Pathogenicity Island Markers, Virulence Determinants *malX* and *usp*, and the Capacity of *Escherichia coli* To Persist in Infants' Commensal Microbiotas[▽]

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Virulence-associated genes in bacteria are often located on chromosomal regions, termed pathogenicity islands (PAIs). Several PAIs are found in *Escherichia coli* strains that cause extraintestinal infections, but their role in commensal bowel colonization is unknown. Resident strains are enriched in adhesins (P fimbriae and type 1 fimbriae), capsular antigens (K1 and K5), hemolysin, and aerobactin and mostly belong to phylogenetic group B2. Here, we investigated whether six pathogenicity islands and the virulence determinants *malX* and *usp* are associated with fitness of *E. coli* in the infant bowel microbiota. *E. coli* strains isolated from stools of 130 Swedish infants during the first year of life were examined for their carriage of PAI markers, *malX*, and *usp* by PCR. Carriage was related to strain persistence: long-term colonizers (≥12 months) carried significantly more of PAI II from strain CFT703 (II_{CFT703}), IV_{536}, and II_{J96} and *malX* and *usp* than intermediate colonizers (1 to 11 months) and transient strains (<3 weeks). The accumulation of PAI markers in each individual strain correlated positively with its time of persistence in the colon. Phylogenetic group B2 accounted for 69% of long-term colonizers, 46% of intermediate colonizers and 14% of transient strains. These results support the hypothesis that some bacterial traits contributing to extraintestinal infections have in fact evolved primarily because they increase the fitness of *E. coli* in its natural niche, the colon; accordingly, they may be regarded as fitness islands in the gut.

Escherichia coli is a normal inhabitant of the large intestine, but certain strains also cause extraintestinal infections when they spread from their primary niche. Such pathogenic *E. coli* strains express several virulence-associated traits that contribute to the disease process, including adhesins, certain O serotypes and capsular antigens, iron-trapping compounds (such as aerobactin), and cytolytic toxins (such as hemolysin) (17).

Pathogenicity-associated islands (PAIs) are particular regions on the bacterial chromosome where virulence genes have accumulated. PAIs, and their associated virulence genes, have spread among bacterial populations by horizontal transfer (15). Several PAIs were previously identified in uropathogenic *E. coli* strains such as *E. coli* 536, *E. coli* J96, and *E. coli* CFT073. PAIs I to IV from strain 536 (I_{536} to IV_{536}) encode a range of virulence factors, including P fimbriae, P-related fimbriae, α-hemolysin, S fimbriae, and the yersiniabactin siderophore system. PAI I_{J96} and II_{J96} encode P fimbriae, P-related fimbriae, and α-hemolysin; PAI I_{CFT073} and II_{CFT073} encode P fimbriae, α-hemolysin, and aerobactin (42) (Table 1). *usp* is prevalent among strains causing urinary tract infections (24). It enhances infectivity in a mouse ascending urinary tract infection (UTI) model (48), displays homology with S-type pyocin, and may function as a bacteriocin (34). *malX* codes for a phosphotransferase system enzyme II that recognizes maltose and glucose (37) and is enriched in strains causing extraintestinal infections (18, 19, 41).

E. coli segregate into four main phylogenetic groups, termed A, B1, B2, and D (16). Strains of group A and B1 seldom cause extraintestinal infections and carry few virulence factor genes (4, 7). Extraintestinal pathogenic *E. coli* strains belong mostly to group B2 (4, 18) and to a lesser extent to group D and usually carry several PAIs (5, 33, 40).

Intestinal *E. coli* strains differ widely in colonization capacity; resident strains have the ability to persist in the gut microbiota, while transient strains disappear from the microbiota within a short time (44–46). Previously, in several epidemiological studies, we demonstrated that genes for certain virulence-associated factors, such as P fimbriae, type 1 fimbriae, hemolysin, capsular polysaccharides (K1 and K5), and aerobactin, are significantly enriched in resident strains (27–29). Furthermore, strains of B2 origin have an enhanced ability to persist in the intestinal microbiotas of infants (32).

The present study was designed to investigate whether several markers for pathogenicity islands and the virulence-associated genes *malX* and *usp* are associated with an increased capacity of *E. coli* to persist in the infantile intestinal microbiota. In regard to this, 273 *E. coli* strains obtained from 130 healthy Swedish infants, monitored longitudinally over the first year of life, were assessed for carriage of markers for PAIs I_{536}, II_{536}, III_{536}, IV_{536}, II_{CFT073}, and II_{J96} and for *malX* and *usp*. Carriage of PAI-associated markers was compared with a strain's persistence.

MATERIALS AND METHODS

***E. coli* strains.** The source population comprised 130 healthy Swedish infants born in 1998 to 2001 at the Sahlgrenska University Hospital, Göteborg, Sweden. They were part of a prospective birth cohort study examining the relation between the intestinal colonization pattern and allergy development,

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TABLE 1. The pathogenicity islands and the functions encoded

Pathogenicity island or gene	Product(s)	Reference(s)
Pathogenicity islands		
II_{CFT073}	P fimbriae, iron-regulated proteins	36
I_{536}	α-Hemolysin, F17-like fimbriae, CS12-like fimbriae	12
II_{536}	Hek adhesin, P-related fimbriae, α-hemolysin, hemagglutinin-like adhesin	12
III_{536}	S fimbriae, salmochelin, HmuR-like heme receptor, Sat toxin, Tsh-like hemoglobin protease, antigen 43	12
IV_{536}	Yersiniabactin siderophore system	12
II_{J96}	α-Hemolysin, Prs fimbriae, cytotoxic, necrotizing factor	3, 6
Virulence-associated genes		
malX	Maltose- and glucose-specific component IIa of a phosphoenolpyruvate-dependent phosphotransferase system	14, 38
usp	Putative bacteriocin	24

the ALLERGYFLORA study (1, 2). *E. coli* strains ($n = 149$) from 70 of these were previously described with regard to *E. coli* colonization pattern, association between certain virulence factor genes, phylogenetic group distribution, and persistence (28, 30). Here in a larger infant cohort, carriage of six PAI markers, including PAI I_{536}, II_{536}, III_{536}, IV_{536}, II_{J96}, and II_{CFT073}, as well as *malX* and *usp*, in the intestinal *E. coli* strains was identified. Phylogenetic group distribution was further determined.

E. coli strains were isolated and quantified in stools as previously described (1). In brief, a rectal swab was obtained at 3 days of age and cultured semiquantitatively under aerobic condition for facultative bacteria. Fecal samples were collected at 1, 2, 4, and 8 weeks and at 6 and 12 months of age. They were diluted serially and cultured on Drigalski's agar for isolation of *Enterobacteriaceae*. One to six colonies with different morphologies were isolated from each positive culture and enumerated, identified to species level by using biotyping (API20E; API Systems SA, La Balme les Grottes, Montalieu-Vercieu, France), and stored at −70°C. CFU with the same morphology within each sample were regarded as the same strain. After species identification, *E. coli* isolates were analyzed by random amplified polymorphic DNA (RAPD) profiling. Isolates from a particular infant that exhibited RAPD patterns differing by no more than three weak bands were considered to be the same strain (28).

The strains were divided into three groups according to the time they persisted in the host: strains colonizing the microbiota for <3 weeks were termed transient strains, strains colonizing for 1 to 11 months were termed intermediate colonizers, and those colonizing for ≥12 months were termed long-term colonizers. Strains appearing only once in the last sample or in samples with long sampling intervals could not be classified according to these definitions and therefore were included only in characterization of the infant *E. coli* microbiota.

Detection of markers for pathogenicity islands and assignment to phylogenetic groups by PCR. Phylogenetic group distribution was determined using triplex PCR as described by Clermont et al. (8) with slight modifications. Bacterial DNA was added to a PCR mixture containing HotStarTaq master mix (Qiagen, Spånga, Sweden) and 20 pmol of each primer pair amplifying the genes *chuA* and *yjaA* and the DNA fragment TspE4.C2.

All primers used for identification of the screened PAI markers have already been described (39). They were selected from PAI-associated sequences (11, 20, 23, 25), and their similarity to *E. coli* and specificity for the PAI sequences have also been screened using BLASTN (39).

To extract genomic DNA, a small amount of bacteria was suspended in Tris-EDTA buffer (Sigma-Aldrich Sweden AB, Stockholm, Sweden) and heated at 95°C for 10 min. The mixture was centrifuged for 5 min at room temperature, and supernatants were used for PCRs. The DNA extraction method was identical in all PCR assays.

PAI IV_{536} (high-pathogenicity island) was identified as previously described (9). The presence of *malX* was confirmed by a PCR assay described elsewhere (31) using the previously published primers (20).

PAI I and II in *E. coli* 536 and PAI II_{J96} were identified by a duplex and a single PCR, respectively. The following PCR procedure was used for both assays: 1.2 U Expand high-fidelity *Taq* DNA polymerase (Roche Diagnostics, Bromma, Sweden), 0.5 mM deoxynucleoside triphosphates (dNTPs), 3 mM MgCl₂, 0.5 mM each primer (primers 1.9, 1.10, orf1 up, and orf1 down for duplex PCR and primers hlyd and cnf for single PCR) in a final volume of 25 µl. The PCR

program was 95°C for 5 min and then 30 cycles of 94°C for 1 min, 57°C for 1 min, and 72°C for 3 min, with a final extension step at 72°C for 10 min.

The presence of PAI II_{CFT073} and PAI III_{536} were verified in a duplex PCR using 12.5 µl HotStarTaq master mix (Qiagen, Spånga, Sweden), 0.1 µM primers cft073.2Ent1 and cft073.2Ent2, 0.5 µM primers sfaAI.1 and sfaAI.2, and 2.0 mM MgCl₂ in a final volume of 25 µl. The PCR program was 95°C for 15 min and then 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension step at 72°C for 10 min. The PCR amplicons were separated on agarose gels and stained with ethidium bromide.

We screened all strains for the presence of the *papG* allele I, which is known as a marker for PAI $_{IJ96}$ (39), using a PCR assay described elsewhere (27). However, we found no *papGI*-positive strain, and we therefore excluded this PAI in the present study.

A real-time PCR was developed to detect *usp*. Primers and a probe were designed using Primer Express 3.0 software (Applied Biosystems, Stockholm, Sweden) based on the sequence for *usp* in *E. coli* strain Z42 as retrieved from DDBJ, accession number AB056434 (26). The primers were USPf (5′-CGGCG CATATTGCTGATAAAT-3′) and USPr (5′-CGCCTCGCGAAACTCATC-3′), and the probe was 5′-ACGGGAGTTTAAAACC-3′. The PCR was performed by adding 2.5 µl of bacterial lysate to a mixture containing 12.5 µl TaqMan universal PCR master mix (Applied Biosystems) and 0.2 µM each primer and probe in a final volume of 25 µl. The PCR was carried out using Applied Biosystems 7500 PCR systems (Applied Biosystems, Stockholm, Sweden). The PCR program was 95°C for 10 min, followed by 95°C for 15 s and 60°C for 1 min for 35 cycles.

To verify the real-time PCR assay, 100 strains were assessed using the conventional primers described by Nakano et al. (26). There was a 99% accordance. Strains CFT073, 536, J96, Z42, Z13, and C10 (26) served as positive controls for PAI markers.

Virulence score representing carriage of PAI markers and *malX* and *usp*. Each strain was assigned a score (from 0 to 8) corresponding to the number of PAI markers, *malX*, and *usp*. This score was used as an X variable (an independent variable) in the multivariate models.

Statistical methods. Proportions were compared using Fisher's exact test. The prevalence of each individual PAI marker, *malX*, and *usp* in transient strains, intermediate colonizers, and long-term colonizers was first analyzed in a 2-by-3 table. The bacterial determinants that showed a significant nonrandom distribution ($P < 0.05$) were further compared between each group in a series of 2-by-2 tables. The virulence score was compared by the Mann-Whitney U test. Multivariate data analysis was performed using Simca-P + 12.0 (Umetrics AB, Umeå, Sweden). Projections to latent structures by means of partial least square (PLS) were used to assess the relationship between carriage of the PAI markers, *usp*, and *malX* and persistence of *E. coli* strains in the gut flora. This method is a regression development of principal component analysis (PCA) in which clustering data are related to a Y variable, in this case, persistence in the gut flora, that was generated by a score setting 0 for transient strains, 1 for intermediate colonizers, and 2 for long-term colonizers. The studied PAI markers, *malX*, *usp*, and phylogenetic group origin were modeled as X variables (independent variables) and persistence as the Y variable (dependent variable). Analyses were made using the default settings in SIMCA-P+ 12.0.

Logistic regression analysis, using stepwise selection, was performed to predict which trait was independently associated with persistence.

TABLE 2. Prevalence of PAI markers, *malX*, and *usp* in the
intestinal *E. coli* strains of healthy Swedish infants[a]

Marker or gene	% positive strains	
	All strains ($n = 273$)	Strains defined by persistence ($n = 100$)
PAI markers		
IV$_{536}$	57	62
II$_{CFT073}$	34	37
I$_{536}$	18	14
II$_{J96}$	17	14
II$_{536}$	12	8
III$_{536}$	3	4
Virulence-associated genes		
malX	44	46
usp	45	52

[a] A total of 273 commensal *E. coli* strains obtained from 130 Swedish infants during the first year of life were assessed for carriage of six PAI markers, *malX*, and *usp*. Of these, 100 were defined according to the time they persisted in the infantile gut.

RESULTS

Prevalence of PAI markers, *malX*, and *usp* among *E. coli* strains from the infantile intestinal microbiota. Overall, 273 distinct fecal *E. coli* strains were identified in samples from the 130 Swedish infants monitored from 3 days to 1 year of age. The median number of *E. coli* strains per infant was 2 (range 0 to 6). Of these, 60% carried at least one of the investigated bacterial traits. The prevalence of the PAI markers, *malX*, and *usp* is shown in Table 2. Markers for PAI IV$_{536}$ were the most abundant, while markers for PAI I$_{536}$, II$_{536}$, and III$_{536}$ were the least frequent. The carriage rates of *malX*, *usp*, and PAI II$_{CFT073}$ were roughly similar.

Presence of PAI markers, *malX*, and *usp* in relation to persistence of *E. coli* strains in the gut microbiota. The time of persistence was determined for 100 of the 273 strains. It was not possible to classify strains ($n = 173$) that appeared only once in the last sample or in a sample with a long sampling interval as long-term colonizers, intermediate colonizers, or transient strains. A total of 32 *E. coli* strains were classified as long-term colonizers (>12 months), 47 as intermediate colonizers (1 to 11 months), and 21 as transient strains (<3 weeks).

Each marker showed a clear tendency to increase in frequency with the time the strains persisted in the microbiota (Fig. 1). There was a significant association between persistence and markers for PAI IV$_{536}$, PAI II$_{CFT073}$, PAI II$_{J96}$, *malX*, and *usp*. Indeed, they were considerably enriched in long-term colonizers compared to intermediate or transient strains (Fig. 1). No significant differences regarding markers for PAI I$_{536}$, II$_{536}$, and III$_{536}$ were observed.

The relationship between virulence score and persistence is shown in Fig. 2. There was a dose-response relationship between the number of PAI markers, *malX*, and *usp* and persistence. Long-term colonizers had on average four virulence traits, compared with one trait in intermediate colonizers and no traits in transient strains ($P = 0.0025$ and $P < 0.0001$, respectively).

PLS regression analysis was used to investigate the relative strength of the associations of the PAI markers, *malX*, and *usp*
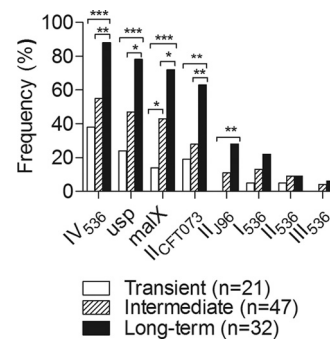

FIG. 1. Prevalence of the PAI markers I$_{536}$, II$_{536}$, III$_{536}$, IV$_{536}$, II$_{CFT073}$, and II$_{J96}$ and the virulence determinants *malX* and *usp* in long-term-colonizer ($n = 32$), intermediate-colonizer ($n = 47$), and transient ($n = 21$) strains. Proportions were compared between groups using Fisher's exact test; asterisks indicate the level of significance: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$.

and phylogenetic group with colonic persistence. The PLS analysis yielded a one-component model that revealed the clustering of *E. coli* strains based on these bacterial traits and phylogenetic group (Fig. 3a). Long-term colonizers were mainly positioned on the positive axis of the component, while transient strains were most often found on the negative axis of the component. In this model, intermediate colonizers were scattered across both axes.

The bacterial determinants that generated the separation are shown in Fig. 3b. According to the model, variables located on the right side of diagram (with positive values on the y axis) were associated with long-term colonizers, while traits enriched in transient strains appear on the left (with negative values on the y axis). The taller the bar, the greater the trait's estimated contribution to persistence, and the smaller the error bar, the more reliable the estimate. The characteristics virulence score, *malX*, *usp*, group B2 origin, PAI II$_{CFT073}$, PAI IV$_{536}$, and PAI II$_{J96}$ were obviously associated with persistence, while markers for PAI I$_{536}$, II$_{536}$, and III$_{536}$ were less associated with persistence (Fig. 3b). Phylogenetic group origins A, B1, and D were linked to transient strains.

In addition, we used a logistic regression analysis with stepwise selection; in this model, transient strains and long term
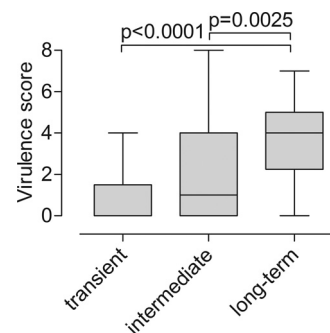

FIG. 2. Virulence score (number of PAI markers or virulence determinants) in long-term-colonizer, intermediate-colonizer, and transient strains in relation to the time of persistence of *E. coli* strains in the microbiotas of Swedish infants. The median values were compared using the Mann-Whitney U test.
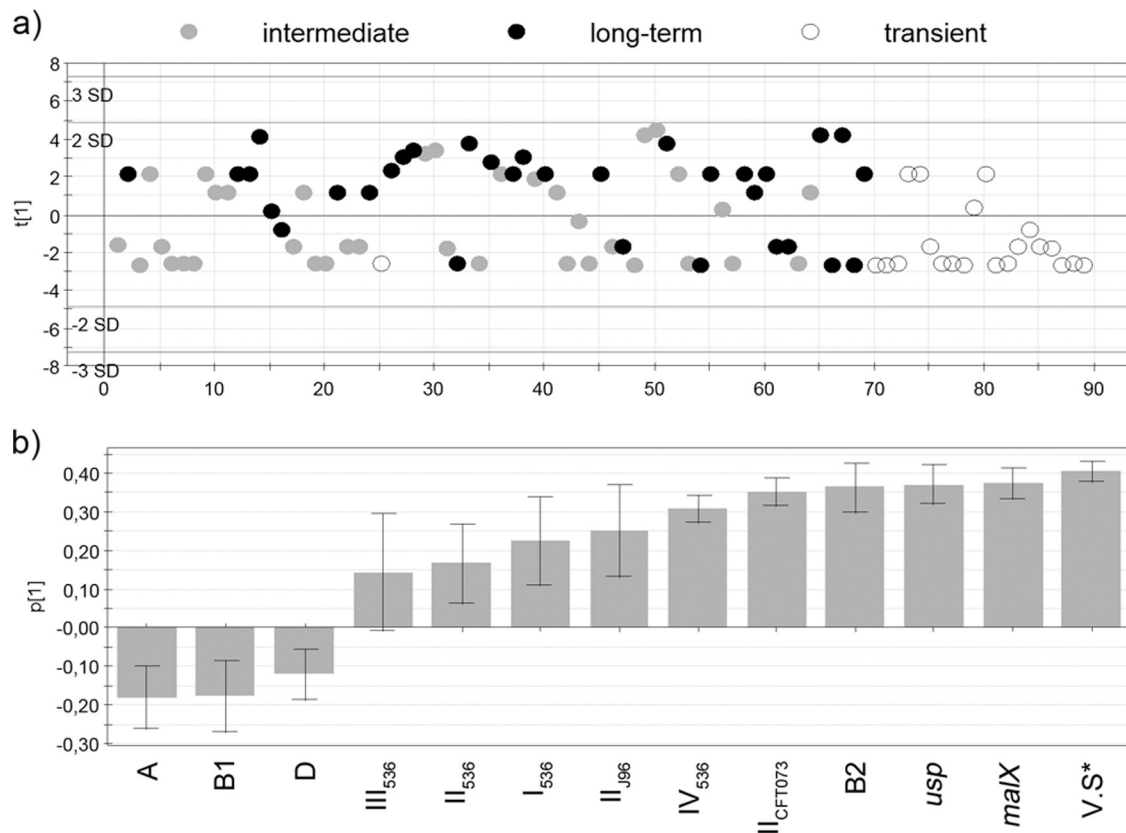
FIG. 3. Projections to latent structures by means of partial least square (PLS) was used to relate two data matrices, i.e., the X variables, including the virulence score and phylogenetic group, and the responder Y variable (persistence) to each other by a linear multivariate model. The model resulted in one component, which is on the y axis. (a) The score plot shows the position of observations (strains) along the principal component (y axis). Long-term colonizers (black dots) were mainly gathered on the positive axis and transient strains (white dots) on the negative axis. Intermediate colonizers (gray dots) appeared at either side. (b) Loadings, i.e., the variables generating the observed separation. PAI markers and virulence determinants, phylogenetic group, and the virulence score (V.S*) are included.

colonizers were included. Here we entered the screened PAI markers, *malX*, *usp*, and phylogenetic group B2 as independent variables and persistence as the dependent variable. *malX* was the only variable that yielded significance in this model (odds ratio, 16.2; 95% confidence interval, 3.8 to 68; $P < 0.000$).

Phylogenetic group in relation to PAI markers, *malX*, *usp*, and persistence. Of the 273 distinct fecal strains, approximately half (45%) belonged to phylogenetic group B2, followed by groups A (28%), D (15%), and B1 (12%). The phylogenetic distribution of the 100 strains characterized regarding time of persistence were similar to that for the whole collection: B2, 46%; A, 23%; D, 18%; and B1, 13%.

Phylogenetic group B2 strains were significantly more common in long-term colonizers than intermediate colonizers (69% versus 46%; $P = 0.01$), and transient strains (69% versus 14%; $P < 0.0001$). In contrast, 32% of transient strains belonged to group B1, compared to 7% of intermediate strains ($P = 0.01$) and 9% of long-term colonizers (not significant).

The presence of PAI markers, *malX*, and *usp* varied by phylogenetic group. Group B2 strains were much more likely to carry each of the 8 investigated traits than all other strains ($P = 0.002$ for PAI III$_{536}$; $P < 0.0001$ for all other markers). The only trait which was common in other phylogenetic groups

was PAI IV$_{536}$ (6% of B1, 25% of A, and 49% of D strains compared to 91% of B2 strains). *malX* was also relatively frequent among group D strains (17%).

DISCUSSION

Pathogenicity islands (PAIs) are enriched among *E. coli* strains causing extraintestinal infections, and extraintestinal pathogenic *E. coli* (ExPEC) strains mostly belong to phylogenetic groups B2 and D. Here, we examined whether carriage of six PAI markers, *malX*, and *usp* was associated with the capacity of *E. coli* strains to persist in the intestinal microbiota of 130 Swedish infants. The infants were monitored over the first year of life with regular quantitative cultures of stool samples. Individual *E. coli* strains were identified and characterized with respect to carriage of PAI markers, *malX*, and *usp*, phylogenetic group origin in each stool sample, and duration of persistence in the microbiota.

The most direct method of detecting PAIs is to determine the physical location of genetic elements representative of specific PAIs (5). Hence, the combination of pulsed-field gel electrophoresis analysis of macrorestricted genomic DNA and Southern hybridization is a reliable method. However, in the

present study, for efficiency we used several PCR assays for detection of six PAI markers, *malX*, and *usp*.

We found that more than half of the intestinal commensal *E. coli* strains presumptively contained at least one of the studied bacterial determinants, including I_{536}, II_{536}, III_{536}, IV_{536}, II_{J96}, I, *malX*, and *usp*, and that each of these was found in a higher frequency than generally reported among commensal strains (22, 24, 39, 43). The high prevalence of PAI markers is likely to reflect the dominance of phylogenetic group B2 strains in the intestinal microbiota of the Swedish infants examined here (32).

There was no significant association between carriage of PAIs I_{536}, II_{536}, and III_{536} and persistence. However, none of the transient strains carried markers for PAI III_{536}, and few carried markers for PAIs I_{536} and II_{536}. This indicates that PAIs I_{536}, II_{536}, and III_{536} possibly have an impact on persistence, although their low prevalence here as a single marker precluded statistical significance. In fact, 96% of PAIs I_{536}, II_{536}, and III_{536} appear with other common markers in combination, i.e., mostly together with 5 to 7 markers. Only two strains carried PAIs I_{536}, II_{536}, and III_{536} as single markers. Notably, acquisition of PAIs is not a random event (13), and here the rarest PAI markers were present only in strains already carrying the more common markers.

PLS analysis demonstrated the internal order of hierarchy in the association between accumulation of pathogenicity traits and persistence. The variable representing the virulence score exhibited the strongest association with persistence. Indeed, the greater the virulence score, the longer the duration of persistence. One may speculate that pathogenicity markers have an additive effect on intestinal colonization. It might be due to genes carried by different pathogenicity islands having an additive and/or synergistic effect. Or it may be that PAIs tend to occur in greater numbers in strains that have other unknown factors which promotes intestinal colonization. In support of the first interpretation, a recent experimental study by Diard et al. demonstrated an additive influence of seven PAIs (I_{536} to VII_{536}) on persistence of *E. coli* in the intestines of mice. No single PAI showed a significant effect on intestinal colonization (10). Similarly, an additive function of the same seven PAIs for stabilizing extraintestinal virulence has been observed in a mouse septicemia model (47).

Many of the investigated markers probably do contribute to persistence, but as known virulence associated genes appear together and this multicollinearity leads to reduced precision in the prediction (35), it is difficult to show using logistic regression. Indeed, *malX* was the only marker that yielded significance, which is in accordance with the PLS results.

The association of phylogenetic group B2 and several extraintestinal virulence genes with persistence has previously been documented among *E. coli* strains from 70 of the 130 infants examined here (28, 32).

Our previous observations suggest that several well-known bacterial traits in *E. coli*, such as P fimbriae, type 1 fimbriae, aerobactin, capsular antigens (K1 and K5 capsule) and hemolysin, are associated with persistence of *E. coli* in its natural ecological niche, the large intestine (27–29).

Notably, *hylA* and *papC* may also be carried by PAI II_{J96}, PAI I_{536}, PAI II_{536}, and PAI II_{CFT073}, respectively (3, 6, 12, 36); these PAIs were associated with persistence in the present

study. We also showed previously that resident *E. coli* strains possessed siderophores such as aerobactin (27, 29) and yersiniabactin (27). Interestingly, PAI IV_{536} codes for yersiniabactin, and a variety of other siderophore systems are also located on several PAIs (3, 6, 12, 36). The virulence determinants *malX*, also found on PAI I_{CFT073} (14), and *usp* are slightly associated with the B2 phylogenetic group (20, 21); strains of this group are, in general, excellent intestinal colonizers. One may speculate that the superior capacity of B2 strains to colonize the human gut is due partly to their accumulation of virulence factor genes, many of which are encoded by PAIs.

In summary, we have confirmed association between additional factors, i.e., six PAI markers, *malX*, and *usp*, with persistence of *E. coli* strains in the intestine in a large infant cohort. Further, this shows that the studied pathogenicity traits exhibit an accumulation effect promoting persistence of group B2 strains in the intestinal microbiota.

Taken together, our findings support the hypothesis that B2 strains may have evolved many of their so-called pathogenic characteristics in order to survive in the complex ecosystem in the human colon, with no reference to virulence. These pathogenicity markers likely cooperate in an additive manner for promoting the intestinal persistence of *E. coli* strains; thus, they may be regarded as "fitness islands."

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