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Cyclomodulins in Urosepsis Strains of *Escherichia coli*▽

Damien Dubois,[1,2] Julien Delmas,[1,2] Anne Cady,[3] Frédéric Robin,[1,2] Adeline Sivignon,[2] Eric Oswald,[4,5,6,7] and Richard Bonnet[1,2]*

CHU Clermont-Ferrand, Centre de Biologie, Laboratoire de Bactériologie, Clermont-Ferrand F-63003,[1] Clermont Université, Université d'Auvergne, Evolution des bactéries pathogènes et susceptibilité de l'hôte, JE 2526 usc INRA 2018, Clermont-Ferrand F-63001,[2] CHU Rennes, Hôpital Pontchaillou, Pôle des Micro-organismes, Service de Bactériologie, Virologie et Hygiène Hospitalière, Rennes F-35033,[3] Université de Toulouse, UPS, Faculté de Médecine, Toulouse F-31400,[4] Laboratoire de Bactériologie-Hygiène, CHU de Toulouse, Institut Fédératif de Biologie, Toulouse F-31059,[5] INRA, UMR 1225, Toulouse F-31076,[6] and Université de Toulouse, ENVT, UMR 1225, Toulouse F-31076,[7] France

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Determinants of urosepsis in *Escherichia coli* remain incompletely defined. Cyclomodulins (CMs) are a growing functional family of toxins that hijack the eukaryotic cell cycle. Four cyclomodulin types are actually known in *E. coli*: cytotoxic necrotizing factors (CNFs), cycle-inhibiting factor (Cif), cytolethal distending toxins (CDTs), and the *pks*-encoded toxin. In the present study, the distribution of CM-encoding genes and the functionality of these toxins were investigated in 197 *E. coli* strains isolated from patients with community-acquired urosepsis ($n = 146$) and from uninfected subjects ($n = 51$). This distribution was analyzed in relation to the phylogenetic background, clinical origin, and antibiotic resistance of the strains. It emerged from this study that strains harboring the *pks* island and the *cnf1* gene (i) were strongly associated with the B2 phylogroup (P, <0.001), (ii) frequently harbored both toxin-encoded genes in phylogroup B2 (33%), and (iii) were predictive of a urosepsis origin (P, <0.001 to 0.005). However, the prevalences of the *pks* island among phylogroup B2 strains, in contrast to those of the *cnf1* gene, were not significantly different between fecal and urosepsis groups, suggesting that the *pks* island is more important for the colonization process and the *cnf1* gene for virulence. *pks*- *or cnf1*-harboring strains were significantly associated with susceptibility to antibiotics (amoxicillin, cotrimoxazole, and quinolones [P, <0.001 to 0.043]). Otherwise, only 6% and 1% of all strains harbored the *cdtB* and *cif* genes, respectively, with no particular distribution by phylogenetic background, antimicrobial susceptibility, or clinical origin.

The bacterial species *Escherichia coli* comprises a wide diversity of strains belonging to the commensal intestinal flora of humans and warm-blooded animals. Among these strains, several pathogenic variants cause intestinal or extraintestinal infections in humans and animals (33). Population genetic studies based on multilocus enzyme electrophoresis and various DNA markers (10, 20, 44) classify the *E. coli* strains into four major phylogenetic groups (A, B1, B2, and D). The groups are diversely associated with certain ecological niches and propensities to cause disease.

Extraintestinal pathogenic *E. coli* (ExPEC) strains are facultative pathogens that are not yet fully described. They are reported to belong mainly to phylogroups B2 and D, and they possess high numbers of virulence genes that belong to a flexible gene pool (43, 53). Among ExPEC strains, uropathogenic *E. coli* (UPEC) strains take advantage of host behavior and susceptibility by employing virulence factors that facilitate bacterial growth and persistence in the urinary tract (5, 28–30). Important virulence mechanisms are adhesion, invasion, subversion of host defenses, and direct interference with host cellular functions via secreted effectors (33, 69).

These effectors include the cyclomodulins (CMs), a functional class of toxins that hijack the cell cycle, a fundamental host cell process (48). In the species *E. coli*, four kinds of CMs have been identified: the rho GTPase-targeting toxins CNF-1 to CNF-3 (cytotoxic necrotizing factors) (34), the cycle-inhibiting factor (Cif) (38), and two kinds of genotoxins, cytolethal distending toxins (CDTs) I to V (19) and the recently discovered colibactin (41). Colibactin is probably a hybrid polyketide-nonribosomal peptide toxin, whose activity is encoded by the genomic *pks* island (41). CDTs, Cif, and colibactin block mitosis, whereas CNFs promote DNA replication without cytokinesis. CM production can therefore be detected by the analysis of the cytopathic effects induced (41, 50).

E. coli CMs are encoded by mobile elements (genomic islands, plasmids, and bacteriophages) that belong to the flexible gene pool of *E. coli* (22). The aim of the present study was to compare the prevalences of CMs in *E. coli* strains that differed in their clinical origins (community-acquired urosepsis or feces), phylogroups, and susceptibilities to antimicrobial agents.

MATERIALS AND METHODS

Recruitment of patients and strains. One hundred forty-six *E. coli* strains were collected from blood cultures of 146 adults with community-acquired urosepsis between May 2006 and July 2008 in two university hospitals in France (Clermont-Ferrand [$n = 86$] and Rennes [$n = 60$]). Community-acquired urosepsis was defined as the association of a urinary tract infection ($>10^4$ leukocytes/ml and $>10^5$ CFU/ml) with bacteremia due to the same *E. coli* strain in patients who had not previously been admitted to hospital and whose hospital stay did not exceed 48 h. Patients with histories of urine disorders (previous hospitalization in a urology or nephrology unit, urethral instrumentation, or nephrostomy) were excluded. Of the 146 isolates, 123 came from samples taken in emergency wards, 5 in intensive care units, and 18 in other medical units. The median age of

* Corresponding author. Mailing address: Laboratoire de Bactériologie, 28 place H. Dunant, 63001 Clermont-Ferrand, France. Phone: 33 4 73 75 49 20. Fax: 33 4 73 75 49 22. E-mail: rbonnet@chu-clermontferrand.fr.

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TABLE 1. Archetypal *E. coli* control strains used in this study

E. coli strain[a]	Gene(s) or classification method	Host origin	Source or reference
28C	*hly*, *cnf1*, *cdt-IV*, *clbA*,[b] *clbK*,[b] *clbJ*,[b] *clbQ*[b]	Porcine septicemia	13
1404	*cnf2*, *cdt-III*	Bovine septicemia	47
IHE3034 Δ*clbP*	*cdt-I*, *clb* Δ*clbP*	Newborn meningitis	41
DH5α(pCP2123)	*cdt-II*	Laboratory strain	54
C48a	*cnf3*, *eae*, *cif*[b]	Healthy lamb	45
DH10β(pBAC*pks*)	*clb*	Laboratory strain	41
E22	*cif*, *eae*	Rabbit, EPEC	38
RS218	PCR-based phylotyping	Newborn meningitis	61
EDL933	*eae*, stx_1, stx_2	EHEC	51
DH5α		Laboratory strain	Novagen

[a] Strain C48a was kindly provided by José Antonio Orden, and strain RS218 was kindly provided by Philippe Bidet and Edouard Bingen.
[b] The *clbA*, *clbK*, *clbJ*, *clbQ*, and *cif* genes were detected during this study.

patients with urosepsis was 76 years (range, 20 to 96 years). There were 38 males (26%) (median age, 71 years; range, 22 to 93 years) and 108 females (74%) (median age, 76 years; range, 20 to 96 years).

Fecal isolates ($n = 51$) were collected by anal swabs from adults in the community who had no evidence of acute infection or other disorders of the gastrointestinal tract and who had not received antibiotics in the preceding month. The healthy individuals had no health care association within the past 6 months. Healthy individuals who had had urinary tract infections in the previous 12 months and patients with benign or malignant gastrointestinal disease were excluded. The median age of the 51 patients was 46.5 years (range, 23 to 62 years). There were 18 males (35%) (median age, 30.5 years; range, 24 to 59 years) and 33 females (65%) (median age, 50 years; range, 23 to 62 years). One arbitrarily selected *E. coli* colony per sample was analyzed. Previous data show that there is an 86% probability that an arbitrarily selected fecal *E. coli* colony represents the quantitatively predominant clone in the sample (35).

The *E. coli* strains were identified with the automated Vitek II system (bioMérieux). The experimental guidelines of the authors' institutions were followed in the conduct of clinical research. Epidemiological and laboratory results for each episode were recorded anonymously in a computer database in accordance with French law. *E. coli* archetypal reference strains and the *E. coli* K-12 strain DH5α, which are used as positive and negative controls, respectively, for genotypic and phenotypic analyses, are given in Table 1. All strains were stored on 15% glycerol-supplemented Luria-Bertani (LB) medium at −80°C.

DNA extraction. Template DNA was extracted by the boiling-water method. Briefly, three to five bacterial colonies from a freshly grown culture were suspended in 150 μl sterile distilled water and were incubated for 15 min at 95°C. After chilling on ice, bacterial debris was removed by centrifugation at 15,000 × *g* for 5 min at 4°C. The supernatant was transferred to a fresh microcentrifuge tube and was stored at −20°C until required.

PCR phylogenetic grouping and fingerprint profiling. *E. coli* strains were classified according to the *Escherichia coli* Reference Collection (ECOR) system (23) into phylogenetic groups A, B1, B2, and D by using a multiplex PCR technique (10). The *uidA* gene was used as an internal control of amplification, and the anonymous gene *svg* was detected to distinguish the $B2_1$ ribotyping/ST29 multilocus sequence type (MLST) isolates from the B2 strains (3). Three subtypes of phylogroup B2 strains were differentiated by this technique and were designated $B2_1$, Tsp-negative B2, and Tsp-positive B2. A DNA positive control was performed with strain RS218, which harbors all the genes targeted by the multiplex PCR. In order to investigate the clonal relationships among the *pks*- and *cnf1*-positive strains, fingerprint profiles had been generated for each isolate using the enterobacterial repetitive intergenic consensus (ERIC)-PCR scheme reported by Adamus-Bialek et al. (1).

Detection of CM-producing genes by dot blot DNA hybridization. CM-encoding genes were detected by dot blot DNA hybridization experiments. The probes were obtained by PCR with previously published primers (Table 2) by using the PCR DIG probe synthesis kit (Roche Molecular Biochemicals, France) according to the manufacturer's instructions. Two-microgram DNA samples were fixed onto positively charged nylon membranes by UV illumination for 20 min. Hybridization was performed using the Roche labeling and detection kit (Roche Molecular Biochemicals) as indicated by the manufacturer. Each spot was checked with a 16S rRNA gene probe. The *pks* island, which contains the

TABLE 2. Primers used in this study

Primer	Oligonucleotide sequence (5′–3′)	T_m of PCR (°C)	Specificity	PCR product size (bp)	Source or reference
91E	TCAAA(G,T)GAATTGACGGGGGC	54	16S rRNA gene	473	58
13B	GCCCGGGAACGTATTAC				18
pksORF9-10.1KJ	ATTCGATAGCGTCACCCAAC	58	*clbK-clbJ*	2,119	41
pksORF9-10.2KJ	TAAGCGTCTGGAATGCAGTG				
IHAPJPN42	CAGATACACAGATACCATTCA	55	*clbA*	1,002	27
IHAPJPN46	CTAGATTATCCGTGGCGATTC				
IHAPJPN55	TTATCCTGTTAGCTTTCGTTC	55	*clbQ*	821	27
IHAPJPN56	CTTGTATAGTTACACAACTATTTC				
CNF-1s	GGGGGAAGTACAGAAGAATTA	48	*cnf1*	1,112	64
CNF-1as	TTGCCGTCCACTCTCACCAGT				
CNF-2s	TATCATACGGCAGGAGGAAGCACC	48	*cnf2*	1,241	66
CNF-2as	GTCACAATAGACAATAATTTTCCG				
CNF3-3D	TAACGTAATTAGCAAAGA	48	*cnf3*	757	45
CNF-3as	GTCTTCATTACTTACAGT				This study
CDT-s1	GAAAGTAAATGGAATATAAATGTCCG	60	*cdtB-II*, *cdtB-III*, *cdtB-V*	467	64
CDT-as1	AAATCACCAAGAATCATCCAGTTA				
CDT-IIas[a]	TTTGTGTTGCCGCCGCTGGTGAAA	62	*cdtB-II*	556	64
CDT-IIIas[a]	TTTGTGTCGGTGCAGCAGGGAAAA	62	*cdtB-III*, *cdtB-V*	555	64
CDT-s2	GAAAATAAATGGAACACACATGTCCG	56	*cdtB-I*, *cdtB-IV*	467	64
CDT-as2	AAATCTCCTGCAATCATCCAGTTA				
CDT-Is	CAATAGTCGCCCACAGGA	56	*cdtB-I*	411	64
CDT-Ias	ATAATCAAGAACACCACCAC				
CDT-IVs	CCTGATGGTTCAGGAGGCTGGTTC	56	*cdtB-IV*	350	64
CDT-IVas	TTGCTCCAGAATCTATACCT				
P105	GTCAACGAACATTAGATTAT	49	*cdtC-V*	748	25
c2767r	ATGGTCATGCTTTGTTATAT				
cif-int-s	AACAGATGGCAACAGACTGG	55	*cif*	383	38
cif-int-as	AGTCAATGCTTTATGCGTCAT				

[a] Used with primer CDT-s1.

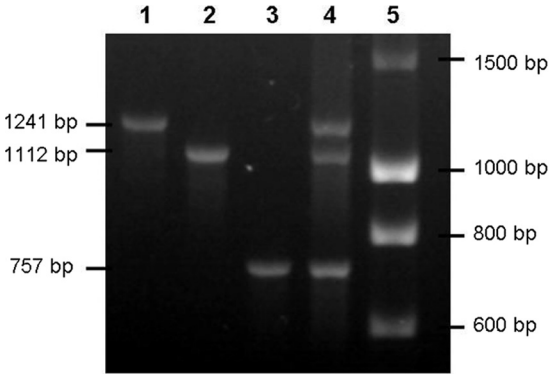

FIG. 1. Standardization of *cnf* gene typing by multiplex PCR. Lanes: 1, strain 1404 (*cnf2*); 2, strain 28C (*cnf1*); 3, strain C48a (*cnf3*); 4, mixture of equal quantities of 1404, 28c, and C48a DNAs (*cnf1*, *cnf2*, *cnf3*); 5, DNA ladder (Eurogentec).

colibactin-producing gene cluster *clb*, was screened with a probe overlapping the *clbK* and *clbJ* genes. The *cnf* genes were detected with a mixture of probes specific to *cnf1*, *cnf2*, and *cnf3*. The *cdtB* genes were detected by two hybridization experiments with the *cdtB-II–cdtB-III* and *cdtB-I–cdtB-IV* probe mixtures. The *cif* gene was detected using an internal specific probe. The sensitivities and specificities of the probes were checked on each membrane by spotting DNA extracts of all CM control strains.

Identification of the CM-producing genes and other virulence factors by PCR assays. Positive hybridizations with a CM probe were subjected to confirmatory PCR assays using the primers given in Table 2. The reaction mixture contained 50 ng DNA sample, 0.2 mM each deoxynucleoside triphosphate (dNTP), 0.4 μM each primer, 3 mM $MgCl_2$, and 1.0 U RedGoldStar DNA polymerase (Eurogentec, France) in the corresponding reaction buffer. Primers located in the 5′ and 3′ regions of the *pks* island (the *clbA* and *clbQ* genes) were used to confirm the full presence of the colibactin-producing island. A multiplex PCR was used to distinguish the *cnf1*, *cnf2*, and *cnf3* genes. Figure 1 shows the standardization of the *cnf* multiplex PCR. *cdtB-I*, *cdtB-II*, *cdtB-III*/*cdtB-V*, and *cdtB-IV* were differentiated by simplex PCR. The *cdt-III* and *cdt-V* operons were differentiated by *cdtC* gene sequencing (Cogenics, Meylan, France) (Table 2) (4). Isolates harboring *cdt* or *cif* were further tested by PCR for the additional virulence factor genes stx_1, stx_2, and *eae*, as previously described (8).

Phenotypic tests. The cytopathic effects of CNF and CDTs were investigated in all strains, and those of colibactin and Cif were investigated in nonhemolytic strains, as previously described (41, 50). Briefly, the effects of CDT and CNF were detected with a cell-lysate-interacting test. After a 48-h culture at 37°C with shaking in Luria-Bertani broth medium, bacterial cells were sonicated and were sterile filtered separately using 0.22-μm-pore-size filters. HeLa cells were treated with the sterile sonicated lysates until analysis. The effects of colibactin and Cif were detected using a cell-bacterium-interacting test, which was based on the interaction between HeLa cells and bacteria. Overnight Luria-Bertani broth cultures of *E. coli* were diluted in interaction medium, and HeLa cell cultures were infected at multiplicities of infection (numbers of bacteria per cell at the onset of infection) of 100 and 200. Cells were washed 3 to 6 times 4 h after inoculation and were incubated in cell culture medium with 200 μg/ml gentamicin until analysis. After 72 h of incubation at 37°C under a 5% CO_2 atmosphere, the medium was removed by several washes of the HeLa cell monolayers. The morphological changes characteristic of CDT, CNF, colibactin, and Cif were observed after staining with Giemsa stain, as described elsewhere (41).

The detection of alpha-hemolysin was performed for all strains studied by overnight growth at 37°C on Columbia sheep blood (5%) agar (Oxoid, Dardilly, France). Antibiotic susceptibility tests were performed by the disc diffusion method (Bio-Rad), using CA-SFM interpretative criteria (7). *E. coli* 25922 (ATCC) was used as the reference strain.

Statistical analysis. Statistical analysis was performed using the Fisher exact and chi-square tests. (For multiple-group comparisons, an initial chi-square test for heterogeneity was done, and only if this yielded a P value of <0.05 were the individual pairwise comparisons tested.) The threshold for statistical significance was a P value of <0.05.

No statistical difference in the distribution of phylogenetic groups, CM genotypes, or antimicrobial resistance phenotypes was observed between strain groups of the same clinical origin from different geographic sources (except for resistance to extended-spectrum cephalosporins [C3G], where the P value was 0.041). The strains were therefore grouped together and were analyzed according to their uroseptic or fecal origin.

TABLE 3. Distribution of phylogenetic groups among 197 *E. coli* isolates recovered from patients with urosepsis and from the feces of healthy individuals

Phylogenetic group	No. (%) of *E. coli* isolates		P^a
	Fecal (n = 51)	Urosepsis (n = 146)	
A	19 (37)	27 (18)	(0.011)
B1	12 (24)	7 (5)	(<0.001)
B2	12 (24)	79 (54)	<0.001
D	8 (16)	33 (23)	

[a] P values (by Fisher's exact test) are shown where P is <0.05. P values given in parentheses indicate negative associations between the prevalence of the urosepsis strains and the particular phylogroup.

RESULTS AND DISCUSSION

***E. coli* phylogroups and clinical origin.** *E. coli* strains isolated from the fecal and urosepsis groups differed significantly in the prevalences of phylogenetic groups A, B1, and B2 but not in that of phylogroup D (Table 3). Phylogroup B2 was predominant in urosepsis strains (54%), followed by phylogroups D (23%), A (18%), and B1 (5%), as previously observed (5, 28–30). Fecal isolates belonged most frequently to phylogenetic group A (37%), followed by phylogroups B1 and B2, with identical prevalences (24%). These results are different from those of other studies, which found B2 strains predominant in fecal samples of healthy subjects, notably in industrialized countries (14, 16, 17, 29, 42, 71). This difference might be explained by the impact of geographic/climatic conditions, dietary factors, and/or the use of antibiotics or host genetic factors on the commensal flora (14, 16, 62). Hence, phylogroup A, and to a lesser extent phylogroup B1, was significantly more prevalent among fecal strains than among urosepsis strains (37% versus 18% [P, 0.011] and 24% versus 5% [P, <0.001], respectively). In contrast, phylogroup B2, known to encompass the most virulent ExPEC strains (15), was significantly more predominant in urosepsis strains than in fecal strains (54% versus 24% [P, <0.001]).

Distribution of CM-encoding genes according to phylogenetic background. Table 4 shows a clear heterogeneity in the

TABLE 4. Phylogenetic distribution of cyclomodulin genes and Hly among 197 *E. coli* isolates recovered from patients with urosepsis and from the feces of healthy individuals

Virulence factor	No. (%) of *E. coli* isolates					P^a
	Total (n = 197)	Group A (n = 46)	Group B1 (n = 19)	Group B2 (n = 91)	Group D (n = 41)	
pks	53 (27)	0	0	53 (58)	0	<0.001
cnf1-Hly	36 (18)	1 (2)	1 (5)	34 (37)	0	<0.001
Hly alone	15 (8)	5 (11)	0	8 (9)	2 (5)	
cnf1-Hly, *pks*	30 (15)	0	0	30 (33)	0	<0.001
cdtB-I	5 (2)	1 (2)	0	4 (4)	0	
cdtB-IV	6 (3)	0	0	5 (5)	1 (2)	
cdtB-V	1 (1)	0	1 (5)	0	0	
cif	2 (1)	0	2 (11)	0	0	

[a] P values (by Fisher's exact test) are shown where P is <0.05 and are for comparisons of group B2 versus all other groups combined.

TABLE 5. Distribution of cyclomodulin genes and Hly among 197 *E. coli* isolates according to phylogenetic group and clinical origin

CM gene(s)	No. (%) of all isolates (n = 197)		P value for all isolates[a]	No. (%) of isolates						P value for group B2[a]	No. (%) of group D isolates (n = 41)	
				Group A (n = 46)		Group B1 (n = 19)		Group B2 (n = 91)				
	Fecal (n = 51)	Urosepsis (n = 146)		Fecal (n = 19)	Urosepsis (n = 27)	Fecal (n = 12)	Urosepsis (n = 7)	Fecal (n = 12)	Urosepsis (n = 79)		Fecal (n = 8)	Urosepsis (n = 33)
pks	6 (12)	47 (32)	0.005	0	0	0	0	6 (50)	47 (59)		0	0
cnf1-Hly	1 (2)	35 (24)	<0.001	0	1 (4)	0	1 (14)	1 (8)	33 (42)	0.028	0	0
Hly alone	3 (6)	12 (8)		2 (11)	3 (11)	0	0	1 (8)	7 (9)		0	2 (6)
cnf1-Hly, *pks*	1 (2)	29 (20)	0.001	0	0	0	0	1 (8)	29 (37)		0	0
cdtB-I	2 (4)	3 (2)		1 (5)	0	0	0	1 (8)	3 (4)		0	0
cdtB-IV	3 (6)	3 (2)		0	0	0	0	2 (17)	3 (4)		1 (12)	0
cdtB-V	1 (2)	0		0	0	1 (8)	0	0	0		0	0
cif	0	2 (1)		0	0	0	2 (29)	0	0		0	0

[a] P values (by Fisher's exact test) are shown where P is <0.05.

prevalences of CM genes, of which the most frequent were the *pks* island (27%) and *cnf1* (18%), and in that of alpha-hemolysin expression (26%), three traits that were strongly associated with phylogroup B2.

All *pks*-harboring strains belonged to phylogroup B2, as previously observed (27, 41, 56). This association was deep-seated (P, <0.001 for B2 versus A, B1, and D combined or separately). Fifty-eight percent of B2 strains possessed this trait, which was observed in all B2 subtypes according to phylogenetic grouping by PCR (3 among 12 $B2_1$ strains, 5 among 7 Tsp-negative B2 strains, and 45 among 75 Tsp-positive B2 strains).

Multiplex PCR-based *cnf* typing revealed only *cnf1* genes. No *cnf2* or *cnf3* genes, which were first observed in *E. coli* strains isolated from animals (12, 45), were detected in our human strains, a finding consistent with the absence or very weak prevalence of these genes observed in previous studies (9, 12, 32, 37, 55, 63, 64). These results suggest that *cnf2*- and *cnf3*-positive strains are almost entirely absent in humans and thus are probably involved only in animal diseases. *cnf1*-harboring strains were highly associated with phylogroup B2, including 37% of *E. coli* B2 strains, but were barely present in the other phylogroups (P, <0.001 for B2 versus other groups), as previously observed (5, 26, 29, 30). Moreover, 33% of B2 strains harbored both the *pks* island and the *cnf1* gene (P, <0.001 for B2 versus other groups), as previously observed (27).

All *cnf1*-positive strains exhibited the alpha-hemolytic phenotype. This association was probably due to the presence of the pathogenicity island (PAI) II_{J96}-like domain, in which the *cnf1* gene is located just downstream of the *hlyCABD* operon, which encodes the alpha-hemolytic phenotype (6, 34). We observed two isolates from one patient, one hemolytic *cnf1*-positive and one nonhemolytic *cnf1*-negative isolate, sharing the same randomly amplified polymorphic DNA (RAPD) 1283 typing and antimicrobial susceptibility profiling, suggesting the spontaneous loss of a PAI II_{J96}-like domain in the second isolate, as a result of the instability of the PAI (39). Only the hemolytic *cnf1*-positive isolate was retained for statistical analysis. Only 8% of strains exhibited an alpha-hemolytic phenotype without the *cnf1* gene. These strains, which accounted for 29% of the alpha-hemolytic strains, had no particular phylogenetic distribution pattern. They probably possessed another PAI containing the *hly* operon, similar to the PAI I_{CFT073}-like domain (21).

cdtB genes were observed in 6% of strains, with only one *cdtB* subtype per strain. Although 75% of *cdtB*-positive strains belonged to phylogroup B2, no significant phylogenetic distribution pattern clearly emerged, even among *cdtB* subtypes. In our study, *cdtB-I* (n = 5) and *cdtB-IV* (n = 6) types were overrepresented compared to the *cdtB-V* (n = 1) type. In contrast, no *cdt-II*- or *cdt-III*-positive strains were found. Of the *cdt* genes, *cdt-I* and *cdt-IV* are the most closely homologous, and both genes, framed with lambdoid prophage genes (65), might have been acquired from a common ancestor by phage transduction. *cdt* genes did not possess any particular association with the *pks* island or the *cnf1* gene (see Table 6). Since *cdt* genes have been extensively investigated in Shiga toxin-producing *E. coli* (STEC) strains, *cdt*-harboring strains were screened for the *eae* and *stx* genes (4, 25, 46, 49). No *eae* or *stx* genes were detected in our *cdt*-encoding strains.

Two *cif*-harboring strains were observed. They belonged to phylogroup B1 and harbored no other CM-encoding gene. Cif is an effector protein of the type 3 secretion system (T3SS) encoded by the locus of enterocyte effacement pathogenicity island, and previous observations of the *cif* gene were restricted to enteropathogenic *E. coli* (EPEC) and enterohemorrhagic *E. coli* (EHEC) strains (36, 38). The two *cif*-positive strains of this study were confirmed as EPEC strains by detection of the *eae* gene but not of *stx* genes.

Distribution of CM-encoding genes according to clinical origin. Overall, the urosepsis strains had a significantly higher prevalence of the *pks* island than the fecal strains (32% versus 12%; P, 0.005) (Table 5). The *pks* island could therefore be involved in uropathogenesis; however, the prevalences of the *pks* island among phylogroup B2 strains were not significantly different between the fecal and urosepsis groups. The urosepsis strains were also significantly more likely to harbor Hly (P, <0.001) and *cnf1* associated with Hly (P, <0.001) than the fecal strains. Likewise, the B2 urosepsis strains harbored *cnf1* associated with Hly significantly more frequently than their B2 fecal counterparts (P, 0.028).

The weak prevalence of *cdtB* genes in urosepsis strains (4%) has been documented elsewhere (28, 29, 31). These observations suggest that CDTs are not major virulence factors for urosepsis. *cdt* genes in *E. coli* urosepsis strains were repre-

TABLE 6. Distribution of phylogenetic group-cyclomodulin gene profiles among 197 *E. coli* isolates recovered from patients with urosepsis and from the feces of healthy individuals

Phylogenetic group	CM gene profile	No. (%) of isolates		P^a
		Fecal (n = 51)	Urosepsis (n = 146)	
B1	None	11 (21.6)	4 (2.7)	(0.003)
	cdtB-V	1 (2)	0	
	cif	0	2 (1.4)	
	cnf1	0	1 (0.7)	
A	None	18 (35.3)	26 (17.8)	(0.018)
	cnf1	0	1 (0.7)	
	cdtB-I	1 (2)	0	
B2	None	4 (7.8)	26 (17.8)	
	cnf1	0	4 (2.7)	
	pks	4 (7.8)	17 (11.6)	
	pks, *cnf1*	1 (2)	26 (17.8)	0.003
	pks, *cnf1*, *cdtB-I*/*cdtB-IV*[b]	0	3 (2)	
	pks, *cdtB-I*	1 (2)	1 (0.7)	
	cdtB-I/*cdtB-IV*[b]	2 (4)	2 (1.4)	
D	None	7 (13.7)	33 (22.6)	
	cdtB-IV	1 (2)	0	

[a] *P* values (by Fisher's exact test) are shown where *P* is <0.05. *P* values given in parentheses indicate negative associations between the prevalence of the urosepsis strains and the particular phylogenetic group-cyclomodulin gene profile.

[b] Because of their small numbers, high level of homology, and close epidemiology, *cdt-I* and *cdt-IV* were aggregated.

sented exclusively by the *cdtB-I* and *cdtB-IV* types. *cdtB* genes were more prevalent in the fecal isolates than in the urosepsis isolates (12% versus 4%), but not to the level of significance. This is in contrast with other studies in which *cdtB* prevalence in fecal strains ranged from 0.9% to 5%, with similar prevalences in urosepsis strains (29, 30, 32, 64). This difference may be explained in part by the exhaustive research into *cdt* subtypes that was performed in our study. In several studies, CDTs were detected in EPEC or STEC/EHEC strains isolated from patients with diarrhea (2, 4, 46, 49). Our CDT-producing strains were not isolated during bouts of enteric disease and did not possess *eae* or *stx* genes. A case-control study of CDT-producing *E. coli* showed no association between CDT-positive *E. coli* and diarrhea (2). Our *cdtB-V*-positive fecal strain did not possess *stx* and *eae* genes, while the *cdtB-V* gene has been reported only in STEC collections (4, 46). Finally, the two *cif*-positive, *eae*-positive urosepsis strains suggest the potential involvement of EPEC as an opportunistic organism in extraintestinal infections. The patients were both female and >80 years old, two risk factors for urinary sepsis.

Distribution of phylogroup-CM gene profiles according to clinical origin. CM gene profiles and phylogroups were coanalyzed to determine whether combinations of CMs and phylogroups can also differentiate between the two clinical groups of strains (Table 6). Phylogroup B1 and A strains with no CM-encoding genes were significantly more prevalent in feces than in the blood cultures of patients with urosepsis (*P*, 0.003 and 0.018, respectively), whereas the association of the CM profile *pks cnf1* with phylogroup B2 strains was more widespread in urosepsis strains (*P*, 0.003). Overall, both the *pks* island and the *cnf1* gene, whether associated with another CM or not, were highly predictive of a urosepsis origin (20% versus 2%; *P*, 0.001) (Table 5). Three fingerprint profiles (encompassing 17, 10, and 3 strains) were obtained from the 30 *pks*-, *cnf1*-, and group B2-positive strains, suggesting that these urosepsis strains can belong to a distinct genetic background.

During urosepsis, colibactin and CNF1 may induce profound changes in cellular signaling pathways. Colibactin induces DNA double-strand breaks (41). CNF1 modulates a high number of cellular functions by hijacking rho GTPases (34), particularly attenuates polymorphonuclear leukocyte functions (11), and induces a severe and controlled inflammatory response (40, 57, 59, 60). By affecting the immune response, CNF1 could lengthen the brief time window between the establishment of bacteriuria and the activation of a host defense during urosepsis, consequently enhancing UPEC survival and allowing invasion of the parenchyma and bacteremia. CNF1 and probably colibactin may also favor host colonization, since their encoding genes have been found together in group B2 strains responsible for asymptomatic bacteriuria (70). In this study, the difference in the prevalences of the *pks* island and the *cnf1* gene in B2 fecal strains suggest that colibactin may act mainly as a colonization factor and CNF-1 as a virulence factor (Table 5). Further experimental investigations are needed to shed light on the pathogenic and/or colonizer roles of these CMs.

Phenotypic detection of CMs. Interpretation of the four CM-related phenotypes in eukaryotic cells was straightforward in all but two strains, which harbored the *pks* island and the *cdt* gene and for which the phenotypes are similar in the cell-bacterium-interacting test. Thus, we were not able to affirm that the *pks* islands of these two strains were functional. Nevertheless, all *cdt*- and *cnf*-positive strains produced a cytopathic effect, and a cytopathic effect induced by the *pks* island was observed in all but three strains. This result suggests nonfunctional *pks* islands. However, we cannot rule out the possibility that other genes, potentially involved in the secretion of colibactin or in the synthesis of precursors used by *pks* island-encoded enzymes, were altered. It is noteworthy that two of these nonfunctional *pks* islands did not belong to ExPEC strains and accounted for 33% of *pks*-positive fecal isolates. Only one of the two *cif* genes produced a cytopathic effect. Loukiadis et al. reported that 66% of *cif*-positive *E. coli* strains did not induce a Cif-related phenotype in eukaryotic cells due to frameshift mutations or an insertion sequence in the *cif* gene (36). However, a nonfunctional T3SS may also explain the absence of a cytopathic effect. In addition, all strains that induced cytopathic effects harbored CM-encoding genes, suggesting that the genomic techniques used had reliable sensitivity.

Antibiotic susceptibilities of urosepsis strains according to CM-encoding genes and phylogenetic background. The prevalence of antibiotic resistance among urosepsis strains was as follows: 59% were resistant to amoxicillin, 5% to extended-spectrum cephalosporins (C3G), 25% to cotrimoxazole, 18% to nalidixic acid and norfloxacin, 14% to ciprofloxacin, 2% to gentamicin, and 0% to amikacin. Table 7 shows the prevalences of CM-encoding genes and phylogenetic groups according to antibiotic susceptibility status. Quinolone susceptibility was associated with phylogenetic group B2 (59% of susceptible versus 33% of resistant isolates; *P*, 0.019) and the *pks* island

TABLE 7. Distribution of antibiotic susceptibility according to cyclomodulin genes/Hly and phylogenetic background among 143 *E. coli* isolates recovered from patients with urosepsis[a]

Variable	Quinolones–fluoroquinolones[b]			Amoxicillin			C3G[c]			Gentamicin		Cotrimoxazole		
	No. (%) of isolates		P^d	No. (%) of isolates		P^d	No. (%) of isolates		P^d	No. (%) of isolates		No. (%) of isolates		P^d
	R (n = 27)	S (n = 119)		R (n = 86)	S (n = 60)		R (n = 7)	S (n = 139)		R (n = 3)	S (n = 143)	R (n = 36)	S (n = 110)	
Phylogenetic groups														
A	11 (41)	16 (13)	(0.002)	24 (28)	3 (5)	(<0.001)	6 (86)	21 (15)	(<0.001)	2 (67)	25 (17)	9 (25)	18 (16)	
B1	2 (7)	5 (4)		5 (6)	2 (3)		1 (14)	6 (4)		1 (33)	6 (4)	3 (8)	4 (4)	
B2	9 (33)	70 (59)	0.019	32 (37)	47 (78)	<0.001	0 (0)	79 (57)	0.003	0 (0)	79 (55)	13 (36)	66 (60)	0.020
D	5 (19)	28 (24)		25 (29)	8 (13)	(0.028)	0 (0)	33 (24)		0 (0)	33 (23)	11 (31)	22 (20)	
CMs														
pks	3 (11)	44 (37)	0.011	18 (21)	29 (48)	<0.001	0	47 (34)		0	47 (33)	2 (6)	45 (41)	<0.001
cnf1-Hly	7 (26)	28 (24)		14 (16)	21 (35)	0.011	2 (29)	33 (24)		0	35 (24)	4 (11)	31 (28)	0.043
Hly alone	1 (4)	11 (9)		8 (9)	4 (7)		0	12 (9)		0	12 (8)	2 (6)	10 (9)	
cdtB-I	0	3 (3)		1 (1)	2 (3)		0	3 (2)		0	3 (2)	0	3 (3)	
cdtB-IV	0	3 (3)		1 (1)	2 (3)		0	3 (2)		0	3 (2)	0	3 (3)	
cif	0	2 (2)		1 (1)	1 (2)		0	2 (1)		0	2 (1)	0	2 (2)	

[a] R, resistant; S, susceptible.
[b] All resistant isolates were resistant both to quinolone (nalidixic acid) and to fluoroquinolones (either norfloxacin alone or norfloxacin and ciprofloxacin).
[c] C3G, extended-spectrum cephalosporins.
[d] P values (by Fisher's exact test) are shown where P is <0.05. P values shown in parentheses indicate negative associations between the prevalence of the susceptible strains and the particular trait (phylogenetic group or CM).

(37% versus 11%; P, 0.011), whereas quinolone resistance was associated with group A (41% of resistant versus 13% of susceptible isolates; P, 0.002). Likewise, amoxicillin susceptibility was associated with group B2 (78% versus 37%; P, <0.001), the combination of the alpha-hemolytic phenotype and the *cnf1* gene (35% versus 16%; P, 0.011), and the *pks* island (48% versus 21%; P, <0.001), whereas amoxicillin resistance was associated with groups A and D (28% versus 5% [P, <0.001] and 29% versus 13% [P, 0.028], respectively). Cotrimoxazole susceptibility was also associated with group B2 (60% versus 36%; P, 0.02), the alpha-hemolytic phenotype (36% versus 17%; P, 0.024), the combination of the alpha-hemolytic phenotype and the *cnf1* gene (28% versus 11%; P, 0.043), and the *pks* island (41% versus 6%; P, <0.001). C3G susceptibility was associated with group B2 (57% versus 0%; P, 0.003), whereas C3G resistance (six strains with overexpression of chromosome-mediated cephalosporinases and one with an extended-spectrum β-lactamase, CTX-M-14) was associated with group A (86% versus 15%; P, <0.001).

These results suggest that resistance to quinolones is associated with the less virulent phylogenetic groups and with a weak prevalence of virulence factors, as previously observed (24, 26, 52, 67, 68). We obtained similar results for resistance against cotrimoxazole, amoxicillin, and C3G. The acquisition of antibiotic resistance by horizontal gene transfers or mutations may therefore require a particular genetic background, as observed for virulence factors (15). Some studies have suggested that the low-virulence group A isolates are more exposed to antibiotic selection pressure within the intestinal tract (24, 26), which indicates the possible importance of environmental factors.

Conclusion. The distribution of CM-encoding genes, including the recently described *pks* genomic island, and the functionality of these toxins were investigated in *E. coli* strains in relation to their phylogenetic background, clinical origin, and antibiotic resistance. One finding to emerge from the present study was the frequent association of the *pks* island with the *cnf1* gene and the alpha-hemolytic phenotype, and their presence in amoxicillin-, cotrimoxazole-, and quinolone-susceptible *E. coli* strains of the B2 phylogenetic background isolated from patients with urosepsis. The widespread diffusion of the *pks* island and the *cnf1* gene in *E. coli* help to distinguish ExPEC from commensal strains and reinforce the idea that these genes of the *E. coli* flexible gene pool are involved in pathogenicity and/or in the ability to survive in new ecological niches, such as the human urinary tract.

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