JOURNAL OF BACTERIOLOGY, Oct. 2010, p. 4885–4893 Vol. 192, No. 19
0021-9193/10/$12.00 doi:10.1128/JB.00804-10

Pathogenicity-Associated Islands in Extraintestinal Pathogenic *Escherichia coli* Are Fitness Elements Involved in Intestinal Colonization▽§

Médéric Diard,[1]‡ Louis Garry,[2] Marjorie Selva,[1] Thomas Mosser,[1] Erick Denamur,[2]† and Ivan Matic[1]†*

INSERM U1001, Faculté de Médecine Paris Descartes, Université Paris Descartes, Paris, France,[1] and INSERM U722, Faculté de Médecine, Université Paris Denis Diderot, Site Xavier Bichat, Paris, France[2]

Received 9 July 2010/Accepted 14 July 2010

The virulence of many human pathogens does not seem to be an evolutionarily selected trait, but an accidental by-product of the selection that operates in another ecological context. We investigated the possibility that virulence of the extraintestinal pathogenic *Escherichia coli* (ExPEC) strains, which frequently cause disease in the host in which they asymptomatically colonize the intestine, is the consequence of commensalism. Most of the ExPEC virulence factors are clustered on genomic islands called pathogenicity-associated islands (PAIs). We constructed and characterized several mutants of the ExPEC 536 strain with either (i) deletions of each single PAI or (ii) a complete deletion of all seven PAIs. *In vitro* phenotypic characterization of 536 mutants showed that the seven PAIs were dispensable for growth in the absence of external stress, as well as under a range of biologically relevant stressors, i.e., serum, bile, and oxidative, nitrosative, hyperosmotic, and acidic stress. However, challenge against the wild-type (WT) strain in a murine model shows that the deletion of all seven PAIs drastically reduces the fitness of 536 during persistent intestinal colonization. This defect seems to be linked to the hypermotility observed for mutants devoid of all seven PAIs. In addition, we show that PAIs diminish fitness of their carrier during growth in urine, suggesting that urinary tract infections are unlikely to provide selective pressure for the maintenance of ExPEC PAIs. Our results are in accordance with the coincidental-evolution hypothesis postulating that extraintestinal *E. coli* virulence is a by-product of commensalism.

Theoretical and experimental investigations suggest that increased virulence, i.e., parasite-mediated morbidity and mortality for infected hosts (38), could be selected for when it enhances the chance of a microorganism to be transmitted to a new host (38). However, virulence and transmission are not always correlated, and some forms of virulence did not coevolve with the host (39). For example, in an anthropo-centered point of view, *Escherichia coli* O157:H7 is an obligate intestinal pathogen. However, the normal habitat of this bacterium is the bovid gastro-intestinal tract, where it lives as a commensal rarely causing disease (24). *E. coli* O157:H7 virulence against humans presumably results from coincidental evolution, i.e., the symptoms provoked by the bacteria seem to be a maladjusted response to its accidental presence in the human gastro-intestinal tract rather than an evolutionarily selected means of transmission. Experimental evidence suggests that some of the genetic determinants of *E. coli* O157:H7 virulence, such as *stx* genes coding for the Shiga toxins, could be selected for to face off predators in the bovid intestinal tract (33, 59). Generally, the *E. coli* species challenges the threshold between commensal and pathogen (for a review, see reference 32). Hence, it constitutes an interesting model to address the question of the evolution of virulence. This is especially true for the extraintestinal pathogenic *E. coli* (ExPEC) strains, as they most frequently cause disease in the host in which they first asymptomatically colonize the intestine as commensal bacteria.

ExPEC strains are defined as bacteria with enhanced ability to cause infection outside the intestinal tract, such as in the bloodstream, the cerebrospinal fluid, or the urinary tract of mammalian and human hosts (29). The ExPEC ability to proliferate in these environments makes them the causative agents of devastating diseases, including septicemia, meningitis, cystitis, and pyelonephritis. ExPEC strains belong mainly to the B2 and D *E. coli* phylogenetic groups to which the prevalent intestinal colonizers of healthy individuals living in industrialized countries also belong (13, 45, 49, 62). Epidemiological studies showed that *E. coli* strains causing extraintestinal diseases carry specific genetic determinants called ExPEC virulence factors (VFs), which code for toxins, fimbriae, adhesins, and iron acquisition systems and confer utilization of alternative nutrients or resistance to biological stresses (reviewed in reference 30).

Most of the ExPEC VFs are clustered on mobile genetic elements, such as plasmids, or on genomic islands called pathogenicity-associated islands (PAIs) (21). PAIs are horizontally

* Corresponding author. Mailing address: INSERM U1001, Faculté de Médecine Paris Descartes, Université Paris Descartes, 156 rue de Vaugirard, 75730 Paris Cedex 15, France. Phone: 33 1 40 61 53 25. Fax: 33 1 40 61 53 22. E-mail: ivan.matic@inserm.fr.

‡ Present address: Institute of Microbiology, ETH Zurich, 8093 Zurich, Switzerland.

† These authors contributed equally to this work.

§ Supplemental material for this article may be found at http://jb.asm.org/.

▽ Published ahead of print on 23 July 2010.

acquired genetic elements, whose involvement in extraintestinal pathogenesis was experimentally demonstrated (41). Under laboratory growth conditions, most PAIs can be spontaneously deleted from the chromosome (44). It is not clear if PAIs were acquired and maintained *in vivo* because they increase the fitness of their carriers in the intestine or in the extraintestinal environments. Having the intestine as a main reservoir provides optimal conditions for transmission because commensal bacteria are shed daily in the external environment and hence can reach new hosts. On the other hand, within-host extraintestinal environments do not provide such efficient modes of transmission. In fact, some can be considered dead ends, e.g., blood and cerebrospinal fluid. Therefore, it is plausible that ExPEC virulence is a by-product of commensalism resulting from an intrahost evolution. According to this hypothesis, the acquisition of PAIs should increase the recipient strains' fitness inside the host intestine but also coincidentally enhance the virulence of their carriers in extraintestinal niches. Such a scenario has previously been proposed (22, 37, 39) and is supported by both epidemiological (49–52, 62) and experimental (1, 18, 25, 26) studies showing that virulence factors such as adhesins could favor intestinal colonization. However, the consequences of the complete elimination of all PAIs have never been determined for commensal and pathogenic lifestyles. To gain insight into the selective pressures that drive the acquisition and/or maintenance of PAIs, we chose the uropathogenic strain *E. coli* 536 (5) as a model organism. Genomic sequencing has revealed the presence of seven PAIs in the chromosome of this bacterium (6).

We constructed seven single-PAI deletion mutants, as well as mutants with the deletion of all seven PAIs (I to VII). These mutants were characterized both *in vitro*, for growth, resistance to biologically relevant stressors, motility, and biofilm formation, and *in vivo*, for virulence and asymptomatic intestinal colonization. Our results strongly suggest that PAIs are fitness elements cooperating to enhance persisting intestinal colonization. This seems to be linked to the regulation of flagellum gene expression.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The uropathogenic strain *E. coli* 536 (O6:K15:H31) was isolated from a patient with acute pyelonephritis (5). This strain is naturally resistant to streptomycin. Therefore, all the strains used in this study are also resistant to streptomycin. Mutants deleted for all seven PAIs, the ΔI-ΔVII mutants, were constructed starting from the *E. coli* 536 ΔIII strain kindly provided by U. Dobrindt (Institut für Molekulare Infektionsbiologie, Universität Würzburg, Germany) (44). Bacteria were grown overnight under aeration at 30°C or at 37°C in LB broth (Difco) from frozen samples kept at −80°C in 20% glycerol. The antibiotics ampicillin, chloramphenicol, and kanamycin were added when needed at final concentrations of 100 μg/ml, 25 μg/ml, or 50 μg/ml, respectively. Serial dilutions of bacteria were performed in 10^{-2} M $MgSO_4$ unless otherwise mentioned. A complete list of strains and plasmids used in this study is provided in Table S1 in the supplemental material.

Construction of deletion mutants. Deletions of *fliC* and PAIs from the chromosome of *E. coli* 536 were performed using gene replacement methods based on the lambda Red recombinase system (11). Primers designed to amplify a recombinogenic PCR fragment consisting of a removable antibiotic resistance cassette flanked by 40-bp sequences homologous to the 5′ (upstream [up]) or 3′ (downstream [dw]) extremity of the target region that they were to replace are listed Table S2 in the supplemental material. The removable chloramphenicol resistance cassettes flanked by flipase recognition target (FRT) sequences were amplified from the template plasmid pKD3 (11). The same cassettes flanked by *loxP* sequences were amplified from an NdeI/XmnI digestion product of the plasmid pLoxPcat. Removable kanamycin resistance cassettes flanked by FRTs were amplified from pKD4. PCR and enzymatic digestion products were purified after electrophoresis on agarose gel with commercial kits (Promega). Strains carrying the temperature-sensitive helper plasmid pKD46 (11) coding the lambda Red recombinase system were treated as described previously (11). Purified recombined clones were verified by genotypic analysis with the PCR primers listed in Table S3 in the supplemental material and tested for the loss of pKD46 on LB agar with ampicillin. These primers were specially designed for multiplex PCR in order to detect several deletions in the same strain (melting temperature [T_m], 55°C, and primers used at a final concentration of 0.4 μM each). In order to remove the antibiotic resistance cassette from the chromosome, resistant clones were transformed with the helper plasmid pCP20 or pMDcre46 encoding the flipase (FLP) or the Cre recombinase, respectively. To remove the cassette flanked by FRT sites, strains transformed with pCP20 were treated as described previously (11). Cre was expressed from pMDcre46 under the control of the P_{ara} promoter inducible by arabinose but presenting an important leakiness. For this reason, removal of cassettes flanked by *loxP* sequences was conducted by incubating cells carrying pMDcre46 at 42°C overnight on nonselective LB agar plates without arabinose. The loss of helper plasmids and resistance cassettes was verified by plating clones on selective medium plates. In addition, each PAI deletion and antibiotic resistance cassette loss was verified by PCR with the primers listed in Table S3. The sequential deletion of the seven PAIs implied the presence of an increasing number of genomic scars consisting of FRT sequences. These scars provide substrates for homologous recombination with the recombinogenic fragment used to delete the next PAI carrying two of these FRT sequences. We noticed that after four successive deletions, the replacement efficiency of the following PAI decreased drastically because of improper insertion of the recombinogenic fragment in a previous scar, giving more than 99.9% false-positive clones. We resolved this problem by adapting the Cre/*loxP* recombination system (20) to the original gene replacement technique, and *loxP* sequences were used for some deletions instead of FRT sites during the construction of the septuple mutants. Because the seven PAIs represent 7.7% of the whole genome of *E. coli* 536, it was technically impossible to complement these deletions. Consequently, to be sure that the observed phenotypes were due to the deletion of the PAIs, two independent ΔI-VII mutants (#1 and #2) were constructed and tested in parallel in all experiments. Moreover, it has to be noticed that six sequential deletions represent 17 rounds of competent-cell preparation and electro-transformation, plating, streaking, overnight growth in liquid medium, and six rounds of expression of the lambda Red recombinase system. During these different steps, with each deletion representing around 200 generations, some mutations could be fixed. For this reason, an *E. coli* 536 strain derivative, referred to as the wild-type (WT) strain in the text, was obtained by submitting the ancestral 536 strain to six blank rounds of deletions in which the preparation of recombinogenic fragments was replaced by distillated sterile water. This evolved WT strain was used as a reference in all experiments performed in this study. A list of characterized and putative functions encoded on *E. coli* 536 PAIs is presented in Table S4 in the supplemental material.

Labeling of strains with fluorescent reporter gene. The ΔI-VII #1 mutant was labeled by integrating the pMD2YFP (derived from pAH63) plasmid carrying the *yfp* gene under the control of the constitutive promoter $P2_{mnB}$ in the phage lambda *attB* site on the chromosome as described in reference 23. The integration of the plasmid was verified as described in reference 23. The resulting strain was named ΔI-VII #1 (L).

Competitions in chemostat. The fitness of the ΔI-VII mutants relative to the WT strain was estimated during competitions in chemostat. The chemostats were constructed as described in reference 9, with some modifications (see http://www.necker.fr/tamara/pages/lab.html for the design and photographs). The growth rate, equal to the dilution rate (48), was set to 1 h, with a maximal volume of 16 ml and a fresh nutrient flow of 16 ml/h. The maximal volume was limited by an overflow consisting of a flushing glass tube linked to a pump sucking up the exhausted medium and alive or dead cells. Agitation was ensured by air or by N_2 bubbling during aerobic or anaerobic experiments, respectively. Maintenance of the temperature at 37°C was ensured by maintaining chemostats in thermostated heating blocks. A total of 200 μl of a 1:1 mixture of mutants and WT strains coming from overnight cultures was added in chemostats continuously supplied with LB broth supplemented with streptomycin to a final concentration of 100 μg/ml. From each chemostat, 1 ml of culture was taken daily, the optical density at 600 nm (OD_{600}) was measured, and the relative proportion of each strain was evaluated by plating dilutions on selective and nonselective LB agar plates. The competitive index (CI) was calculated as the logarithm of the number of antibiotic-resistant CFU divided by the inferred number of sensitive CFU after a given time and divided again by the same ratio in the inoculum for each chemostat.

Competitions in batch cultures. We determined the relative fitness of the ΔI-VII mutants compared to the WT in LB and human urine over 100 generations in batch cultures. The human urines correspond to a pool of 24-h urines obtained from several healthy male donors that did not take any drug and were devoid of urinary tract infection (UTI) history. Overnight cultures of *E. coli* 536 WT and the ΔI-VII mutants were diluted a thousandfold in LB or urine at a final ratio of 1:1. Every 24 h, cultures were diluted a thousandfold in fresh medium during 10 days. Each of these cycles corresponds to 10 generations. The relative amount of each strain was determined after plating on selective agar plates. Neutrality of the chloramphenicol resistance cassette carried by the ΔI-VII mutants was estimated by using competing ΔI and ΔI::Cm strains (see Table S1 in the supplemental material).

Motility. We measured the impact of PAIs on the motility of *E. coli* 536 by measuring both the abilities to swarm and to swim as described in reference 19. Overnight cultures were inoculated on swimming and swarming plates with a sterile toothpick. The swimming motility plates were prepared with 0.3% agar (Difco), 1.0% Bactotryptone (Difco), 0.5% yeast extract (Difco), and 0.5% NaCl (Sigma). The swarming motility plates were prepared with 0.5% agar (Difco), 1.0% Bactotryptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl (Sigma), and 0.5% D-(+)-glucose (Sigma). The plates were air dried at room temperature for 30 min under a laminar-flow hood before being inoculated. The plates were photographed after incubation for 8 h for swimming and 24 h for swarming at 37°C. Competitions in structured medium (LB 0.3% agar or 0.75% agar) allowing or not allowing for swimming were performed as described in reference 14 between the WT strain and the ΔI-VII #1 (L) mutant labeled with the *yfp* gene.

Quantitative reverse transcriptase (qRT)-PCR. For real-time PCR, the cDNA synthesis was performed using 2 μg RNA with random hexamers (12.5 ng/ml) and the Superscript II RNase H^- kit 5 (Invitrogen) according to the manufacturer's instructions. The real-time PCR experiments were performed using the SYBR green PCR Master Mix (Applied Biosystems) to quantify the expression level of the *fliA* and *motA* genes. The *rpoD* gene was chosen as a reference gene for data normalization. Primer pairs were designed using the Primer Express software (Applied Biosystems): for *motA*, 5′-GCGATTAAAGGCACGCTG AAGG and 5′-GAAGGTGTTCATGTGACCGCTG; for *fliA*, 5′-GCGTGG AACTTGACGATCTGC and 5′-TCCGCTACCTCAGTTTCCGTG; and for *rpoD*, 5′-CGTCTGATCATGAAGCTCTG and 5′-GTATCGCTGGTTTCGTTG. Amplification and detection of gene-specific products were carried out with the 7300 real-time PCR system (Applied Biosystems). Data analysis was performed with the 7300 system software. For each target gene, the average cycle threshold (C_T) value was calculated from triplicate RNA samples. The difference between the C_T value of the target gene and the C_T value of the endogenous reference gene (*rpoD*) was defined as the ΔC_T. The $\Delta\Delta C_T$ value described the difference between the ΔC_T of the WT strain and the mutant strain. The difference in expression levels was calculated as $2^{\Delta\Delta CT}$.

Biofilm formation. To assess the ability of the bacterial strains under study to form biofilm, an *in vitro* test was conducted as described in reference 12. Briefly, bacteria from overnight cultures were diluted in minimal medium supplemented with glucose and grown for 24 h at 37°C without shaking in 96-well polystyrene plates. Planktonic bacteria were washed out with distilled water, and bacteria that remained attached to the well walls were dried and fixed for 30 min at 80°C. The amount of biofilm was estimated using 1% crystal violet (Sigma) solution. The crystal violet trapped in biofilms was solubilized with dimethyl sulfoxide (Sigma). The concentration of crystal violet was then measured with a spectrophotometer at 600 nm. For each strain, at least six independent experiments were performed.

Septicemia. The ability of bacterial strains to cause sepsis was determined using 16- to 18-g female mice (Charles River OF1). A total of 200 μl of a suspension of 10^9 bacteria/ml in physiological serum was inoculated by subcutaneous injection, and the number of surviving mice was monitored during 7 days (53).

Intestinal colonization. Six-week-old female mice (Charles River CD-1) treated with streptomycin were used to monitor the ability of the different strains to colonize the intestine of a mammalian host as described in reference 46, with modifications. Five days before inoculation, mice were isolated and streptomycin was added to the sterile drinking water at a final concentration of 5 g/liter. Streptomycin was maintained until the end of the experiment. The antibiotic treatment efficiency against the coliform intestinal population was controlled by plating a pure suspension of feces in physiological water on Drigalski selective agar medium. Mice free of coliform flora were inoculated *per os* with 10^6 bacteria in 200 μl of physiological water. Every day postinoculation, the intestinal population of *E. coli* was estimated by plating dilutions of weighted fresh feces on LB agar with or without appropriate antibiotic. All animal experiments were performed in compliance with the recommendations of the French Ministry of Agriculture and approved by the French Veterinary Services (accreditation A 75-18-05).

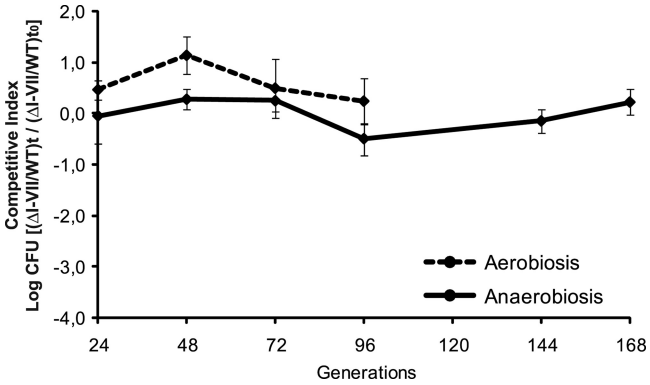

FIG. 1. Competitions in chemostat. WT strain and ΔI-VII mutants were inoculated at an initial ratio of 1:1 in LB medium supplemented with 100 μg/ml streptomycin and maintained at 37°C. Homogenization was ensured by regular bubbling of filtered air (aerobic conditions, dashed line) or N_2 (anaerobic conditions, solid line). The generation time was fixed at 1 h, and the competitive index was determined as described in Materials and Methods. Error bars represent the standard error of the mean of at least four experiments. No significant difference was observed between the two strains at each time point (Wilcoxon signed-rank test, all P values were >0.05).

Statistics. Statistical analyses were performed using JMP IN (SAS Institute, Inc.) and Prism (GraphPad Software, Inc.).

RESULTS

***In vitro* fitness.** Deletion of the seven PAIs reduced the *E. coli* 536 genome size by 7.7%. The loss of some PAI-encoded functions and/or such an important reduction of the genome size could modify the fitness of the ΔI-VII mutants relative to the WT strain. Therefore, we tested the ability of these mutants to grow in LB medium or in human urine in competition against the WT. In order to follow bacterial growth at constant rates during several days, we first conducted experiments in chemostat. Growth conditions were set to obtain a generation time of about 60 min, which is close to the generation time of bacteria growing in the intestinal tract of streptomycin-treated mice (54). Growth under anaerobic conditions, mimicking the lack of oxygen in the intestinal mucus (31), was performed in chemostat in which air bubbling was replaced by N_2 bubbling. After 4 days of competition in the presence of oxygen (about 96 generations) or 7 days of competition under anaerobic conditions (about 168 generations), there was no significant deviation from the initial ratio between the WT strain and the ΔI-VII mutant strains (Fig. 1; $CI_{O2} = 0.23 \pm 0.45$; $CI_{N2} = 0.22 \pm 0.25$; Wilcoxon signed-rank test, $P > 0.05$).

We also performed competitions with aerobic batch cultures in LB or in human urine (Fig. 2). This experimental setup allowed for testing of bacterial strain fitness during 10 cycles of passages through lag phase, exponential growth, and stationary phase. Under such competition conditions, after about 100 generations, the complete PAI deletion was advantageous in LB medium and in urine ($CI_{LB} = 0.51 \pm 0.08$; $CI_{urine} = 1.32 \pm 0.18$; Wilcoxon signed-rank test, $P < 0.05$). It should be noted that the relative fitness of the ΔI-VII mutants compared to the WT was significantly higher in human urine than in LB (Mann

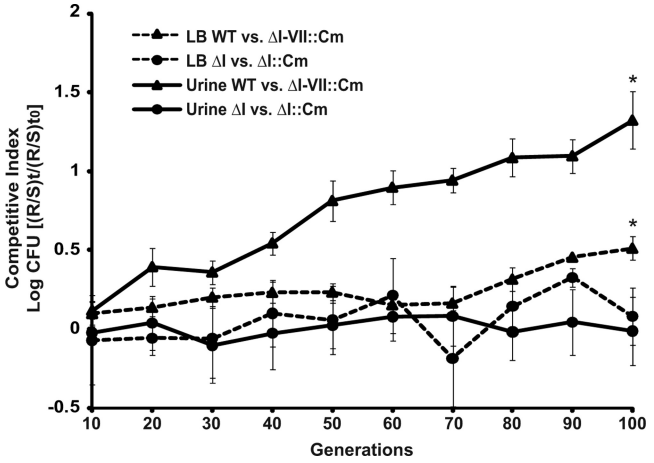

FIG. 2. Competitions in batch cultures. The relative fitness of the ΔI-VII mutants compared to *E. coli* 536 WT in LB (dashed lines) and urine (solid lines) over 10 cycles (i.e., 100 generations) of growth in batch cultures was determined as described in Materials and Methods. The competition opposing the ΔI strain to the ΔI::Cm strain was used as a control for the cost of the chloramphenicol resistance cassette present in the ΔI-VII mutants. Error bars represent the standard error of the mean of at least three experiments. ΔI-to-VII mutants significantly outcompeted the WT in each medium after about 100 generations (*, $P < 0.05$, Wilcoxon signed-rank test). R, antibiotic-resistant strain; S, antibiotic-sensitive strain.

Whitney test, $P < 0.01$). This effect was not due to the presence of the chloramphenicol resistance cassette in the ΔI-VII mutants, as the ΔI::Cm strain had the same fitness relative to the isogenic ΔI mutant ($CI_{LB} = 0.08 \pm 0.18$; $CI_{urine} = -0.01 \pm 0.21$; Wilcoxon signed-rank test, $P > 0.05$). The fact that PAIs diminish the fitness of its carrier during growth in human urine is in line with the hypothesis according to which ExPEC PAIs could be selected and maintained in an ecological context other than the urinary tract.

Metabolic competence. The ability of the WT and ΔI-VII mutant strains to use 95 substrates as a sole carbon source was determined using Biolog GN2 galleries. Bacterial growth was evaluated by measuring the increase of the OD_{600} due to tetrazolium reduction after incubation for 24 h and 48 h at 37°C. A given substrate was considered metabolized when the OD_{600} was >0.2 for at least two repetitions. WT and ΔI-VII mutant strains differed only in their abilities to use D-serine, as it took more time for the ΔI-VII mutants to reach the threshold OD_{600} than for the WT strain (see Table S5 in the supplemental material). This presumably results from the absence in the ΔI-VII mutants of the *dsd* gene cluster carried by PAI II. This gene cluster codes for DsdA, a D-serine dehydratase, DsdX, a transporter, and DsdC, a transcriptional regulator. Nevertheless, the duplication of *dsdA* and the presence of an alternative transporter coded by *cycA* (3) in the chromosome of *E. coli* 536, outside the seven PAIs, should allow ΔI-VII mutants to use D-serine, albeit at a lower rate.

Stress resistance. In order to identify selective pressures that might lead to the acquisition and maintenance of PAIs, several *in vitro* stress resistance tests were conducted. All of the chosen stressors were of biological relevance, i.e., the ability to grow in bovine bile or human serum, the resistance to oxidative and nitrosative stress generated, respectively, by paraquat or $NaNO_2$, and resistance to hyperosmotic stress and acidic pH. For all conditions, the responses of the ΔI-VII mutants and of the WT strain were compared. For some stressors, resistance of the mutants with a single PAI deletion was also tested. No significant difference between resistance levels of the ΔI-VII mutants, single mutants, and WT strain to any of these stressors was observed (see Fig. S1, S2, and S3 in the supplemental material). Therefore, among the genes carried on the seven PAIs of *E. coli* 536, none seemed to play an essential role in the resistance to tested biological stressors which are normally encountered by this bacterium during both commensal and pathogenic life cycles.

Motility. Motility is a major trait involved in both the pathogen and commensal lifestyles of *E. coli* (16, 34). Different orthologous genes present on several PAIs of *E. coli* 536 were shown to be involved in the regulation of motility in other ExPEC strains (57, 58). These genes encode putative MarR-like transcriptional regulators. They are located downstream of the adhesin-coding gene in some fimbrial operons. According to the nucleotide sequence homology and synteny (determined using Microbial Genome Annotation System; https://www.genoscope.cns.fr), at least three of such genes are present in *E. coli* 536, i.e., ECP_3776 on PAI I, *papX* (ECP_4531) on PAI II, and *sfaX* (ECP_0301) on PAI III. The role of PAIs in the motility of *E. coli* 536 was addressed by measuring the swarming and the swimming abilities of the WT and the mutant strains. Single mutants did not present any alteration of their swimming and swarming abilities compared to the WT (Fig. 3A and B). On the other hand, the swimming and swarming abilities of the ΔI-VII mutants were clearly enhanced (Fig. 3A and B). Redundancy may explain why only the ΔI-VII mutants had an altered motility, unlike the single PAI deletion mutants. This result was confirmed by placing the ΔI-VII #1 (L) mutant in competition against the WT strain in a structured environment. When the agar percentage was low enough to allow for cell motility, the WT strain was rapidly outcompeted by the mutant (Fig. 3C). On solid LB agar, an environment on which bacteria cannot swim, no difference in fitness between the ΔI-VII #1 (L) and WT strains was observed. Because regulators potentially responsible for alteration of *E. coli* 536 motility act at the transcriptional level, the expression of the class II flagellar gene *fliA* and the *motA* gene coding for the proton conductor component of the flagellum motor was quantified using qRT-PCR (Fig. 3D). The expression of *fliA* was found to be upregulated >4-fold and that of *motA* >2-fold in the ΔI-VII mutants compared to the WT.

Biofilm formation. In both the gastrointestinal (42) and urinary tracts (2), ExPEC cells are found in biofilm-like communities, which could ensure their persistence. Therefore, we assayed the ability of the mutants deleted for one or the seven PAIs to form a biofilm in polystyrene 96-well plates during overnight growth in minimal medium glucose at 37°C (Fig. 4). The amount of biofilm was revealed using crystal violet. The results obtained for the different strains show that only genes carried by PAI II and PAI III play a significant role in biofilm formation by *E. coli* 536. Both the ΔII and ΔIII mutants formed thinner biofilms than the WT (Mann Whitney test, *P* values corrected for multiple comparisons by the Bonferroni-Holm procedure were <0.0001).

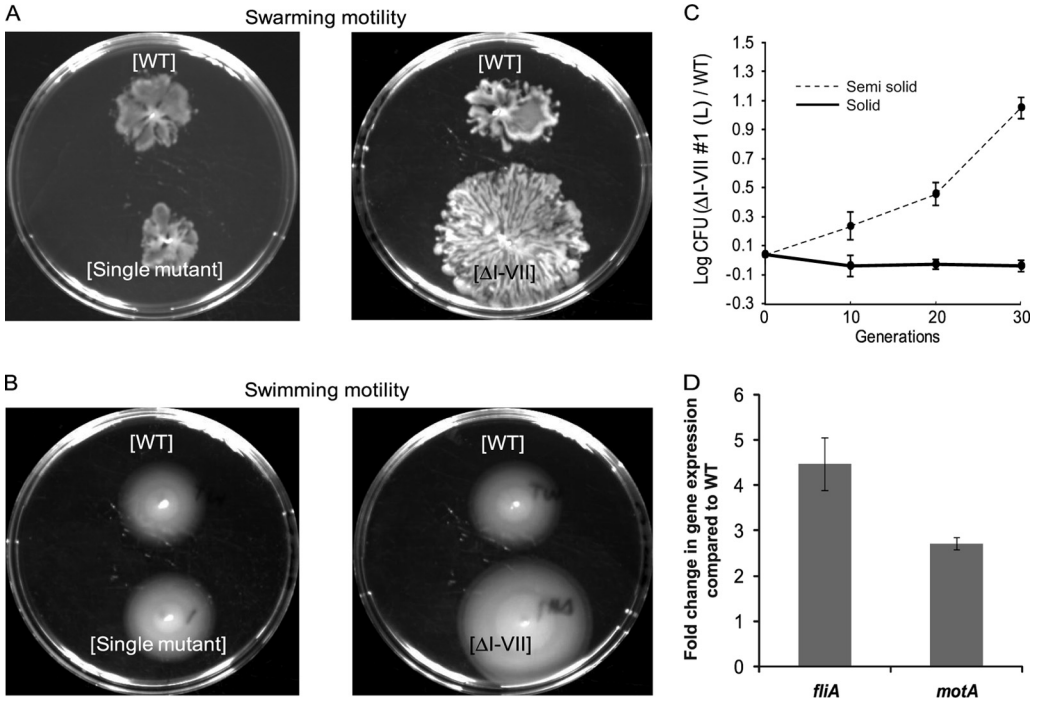

FIG. 3. Effect of PAI deletion on *E. coli* 536 motility. Swarming (A) and swimming (B) colonial pattern developed on semisolid agar plates after incubation for 24 h and 8 h at 37°C h, respectively. Representative results obtained for the WT, ΔI-VII mutants, or single-deletion mutants are presented. (C) Competitions between WT strain and ΔI-VII #1 (L) mutant inoculated at an initial ratio of 1:1 in solid (0.75% agar, solid line) or semisolid (0.3% agar, dashed line) minimal medium glucose at 37°C. Mutant was labeled with the *yfp* gene as described in Materials and Methods, rendering colonies fluorescent. The issue of the competition was determined by dividing the number of CFU of the ΔI-VII #1 (L) mutant by the number of CFU of the WT strain and log transformed. Error bars represent the standard error of the mean of four experiments. (D) Relative expression of motility genes *fliA* and *motA* in ΔI-VII mutants compared to the WT strain determined by qRT-PCR analysis. Error bars represent the standard error of the mean of three experiments.

The impact of those genes seems cumulative, as the ΔII-ΔIII double mutant and ΔI-VII strain were equivalently impaired in their capacities to form biofilm (Mann Whitney test, $P > 0.05$).

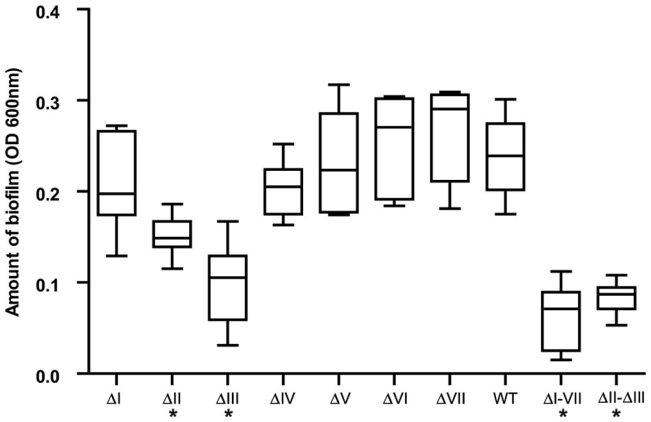

FIG. 4. Biofilm formation ability. Whiskers box plot showing the amount of biofilm formed by each mutant and the WT strain, measured after growth on minimal medium in 96-well plaques as described in Materials and Methods. An asterisk bellow the name of a mutant stands for values significantly different from those obtained with the WT strain (Mann Whitney test, *P* values corrected for multiple comparisons by Bonferroni-Holm procedure, <0.0001). ΔII, ΔIII, ΔII-ΔIII, and ΔI-VII mutants formed significantly less biofilm than the WT strain.

Septicemia. The pathogenicity of different mutants relative to the WT strain was evaluated using the murine septicemia model (53). Analysis of the survival data by the Kaplan-Meyer method showed that the ΔI-VII mutants had significantly attenuated virulence compared to the WT strain (Fig. 5, log rank test, $P < 0.0001$). This result confirms that some PAIs carry genes that enhance the capacity of the WT strain to kill mice during sepsis. However, none of the mutants deleted for a single PAI had a significant defect in their ability to kill mice under the experimental conditions used. This could be explained by the redundancy of functions encoded on several different PAIs (e.g., the alpha-hemolysin encoded on both PAI I and PAI II [47]) and/or the additive effects of VFs on virulence (28, 60). It should be noted that the attenuation of the ΔI-VII mutants cannot be related to a loss of serum resistance (see Fig. S1B in the supplemental material).

Intestinal colonization. The hypothesis that the genes on PAIs could be involved in intestinal colonization was verified using streptomycin-treated mice. This is a well-established model for determination of the fitness of enteric bacteria during intestinal colonization (8). The efficiency of the streptomycin treatment in elimination of the *Enterobacteriaceae* from the mice intestine was verified by plating feces on Drigalski medium plates.

First, the ability of individual bacterial strains to colonize the gut of mice without interference from coliform competitors was determined. Pure cultures of WT or ΔI-VII mutant strains

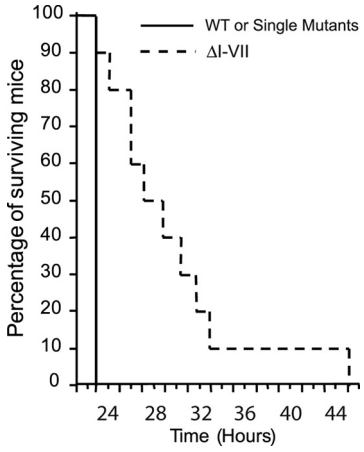

FIG. 5. Virulence during septicemia. Ten mice were inoculated per condition as described in Materials and Methods. Survival data of infected OF1 mice were analyzed by Kaplan-Meyer method, and a nonparametric log rank test was used to test statistical significance of observed differences. The WT strain and each single mutant (solid line) killed mice during the first 12 h. However, ΔI-VII mutants (dashed line) were significantly attenuated in their killing ability compared to WT strain ($P < 0.0001$).

were inoculated in streptomycin-treated mice by intragastric administration of 10^6 CFU per animal. During 1 week, the amount of *E. coli* bacteria in the intestine was evaluated daily by plating serial dilutions of fresh feces on LB agar plates. The WT and the mutant strains colonized the gut of mice with the same efficiency. After 48 h, the amount of 10^9 to 10^{10} CFU/g of feces was achieved and maintained throughout the following period of 5 days (Fig. 6A). Therefore, without coliform competitors and the streptomycin-sensitive part of the intestinal flora, the absence of PAIs did not affect the ability of *E. coli* 536 to initiate and maintain intestinal colonization.

In natura, the intestinal ecological niche never stays free of competitors. Thus, in a second experiment, we measured the fitness of mutants without one or the seven PAIs relative to the WT strain during competitions for intestinal colonization (Fig. 6B). A nonparametric Wilcoxon test on paired data comparing the number of CFU of antibiotic-resistant strain with the number of CFU of sensitive strain was conducted for each competition, and the *P* values was corrected for multiple comparisons by the Bonferroni-Holm procedure (27). The expression of the cassettes conferring resistance to chloramphenicol or to kanamycin that were used to differentiate strains during competitions did not have any significant cost during intestinal colonization (Fig. 6B).

No defect due to the absence of PAIs for the initial phase of the colonization, i.e., 24 h postinoculation, was observed (Fig. 6B). Forty-eight hours postinoculation, populations of both competing strains reached 10^9 to 10^{10} CFU/g of feces (data not shown). However, the mean competitive index of the ΔI-VII mutants decreased by three orders of magnitude 7 days postinoculation (Fig. 6B). Both ΔI-VII mutants (#1 and #2) gave similar results against the WT (Mann Whitney test, $P > 0.05$). Therefore, no mutation elsewhere in the core genome other than the PAI deletions seemed to be responsible for the ΔI-VII colonization defect. None of the single PAI deletion mutants showed significant intestinal fitness loss. This suggests that the

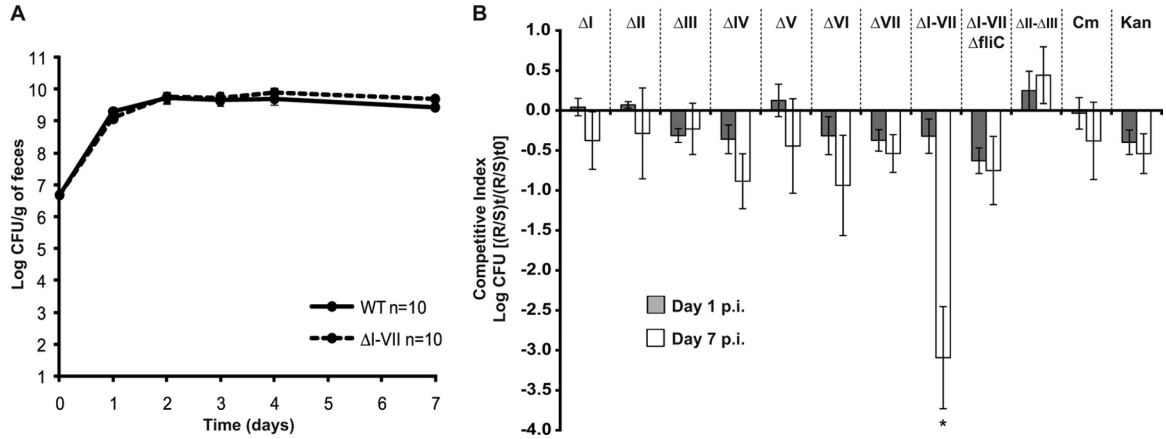

FIG. 6. Intestinal colonization. (A) Monocolonization of the intestine of streptomycin-treated CD1 mice by the WT strain (solid line) and ΔI-VII mutants (dashed line). At day 0, 5 mice were inoculated by intragastric inoculation of around 10^6 bacteria of a given strain. Each day, the size of bacterial populations in the intestine of mice was evaluated by plating dilutions of fresh feces. Two independent experiments were performed. Error bars represent the standard error of the mean. No significant difference was observed between the two strains at each time point (two-sample Wilcoxon test, all *P* values were >0.05). (B) Intestinal competitions were performed in streptomycin-treated CD1 mice. Competitive indexes are given for day 1 (filled bars) and for day 7 postinoculation (p.i.) (open bars). Before inoculation, each mutant, labeled with a cassette conferring resistance to chloramphenicol (the ΔI, ΔII, ΔIII, ΔIV, ΔVI, ΔVII, ΔI-VII, ΔI-VII *fliC*, and ΔII-ΔIII mutants) or kanamycin (the ΔV mutant), was mixed at a ratio of 1:1 with the WT strain. Mice were inoculated by gavage with 10^6 bacteria. At day 1 and 7, the sizes of bacterial populations in the intestine of mice were evaluated by plating dilutions of fresh feces on LB agar plates supplemented with the appropriate antibiotic when needed. The proportion of antibiotic-sensitive bacteria was inferred by subtracting the number of CFU obtained on a nonselective plate by the number of CFU obtained on a plate with antibiotic. At least three mice were used in competition in at least two independent experiments. Error bars represent the standard error of the mean. ΔI to ΔII-ΔIII, antibiotic-resistant mutants in competition with the WT sensitive strain; Cm, control competition evaluating the cost of the chloramphenicol resistance cassette and opposing ΔI to ΔI::Cm mutants; Kan, control competition evaluating the cost of the kanamycin resistance cassette and opposing ΔV to ΔV::Kan mutants; *, $P < 0.05$, generated by the Wilcoxon signed-rank test and corrected for multiple comparisons by the Bonferroni-Holm procedure.

maintenance of the colonization was compromised only by the absence of several PAIs. Redundant functions encoded by different PAIs promoting intestinal maintenance could render difficult the detection of effects resulting from the deletion of a single PAI. It is also again possible that genes on different PAIs encode proteins with additive and/or synergistic activities.

Biofilm formation, motility, and intestinal colonization. *In vitro* characterization of different PAI mutants showed that the ΔI-VII mutant had two phenotypes that differ significantly from those of the WT, i.e., a reduced capacity to form biofilm and an enhanced motility. We tested a possible involvement of these phenotypes in the intestinal colonization. First, we used the double PAI II-PAI III deletion mutant (the ΔII-ΔIII mutant), which is as deficient in its capacity to form biofilms as the ΔI-VII mutant (Fig. 4), to test the involvement of this phenotype in intestinal colonization. This strain did not show any intestinal colonization defect relative to the WT (Fig. 6B), suggesting that the capacity to form biofilm on an abiotic surface is not correlated with the ability to durably colonize the gut of mice. Second, we deleted *fliC*, coding for flagellin, from the genomes of the WT and the ΔI-VII mutant in order to test the role of motility in the intestinal colonization. Both strains were completely immobile (data not shown). Competition between the *fliC* and ΔI-VII *fliC* mutants showed that there is no difference in their capacities to colonize and persist in intestine (Fig. 6B), indicating a role of flagella in intestinal colonization by *E. coli*.

DISCUSSION

ExPEC strains are characterized by an enhanced ability to cause infections outside the intestinal tract compared to other *E. coli* strains (29). Most of these strains belong to the *E. coli* phylogenetic groups B2 and D (53). The virulence of ExPEC strains depends on VFs, most of which are clustered on mobile genetic elements, such as plasmids, or on PAIs (reviewed in reference 30). Nevertheless, *E. coli* strains from groups B2 and D, carrying ExPEC VFs, are prevalent in the feces of healthy individuals living in industrialized countries (13, 45, 49, 62). These observations indicate that the intestine could be the primary reservoir of ExPEC strains. As a corollary, it has been proposed that ExPEC PAIs could be fitness elements in the intestinal ecological context. Consequently, the virulence of ExPEC strains could be seen as the by-product of the commensal lifestyle (37, 39). The aim of this study was to provide experimental demonstration of this hypothesis. The archetypal uropathogenic strain *E. coli* 536 was chosen as a model organism (21). The vast majority of 536 VFs are clustered on seven PAIs. Mutants with a complete deletion of all seven PAIs (the ΔI-VII mutants) and a set of mutants with only one or two PAIs deleted were constructed and characterized both *in vitro* and *in vivo*.

The capacity of the ΔI-VII mutants to colonize the host intestine relative to the WT strain was evaluated using streptomycin-treated mice. For more than 7 days after intragastric inoculation, the WT strain colonized the mice, reaching populations of 10^9 to 10^{10} bacteria per gram of feces, without any evidence of clinical symptoms. The ΔI-VII mutants colonized the mice intestine with the same efficiency as that of the WT strain (Fig. 6A). We concluded that PAI-encoded functions are not essential for the colonization of the streptomycin-treated mice. In order to colonize the mice intestine, bacteria must survive acidic pH in the stomach and then the bile unloaded in the duodenum. Therefore, it is not surprising that the ΔI-VII mutants and the WT strain resist equivalently to both stresses *in vitro* (see Fig. S1 and S3 in the supplemental material). In competition against the WT strain, the ΔI-VII mutants were able to initiate colonization, reaching the intestinal carrying capacity corresponding to 10^9 to 10^{10} bacteria/g of feces. However, 7 days after inoculation, the population size of the ΔI-VII mutants decreased to a level on average three orders of magnitude lower than that of the WT (Fig. 6B). These results clearly showed that ΔI-VII mutants were impaired in their ability to maintain intestinal colonization but only in competition with the WT strain.

Maintenance of intestinal colonization requires many properties, among which metabolic competence is of the utmost importance. When two strains are in competition for a limited nutrient, the one that is able to use it more efficiently should outcompete the other (15). However, several pieces of evidence rule out metabolic competence as an important factor in the outcome of the intestinal competition between the ΔI-VII mutants and the WT strain. First, *in vitro* competitions in rich medium in chemostat showed that the ΔI-VII mutants were at least as fit as the WT under both aerobic and anaerobic conditions (Fig. 1) and in batch culture competitions the ΔI-VII mutants were even fitter than the WT (Fig. 2). Second, comparison of the metabolic capacities of both strains in Biolog GN2 galleries (see Table S5 in the supplemental material) showed that the ΔI-VII mutants were impaired only in their ability to use D-serine as a sole carbon source. This phenotype probably results from the deletion of PAI II that carries the *dsd* gene cluster as published for *E. coli* CFT073 (3). However, the WT strain did not outcompete the ΔII mutant in the intestine. Finally, the WT strain and the ΔI-VII mutants grow equally well in minimal medium with gluconate, i.e., the principal carbon source used by *E. coli* in the intestine (7) (see Table S5).

When competitors have the same ability to use available nutrients, the issue of competition can be determined by the ability to resist different stresses or the outflow by, for example, forming biofilms. The WT strain and the ΔI-VII mutants showed no significant difference in their abilities to resist different biologically relevant stressors (see Fig S1, S2, and S3 in the supplemental material). However, the ΔI-VII mutants had a completely abolished ability to form biofilm on an abiotic surface compared to the WT strain (Fig. 4). Some of the genetic determinants allowing for biofilm formation *in vitro* seemed located on PAI II and III (Fig. 4). Several genes on PAI III were known to play a role in biofilm formation, i.e., *flu* coding for antigen 43 (10) and the *foc* operon coding for the F1C fimbriae (35), while further work must be done to identify genes in PAI II involved in biofilm formation. F1C fimbriae were shown to improve the maintenance of the *E. coli* commensal strain Nissle 1917 in the intestine of infant mice during competition (35). However, the capacity to form biofilms did not contribute to fitness during intestinal colonization, with the ΔII-ΔIII double mutant being as fit as the WT, even though it was unable to form biofilm (Fig. 4 and 6B).

In vitro motility was enhanced in the ΔI-VII mutants, but not

in single mutants (Fig. 3). Despite the fact that their enhanced motility provided a selective advantage to ΔI-VII mutants in competition with the WT strain in an *in vitro* structured environment (Fig. 3C), it did not contribute to any fitness increase during intestinal colonization (Fig. 6B). Actually, when both the WT and ΔI-VII mutants were deleted for *fliC*, the gene encoding flagellin, no difference between the two strains was observed regarding their relative fitness in the mouse intestinal tract. This suggests that several PAIs could be maintained in the chromosome of *E. coli* 536 because they carry genes negatively regulating flagellum expression. This result corroborates the previous observation that motility is dispensable for intestinal colonization by *E. coli* F18 (43). Furthermore, selective pressures in the intestinal tract tend to counterselect the motility of *E. coli*. Hence, it was shown that mutations in the *flhDC* genes and in the two-component system *envZ-ompR*, drastically affecting the expression of the flagella, give a selective advantage in the intestine of mice (17, 36). A parsimonious regulation of the expression of genes involved in motility is required probably because proteins constituting the flagella are immunogenic (4) and costly to produce (14). However, neither the cost of motility nor the potential immunogenicity of the flagella seemed to be involved in the reduction of fitness of the ΔI-VII mutants relative to the WT strain during intestinal colonization as suggested by two observations: (i) the cost of hypermotility for the ΔI-VII mutants was not detected under conditions allowing elevated growth rates *in vitro* (Fig. 1 and 2) and (ii) no macroscopic or microscopic cecal inflammation was observed during colonization with either the ΔI-VII mutant or the WT strain (data not shown). Hence, the fact that *fliC*-dependent hypermotility reduces fitness of the ΔI-VII mutant remains to be explained.

The purpose of this study was to show that PAIs could be selected in the intestinal ecological context. Our results strongly suggest that this could be the case. The ability to durably colonize the intestinal tract guaranties the long-term fitness of commensal *E. coli* in terms of transmission. In adult humans, *E. coli* is the main facultative anaerobic species, reaching around 10^9 bacteria per gram of colonic content (56), meaning that up to 200 g of feces per individual per day, containing about 10^{11} *E. coli* bacteria, reach the external environment. It is undeniable that PAIs are an important contributor to the pathogenicity of ExPEC strains in extraintestinal niches. The loss of the seven PAIs clearly affected the ability of *E. coli* 536 to kill mice during sepsis (Fig. 5). However, extraintestinal infections are unlikely to provide a mode of transmission as efficient as intestinal colonization. The impact of the extraintestinal infections on the transmission of bacteria between hosts depends on the type of infection. Host death resulting from sepsis or neonatal meningitis is an evolutionary dead end for bacteria. Urinary tract infections could provide means of transmission via urine. On the other hand, induction of the host immune response results in clearance of the bacteria, thus limiting the time of shedding. Some *E. coli* strains provoking urinary tract infections can stay hidden in a quiescent state in the bladder urothelium, from which they can spread and trigger new infections once conditions are again favorable (55). However, even when urinary tract infections are recurrent, the quantity of bacterial cells shed should be several orders of magnitude lower than that disseminated via feces. Some *E. coli* strains can durably and asymptomatically colonize the human urinary tract and can be shed daily in quantities up to 10^9 bacteria/ml of urine (40). Such adaptation to the human urinary tract could increase transmission. However, full armament of ExPEC VFs seems not to be selected for during the evolution of urinary tract commensalism, as it was reported that some VFs of these strains are inactivated by point mutations and recombination events (61). Last, our results showed that PAIs represented a burden when *E. coli* 536 was cultivated in urine (Fig. 2).

In conclusion, we demonstrated that ExPEC PAIs are fitness elements involved in intestinal colonization. Our results are in accordance with the coincidental evolution hypothesis that regards extraintestinal *E. coli* virulence as a by-product of commensalism. This work lays the ground for further research on new strategies preventing extraintestinal diseases by acting directly on *E. coli* inside the gut before it can reach extraintestinal sites.

ACKNOWLEDGMENTS

We are grateful to U. Dobrindt for the generous gift of a strain. We thank A. Swidsinski for his technical help, O. Bouvet for useful discussions, and M. Leroy and L. Robert for comments on the manuscript.

M. Diard was supported by a grant from Fondation pour la Recherche Médicale.

REFERENCES

1. **Adlerberth, I., L. A. Hanson, C. Svanborg, A. M. Svennerholm, S. Nordgren, and A. E. Wold.** 1995. Adhesins of *Escherichia coli* associated with extraintestinal pathogenicity confer binding to colonic epithelial cells. Microb. Pathog. **18:**373–385.
2. **Anderson, G. G., J. J. Palermo, J. D. Schilling, R. Roth, J. Heuser, and S. J. Hultgren.** 2003. Intracellular bacterial biofilm-like pods in urinary tract infections. Science **301:**105–107.
3. **Anfora, A. T., and R. A. Welch.** 2006. DsdX is the second D-serine transporter in uropathogenic *Escherichia coli* clinical isolate CFT073. J. Bacteriol. **188:** 6622–6628.
4. **Bambou, J.-C., A. Giraud, S. Menard, B. Begue, S. Rakotobe, M. Heyman, F. Taddei, N. Cerf-Bensussan, and V. Gaboriau-Routhiau.** 2004. In vitro and ex vivo activation of the TLR5 signaling pathway in intestinal epithelial cells by a commensal *Escherichia coli* strain. J. Biol. Chem. **279:**42984–42992.
5. **Berger, H., J. Hacker, A. Juarez, C. Hughes, and W. Goebel.** 1982. Cloning of the chromosomal determinants encoding hemolysin production and mannose-resistant hemagglutination in *Escherichia coli*. J. Bacteriol. **152:**1241–1247.
6. **Brzuszkiewicz, E., H. Bruggemann, H. Liesegang, M. Emmerth, T. Olschlager, G. Nagy, K. Albermann, C. Wagner, C. Buchrieser, L. Emody, G. Gottschalk, J. Hacker, and U. Dobrindt.** 2006. How to become a uropathogen: comparative genomic analysis of extraintestinal pathogenic *Escherichia coli* strains. Proc. Natl. Acad. Sci. U. S. A. **103:**12879–12884.
7. **Chang, D. E., D. J. Smalley, D. L. Tucker, M. P. Leatham, W. E. Norris, S. J. Stevenson, A. B. Anderson, J. E. Grissom, D. C. Laux, P. S. Cohen, and T. Conway.** 2004. Carbon nutrition of *Escherichia coli* in the mouse intestine. Proc. Natl. Acad. Sci. U. S. A. **101:**7427–7432.
8. **Cohen, P. S., R. Rossoll, V. J. Cabelli, S. L. Yang, and D. C. Laux.** 1983. Relationship between the mouse colonizing ability of a human fecal *Escherichia coli* strain and its ability to bind a specific mouse colonic mucous gel protein. Infect. Immun. **40:**62–69.
9. **Cornejo, O. E., D. E. Rozen, R. M. May, and B. R. Levin.** 2009. Oscillations in continuous culture populations of *Streptococcus pneumoniae*: population dynamics and the evolution of clonal suicide. Proc. Biol. Sci. **276:**999–1008.
10. **Danese, P. N., L. A. Pratt, S. L. Dove, and R. Kolter.** 2000. The outer membrane protein, antigen 43, mediates cell-to-cell interactions within *Escherichia coli* biofilms. Mol. Microbiol. **37:**424–432.
11. **Datsenko, K. A., and B. L. Wanner.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc. Natl. Acad. Sci. U. S. A. **97:**6640–6645.
12. **Djordjevic, D., M. Wiedmann, and L. A. McLandsborough.** 2002. Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. Appl. Environ. Microbiol. **68:**2950–2958.
13. **Escobar-Páramo, P., K. Grenet, A. Le Menac'h, L. Rode, E. Salgado, C. Amorin, S. Gouriou, B. Picard, M. C. Rahimy, A. Andremont, E. Denamur,**

and R. Ruimy. 2004. Large-scale population structure of human commensal *Escherichia coli* isolates. Appl. Environ. Microbiol. **70:**5698–5700.
14. **Fontaine, F., E. J. Stewart, A. B. Lindner, and F. Taddei.** 2008. Mutations in two global regulators lower individual mortality in *Escherichia coli*. Mol. Microbiol. **67:**2–14.
15. **Freter, R., H. Brickner, J. Fekete, M. M. Vickerman, and K. E. Carey.** 1983. Survival and implantation of *Escherichia coli* in the intestinal tract. Infect. Immun. **39:**686–703.
16. **Gauger, E. J., M. P. Leatham, R. Mercado-Lubo, D. C. Laux, T. Conway, and P. S. Cohen.** 2007. Role of motility and the flhDC operon in *Escherichia coli* MG1655 colonization of the mouse intestine. Infect. Immun. **75:**3315–3324.
17. **Giraud, A., S. Arous, M. De Paepe, V. Gaboriau-Routhiau, J. C. Bambou, S. Rakotobe, A. B. Lindner, F. Taddei, and N. Cerf-Bensussan.** 2008. Dissecting the genetic components of adaptation of *Escherichia coli* to the mouse gut. PLoS Genet. **4:**e2.
18. **Goetz, G. S., A. Mahmood, S. J. Hultgren, M. J. Engle, K. Dodson, and D. H. Alpers.** 1999. Binding of pili from uropathogenic *Escherichia coli* to membranes secreted by human colonocytes and enterocytes. Infect. Immun. **67:**6161–6163.
19. **Gómez-Gómez, J. M., C. Manfredi, J. C. Alonso, and J. Blazquez.** 2007. A novel role for RecA under non-stress: promotion of swarming motility in *Escherichia coli* K-12. BMC Biol. **5:**14.
20. **Güldener, U., S. Heck, T. Fielder, J. Beinhauer, and J. H. Hegemann.** 1996. A new efficient gene disruption cassette for repeated use in budding yeast. Nucleic Acids Res. **24:**2519–2524.
21. **Hacker, J., G. Blum-Oehler, I. Muhldorfer, and H. Tschape.** 1997. Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. Mol. Microbiol. **23:**1089–1097.
22. **Hacker, J., and E. Carniel.** 2001. Ecological fitness, genomic islands and bacterial pathogenicity. A Darwinian view of the evolution of microbes. EMBO Rep. **2:**376–381.
23. **Haldimann, A., and B. L. Wanner.** 2001. Conditional-replication, integration, excision, and retrieval plasmid-host systems for gene structure-function studies of bacteria. J. Bacteriol. **183:**6384–6393.
24. **Hancock, D. D., T. E. Besser, and D. H. Rice.** 1998. Ecology of *Escherichia coli* O157:H7 in cattle and impact of management practices, p. 85–91. *In* J. B. Kaper and A. D. O'Brien (ed.), *Escherichia coli* O157:H7 and other Shiga toxin-producing *E. coli* s trains. ASM Press, Washington, DC.
25. **Herías, M. V., T. Midtvedt, L. A. Hanson, and A. E. Wold.** 1995. Role of *Escherichia coli* P fimbriae in intestinal colonization in gnotobiotic rats. Infect. Immun. **63:**4781–4789.
26. **Hérias, M. V., T. Midtvedt, L. A. Hanson, and A. E. Wold.** 1997. *Escherichia coli* K5 capsule expression enhances colonization of the large intestine in the gnotobiotic rat. Infect. Immun. **65:**531.
27. **Holm, S.** 1979. A simple sequentially rejective multiple test procedure. Scand. J. Stat. **6:**65–70.
28. **Johnson, J. R., and M. Kuskowski.** 2000. Clonal origin, virulence factors, and virulence. Infect. Immun. **68:**424–425.
29. **Johnson, J. R., and T. A. Russo.** 2002. Extraintestinal pathogenic *Escherichia coli*: "the other bad *E. coli*." J. Lab. Clin. Med. **139:**155–162.
30. **Johnson, J. R., and T. A. Russo.** 2005. Molecular epidemiology of extraintestinal pathogenic (uropathogenic) *Escherichia coli*. Int. J. Med. Microbiol. **295:**383–404.
31. **Jones, S. A., F. Z. Chowdhury, A. J. Fabich, A. Anderson, D. M. Schreiner, A. L. House, S. M. Autieri, M. P. Leatham, J. J. Lins, M. Jorgensen, P. S. Cohen, and T. Conway.** 2007. Respiration of *Escherichia coli* in the mouse intestine. Infect. Immun. **75:**4891–4899.
32. **Kaper, J. B., J. P. Nataro, and H. L. Mobley.** 2004. Pathogenic *Escherichia coli*. Nat. Rev. Microbiol. **2:**123–140.
33. **Lainhart, W., G. Stolfa, and G. B. Koudelka.** 2009. Shiga toxin as a bacterial defense against a eukaryotic predator, *Tetrahymena thermophila*. J. Bacteriol. **191:**5116–5122.
34. **Lane, M. C., V. Lockatell, G. Monterosso, D. Lamphier, J. Weinert, J. R. Hebel, D. E. Johnson, and H. L. Mobley.** 2005. Role of motility in the colonization of uropathogenic *Escherichia coli* in the urinary tract. Infect. Immun. **73:**7644–7656.
35. **Lasaro, M. A., N. Salinger, J. Zhang, Y. Wang, Z. Zhong, M. Goulian, and J. Zhu.** 2009. F1C fimbriae play an important role in biofilm formation and intestinal colonization by the *Escherichia coli* commensal strain Nissle 1917. Appl. Environ. Microbiol. **75:**246–251.
36. **Leatham, M. P., S. J. Stevenson, E. J. Gauger, K. A. Krogfelt, J. J. Lins, T. L. Haddock, S. M. Autieri, T. Conway, and P. S. Cohen.** 2005. Mouse intestine selects nonmotile *flhDC* mutants of *Escherichia coli* MG1655 with increased colonizing ability and better utilization of carbon sources. Infect. Immun. **73:**8039–8049.
37. **Le Gall, T., O. Clermont, S. Gouriou, B. Picard, X. Nassif, E. Denamur, and O. Tenaillon.** 2007. Extraintestinal virulence is a coincidental by-product of commensalism in B2 phylogenetic group *Escherichia coli* strains. Mol. Biol. Evol. **24:**2373–2384.
38. **Levin, B. R.** 1996. The evolution and maintenance of virulence in microparasites. Emerg. Infect. Dis. **2:**93–102.
39. **Levin, B. R., and C. Svanborg Eden.** 1990. Selection and evolution of virulence in bacteria: an ecumenical excursion and modest suggestion. Parasitology **100**(Suppl.)**:**S103–S115.
40. **Lindberg, U., I. Claesson, L. A. Hanson, and U. Jodal.** 1975. Asymptomatic bacteriuria in schoolgirls. I. Clinical and laboratory findings. Acta Paediatr. Scand. **64:**425–431.
41. **Lloyd, A. L., T. A. Henderson, P. D. Vigil, and H. L. Mobley.** 2009. Genomic islands of uropathogenic *Escherichia coli* contribute to virulence. J. Bacteriol. **191:**3469–3481.
42. **Macfarlane, S.** 2008. Microbial biofilm communities in the gastrointestinal tract. J. Clin. Gastroenterol. **42**(Suppl. 3)**:**S142–S143.
43. **McCormick, B. A., D. C. Laux, and P. S. Cohen.** 1990. Neither motility nor chemotaxis plays a role in the ability of *Escherichia coli* F-18 to colonize the streptomycin-treated mouse large intestine. Infect. Immun. **58:**2957.
44. **Middendorf, B., B. Hochhut, K. Leipold, U. Dobrindt, G. Blum-Oehler, and J. Hacker.** 2004. Instability of pathogenicity islands in uropathogenic *Escherichia coli* 536. J. Bacteriol. **186:**3086–3096.
45. **Moreno, E., J. R. Johnson, T. Perez, G. Prats, M. A. Kuskowski, and A. Andreu.** 2009. Structure and urovirulence characteristics of the fecal *Escherichia coli* population among healthy women. Microbes Infect. **11:**274–280.
46. **Myhal, M. L., D. C. Laux, and P. S. Cohen.** 1982. Relative colonizing abilities of human fecal and K 12 strains of *Escherichia coli* in the large intestines of streptomycin-treated mice. Eur. J. Clin. Microbiol. **1:**186–192.
47. **Nagy, G., A. Altenhoefer, O. Knapp, E. Maier, U. Dobrindt, G. Blum-Oehler, R. Benz, L. Emody, and J. Hacker.** 2006. Both alpha-haemolysin determinants contribute to full virulence of uropathogenic *Escherichia coli* strain 536. Microbes Infect. **8:**2006–2012.
48. **Novick, A., and L. Szilard.** 1950. Description of the chemostat. Science **112:**715–716.
49. **Nowrouzian, F., I. Adlerberth, and A. E. Wold.** 2001. P fimbriae, capsule and aerobactin characterize colonic resident *Escherichia coli*. Epidemiol. Infect. **126:**11–18.
50. **Nowrouzian, F., A. E. Wold, and I. Adlerberth.** 2001. P fimbriae and aerobactin as intestinal colonization factors for *Escherichia coli* in Pakistani infants. Epidemiol. Infect. **126:**19–23.
51. **Nowrouzian, F. L., I. Adlerberth, and A. E. Wold.** 2006. Enhanced persistence in the colonic microbiota of *Escherichia coli* strains belonging to phylogenetic group B2: role of virulence factors and adherence to colonic cells. Microbes Infect. **8:**834–840.
52. **Nowrouzian, F. L., A. E. Wold, and I. Adlerberth.** 2005. *Escherichia coli* strains belonging to phylogenetic group B2 have superior capacity to persist in the intestinal microflora of infants. J. Infect. Dis. **191:**1078–1083.
53. **Picard, B., J. S. Garcia, S. Gouriou, P. Duriez, N. Brahimi, E. Bingen, J. Elion, and E. Denamur.** 1999. The link between phylogeny and virulence in *Escherichia coli* extraintestinal infection. Infect. Immun. **67:**546–553.
54. **Poulsen, L. K., T. R. Licht, C. Rang, K. A. Krogfelt, and S. Molin.** 1995. Physiological state of *Escherichia coli* BJ4 growing in the large intestines of streptomycin-treated mice. J. Bacteriol. **177:**5840–5845.
55. **Rosen, D. A., T. M. Hooton, W. E. Stamm, P. A. Humphrey, and S. J. Hultgren.** 2007. Detection of intracellular bacterial communities in human urinary tract infection. PLoS Med. **4:**e329.
56. **Savage, D. C.** 1977. Microbial ecology of the gastrointestinal tract. Annu. Rev. Microbiol. **31:**107–133.
57. **Simms, A. N., and H. L. Mobley.** 2008. PapX, a P fimbrial operon-encoded inhibitor of motility in uropathogenic *Escherichia coli*. Infect. Immun. **76:**4833–4841.
58. **Sjöström, A. E., C. Balsalobre, L. Emody, B. Westerlund-Wikstrom, J. Hacker, and B. E. Uhlin.** 2009. The SfaXII protein from newborn meningitis *E. coli* is involved in regulation of motility and type 1 fimbriae expression. Microb. Pathog. **46:**243–252.
59. **Steinberg, K. M., and B. R. Levin.** 2007. Grazing protozoa and the evolution of the *Escherichia coli* O157:H7 Shiga toxin-encoding prophage. Proc. Biol. Sci. **274:**1921–1929.
60. **Tourret, J., M. Diard, L. Garry, I. Matic, and E. Denamur.** 2010. Effects of single and multiple pathogenicity island deletions on uropathogenic *Escherichia coli* strain 536 intrinsic extra-intestinal virulence. Int. J. Med. Microbiol. [Epub ahead of print.] doi:10.1016/j.ijmm.2010.04.013.
61. **Zdziarski, J., C. Svanborg, B. Wullt, J. Hacker, and U. Dobrindt.** 2008. Molecular basis of commensalism in the urinary tract: low virulence or virulence attenuation? Infect. Immun. **76:**695–703.
62. **Zhang, L., B. Foxman, and C. Marrs.** 2002. Both urinary and rectal *Escherichia coli* isolates are dominated by strains of phylogenetic group B2. J. Clin. Microbiol. **40:**3951–3955.