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Production of Cellulose and Curli Fimbriae by Members of the Family *Enterobacteriaceae* Isolated from the Human Gastrointestinal Tract

Xhavit Zogaj,[1,2] Werner Bokranz,[2] Manfred Nimtz,[3] and Ute Römling[1]*

Microbiology and Tumorbiology Center, Karolinska Institutet, SE-171 77 Stockholm, Sweden,[1] and Divisions of Cell Biology and Immunology[2] and Structural Biology,[3] Gesellschaft für Biotechnologische Forschung, D-38124 Braunschweig, Germany

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***Citrobacter* spp., *Enterobacter* spp., and *Klebsiella* spp. isolated from the human gut were investigated for the biosynthesis of cellulose and curli fimbriae (*csg*). While *Citrobacter* spp. produced curli fimbriae and cellulose and *Enterobacter* spp. produced cellulose with various temperature-regulatory programs, *Klebsiella* spp. did not show pronounced expression of those extracellular matrix components. Investigation of multicellular behavior in two *Citrobacter* species and *Enterobacter sakazakii* showed an extracellular matrix, cell clumping, pellicle formation, and biofilm formation associated with the expression of cellulose and curli fimbriae. In those three strains, the *csgD-csgBA* region and the cellulose synthase gene *bcsA* were conserved. PCR screening for the presence of *csgD, csgA* and *bcsA* revealed that besides *Klebsiella pneumoniae* and *Klebsiella oxytoca,* all species investigated harbored the genetic information for expression of curli fimbriae and cellulose. Since *Citrobacter* spp., *Enterobacter* spp., and *Klebsiella* spp. are frequently found to cause biofilm-related infections such as catheter-associated urinary tract infections, the human gut could serve as a reservoir for dissemination of biofilm-forming isolates.**

Besides *Escherichia coli,* other genera of the family *Enterobacteriaceae,* mainly *Citrobacter, Enterobacter, Klebsiella,* and *Proteus* spp., are regularly recovered from the human gastrointestinal tract, where they are members of the normal fecal floras or transient colonizers (14). Besides inhabitants of the human gastrointestinal tract, the *Enterobacteriaceae,* and primarily the above-listed genera, are the most prominent family of gram-negative bacteria that cause biofilm-related infections such as biliary tract infections, bacterial prostatitis, and catheter-associated urinary tract infections, the most common type of nosocomial infection (4). The gut is considered a primary source for dissemination and transmission of those potential pathogens to susceptible sites.

The rdar morphotype is a multicellular behavior commonly expressed by *Salmonella enterica* serotype Typhimurium isolates (26) and certain isolates of *Escherichia coli* (2, 24). The rdar morphotype mediates different types of multicellular behavior, for example, cell aggregation in liquid culture, pellicle formation at the air-liquid interface, and biofilm formation at liquid-solid interfaces (17). The rdar morphotype produces an extracellular matrix consisting of cellulose and curli fimbriae, which is the major determinant of cell-cell interactions and cell adherence to hydrophilic and hydrophobic abiotic surfaces. Besides their distinct roles in bacterial self-organization, features related to virulence and transmission such as adherence and invasion of epithelial cells and chlorine resistance have been assigned to curli fimbriae and cellulose, respectively (3, 8, 13, 21, 22).

Gene required for cellulose biosynthesis are encoded by the *bcsABZC* operon. While *bcsA* encodes the cellulose synthase, the exact functions of the other genes remain to be determined. Structural genes for curli fimbriae are encoded by the *csgBA*(*C*) operon. Cellulose and curli fimbria biosynthesis is commonly regulated, either through *adrA* or directly by *csgD,* a transcriptional activator, divergently transcribed to *csgBA*(*C*).

Performing this study, we wanted to gain insights into the ability of clinically important *Enterobacteriaceae* besides *E. coli* and serotype Typhimurium to express cellulose and curli fimbriae. The isolates were collected from the gastrointestinal tract as the major human reservoir for dissemination of isolates.

Isolation and phenotypic characterization of bacterial strains. Since we suspected that cellulose and curli fimbria expression are unstable phenotypes upon subculture, we collected primary isolates of bacterial strains from 21 volunteers who provided fecal samples. Fecal swabs were immediately inoculated on MacConkey agar plates (Oxoid) and incubated overnight at 37°C. The confluent microbial outgrowth was collected and resuspended, and appropriate dilutions were plated on MacConkey agar plates for single colonies. A total of 25 non-*E. coli* isolates which belonged to 13 species were identified with the API 20E kit (BioMerieux, Nürtingen, Germany): *Citrobacter* sp. (isolated independently two times), *Citrobacter freundii* (n = 3), *Citrobacter koseri*/*farmeri* (n = 1), *Enterobacter* sp. (n = 1), *Enterobacter aerogenes* (n = 2), *Enterobacter cloacae* (n = 3), *Enterobacter sakazakii* (n = 2), *Klebsiella* sp. (n = 2), *K. oxytoca* (n = 2), *K. pneumoniae* (n = 2), *Klyvera* sp. (n = 2), *Proteus mirabilis* (n = 1), and *Raoultella ornithinolytica* (n = 2). When the fecal strains were grown on Congo Red (CR) plates (17), we observed that several of the isolates selectively bound CR, thereby resembling the rdar, bdar, or pdar morphotype of serotype Typhimurium reference strains (Fig.

* Corresponding author. Mailing address: Karolinska Institute, Microbiology and Tumorbiology Center (MTC), Box 280, SE-171 77 Stockholm, Sweden. Phone: 46-8-728-7319. Fax: 46-8-330744. E-mail: ute.romling@mtc.ki.se.

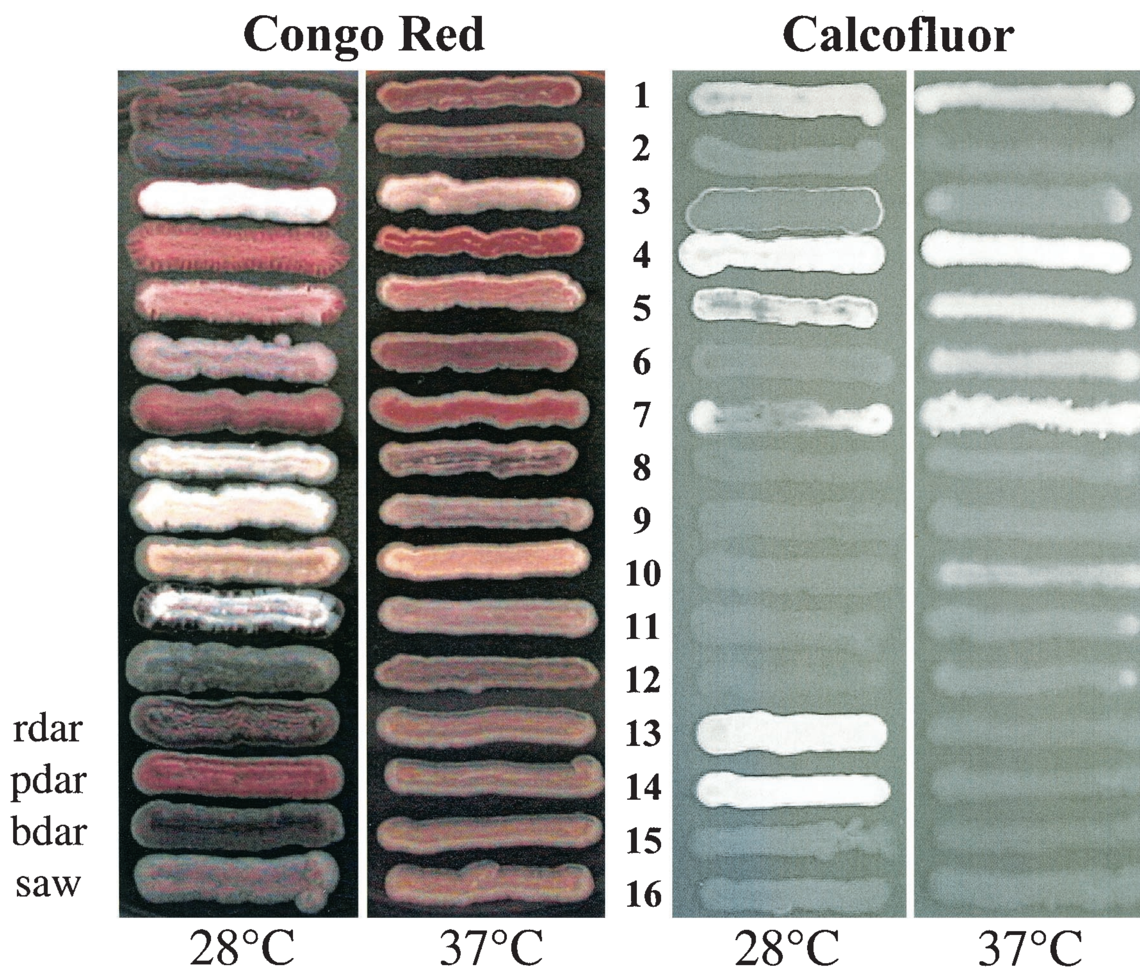

FIG. 1. Congo red and Calcofluor staining of morphotypes of representatives of enterobacterial species isolated from feces. The strains were grown for 48 h at 28°C and for 24 h at 37°C. Morphotypes were compared with the phenotype of serotype Typhimurium UMR1 (ATCC 14028-1s, Nal ^{r}) and its mutants, which have been streaked at the lower part of the panel. ROWS: 1, *Citrobacter* sp. strain Fec2; 2, *C. freundii* Fec4; 3, *C. koseri/farmeri* Fec157; 4, *Enterobacter* sp. strain Fec125; 5, *E. aerogenes* Fec135; 6, *E. cloacae* Fec36; 7, *E. sakazakii* Fec39; 8, *Klebsiella* sp. strain Fec164; 9, *K. oxytoca* Fec139; 10, *K. pneumoniae* Fec141; 11, *R. ornithinolytica* Fec153; 12, *P. mirabilis* Fec162; 13, serotype Typhimurium UMR1 (cellulose $^{28+}$, curli $^{28+}$); 14, serotype Typhimurium MAE1 (cellulose $^{28+}$, curli $^{-}$); 15, serotype Typhimurium MAE222 (cellulose $^{-}$, curli $^{28+}$); 16, serotype Typhimurium MAE51 (cellulose $^{-}$, curli $^{-}$).

1). Serotype Typhimurium colonies which show the rdar morphotype express cellulose and curli fimbriae, while colonies which show the pdar morphotype express cellulose and colonies which show the bdar morphotype express curli fimbriae (17, 26).

Citrobacter spp. regularly expressed the rdar or the bdar morphotype at 28°C, while *Enterobacter* spp. commonly expressed the pdar morphotype at either 28°C and 37°C or only at 37°C. *Klebsiella* spp. did not show characteristic morphotypes that indicated pronounced expression of cellulose or curli fimbriae.

Expression of curli fimbriae and cellulose. Three strains with characteristic morphotypes, *Citrobacter* sp. strain Fec2, *C. freundii* Fec4, and *E. sakazakii* Fec39, were chosen for more detailed analyses. *Citrobacter* sp. strain Fec2 expressed the bdar morphotype at 28°C, *C. freundii* Fec4 expressed the rdar morphotype at 28°C, and *E. sakazakii* Fec39 expressed the pdar morphotype at 28°C and 37°C (Fig. 1). However, as displayed in Fig. 1, during storage for several months at −70°C, *Citrobacter* sp. strain Fec2 changed morphotype to rdar at 28°C and pdar at 37°C, indicating enhanced cellulose biosynthesis. However, since those changes occurred after the molecular study of the isolates, they will not be considered here.

After an enrichment procedure employed for plate-grown cultures of *Citrobacter* sp. strain Fec2 (18a), the putative curli fimbria subunit was detected as a prominent band at ~16 kDa that was cut out, trypsin digested, and subjected to sequence analysis by quadruple time-of-flight mass spectrometry (20). Sequence comparison of three peptides with the database indicated that the protein was homologous to CsgA, the structural subunit of curli fimbriae expressed by *E. coli* and serotype Typhimurium. Western blot analysis detected curli fimbriae after formic acid treatment (5) produced by *Citrobacter* sp. strain Fec2 and *C. freundii* Fec4 at 28°C, while *E. sakazakii* Fec39 did not produce any curli fimbriae (data not shown).

Calcofluor binding indicated variable production of cellu-

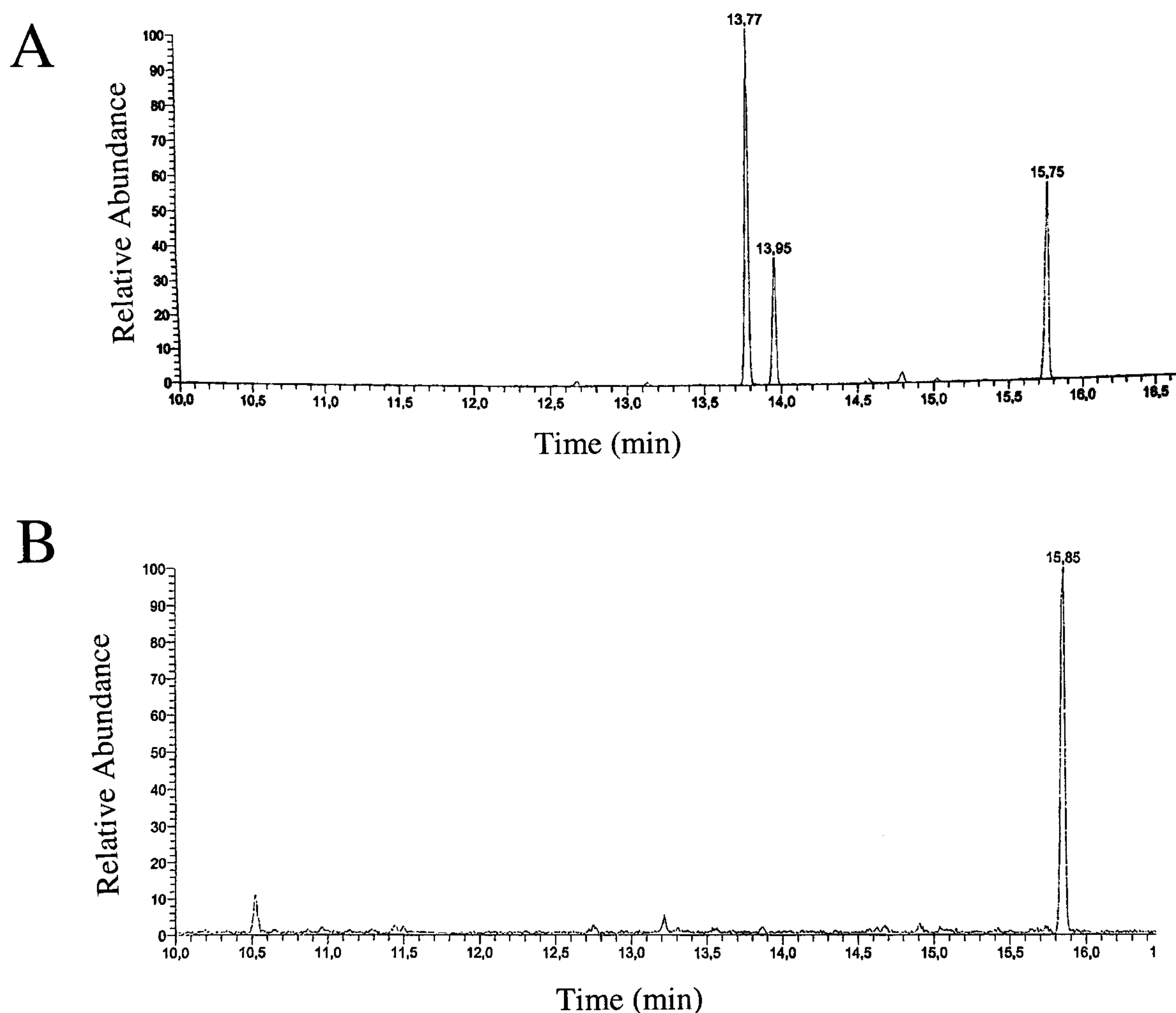

FIG. 2. Detection of glucose monomers by HPLC after isolation of crystalline cellulose. Results for (A) *E. sakazakii* Fec39 and (B) serotype Typhimurium MAE51 are shown as examples of positive and negative outcomes, respectively. Crystalline cellulose was hydrolyzed and sugar monomers were detected by HPLC. Glucose shows two peaks representing the α- and β-anomers (retention time, 13.77 and 13.95 min, respectively); 1 μg of myoinositol was used as an internal standard (retention time, 15.75 min).

lose, a 1,4-β-glucan, in the three strains (Fig. 1). Since Calcofluor binding is not absolutely specific for cellulose, we confirmed cellulose production by isolating crystalline cellulose by the Updegraff method with a mixture of hot 58% acetic acid and 19% nitric acid (25, 26). After hydrolysis of the isolated polymer, the glucose monomers detected by high-pressure liquid chromatography (HPLC) (Fig. 2) were indicative of cellulose production. While glucose was detected when samples from *E. sakazakii* Fec39 grown at 28°C and 37°C and *C. freundii* Fec4 grown at 28°C were analyzed, *Citrobacter* sp. strain Fec2 samples did not show any significant glucose peak.

In conclusion, consistent with the expression of the pdar morphotype expressed at 28°C and 37°C, *E. sakazakii* Fec39 produced cellulose but not curli fimbriae at both temperatures. *C. freundii* Fec4, which showed the rdar morphotype at 28°C, produced cellulose and curli fimbriae at 28°C, while *Citrobacter* sp. strain Fec2, which showed the bdar morphotype, produced only curli fimbriae.

Multicellular behavior of isolates. The expression of the extracellular matrix components cellulose and curli fimbriae is associated with biofilm formation and other modes of multicellular behavior (17). We used a steady-state model, incubation of the strains in glass tubes (17), to test biofilm formation by *Citrobacter* sp. strain Fec2, *C. freundii* Fec4, and *E. sakazakii* Fec39. Optimal biofilm formation was achieved with different incubation conditions for each strain (Fig. 3A and data not shown), which indicated that the regulatory patterns of biofilm formation with respect to oxygen tensions were different between the strains. Pellicle formation was observed for all three strains (data not shown). While *E. sakazakii* Fec39 formed a pellicle after just 24 h of incubation at 28°C in standing cultures, pellicle formation in cultures of *Citrobacter* sp. strain Fec2 and *C. freundii* Fec4 required 48 h to develop. In liquid culture at 28°C after incubation for 24 h, all three strains formed clumps, although the extent of clumping and the consistency of the clumps varied between the strains (Fig. 3B and

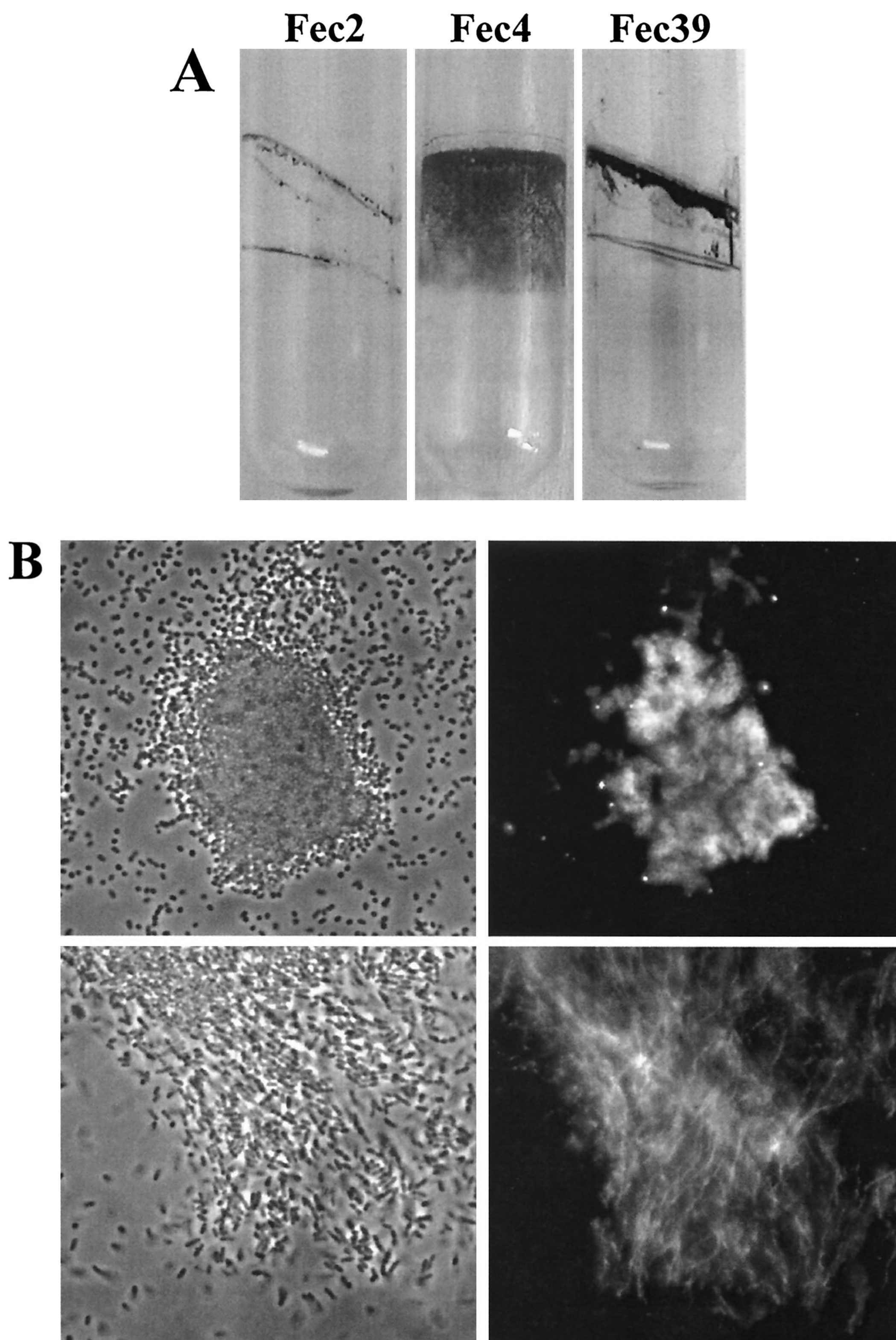

FIG. 3. Phenotypes of *Citrobacter* sp. strain Fec2, *C. freundii* Fec4, and *E. sakazakii* Fec39. (A) Biofilm formation. Cultures were grown at 28°C for 48 h in Luria broth without salt and with shaking. Optimal biofilm formation was achieved at 180 and 210 rpm for *Citrobacter* sp. strain Fec2, *C. freundii* Fec4, and *E. sakazakii* Fec39, respectively. (B) Cell clumping and arrangement of Calcofluor-stained cellulose fibrils in *C. freundii* Fec4 (top) and *E. sakazakii* Fec39 (bottom). Note that *C. freundii* Fec4, due to the expression of cellulose and curli fimbriae, formed tighter clumps than *E. sakazakii* Fec39, which only expressed cellulose. Left, phase contrast; right, fluorescence microscopy. Magnification, ×600. (C) Scanning electron microscopy of plate-grown cells. Upper panel: *C. freundii* Fec4 (left) in comparison with serotype Typhimurium MAE52 (right; cellulose $^{+}$, curli $^{+}$). Lower panel: *E. sakazakii* Fec39 (left) in comparison with serotype Typhimurium MAE97 (right; cellulose $^{+}$, curli $^{-}$). Bars, 1 μm.

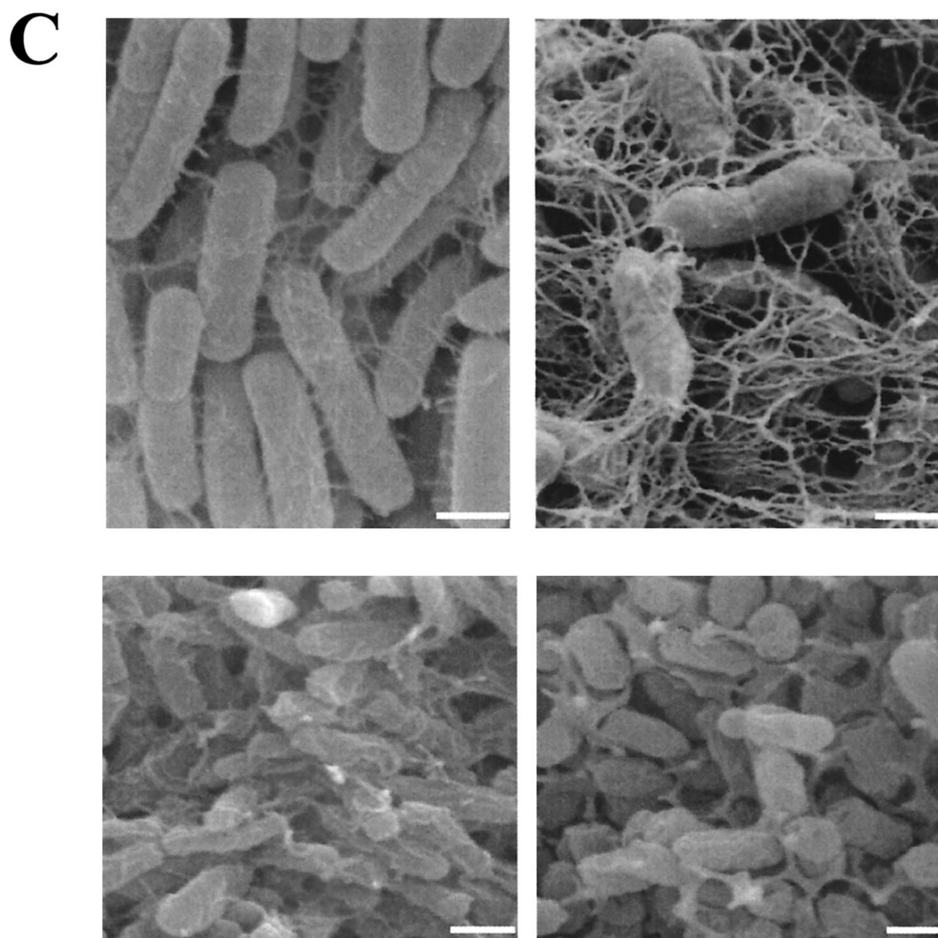

FIG. 3—*Continued*.

data not shown). When observed by fluorescence microscopy, Calcofluor-stained cellulose fibrils could be observed for *E. sakazakii* Fec39 and *C. freundii* Fec4 (Fig. 3B). Free-floating cellulose fibrils associated with cell clumps were observed for *E. sakazakii* Fec39, as in serotype Typhimurium when cellulose is the sole extracellular matrix component (26). On the other hand, *C. freundii* Fec4 produced cellulose fibrils which were tightly wrapped around the cells, as in serotype Typhimurium when cellulose is coexpressed with curli fimbriae (17). Electron microscopy studies of the extracellular matrix of plate-grown colonies of *E. sakazakii* Fec39 and *C. freundii* Fec4 further supported the view that the extracellular matrix components cellulose and curli formed structures similar to those in serotype Typhimurium (Fig. 3C).

Sequence analysis of curli and cellulose biosynthesis genes. Using conserved and strain-specific primers, we sequenced structural genes and regulatory regions involved in cellulose and curli fimbria production in the two *Citrobacter* strains and *E. sakazakii.* In particular, *csgA* was amplified with primers AGFA66 (ATGATGTTAACAATACTGGGTGC) and AGFA60 (CGGC CATTGTTGTGATAAATG), *csgD* was amplified with primers EC-AGFD1 (CAGCAGTGCAACATCTGTCAG) and EC-AGFD2 (AAAGTCTGGAAAATAACGTCCTG), and the intergenic region between *csgA* and *csgD* was amplified with primers FEC-IRD (GATCAACAATAATGTATGACCA) and FEC-IRB (GCTGCCTGATTAAATGAAGAC).

For *bcsA,* the following consensus primers were used: BCSA74A (CTTCCGTATTGGCAGTCAGGTTCAGGACG) and BCSA70 (GCGCCAGCGGGTTAAACGGCTG) for the N terminus, BCSA74 (GCAACAGATTCAATTTCTGCCCTTC) and BCSA86 (GCACCCGC-GCTGGCAGCGTATTCG) for the middle part of the gene, and BCSA62 (TGGGTCTTCTAC AACCTGATTA) and BCSA62A (GCGGCGGTGCAATTTGC GCAAAGGT) for the C terminus.

Sequence data were submitted to the EMBL data library under the following accession numbers: *Citrobacter* sp. strain Fec2, *csgD-csgBA,* AJ515700; *bcsA,* AJ515698; *C. freundii* Fec4, *csgD-csgBA,* AJ515701; *E. sakazakii* Fec39, *csgD-csgBA,* AJ515702; *bcsA,* AJ515699. Although *E. sakazakii* Fec39 did not express curli fimbriae, it contained the *csgD-csgBA* region with intact genes.

CsgD, the central regulator for multicellular behavior, was highly conserved among the species (data not shown). Most diverse were the CsgD proteins from *E. sakazakii* Fec39 and serotype Typhimurium ATCC 14028, with a homology of 94%. While several amino acid substitutions were found in the N-terminal domain, a single amino acid substitution was found in the DNA binding domain.

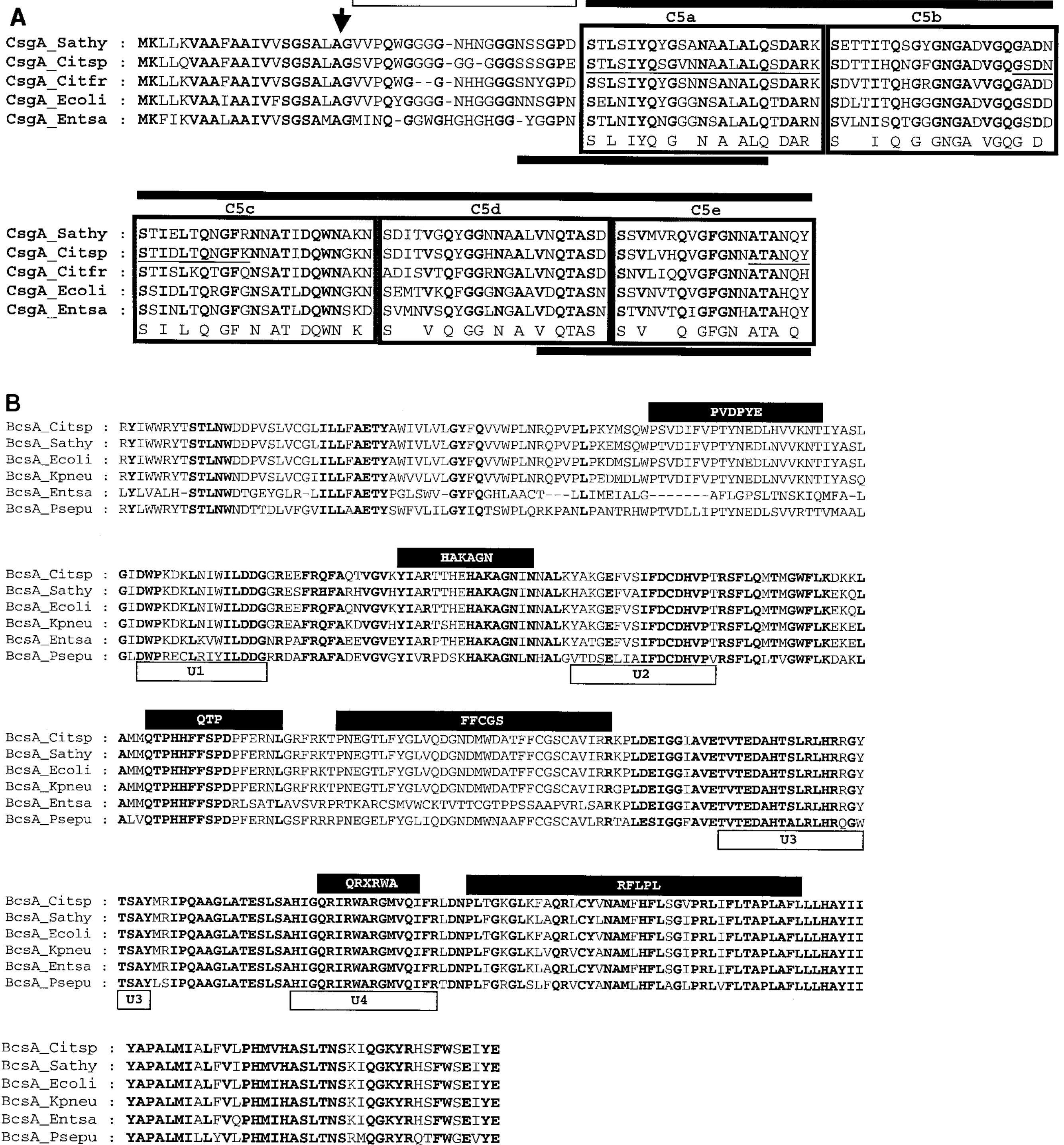

FIG. 4. Comparative analysis of structural genes required for curli and cellulose biosynthesis. (A) Multiple alignment of CsgA sequences from different *Enterobacteriaceae.* The alignment was carried out with ClustalX in default mode (23). Amino acids identical in all the proteins are displayed in bold. An arrow indicates the cleavage site for the signaling peptide. The five internal repeats (C5a to C5e) are boxed. Proteinase-sensitive (light bar) and proteinase-resistant (dark bar) regions are indicated above the alignment. N- and C-terminal amino acid stretches identified in *E. coli* to mediate protein binding (15) are indicated by bars under the alignment. Three tryptic peptides of CsgA from *Citrobacter* sp. strain Fec2 were subjected to mass spectrometric sequence analysis, and the partial sequences (underlined), identical within the limits of technical accuracy to the translated *csgA* gene product, were detected. From the molecular mass of tryptic peptide 1, M+H: 3,990.7 (theoretic mass: 3,990.9), the amino terminus of the protein could be deduced as GSVP. CsgA sequences of *E. coli* K-12 (P28307) and serotype Typhimurium ATCC 14028 (P55225) were taken from the Swiss Prot database. Abbrevations: Sathy, serotype Typhimurium UMR1; Ecoli, *E. coli* K-12; Citsp, *Citrobacter* sp. strain Fec2; Citfr, *C. freundii* Fec4; Entsa, *E. sakazakii.* (B) Multiple alignment of the core region of enterobacterial BcsA, the catalytic subunit of the cellulose synthase. Conserved amino acids are displayed as described for panel A. Conserved motifs are indicated by boxes above and below the sequence. Abbrevations: Kpneu, *K. pneumoniae;* Psepu, *Pseudomonas putida* KT2440. BcsA sequences of *E. coli* K-12 (P37653) and serotype Typhimurium ATCC14028 (Q93IN2) were taken from the Swiss Prot database. BcsA from *P. putida* (http://www.tigr.org) and *K. pneumoniae* (http://genome.wustl.edu/projects/bacterial/?kpneumoniae=1) were from unfinished genome projects. (C) Phylogeny for BcsA sequences with BcsA from *P. putida* as an outgroup. After alignment of sequence, the tree was constructed by the neighbor-joining method subjected to 1,000 bootstrap trials. The tree was drawn with TreeView.

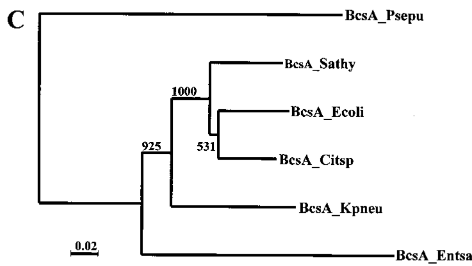

FIG. 4—*Continued*.

csgD expression is highly regulated by environmental conditions mediated by response regulators and DNA architectural proteins (9, 12), which bind in the intergenic region. Surprisingly, binding sites for OmpR and IHF are not located in the most conserved regions (data not shown). This finding suggested that OmpR and IHF binding might differ among the species and consequently the response to environmental conditions. The 521-bp *csgD-csgBA* intergenic region can be divided into four regions, IR1 to IR4 (17). The sequence of the IR3 region is highly diverse among the species, but its length is conserved in all strains, suggesting structural importance.

CsgA and CsgB, the structural proteins of curli fimbriae, were also highly conserved (Fig. 4A and data not shown). Most diverse were the CsgA and CsgB proteins from *C. freundii* Fec4 and *E. sakazakii* Fec39 with a homology of 80% and 78%, respectively. Polymerized CsgA of *Salmonella enteritidis* possesses two domains, an N-terminal domain of 22 residues, which is proteinase K susceptible, and a C-terminal core domain, which is proteinase K resistant (6). The highest sequence diversity among the CsgA proteins was found in the glycine-rich N-terminal domain, which is proteinase K susceptible. The C-terminal domain of each protein consisted of five tandemly arranged sequences each containing the $SX_5QXGX_2NXAX_3Q$ consensus sequence (6).

Phylogenetic analysis of the CsgD, CsgB, and CsgA proteins showed that, for all three proteins, the *E. sakazakii* Fec39 sequences developed fastest (Fig. 4 and data not shown). Protein sequences from *Citrobacter* sp. strain Fec2 and *C. freundii* Fec4 were usually more closely related to other species than to each other (Fig. 4 and data not shown).

Sequence alignment of BcsA, the cellulose synthase, is shown in Fig. 4B. Previously, sequence comparison of all available bacterial cellulose synthases detected a highly conserved core region of 350 residues which contains the D_3D_2 D35QRXRWA motif, common to processive β-glycosyltransferases, and five additional motifs (16). Although the overall sequence was highly conserved among the species over the whole length of the protein, again BcsA of *E. sakazakii* Fec39 showed the highest sequence diversity (Fig. 4C). Most remarkable, the PVDPYE and the FFCGS motif were replaced by a different sequence (Fig. 4B).

Detection of *csgA, csgD,* and *bcsA* in other enterobacterial species isolated from feces. Conserved primers were used to detect the presence of *csgA, csgD, and bcsA* genes in other species isolated from feces (Fig. 5 and data not shown). *bcsA* was present in all species. In *E. aerogenes* Fec135 and *R. ornithinolytica* Fec153, *csgD* could not be detected, while *csgA* was present. We expect *csgD* to be present but not detectable by the primer pair used, because *csgA* is present. In summary, the genes required for the biosynthesis of curli fimbriae were present in all species investigated besides *K. pneumoniae* and *K. oxytoca. Klebsiella ornithinolytica,* which has recently been shown to belong to a phylogenetic line distinct from *K. pneumoniae* and *K. oxytoca* and therefore has been renamed *R. ornithinolytica* (10), harbors the gene cluster for curli biosynthesis.

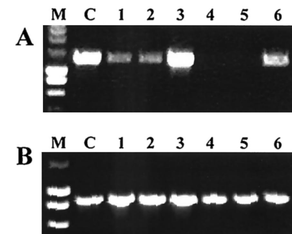

FIG. 5. Amplification of *csgA* (A) and *bcsA* (B) genes by PCR in enterobacterial species isolated from feces. PCR products of 1,301 bp and 860 bp were detected for *csgA* and *bcsA,* respectively. Lanes: M, size markers (SmartLadder), 200 to 10,000 kbp (Eurogentec); C, control serotype Typhimurium MAE52; 1, *E. cloacae* Fec36; 2, *E. aerogenes* Fec135; 3, *C. koseri/farmeri* Fec157; 4, *K. oxytoca* Fec139; 5, *K. pneumoniae* Fec141; 6, *R. ornithinolytica* Fec153. Primers for *csgA* were AGFA66 and AGFA60. *bcsA* primers were ES-BCSA162 (GACGAT CTCTACCAGGTCTGG) and ES-BCSA681 (GTAACGCCACCAG ATATAGCGG).

Temperature-regulated expression of rdar morphotype. In this work, we showed that several non-*E. coli* enterobacterial isolates recovered from the gastrointestinal tract of humans produced either cellulose or curli fimbriae or both compounds under various temperature regulation programs. Different temperature regulation patterns of the rdar morphotype had been observed within clonal variants of serotype Typhimurium strains (17). *Citrobacter* species produced curli fimbriae at ambient temperature, but we did not investigate whether expression of curli fimbriae at 37°C might be triggered by iron depletion, as has been reported for serotype Typhimurium (17).

In the family *Enterobacteriaceae,* cellulose biosynthesis has been shown to occur in *Salmonella* spp., *E. coli,* and *K. pneumoniae* (21, 26). In this study, we found that most of the *Enterobacter* isolates produced cellulose. In particular, two unrelated isolates of *E. sakazakii* showed pronounced cellulose production at 28°C and 37°C (Fig. 1 and data not shown). Clinical isolates of *E. sakazakii* have been reported to display a peculiar wrinkled, leathery colony morphology, which, upon storage as agar stocks, readily dissociated into the standard smooth colony morphology (11). Based on the results from this study, this colony morphology is due to cellulose production, which is retained by *E. sakazakii* upon infection. Species from the genus *Klebsiella* and *R. ornithinolytica* isolated from feces did not show elevated expression of cellulose (Fig. 1), although the genetic information for the biosynthesis of cellulose is present (Fig. 5 and data not shown). However, as a possibility, cellulose biosynthesis might be induced in response to specific environmental signals, as shown for the plant symbiont *Rhizobium leguminosarum* bv. *trifolii* by contact with roots (1, 7). The observed species-specific expression pattern of curli fimbriae and cellulose in fecal strains raises the question of whether species-specific and consequently morphotype-specific niches exist in the gastrointestinal tract. Alternatively, a habitat-specific morphotype for each species is already determined outside the human body and retained when a strain passes through the gastrointestinal tract.

Multicellular behavior, including biofilm formation, is a fundamental life style of bacteria. It is reasonable to assume that such ancient behavior has common principles among bacteria. The rdar morphotype, defined as the expression of cellulose and curli fimbriae, represents a basic multicellular behavior of *Enterobacteriaceae* closely related to serotype Typhimurium and *E. coli,* but individual components of the rdar morphotype and its expression pattern are subject to species-specific adaptation for which no simple pathogen-commensal soil organism classification scheme can be proposed. For example, although semiconstitutive expression of the rdar morphotype is observed (17), the vast majority of pathogenic serovar Typhimurium and serotype Enteritidis strains express the rdar morphotype highly regulated by environmental conditions and only at ambient temperature (18, 18a). Otherwise, *Shigella* spp. and enteroinvasive *E. coli,* which cause invasive gastrointestinal disease (19), have lost the rdar morphotype and multicellular behavior. The rdar morphotype was also not found in the *Klebsiella* species analyzed in this study. However, constitutive expression of cellulose was observed in *E. sakazakii,* which can cause newborn meningitis, and the majority of *E. coli* strains from sepsis produce curli fimbriae or the rdar morphotype constitutively (2). Therefore, the expression of cellulose and curli fimbriae is complex and might be highly determined by the environmental microniche of the organism under study. In any case, the capacity to produce cellulose and curli fimbriae in *Citrobacter, Enterobacter,* and potentially *Klebsiella* species might contribute to the significant role those species play in biofilm-related infections.

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