence of a PCR product, whatever its size. The integrity score of a locus was then calculated by summing the values obtained for this locus in all 54 strains.

ECOR grouping. Classification into phylogenetic groups A, B1, B2 and D was based on the amplification of genes *chuA* and *yjaA*, and of fragment TspE4C2.1, with three primer pairs: *chuA1/chuA2*, *yjaA1/yjaA2* and TspE4C2.1/TspE4C2.2, as described by Clermont *et al.* (2000).

Statistical analysis. The probability of a tDNA locus being targeted by an ECDNA insertion was equal to 1/(score of locus). When fragments of different size were observed at one locus, the distribution of these sizes was compared to the ECOR group of strains using a χ^2 test and exact *P*-values (referred to as 'Size/ECOR group *P*-value') were calculated with the StatXact software (version 5.0, Cytel Software).

tDNA usage. Data concerning the codons recognized by one tRNA were collected from the *E. coli* MG1655 annotated genome (GenBank accession no. U00096). The frequency of each codon per thousand codons was calculated based on the frequency of codons in a set of 27 very highly expressed genes described by Sharp & Li (1986). tDNA usage was then calculated by summing the frequency of the codons recognized by the corresponding tRNA.

Multiple factorial analysis of correspondences (MFCA) and hierarchical clustering. A factorial analysis of correspondences of the PCR results data was undertaken using the SPAD 6.0 software (Decisia, France). For each locus, the value corresponding to the approximate size of the PCR product was used; an absence of PCR product was given the value 0. A modality was defined as a size in bp that a PCR product can take at one locus (e.g. *asnUVW* = 5300). A strain and a modality were defined by their coordinates, the coordinates of a strain being its 34 modalities (i.e. the size of the PCR product obtained at each locus) and the coordinates of a modality being whether or not it is obtained for each of the 54 strains. The coordinates obtained for each strain and each modality were then transformed using matrices into new coordinates onto several factorial axes, each strain (or modality) having one coordinate on each factorial axis. For each factorial axis, using the coordinate of each strain (or modality) on this axis, a variance of the distribution of the strains (or modalities) was then calculated. Factorial axes were

then ordered by decreasing level of variance, axis F1 being the axis for which the variance in the distribution of strains was the highest, F2 being the axis with the second highest variance, and so on.

Results were then represented graphically onto planes described by the F1/F2 axis and F1/F3 axis using the factorial coordinates of each modality. Data concerning the ECOR group and the pathotype were considered as supplementary observations and projected on the F1/F2 plane. From the factorial coordinates on the first five factorial axes obtained by the MFCA analysis, distances between strains were calculated and an ascending hierarchical clustering was performed using Ward's criteria (SPAD 6.0).

RESULTS

Identification of tDNA targeted by ECDNA insertions

Our analysis was restricted to a subset of 36 tDNAs belonging to the core genome, not located inside rDNA operons, and either monocistronic or the last tDNA of a polycistronic operon.

Complete results of the tDNA screening are summarized in Tables 1 and 2. Profiles obtained with strains MG1655 (line 11), CFT073 (line 33) and EDL933 (line 50) were consistent with the published sequences (Hayashi *et al.*, 2001; Perna *et al.*, 2001; Welch *et al.*, 2002) (see Tables 1, 2 and S3).

Except for *pheV*, a positive PCR result was considered as reflecting the absence of insertion of ECDNA region. Conversely, we assumed that a negative PCR most likely indicated that primers were too far apart to allow any amplification in our conditions, thus suggesting the presence of an ECDNA region. The integrity score calculated for each locus was consequently an indication of how frequently this locus was a site for ECDNA insertions. Loci with a high score are presented in Table 1: actually, a majority of loci (19/36) had high scores (≥ 47). For these loci the probability that a negative PCR was observed was below 1/47, i.e. 2.2 %. These loci are therefore unlikely to be targeted by ECDNA insertions.

Loci with a low score are presented in Table 2 and can be considered preferred sites for ECDNA insertions; the six loci with the lowest scores were *aspV*, *pheV*, *leuX*, *thrW*, *metV* and *asnT*. Our scoring scheme was validated by the fact that tDNA loci known as ECDNA insertion hot-spots such as *leuX* (score = 13), *metV* (score = 14), *asnT* (score = 17) and *selC* (score = 36) were given low scores (Redford & Welch, 2002).

In most cases high scores were correlated with a low level of variability of the tDNA, with no more than two or three different sizes of PCR product. However, a high diversity in PCR product size was observed for loci with high scores such as *lysQ* (13 different sizes), *tyrV* (10 different sizes), *argQ* (9 different sizes) and *leuV* (5 different sizes) (see Supplementary Fig. S1 for examples). This suggests that these loci, while not being frequent sites of ECDNA

insertions, are nevertheless highly variable regions. Such polymorphism is observed when comparing these same regions in strains MG1655, CFT073 and EDL933 (see Table S3).

Polycistronic tDNAs have high integrity scores

In order to explain why certain tDNAs were generally intact while others seemed to be frequently targeted by ECDNA insertions we investigated whether this could be related to codon usage, tDNA transcription rate or organization of the corresponding region. Indeed one hypothesis could be that tDNAs with the lowest scores encoded tRNAs recognizing rare codons or less transcribed tRNAs. For that purpose, a tDNA usage score was calculated based on data from Sharp & Li (1986) (see Methods) and estimated transcription rate data from Ardell & Kirsebom (2005) were used. According to our results highly transcribed tDNAs or tDNAs encoding tRNA recognizing frequently used codons (high tDNA usage) tend to have high integrity scores (Fig. 1a, b). However, we could not show a direct relationship between integrity score and either codon usage or tDNA transcription rate. More striking differences were seen when we compared the organization of the tDNAs (monocistronic vs polycistronic): we found that polycistronic tDNAs generally had high scores, indicating that they were unlikely targets for ECDNA insertion (Fig. 1b). Indeed, *metV* was the only tDNA with a low score (score = 14) and a polycistronic organization (Fig. 1b, arrowhead). Interestingly, the highly variable high-integrity loci mentioned above (i.e. *argQ*, *leuV*, *lysQ* and *tyrV*) are all polycistronic loci.

tDNA screening profiles reflect the diversity of *E. coli*

We next wanted to know if the pattern of amplification at tDNA loci for a particular strain was related to either its pathotype or its ECOR group. A χ^2 test was performed to compare the distribution of PCR product sizes between the four ECOR groups. For loci *lysV*, *lysQ*, *argQ*, *tyrV*, *leuV*, *proL*, *leuZ*, *serW*, *selC*, *serX*, *asnUVW*, *glyU*, *asnT*, *serU*, *argW*, *argU* and *metV* this distribution was very unlikely to be random ($P < 0.001$ or $P < 0.01$). Therefore, strains from different ECOR groups are significantly different at several tDNA loci.

A multiple factorial analysis of correspondences (MFCA) was performed to unravel potential associations (Fig. 2). Figs 2(a) and 2(b) depict the distribution of the different PCR product sizes listed in Tables 1 and 2 in two factorial planes: plane F1/F2 defined by the two factorial axes F1 and F2, and plane F1/F3 defined by axes F1 and F3. Axes F1, F2 and F3 were the three most discriminating factorial axes, explaining respectively 12.14 %, 7.96 % and 6.22 % of the distribution. In both Fig. 2(a) and Fig. 2(b), two modalities being close to each other indicates an association of these two modalities; the larger the symbols of these two modalities, the stronger the association. For example, Fig. 2(a) indicates that a fragment of 2700 bp at *glyU* is frequently

Table 1. tDNA screening results (integrity score ≥ 47)

Approximate sizes of PCR products in bp are indicated. tDNAs are ordered by decreasing score values (see Methods). '–' indicates no amplification. When an amplification was observed, the size of the PCR product in bp is indicated. In a few cases two PCR products were observed: both sizes are indicated. The smallest fragment obtained for each locus is in bold. Strains are sorted according to their ECOR group. Size/ECOR group *P*-value indicates whether the distribution of the different PCR sizes products between the different ECOR groups is random or not. *P*-values were calculated using a χ^2 test and the StatXact software (version 5.0, Cytel Software). NA, Not applicable; NS, non-significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$.

	Strain	ECOR	Pathotype	<i>valW</i>	<i>thrT</i>	<i>proM</i>	<i>proK</i>	<i>metY</i>	<i>lysV</i>	<i>lysQ</i>	<i>glyY</i>	<i>aspU</i>	<i>argQ</i>	<i>alaX</i>	<i>glnX</i>	<i>leuU</i>	<i>ileX</i>	<i>tyrV</i>	<i>leuV</i>	<i>serT</i>	<i>proL</i>	<i>leuZ</i>
1	BEN 0014	A	APEC	630	580	550	890	630	5000	1750	600	300	1450	630	600	690	1100	1000	1230	630	900	670
2	BEN 0278	A	APEC	630	580	550	890	630	5200	1700	600	300	1450	630	600	690	1100	1200	1230	630	900	670
3	BEN 1588	A	APEC	630	580	550	890	630	5200	1850	600	300	1450	630	600	690	1100	1200	1250	630	900	–
4	MT458	A	APEC	630	580	550	1050	630	5200	1750	600	300	1450	630	700	690	–	1000	1230	630	900	670
5	DPA065	A	EAEC	630	580	550	890	630	5000	1850	600	300	1450	630	600	690	1100	1200	1150	630	–	670
6	5720/96	A	EHEC	630	580	550	890	630	5000	1850	600	300	1450	630	600	690	1100	1200	1230	630	900	670
7	EDL1284	A	EIEC	630	580	550	890	630	5000	1330	600	300	1600	700	600	690	1100	2000	–	630	900	670
8	C9221a	A	ETEC	630	580	550	890	630	5000	1330	600	300	1450	630	600	690	1100	1050	1250	630	900	670
9	ECOR1	A	Faecal	630	580	550	1050	630	5000	1850	600	300	1450	630	600	690	1100	1200	1250	630	900	670
10	ECOR25	A	Faecal	630	580	550	890	630	5000	1850	600	300	1450	630	600	690	1100	1200	1250	630	900	670
11	MG1655	A	NP	630	580	550	1050	630	5200	1850	600	300	1480	630	600	690	1100	1200	1230	630	900	670
12	1639/77	B1	EHEC	630	580	550	890	630	5200	1750	600	300	1400	630	600	690	1100	870	1230	–	–	670
13	4797/97	B1	EHEC	630	580	550	890	630	1000	1750	600	300	1450	630	600	690	1100	500	1230	–	–	670
14	ECOR28	B1	Faecal	630	580	550	1050	630	1000	1750	600	300	1350	630	600	690	1100	870	1350	630	900	670
15	ECOR58	B1	Faecal	630	580	550	1050	630	1000	1620	600	300	1450	630	–	690	1100	1000	1230	630	900	670
16	ECOR66	B1	Faecal	630	580	550	890	630	5000	1330	600	300	1200	630	600	690	1100	1250	1150	630	250	670
17	BEN 0079	B2	APEC	630	580	550	890	630	5000	1620	600	300	1200	630	600	690	1100	2000	1150	630	250	670
18	BEN 0098	B2	APEC	630	580	550	890	630	5000	1150	600	300	1200	630	600	690	1100	2000	1230	630	250	670
19	BEN 0100	B2	APEC	630	580	550	890	630	5000	1150	600	300	1200	630	600	690	1100	2000	1150	630	900	670
20	BEN 0134	B2	APEC	630	580	550	890	630	5000	1330	1000	300	1200	500	600	690	1100	2000	1150	630	250	670
21	BEN 0277	B2	APEC	630	580	550	890	630	5000	1330	600	300	1200	630	600	690	1100	2000	–	630	250	670
22	BEN 0288	B2	APEC	630	580	550	880/1050	630	5000	1330	600	300	1200	630	600	690	1100	2000	–	630	250	670
23	BEN 0338	B2	APEC	630	580	550	1050	630	5000	1260	600	300	1050	630	600	690	1100	1850	1230	630	250	670
24	BEN 0371	B2	APEC	630	580	550	890	630	5000	1330	600	300	1200	500	600	690	1100	2000	1150	630	250	670
25	BEN 0374	B2	APEC	630	580	550	1050	630	5000	1330	600	300	1200	630	600	690	1100	2000	1150	630	250	670
26	BEN 0591	B2	APEC	630	580	550	890	630	5200	1080	600	300	1200	630	600	690	1100	2000	1230	–	250	670
27	BEN 1567	B2	APEC	630	580	550	890	630	5000	1330	600	300	1200	630	600	690	–	2000	1150	630	250	670
28	BEN 2908	B2	APEC	630	580	550	890	630	5000	1470	600	300	1200	630	600	690	1100	1850	1150	630	250	670
29	MT 140	B2	APEC	630	580	550	1050	630	5000	1470	600	300	1050	500	600	690	1100	1850	1150	630	250	670
30	E2348/69	B2	EPEC	630	580	550	890	630	5000	1620	600	300	1100	630	600	690	1100	–	1250	630	250	670
31	536	B2	ExPEC	630	580	550	890	630	5000	1330	600	300	1600	700	600	690	1100	2000	–	630	250	670
32	ABU83972	B2	ExPEC	630	580	550	890	630	5000	1300	600	300	1200	630	600	690	1100	–	1150	630	–	670
33	CFT073	B2	ExPEC	630	580	550	890	630	5000	1310	600	300	1350	630	600	690	1100	–	1150	630	250	670
34	IHE3034	B2	ExPEC	630	580	550	890	630	5000	1330	600	300	1200	630	600	690	1100	2000	1150	630	250	670

Table 1. cont.

	Strain	ECOR	Pathotype	valW	thrT	proM	proK	metY	lysV	lysQ	glyY	aspU	argQ	alaX	glnX	leuU	ileX	tyrV	leuV	serT	proL	leuZ
35	J96	B2	ExPEC	630	580	550	890	630	5000	1330	650	300	1600	700	600	690	1100	2000	–	630	250	670
36	P42	B2	ExPEC	630	580	550	890	630	5000	1330	600	300	1450	630	600	690	1100	–	1150	630	250	670
37	RS218	B2	ExPEC	630	580	550	890	630	5000	1330	600	300	1100	630	600	690	1100	2000	1150	630	250	670
38	W1825	B2	ExPEC	630	580	550	890	630	5000	1330	600	300	1200	630	600	690	1100	2000	1150	630	250	670
39	764	B2	Faecal	630	580	550	890	630	5000	1330	600	300	1200	630	600	690	1100	2000	1150	630	250	670
40	ECOR51	B2	Faecal	630	580	550	890	630	5000	1450	600	300	1200	630	600	690	1100	–	1150	630	250	670
41	F54	B2	Faecal	630	580	550	890	630	5000	1330	600	300	1200	500	600	690	1100	1250	1150	630	–	670
42	BEN 0071	D	APEC	630	580	550	1050	630	5000	1470	600	300	1250	630	600	690	1100	1000	1150	630	900	–
43	BEN 0280	D	APEC	630	580	550	890	630	5000	1450	600	300	1200	630	600	690	1100	400	1250	630	900	–
44	BEN 0955	D	APEC	630	580	550	890	630	5200	1470	600	300	1450	630	600	–	1100	500	1150	630	250	670
45	BEN 0961	D	APEC	630	580	550	2300	630	5200	1470	600	300	1400	630	600	–	1100	500	1150	630	250	670
46	BEN 1189	D	APEC	630	580	550	2300	630	5200	1470	600	300	1100	630	600	690	–	500	1150	630	250	670
47	493bi	D	EHEC	630	580	550	890	630	4500	1260	600	300	1100	630	600	690	1100	500	900	–	900	–
48	5714/96	D	EHEC	630	580	550	890	630	5000	1620	600	300	1450	630	600	690	1100	870	1230	–	900	–
49	86-24	D	EHEC	630	580	550	890	630	1000	1310	600	300	1250	630	600	690	1100	1000	900	–	900	670
50	EDL933	D	EHEC	630	580	550	890	630	1000	1300	600	300	1250	500	600	690	1100	1000	900	–	900	–
51	ECOR40	D	ExPEC	630	580	550	890	630	5000	1310	600	300	1100	630	700	690	1100	400	1150	630	–	670
52	ECOR50	D	ExPEC	630	580	550	890	630	5000	1670	600	300	1250	630	600	690	–	2000	–	630	900	–
53	ECOR31	D	Faecal	630	580	550	890	630	1200	1470	600	300	1350	630	600	690	1100	830	1230	630	900	670
54	ECOR42	D	Faecal	630	580	550	890	630	1400	1620	600	300	1400	630	600	690	1100	500	1230	630	–	670
	Score			54	54	54	54	54	54	54	54	54	54	54	53	52	50	49	48	47	47	47
	Size/ECOR			NA	NA	NA	NS	NA	***	***	NS	NA	***	NS	*	NS	NS	***	***	*	***	***
	group																					
	P-value																					

Table 2. tDNA screening results (integrity score <47)

See the legend of Table 1 for details.

	Strain	ECOR	Pathotype	<i>serW</i>	<i>selC</i>	<i>serX</i>	<i>serU</i>	<i>pheU</i>	<i>asnUVW</i>	<i>glyU</i>	<i>argW</i>	<i>argU</i>	<i>asnT</i>	<i>metV</i>	<i>thrW</i>	<i>leuX</i>	<i>pheV</i>	<i>aspV</i>
1	BEN 0014	A	APEC	600	2300	3590	5000	850	5300	–	–	1190	–	–	6200	–	–	–
2	BEN 0278	A	APEC	600	2300	3590	800	–	5300	–	6200	1190	–	–	–	–	–	–
3	BEN 1588	A	APEC	–	2300	3590	–	850	5300	–	–	1190	730	970	6200	–	600	–
4	MT458	A	APEC	600	2300	3700	1600	850	5300	–	6200	–	–	–	–	–	–	–
5	DPA065	A	EAEC	600	2300	3590	–	–	5300	–	2900	–	–	970	–	–	–	–
6	5720/96	A	EHEC	600	2300	3590	1600	–	5300	–	–	1190	730	970	–	–	–	–
7	EDL1284	A	EIEC	600	2700	–	800	850	–	–	2900	1190	–	–	–	–	–	–
8	C9221a	A	ETEC	750	2300	3250	400/1500	–	–	3500	2900	–	–	–	–	–	–	–
9	ECOR1	A	Faecal	600	2300	3590	1600	850	5300	–	2900	1190	730	970	–	–	–	–
10	ECOR25	A	Faecal	600	2300	3700	1600	850	–	–	–	1190	730	970	–	5200	–	–
11	MG1655	A	NP	600	2300	3590	1600	850	5300	–	–	–	730	970	–	–	600	2700
12	1639/77	B1	EHEC	600	2300	–	–	–	5300	–	6200	–	–	–	–	–	–	–
13	4797/97	B1	EHEC	600	–	3590	1600	–	5300	–	–	–	730	–	–	–	–	–
14	ECOR28	B1	Faecal	600	2300	3700	–	850	–	–	6200/2900	–	730	970	–	5500	600	–
15	ECOR58	B1	Faecal	600	2300	–	–	850	–	–	2900	1190	730	970	–	–	600	–
16	ECOR66	B1	Faecal	600	2700	3590	800	850	5300	2700	2900	–	–	970	–	–	–	–
17	BEN 0079	B2	APEC	–	2700	–	600/800	850	–	2700	–	–	–	–	–	4800	–	–
18	BEN 0098	B2	APEC	–	2700	400	300/800	–	5300	2700	2900	–	–	–	8300	–	–	–
19	BEN 0100	B2	APEC	–	2700	400	600/800	–	5300	2700	–	3700	–	–	8300	–	–	–
20	BEN 0134	B2	APEC	–	2700	–	600	–	–	2700	–	–	–	–	8300	5500	–	–
21	BEN 0277	B2	APEC	–	2700	400	–	–	–	2700	–	3700	–	–	–	–	–	–
22	BEN 0288	B2	APEC	–	2700	400	600/800	–	–	2700	–	3700	–	–	–	–	–	–
23	BEN 0338	B2	APEC	–	2700	400	800	–	5300	2700	2900	3700	–	–	8300	–	–	–
24	BEN 0371	B2	APEC	950	–	400	800	850	–	2700	2900	–	–	–	–	4800	–	–
25	BEN 0374	B2	APEC	–	2700	–	600/800	–	–	2700	–	–	–	–	6200	4800	–	–
26	BEN 0591	B2	APEC	–	2700	400	600/800	–	5300	2700	2900	–	–	–	8300	–	–	–
27	BEN 1567	B2	APEC	–	2700	400	–	850	–	2700	2900	3700	–	–	–	–	–	–
28	BEN 2908	B2	APEC	–	–	400	–	–	5300	2700	2900	1190	–	–	6200	–	–	–
29	MT 140	B2	APEC	–	–	400	–	–	5300	2700	2900	1190/3700	–	–	–	–	–	–
30	E2348/69	B2	EPEC	700	–	–	800	850	–	2700	–	–	–	970	–	–	–	–
31	536	B2	ExPEC	600	2700	–	–	850	–	–	2900	–	–	–	–	–	–	–
32	ABU83972	B2	ExPEC	600	–	–	–	850	–	2700	–	–	–	–	–	–	–	–
33	CFT073	B2	ExPEC	600	–	–	–	–	–	2700	–	3700	–	–	8300	–	–	–
34	IHE3034	B2	ExPEC	–	2700	–	–	850	–	2700	–	–	–	–	–	4800	–	–
35	J96	B2	ExPEC	600	2700	–	–	850	–	–	2900	–	–	–	–	–	–	–
36	P42	B2	ExPEC	600	–	–	–	850	–	2700	–	–	–	–	–	–	–	–
37	RS218	B2	ExPEC	–	2700	–	800	850	–	2700	–	–	–	–	–	4800	–	–
38	W1825	B2	ExPEC	–	–	400	800	850	–	2700	–	–	–	–	–	–	–	–

Table 2. cont.

Strain	ECOR	Pathotype	serW	selC	serX	serU	pheU	asnUVW	glyU	argW	argU	asnT	metV	thrW	leuX	pheV	aspV
39 764	B2	Faecal	650	—	1300	—	850	—	4200	6200	—	—	—	—	—	—	—
40 ECOR51	B2	Faecal	600	—	—	—	850	—	2700	—	—	—	—	—	—	—	—
41 F54	B2	Faecal	600	2700	400	—	850	5300	2700	—	—	—	—	—	5200	—	—
42 BEN 0071	D	APEC	950	2300	1000	—	—	5300	—	6200	1190	—	970	—	—	—	—
43 BEN 0280	D	APEC	650	2300	400	800	850	5300	—	—	1190	730	—	—	—	—	—
44 BEN 0955	D	APEC	600	2700	3590	600	850	5300	2700	—	3700	730	—	6200/8300	5200	—	—
45 BEN 0961	D	APEC	950	2700	—	600	—	5300	2700	6200	3700	730	—	8300	5200	—	—
46 BEN 1189	D	APEC	950	2700	400	600/800	—	5300	2700	6200	3700	—	—	8300	5200	—	—
47 493bi	D	EHEC	600	—	—	600	850	5300	—	—	1190	730	970	—	—	—	—
48 5714/96	D	EHEC	600	2300	3250	—	850	5300	—	6200	—	730	—	—	—	600	—
49 86-24	D	EHEC	600	—	—	600	850	5300	—	—	1190	730	970	—	—	—	—
50 EDL933	D	EHEC	—	—	—	—	850	5300	—	—	1190	730	—	—	—	—	—
51 ECOR40	D	ExPEC	820	—	400	—	—	5300	—	—	—	—	970	—	—	—	—
52 ECOR50	D	ExPEC	950	—	1300	—	—	5300	—	6200	—	—	—	—	—	—	—
53 ECOR31	D	Faecal	650	—	—	800	—	—	—	—	—	730	—	—	—	—	—
54 ECOR42	D	Faecal	650	—	—	800	850	—	—	6200	1190	730	—	—	5200	600	—
Score			37	36	33	31	31	30	28	26	25	17	14	13	13	6	1
Size/ECOR group			***	***	***	**	NS	***	***	**	**	***	**	NS	*	*	NS
P-value

Fig. 1. Highly transcribed and/or polycistronic tDNAs frequently have high integrity scores. Correlations between integrity score and tDNA usage (a) or transcription rate (b) are shown. In (b), polycistronic tDNAs are shown as open symbols, monocistronic tDNAs as closed symbols. The only polycistronic tDNA locus with a low integrity score (*metV*) is indicated by an open arrowhead. tDNA usage was calculated as described in Methods using data from Sharp & Li (1986). Transcription rate data were from Ardell & Kirsebom (2005).

associated with a fragment of 2700 bp at *selC*, of 400 bp at *serX* and of 2000 bp at *tyrV* (Fig. 2a, circled area). From this analysis, it can be concluded that certain associations, such as that mentioned above, are favoured.

The ECOR group of strains was then superimposed upon the distribution of modalities. This indicated that strains from different ECOR groups were associated with different combinations of PCR product sizes: the four PCR products described above were associated with strains of the B2 group. Similarly, fragments of 500 bp at *tyrV*, 5200 bp at *lysV*, 6200 bp at *argW* and 5300 bp at *asnUVW* were more frequently associated with strains of ECOR group D. Strains of ECOR groups A and B1 were not clearly separated in the F1/F2 plane. However, projection on the F1/F3 plane unravelled additional associations that allowed the distinction of strains from ECOR groups A and B1. Indeed, in contrast to strains of the B1 group, group A strains were

Fig. 2. Graphical representation of the results of the MFCFA analysis. MFCFA analysis was performed using the SPAD software. A modality was defined as a size in bp that a PCR product can take at one locus (e.g. *asnUVW*=5300). See Methods for details. The distribution of modalities was represented graphically onto planes described by the F1/F2 axis (a) and F1/F3 axis (b). The size of the squares is proportional to the contribution of the modality to the variance of the axis: the bigger the square, the more this modality contributes to the variance of the axis. Data concerning the ECOR group (black circles) and the pathotype (black triangles) were considered supplementary observations and projected on the F1/F2 and F1/F3 planes. The size of the circles and triangles is proportional to how well the distribution of these ECOR groups or pathotypes is explained by the projection on the F1/F2 or F1/F3 planes.

Fig. 3. Dendrogram obtained after ascending hierarchical classification of strains. Factorial coordinates on the five main axes resulting from the MFCA analysis were used to calculate distances between strains and cluster strains using Ward's criteria. The distance scale is shown at the bottom of the figure. The four classes that could be distinguished at a distance of 0.109 are represented in the first column at the right of the dendrogram. The phylogenetic (ECOR) group and the pathotype of strains are given in the second and third columns, respectively, at the right of the dendrogram.

Interesting differences were seen between APEC and human ExPEC strains: most B2 strains showing no amplification at *serW* were APEC strains. Similarly, most strains yielding a 2700 bp product at *serC* and a 400 bp fragment at *serX* were APEC strains. However, the small number of strains in the other pathotypes limited the conclusions that could be made concerning these pathotypes.

From the MFCA analysis, an ascending hierarchical clustering was then performed (Fig. 3). Interestingly, the clustering we obtained overlapped quite well with the ECOR clusters. Strains were separated into four major clusters: cluster 1 contained mostly group B2 strains and cluster 4 group A strains, cluster 2 contained only APEC strains belonging to both the D and B2 groups, while cluster 3 was the most heterogeneous, containing A, B1 and D strains. Interestingly, cluster 2 was the only one to contain strains from only one pathotype, i.e. APEC strains; the other clusters contained strains from several pathotypes.

As a whole, these results showed that significant differences exist in the immediate vicinity of tDNA loci between strains of different phylogenetic groups and possibly between strains of different pathotypes.

DISCUSSION

This paper describes the first large-scale PCR-based screening to identify tDNA loci in the *E. coli* genome that are generally intact and those that are preferentially associated with ECDNA regions.

In this study, an absence of amplification was taken as an indication of the presence of an ECDNA region. A negative PCR result could also have occurred because of the absence of the sequence complementary to one of the primers or because of PCR failure. The first possibility was considered unlikely: primers were designed so that they are present in the genome of four *E. coli* strains. Moreover, Ou *et al.* (2006) have shown that upstream and downstream regions of a subset of 20 tDNA genes were highly conserved. The likelihood of PCR failure was also greatly reduced by the use of chromosomal DNA suitable for amplification of large PCR fragments as shown with primer pairs *lysV1/lysV2bis* or *asnV2/asnW2*. Additionally, control PCR reactions were performed in all experiments using chromosomal DNA from strains MG1655, CFT073 and EDL933 in parallel with

associated with fragments of 1450 bp at *argQ*, 1250 bp at *leuV*, 1850 bp at *lysQ*, 1200 bp at *tyrV* and 2300 bp at *serC*.

We also investigated whether the distribution of tDNA PCR product sizes was related to the pathotype of strains.

reactions containing chromosomal DNA from the other strains studied.

The results of this study clearly indicate that a majority of tDNA loci are unlikely ECDNA insertion sites. Since ECDNA regions are frequently genomic islands associated with integrase genes, it is not surprising that, except for *metV*, our results confirm that of Williams (2002) showing that no integrase had ever been described downstream of tDNA encoding Glu, His, Gln and Met isoacceptors. While *metV* might not be associated with genomic islands containing integrase genes as described by Williams (2002), our results indicate that it can still serve as an ECDNA insertion site as is observed in strain CFT073. In addition, Fouts (2006) recently showed that prophages, which are potential ECDNA regions, are more frequently found downstream of Arg, Leu, Thr and Ser tDNA loci: in our study these tDNA loci were found to have low integrity scores. Interestingly, although the presence of ECDNA regions downstream of *pheU* and *pheV* is suggested by our results and was detected in several sequenced *E. coli* strains, Fouts (2006) did not find any prophage downstream of Phe tDNA.

Although several hypotheses have been made to explain the frequent association of ECDNA regions with tDNA loci (Cheetham & Katz, 1995; Hou, 1999; Reiter *et al.*, 1989; Saier, 1995; Williams, 2002), it is still unclear why certain tDNAs are preferred ECDNA insertion sites. We can suggest several reasons why a tDNA is not an ECDNA insertion site, although none of them can fully explain our results. First, we have shown that, with the exception of *metV*, tDNAs organized as polycistronic operons are intact in a majority of strains. We hypothesize that disruption of these loci by ECDNA insertion might prevent the processing of the RNA transcript required to form individual tRNA molecules. This would consequently affect the amount of several tRNAs and have a more severe effect on bacterial growth. Second, our data support the hypothesis that insertion of ECDNA downstream of a tDNA might affect its functionality and therefore be disadvantageous. Indeed we found that tDNAs where insertions were less frequently found had a high estimated transcription rate or encoded tRNA recognizing frequently used codons. These results may mirror the cellular requirement not to impair efficient translation by disruption of tDNA loci that encode isoacceptors present in higher amounts in the tRNA pool of the cell or recognizing frequently used codons.

To date we have no indication as to which of these criteria is the most important in determining the extent to which a tDNA can be targeted by ECDNA insertion. To strengthen our results, data concerning the affinity of one tRNA for the different anticodon it recognizes would be required to calculate the tDNA usage more precisely. Similarly, data from Ardell & Kirsebom (2005) are only estimated transcription rates of tDNAs: availability of the actual tDNA expression level for each individual tDNA would allow more precise calculations, which might affect our conclusions. Another hypothesis that we could not test because of the lack

of data in the literature is that the conditions in which a tDNA is expressed can influence its role as an ECDNA insertion site. In that respect, it is worth mentioning that four tDNAs encoding tRNA^{Leu} were found to be differently expressed depending on the growth conditions (Dobrindt & Hacker, 2001; Rowley *et al.*, 1993) and that only the transcription of the *leuX* tDNA, which had the lowest score of all *leu* tDNA loci, was not growth phase regulated (Rowley *et al.*, 1993). Whether this is also the case for other tDNA loci remains to be elucidated.

The flanking sequence context of each tDNA is also an important determinant. Several tDNA loci (either monocistronic or polycistronic) have been shown to be co-transcribed with downstream genes (Bosl & Kersten, 1991; Fournier & Ozeki, 1985; Li & Deutscher, 2002). Disruption of such transcriptional units could therefore be detrimental to the bacteria. For example, it is not surprising that the *thrT* tDNA had a high score since it is co-transcribed with *tufB*, the product of which is an important element of the translation machinery and whose deletion in certain genetic backgrounds significantly reduces the growth rate of *E. coli* (Lee *et al.*, 1981; Schnell *et al.*, 2003).

The local DNA structure of a tDNA, or the binding of proteins modifying the DNA conformation, might also influence the ability of a tDNA to serve as an ECDNA insertion target. Indeed, the activity of integrase genes found in several genomic islands and involved in genomic islands mobility is likely to depend upon the DNA structure (Cali *et al.*, 2004; Hochhut *et al.*, 2006; Lesic *et al.*, 2004; Pribil & Haniford, 2003; Radman-Livaja *et al.*, 2006). Interestingly, several tDNA loci with high scores such as *alaX*, *glyY*, *lysQ*, *proM*, *thrT*, *valW* or *glnX* had DNA curvature profiles different from those of a number of loci with low scores such as *asn* loci, *pheU*, *metV*, *pheV* and *serW*. In these latter cases, the DNA curvature was found to sharply decrease inside the tDNA locus and then sharply increase at the end of the locus (see Supplementary Fig. S2 – curvature data are from Pedersen *et al.*, 2000). Whether this is linked to the use of tDNA loci as ECDNA insertion hot-spots remains to be clarified.

A major finding of this work is the fact that the tDNA pattern of one strain is related to its phylogenetic (ECOR) group. The MFCA analysis performed in this study separated B2 strains from strains of the three other ECOR groups as well as strains from ECOR groups A and B1 (Fig. 2a, b). This was further illustrated by the hierarchical clustering performed from the MFCA results (Fig. 3). This distinction based on the ECOR group is reminiscent of the hypothesis made by Escobar-Paramo *et al.* (2004), who showed that the genetic background of strains belonging to the B2 ECOR group might be a critical player in the acquisition of ExPEC virulence factors. Since tDNAs are ECDNA insertion hot-spots, the tDNA pattern of one strain is also likely to have an influence on the acquisition/loss of ECDNA regions and could explain the specific properties of B2 strains, including their pathogenic properties (Picard

et al., 1999). Furthermore, ExPEC strains mostly belong to the B2 group and to a lesser extent to the D group (Picard *et al.*, 1999; Sannes *et al.*, 2004); it is therefore possible that tDNA combinations preferentially associated with B2 or D strains detected in our study are necessary for the expression of the full virulence of these strains.

The third cluster of strains identified in the hierarchical clustering was found to be very heterogeneous. Actually, Escobar *et al.* (2004) identified two subgroups (groups C and E) that are not discriminated by the PCR described by Clermont *et al.* (2000) used in this study. It is therefore possible that the heterogeneity of cluster 3 is linked to the fact that it contains strains from groups C and E.

Our results also indicate that some differences may exist between strains of different pathotypes. Analysis of our results unravelled some differences between APEC and human ExPEC strains. Among these differences, we found that the *serW* tDNA was a preferred ECDNA insertion hot-spot in APEC but not in other ExPEC strains. It will therefore be interesting to characterize the sequence present downstream of *serW* in these APEC strains and investigate whether it can encode host specificity factors.

Complementary to these conclusions, our data give an indication as to which tDNAs can serve as ECDNA insertion sites. Chouikha *et al.* (2006) recently used the lack of amplification of a PCR product at *selC* in an APEC strain to identify and characterize a new genomic island (AGI-3). A similar PCR-based strategy was designed by Ou *et al.* (2006) to search for new genomic islands and tested *in silico*, thus validating the PCR-based approach for the identification of new genomic islands. The 15 tDNA loci that we have identified as frequent insertion sites (score < 47) were found by these authors as frequent genomic island insertion sites.

In conclusion, this article describes the first large-scale study of the sequence context of tDNA loci in different *E. coli* isolates. We show that only a few tDNA loci are potential chromosomal insertion sites for large ECDNA regions. In fact, our results show that a majority of tDNAs are rarely sites of ECDNA insertion and provide evidence that this might be related to the organization of the loci, to their transcription rate and to their usage. Most interestingly, we demonstrated that the tDNA organization of a particular strain is related to the phylogenetic group it belongs to.

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