Broad-spectrum biofilm inhibition by a secreted bacterial polysaccharide

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The development of surface-attached biofilm bacterial communities is considered an important source of nosocomial infections. Recently, bacterial interference via signaling molecules and surface active compounds was shown to antagonize biofilm formation, suggesting that nonantibiotic molecules produced during competitive interactions between bacteria could be used for biofilm reduction. Hence, a better understanding of commensal/pathogen interactions within bacterial community could lead to an improved control of exogenous pathogens. To reveal adhesion or growth-related bacterial interference, we investigated interactions between uropathogenic and commensal *Escherichia coli* in mixed *in vitro* biofilms. We demonstrate here that the uropathogenic strain CFT073 and all *E. coli* expressing group II capsules release into their environment a soluble polysaccharide that induces physicochemical surface alterations, which prevent biofilm formation by a wide range of Gram-positive and Gram-negative bacteria. We show that the treatment of abiotic surfaces with group II capsular polysaccharides drastically reduces both initial adhesion and biofilm development by important nosocomial pathogens. These findings identify capsular polymers as antiadhesion bacterial interference molecules, which may prove to be of significance in the design of new strategies to limit biofilm formation on medical in dwelling devices.

bacterial interference | *Escherichia coli* | group II capsule | extraintestinal

In most ecological niches, bacteria grow on natural or artificial surfaces as single- or multiple-species communities known as biofilms. This bacterial lifestyle often involves the expression of envelope proteinaceous adhesins and the production of extracellular polysaccharidic material, which promote both initial surface contacts and bacterial–bacterial interactions during the 3D development of the biofilm (1–3). Bacterial growth on surfaces induces novel behavior as compared with free-swimming organisms, such as characteristic increased tolerance to stress, biocides, and host immunological defenses (1). Therefore, biofilms formed by potentially pathogenic bacteria is considered an important cause of chronic and recurrent infections, in particular because of their capacity to form and persist on medical surfaces and indwelling devices.

Current biofilm preventive strategies are essentially aimed at coating medical surfaces with antimicrobial agents. However, recent studies suggested that nonantibiotic molecules naturally produced within bacterial communities, including secreted signaling molecules or surface active biosurfactant, could also interfere with biofilm formation, modulating microbial interaction with interfaces (4–6). Thus, besides growth inhibition, direct limitation of bacterial surface adhesion could also occur during negative competitive interactions within bacterial communities. However, few studies have addressed these issues in a multispecies biofilm context, and new approaches allowing detailed molecular studies of bacterial interactions within mixed biofilm communities therefore are needed (7–9).

Escherichia coli is a highly versatile bacterium, which exists as a harmless commensal or as an intra- or extraintestinal pathogen (10). In most *in vivo* situations such as the human intestine, *E. coli* is likely to compete and interact with other transient or resident bacterial species, including other commensal or pathogenic *E. coli*. Here, we investigated the competitive interactions between uropathogenic *E. coli* (UPEC) and commensal *E. coli* bacteria and demonstrated a new role for capsular polysaccharidic polymers in mixed biofilm interaction. *E. coli* capsules constitute the outermost protective layer of the cell surface that are classified into four groups based on genetic and biosynthetic criteria (11). We demonstrate here that group II capsule, composed of high molecular weight and negatively charged polysaccharidic polymers, produced by most UPEC and other extraintestinal *E. coli*, are actually released into the bacterial environment. We show that secreted group II capsule modulates bacterial adhesion and prevents biofilm formation by both Gram-positive and Gram-negative bacteria. This property could contribute to competitive interactions within bacterial communities and lead to the development of a new, nonantibiotic tool for bacterial biofilm control.

Results

Inhibition of Commensal *E. coli* Biofilm Formation by the UPEC Strain CFT073. To reveal adhesion or growth-related bacterial interactions in mixed commensal/pathogen biofilms, we developed an *in vitro* model in microfermentors in which an 8-h biofilm formed by commensal *E. coli* strain MG1655 F′ was inoculated with different titers of UPEC strain CFT073 and was further cultured for 24 h (12, 13). With increasing titers of CFT073, we observed a strong reduction in MG1655 F′ biofilm development, which was not observed when we used the commensal *E. coli* strain KS272, a motile strain with biofilm formation capacity similar to CFT073 (Fig. 1*A*). This result suggested that CFT073 could prevent MG1655 F′ biofilm formation, either by direct contact or secretion of an inhibitory molecule (14, 15). To distinguish between these two possibilities, we conducted *in vitro* adhesion assays in poly(vinyl chloride) microtiter plates and microfermentors, containing filter-sterilized CFT073 supernatant of a stationary phase culture. In the presence of CFT073 supernatant, the MG1655 F′ biofilm was severely affected both in steady-state and in continuous-flow conditions (Fig. 1 *B* and *C*). This biofilm inhibition did not result from a growth defect due to bactericidal or bacteriostatic activity, because MG1655 F′ growth rate and cell viability were not affected by the CFT073 supernatant (Fig. 1 *D* and *E*).

CFT073 Supernatant Displays Antibiofilm Activity Toward both Gram-Positive and Gram-Negative Bacteria. To determine the spectrum of the CFT073 antibiofilm activity, we tested the effect of the CFT073

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Abbreviation: UPEC, uropathogenic *Escherichia coli*.

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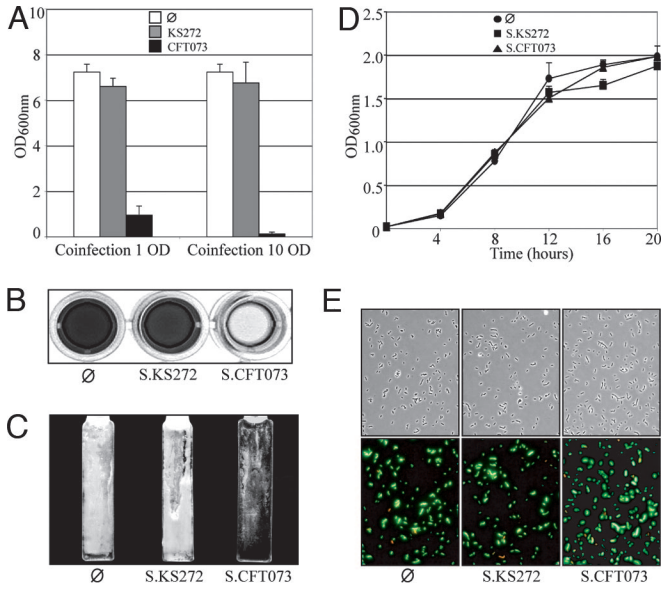

Fig. 1. Biofilm inhibitory effect of CFT073. (*A*) Biofilm formation of MG1655 F′ in microfermentors inoculated with 1 or 10 OD_{600} equivalent of KS272 (gray bars) or CFT073 (black bars) cells. White bars indicate MG1655 F′ biofilm alone (Ø). Results are the average of six replicates ± SD. $P < 0.001$ compared with MG1655 F′ biofilm. (*B*) Microtiter plate MG1655 F′ biofilm alone (Ø) or in the presence of KS272 or CFT073 supernatant (S.KS272 and S.CFT073, respectively). (*C*) MG1655 F′ biofilm in microfermentors perfused with medium without supernatant (Ø) or with S.KS272 or S.CFT073. (*D*) Growth curves of MG1655 F′ alone (Ø) or with S.KS272 or S.CFT073. (*E*) MG1655 F′ cell viability alone (Ø) or with S.KS272 or S.CFT073 visualized with BacLight staining.

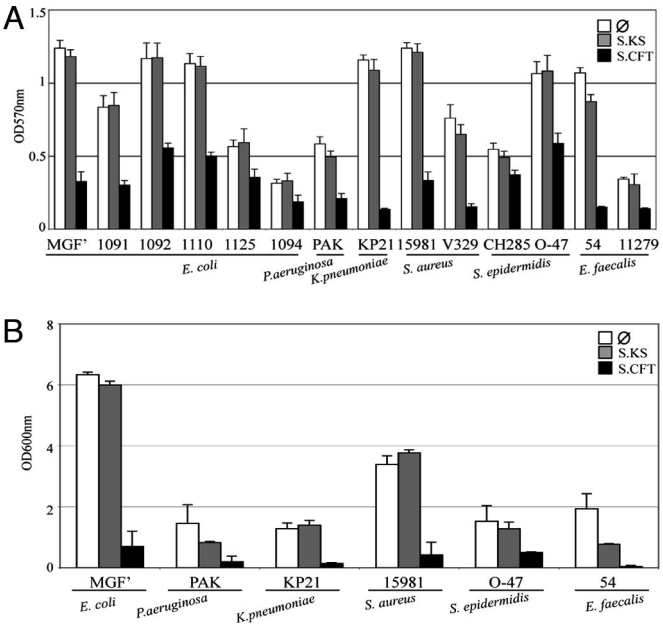

Fig. 2. Effect of CFT073 supernatant on Gram-positive and Gram-negative bacterial biofilm formation. (*A*) Quantification of the microtiter plate biofilm formation of different bacteria alone (Ø) or with KS272 (S.KS) or CFT073 (S.CFT) supernatant. Levels of crystal violet retained were measured spectrophotometrically (OD_{570}). (*B*) Quantification of biofilm formed by several pathogenic bacteria in microfermentors by using media not supplemented (Ø) or supplemented with S.CFT or S.KS. Error bars represent SD of two independent experiments.

supernatant on biofilm formation of several adherent bacteria (*E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Enterococcus faecalis*). This analysis showed that, in the presence of CFT073 supernatant, the biofilm capacity of all bacteria was significantly reduced, whereas this biofilm inhibition was not observed with KS272 supernatant (Fig. 2*A*). A significant reduction of the biofilm formation was also observed when CFT073 supernatant was perfused in the microfermentor model (Fig. 2*B*). These results showed that CFT073 supernatant has a biofilm inhibitory activity against both Gram-negative and Gram-positive biofilm-forming bacteria.

Correlation Between the Antibiofilm Effect and the Release of CFT073 Capsule. To elucidate the genetic basis of the antibiofilm effect, we tested the supernatant activity of ≈10,000 CFT073 random *mariner* transposon insertion mutants. We identified seven candidates impaired in their ability to inhibit MG1655 F′ biofilm formation. All of these mutants mapped in genes involved in the expression of the group II capsular polysaccharides (11, 16). Group II capsule is one of the four capsular types described in *E. coli*, and it displays a conserved modular genetic organization characterized by three functional regions (ref. 17; Fig. 3*A*). Region 1 (*kpsFEDCUS*) and region 3 (*kpsMT*) are conserved in all group II capsulated bacteria and encode proteins required for ABC-dependent export. Region 2 encodes a diversity of polysaccharidic structural components such as K1, K2 (CFT073), K5 and K96 capsular serotypes (11, 17). We deleted the R1, R2, or R3 region or individual CFT073 *kps* gene and showed that all mutants that lost the ability to inhibit *E. coli* biofilm formation also displayed reduced amounts of precipitated capsular sugars in the supernatant (Fig. 3 *B* and *C*). Although a ferritin-stained capsule still could be detected around CFT073 cells (Fig. 3*D*), our results indicated that the CFT073 capsule nevertheless undergoes a significant release into the medium supernatant that is responsible for the observed antibiofilm effect.

To investigate further the relationship between capsule and biofilm inhibition, we purified the polysaccharidic fraction from the CFT073 supernatant by ethanol precipitation, followed by standard ion-exchange chromatography. We purified an active fraction of 500 kDa (FR2), which displayed the previously determined K2 serotype group II capsule composition with galactose, glycerol, phosphate, and acetate at a molar ratio of 1:2:1:1, respectively (18). The FR2 fraction was added to the MG1655 F′ culture in concentrations ranging from 0.5 to 500 μg/ml, and we found that a concentration of 50 μg/ml inhibited MG1655 F′ biofilm formation similarly to CFT073 filtered supernatant (data not shown). We also performed a chemical cleavage of the FR2 fraction into its monomeric compounds, either by total acid hydrolysis or by aqueous hydrofluoric acid. The analysis of the biofilm effect of the FR2 subfractions showed that the chemical cleavage abolished the biofilm inhibitory effect (data not shown), indicating that full-length CFT073 capsular polysaccharide was critical for biofilm inhibition activity.

Antibiofilm Activity Is a Property of Bacterial Strains Expressing Group II Capsular Polysaccharides. To determine whether biofilm inhibition was an exclusive property of the *E. coli* CFT073 supernatant, we screened several clinical uropathogenic bacterial isolates of *Klebsiella*, *Proteus*, *Enterobacter*, *Morganella*, *Citrobacter*, and *Serratia* and a collection of 110 *E. coli* strains of diverse origin. We found that only the filtered supernatant of 39 *E. coli* inhibited biofilm formation on a wide range of bacteria without affecting the growth rate (Fig. 7*A*, which is published as supporting information on the PNAS web site). Using specific PCR probes (19), we showed that the 39 active *E. coli* strains carried group II capsule genes. Consistently, the introduction of a *kpsD* mutation into clinical UPEC isolates U-9 and U-15 abolished the biofilm inhibitory effect of their supernatants (Fig. 7*B*), indicating a high correlation between the antibiofilm effect and expression of the group II capsule. Interestingly, although CFT073, U-9, and U-15 displayed a very limited

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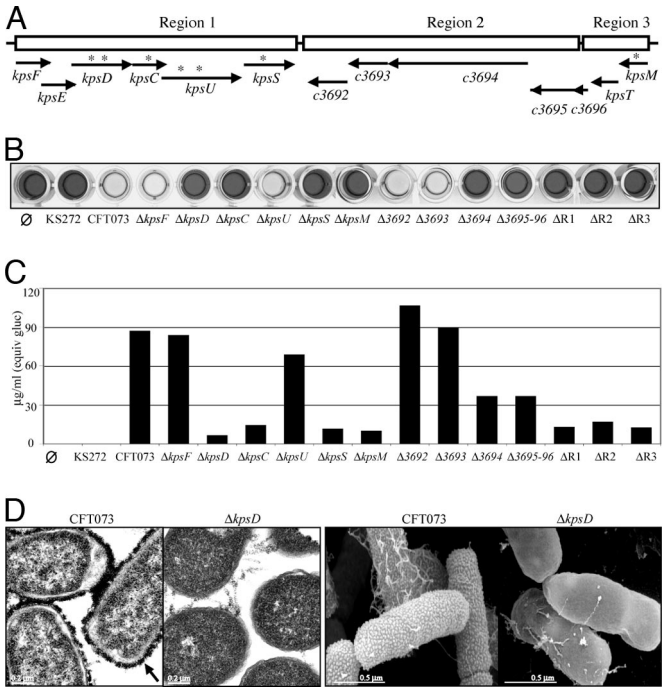

Fig. 3. Relationship between capsule production and CFT073 anti-biofilm activity. (*A*) Genetic organization of the CFT073 capsule R1, R2, and R3 regions. Genes with transposon insertions are marked with an asterisk. (*B*) Biofilm formation of MG1655 F′ cultivated in the presence of capsule mutant supernatants. (*C*) Hexose levels in supernatants. *kpsF*, *kpsU*, *c3692*, and *c3693* correspond to mutants that do not impair capsule production. (*D*) Stationary-phase CFT073 or CFT073Δ*kpsD* bacterial cell capsules stained with ferritin and examined by transmission electron microscopy (×100,000). (Scale bar: 0.2 μm.) (*Left*) A total of 125 and 105 cells were observed, respectively. Stained CFT073 capsule is indicated by an arrow. (*Right*) Scanning electron micrographs of stationary-phase CFT073 or CFT073Δ*kpsD* (×50,000). (Scale bar: 0.5 μm.) A total of 45 and 37 cells were observed, respectively.

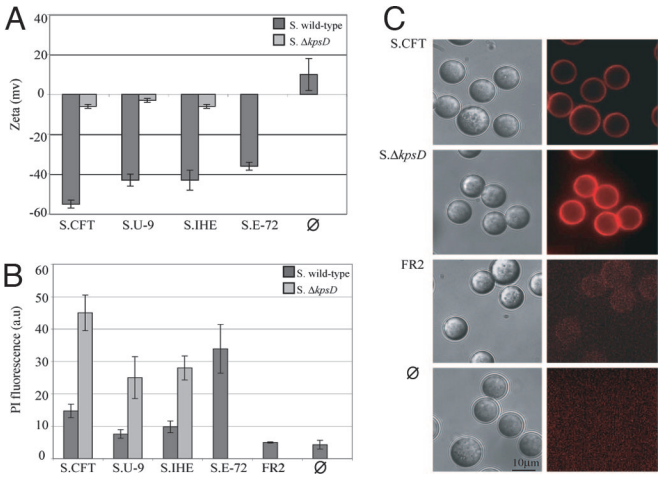

Fig. 4. Physicochemical properties of CFT073 supernatant. (*A*) ζ Potential of cationic colloids incubated with dialyzed supernatants from: CFT073 (S.CFT), U-9, (S.U9), IHE3034 (S.IHE), ECOR72 (S.E-72), and their respective capsule *kpsD* mutants when applicable. Ø corresponds to M63B1glu medium. (*B*) Propidium iodide adsorption onto cationic particles incubated with supernatants of S.CFT, S.U-9, S.IHE, S.E-72, and their respective capsule mutants when applicable. FR2:CFT073 supernatant purified fraction. The extent of adsorption is given by the fluorescent intensity (>670 nm). Error bars represent SD of the mean. (*C*) Fluorescence microscopy of cationic particles incubated with S.CFT, S.Δ*kpsD*, FR2, and M63B1 (Ø).

ability to form biofilm in our microfermentor biofilm model, their respective *kpsD* mutants displayed an increased biofilm phenotype. This phenotype could be reversed upon the addition of their own supernatant, suggesting that these strains also could self-inhibit their own adhesion (Fig. 7*C*). Moreover, a similar capsule expressed by the mucosal pathogen *Neisseria meningitidis* also was able to inhibit biofilm formation (data not shown; refs. 20 and 21). Taken together, these results showed that the group II capsule, commonly expressed by extra intestinal *E. coli* and other mucosal pathogens, displays antibiofilm activity.

Group II Capsular Polysaccharides Induce Physicochemical Alterations of Abiotic Surfaces. The analysis of the polysaccharides precipitated from active supernatants of several group II capsular serotypes, including CFT073 (K2 serotype), U-9 (non-K2), and IHE3034 (K1), confirmed that they displayed significantly different sugar compositions (data not shown; ref. 17). This observation suggested that, although biochemically distinct, group II capsule released by these strains could share a similar mode of action leading to biofilm inhibition. To further study the mechanisms by which group II capsule inhibits bacterial biofilm formation, we brought these fractions into contact with cationic colloids composed of 10-μm (diameter) latex particles bearing a permanent net positive charge because of their polyethylenimine coating. The determination of the interface ζ (zeta) potential showed that wild-type supernatants induced a strong charge inversion of cationic colloids, indicative of their highly anionic nature compared with supernatants of their respective capsule mutants (Fig. 4*A*). Moreover, treatment of acid-cleaned glass slides with active supernatant lowered the water-slide interfacial energy, which is indicative of their hydrophilic nature (data not shown).

To analyze whether the group II capsule could induce surface modifications and affect intermolecular forces on treated surfaces, we monitored the adsorption of propidium iodide, a fluorescent amphiphilic cationic ion, on colloids negatively charged after coating with active or inactive supernatants. We first observed that the anionic but inactive supernatant of the non-group II capsulated strain ECOR72 displayed strong affinity for the cationic fluorescent probe (Fig. 4 *A* and *B*). Despite their high negative charge, active supernatants displayed significantly lower probe affinity than inactive, but less negatively charged, capsule mutant supernatants. This effect was even more pronounced with the purified CFT073 FR2 fraction (Fig. 4 *B* and *C*). These results therefore showed that, in addition to electrostatic modifications, active supernatants also induced remodeling of the colloid surface properties, possibly including surface hydration and steric repulsion.

CFT073 Supernatant Impairs Cell-Surface Initial Interactions. The physicochemical properties displayed by the group II capsule might sharply alter bacterial ability to interact with surfaces and, therefore, drastically reduce adhesion (4). To test this hypothesis, we first analyzed the primary adherence capacity of both *E. coli* and *S. aureus* to glass surfaces pretreated with CFT073 supernatant. We observed that after 1 h of incubation, *E. coli* MG1655 F′ and *S. aureus* 15981 exhibited a 3-fold reduction in their initial adhesion on treated surfaces (data not shown). Consistently, pretreatment of the internal microfermentor glass slide with CFT073 supernatant drastically reduced biofilm formation by *E. coli* and by several Gram-positive and Gram-negative pathogens (Fig. 5). No effect was observed when a similar treatment was performed with CFT073Δ*kpsD* supernatant (Fig. 5). These results therefore suggested that the surface modifications induced by capsular polysaccharides released in the CFT073 supernatant could interfere with biofilm formation by impairing initial bacterial–surface interactions.

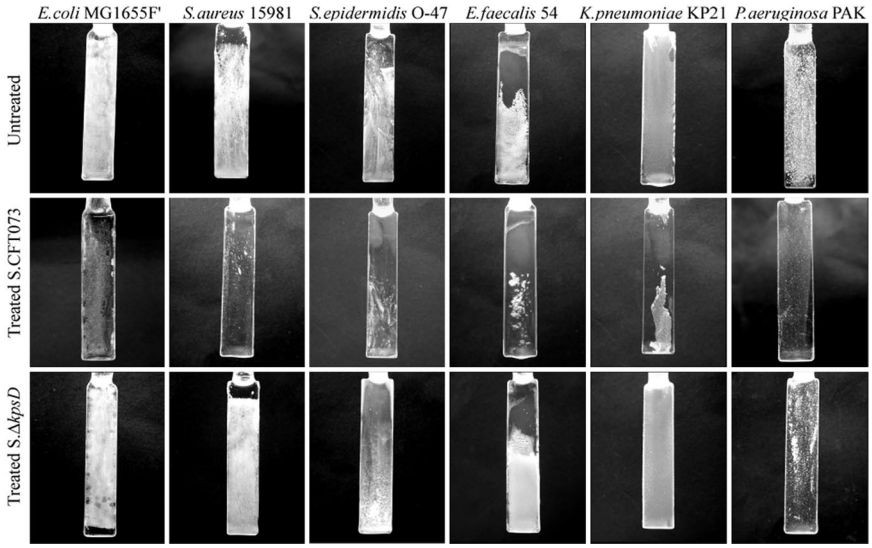

Fig. 5. Biofilm inhibition effect of CFT073 supernatant on treated surfaces. Biofilm formation in microfermentors by several bacteria by using the following: untreated glass slides (*Top*), glass slides treated with CFT073 supernatant (*Middle*), and glass slides treated with CFT073Δ*kpsD* supernatant (*Bottom*).

CFT073 Supernatant Also Inhibits Biofilm Formation by Reducing Cell–Cell Interactions. To investigate the effect of CFT073 supernatant on already existing biofilms, microfermentors inoculated with MG1655 F′ at different stages of biofilm maturation were supplemented with filtered CFT073 supernatant. This analysis showed that, whereas the treatment of a mature 24-h biofilm did not induce biofilm dispersal, addition of the CFT073 supernatant at 0, 1, and 6 h after MG1655 F′ biofilm initiation blocked its further development (Fig. 6*A*). We then examined the *in vitro* biofilm characteristics of a GFP-tagged MG1655 F′ after the addition of CFT073 supernatant and confocal laser scanning microscopy analysis. After 3 h of postinitial inoculation, the addition of active CFT073

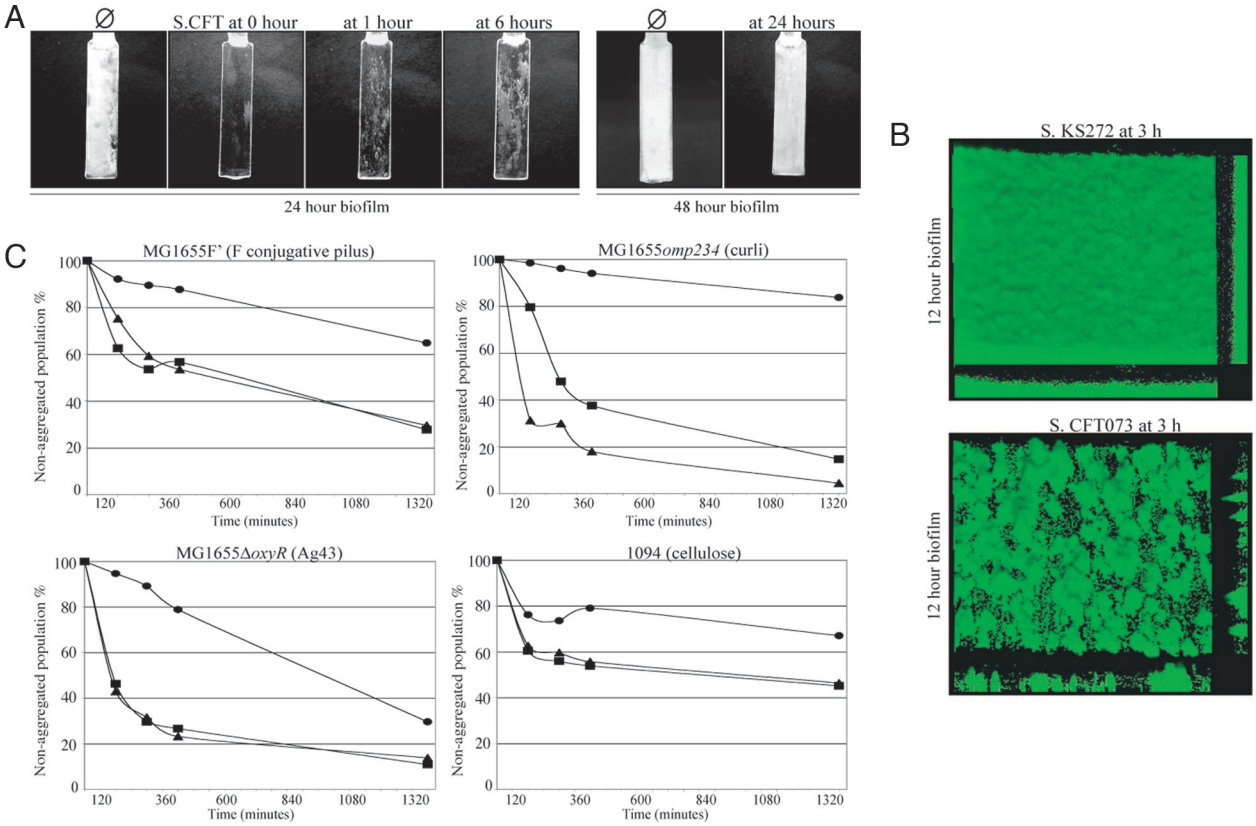

Fig. 6. CFT073 supernatant affects cell–cell interaction. (*A*) MG1655F′ biofilm formation in microfermentors with media supplemented with CFT073 supernatant (S.CFT) at times 0, 1, 6 (24 h of culture), and 24 h (48 h of culture). Ø, no addition of S.CFT. (*B*) GFP-tagged MG1655 F′ inoculated in a flow cell and monitored by confocal microscopy. CFT073 or KS272 supernatants were supplemented after 3 h of culture, and biofilms were grown for 12 h total. (*C*) Autoaggregation assay with strains that aggregate via different mechanisms: MG1655 F′ (F conjugative pilus expression); MG1655*ompR234* (curli overexpression); MG1655Δ*oxyR* (Ag43 autotransporter adhesin overexpression); and 1094 (cellulose production). Cells were diluted to $OD_{600} = 2$ in 3 ml of M63B1 (▲), CFT073 supernatant (●), and Δ*kpsD* supernatant (■).

exogenous supernatant on a regularly covered surface profoundly affected MG1655 F′ mature biofilm structure development (Fig. 6*B*). This effect was not observed upon control KS272 supernatant treatment.

The direct contribution of bacterial surface structures to the 3D *E. coli* biofilm structure has been amply demonstrated (22). These structures also have been shown to mediate bacterial aggregation and clumping in standing cultures. To further characterize the role of group II capsule in biofilm maturation, we tested its effects on bacterial aggregation mediated by several different surface-exposed factors also involved in biofilm formation. We showed that CFT073 supernatant reduces formation of bacterial aggregates induced by bacterial surface structures such as conjugative pili, curli, Ag43 adhesin, or cellulose production (Fig. 6*C*; ref. 22). Taken together, these results suggest that the physicochemical properties of the group II capsular polysaccharides affect biofilm formation by weakening cell-surface contacts (initial adhesion) but also by reducing cell–cell interactions (biofilm maturation).

Discussion

E. coli polysaccharides such as colanic acid polymer, cellulose, and (1–6)-β-*N*-acetyl-glucosamine have been identified as part of the extracellular matrix, which plays an important positive role in building the mature biofilm structure. Besides these polymers, *E. coli* isolates also produce two serotype-specific surface polysaccharides: the lipopolysaccharide O antigen and capsular polysaccharide K antigen. These two classes of surface-exposed polysaccharidic polymers have been shown to play indirect roles in biofilms by the shielding of bacterial surface adhesin (23). Our study demonstrates a previously uncharacterized direct property of the capsule that antagonizes biofilm formation. We show that group II capsular polysaccharides actually are significantly released into the culture supernatant and display antiadhesion activities toward both Gram-positive and Gram-negative bacteria, including important nosocomial pathogens.

Capsule has been shown to be a cell surface structure involved in virulence and colonization by creating a hydrated protective layer around the bacteria and increasing their resistance to phagocytosis and to the bactericidal effects of human serum (10, 24–27). Here we show that, among the tested *E. coli* strains, 17 of the 39 active *E. coli* identified were UPEC isolated from symptomatic urinary tract infections, and 13 others corresponded to ECOR strains of the phylogenetic group B2 or D, also indicative of extraintestinal pathogenic *E. coli* (28). This high percentage of extraintestinal *E. coli* among the active strains (75%) suggests that the antibiofilm property of group II capsular polysaccharides also could play a role in the biology of these pathogens. The capsule-mediated biofilm inhibition may contribute to competitive interactions (bacterial interference) within bacterial communities encountered during the colonization process, which occurs as a continuum from initial entry via the oral route to progression through the intestine and urinary tract (10, 29).

UPEC have been shown to form biofilm-like structures (pods) in the urinary tract (30, 31). By contrast, we made the observation that capsule-producing strains have a very limited ability to form biofilm in our microfermentor model. However, their respective capsule mutants displayed an enhanced biofilm phenotype that could be reversed upon the addition of their own supernatant (Fig. 7*C*). Although the biofilm inhibitory effect was observed in all growth phases and a quorum-sensing Δ*luxS* mutant of CFT073 (data not shown), the *in vivo* regulation of group II capsule expression may contribute to modulate bacterial own adhesion to mucosal surfaces encountered by UPEC in the urinary tract (23, 32, 33).

Capsular polysaccharides are linked to the cell surface of the bacterium via covalent attachments (11, 16). However in still-undetermined conditions, the capsule can be released into the growth medium as a consequence of the instability of the phosphodiester linkage between the polysaccharide and the phospholipid membrane anchor (17). Whereas our analysis revealed that all active strains expressed only group II capsule, we also identified 7 inactive strains carrying the group II capsule genes. These strains may not produce or release the capsule. In addition, our data indicate that non-group II capsulated strains such as ECOR72 also release an anionic hydrophilic capsular polymer that does not induce biofilm inhibition (Fig. 4). Thus, whereas capsule release may not be a hallmark of the group II capsule, not all released capsules display the described antibiofilm activity.

Polymers assembling on surfaces are known to cause strong physical repulsion, depending on their density, size, solvation, and structure (34). Such repulsive forces created by a group II capsule could modulate microbial interaction with interfaces by limiting initial bacterial adhesion and by interfering with subsequent cell–cell contact within the biofilm. Our data show that distinct serotypes of group II capsular polysaccharides, but not their chemically cleaved product, behave like surface active polymers that display antiadhesion properties, suggesting that full-length integrity of group II capsular polymers is more critical to the inhibitory effect than their primary biochemical composition.

Finally, we also found that treatment of abiotic surfaces with group II capsular polysaccharides has a long-lasting effect sufficient to significantly inhibit mature biofilm development of a broad-spectrum of bacteria. Therefore, while contributing to a better understanding of competitive interactions that could occur within mucosal flora, this study may have far-reaching implications regarding the control of pathogenic biofilm development.

Materials and Methods

Bacterial Strains, Growth Conditions, and Microscopic Analysis. Bacterial strains are listed in Table 1, which is published as supporting information on the PNAS web site (also available upon request). Gram-negative bacteria were grown at 37°C in M63B1 0.4% glucose-minimal medium (M63B1glu) or in LB-rich medium. Gram-positive bacteria were grown in trypticase soy broth with 0.25% glucose (TSBglu) at 37°C. The effect of CFT073 supernatant on bacterial growth and viability was evaluated by using growth-curve determination, colony-forming unit count on LB plate, and BacLight Live/Dead viability stain (Molecular Probes, Eugene, OR). Ferritin staining and scanning electronic microscopy were performed as described in ref. 35. Epifluorescence and transmitted light microscopy were performed as described in ref. 36. Scanning laser electronic microscopy was performed on biofilm grown in microfermentors on thermanox slide (Nalgen Nunc International Co., Naperville, IL) fixed on the internal removable glass slide at the Unité de Formation et de Recherche Médecine, Tours, France.

Biofilm Formation Procedures. ***Microfermentor experiments.*** Biofilms were produced as described in ref. 12 and in methods available upon request.

Mixed biofilm cultures. An 8-h MG1655 F′ biofilm formed on the internal microfermentor glass slide was infected with 1 $OD_{600\ nm}$ equivalent of CFT073-gfp overnight culture. After 24 h of continuous culture in M63B1glu, pictures of the glass slides were taken. Biofilm biomass was estimated by determining the $OD_{600\ nm}$ of the biofilm resuspended from the internal microfermentor glass slide (12).

Biofilm inhibition assays. The incoming medium was mixed in a 1:1 ratio with filtered supernatants, adjusted to 0.4% glucose, and brought into the microfermentors at different times after inoculation (0, 1, 6, or 24 h). The biofilm was cultivated further for an additional 24 h.

Analysis of bacterial interaction with treated surfaces. The glass slides were incubated for 5 min with filtered CFT073 supernatant and rinsed once in deionized water before inoculation. Biofilm formation on the slide was determined after 24 h.

Microtitre plate experiments. Static biofilm formation assays were performed in 96-well poly(vinyl chloride) microtiter plates (Falcon;

Becton Dickinson Labware, Oxnard, CA) for 24 h as described in ref. 37.

Biofilm inhibition assays. Overnight cultures were adjusted to OD_{600} = 0.04 before inoculating 100 μl in 96-well poly(vinyl chloride) plates in the presence or absence of 50 μl of supernatant.

Flow-cell experiments. Biofilms were performed in M63B1glu at 37°C in flow cell (3 channels: 1 × 4 × 40 mm each). The flow system was assembled and prepared as described in ref. 38. Inocula were prepared as follows: 16- to 20-h-old overnight cultures in M63B1glu were harvested and resuspended as normalized dilutions (OD_{600} = 0.05). Three hundred microliters was injected into each flow channel. Flow was started 1 h after inoculation at a constant rate of 3 ml·h^{-1} by using a Watson Marlow 205S peristaltic pump. Input medium was changed after 3 h and replaced by a mixed medium with a 1:1 ratio with filtered supernatant. Autoaggregation assays were performed as described in ref. 36. All assays were at least performed in triplicate.

Handling of Culture Supernatants and Polysaccharide Analysis. Overnight cultures in M63B1glu were centrifuged for 30 min at 3,800 × *g* at 4°C, filtered through a 0.2-μm filter, precipitated with 3 volumes of ethanol, and dialyzed against deionized water (10 kDa cassettes; Pierce, Rockford, IL). Total amounts of phosphate and neutral sugars were determined by ammonium molibdate/ascorbic acid and phenol/sulfuric acid methods, respectively. Polysaccharide composition was determined by HPLC (ion-exclusion column) and gas liquid chromatography as in refs. 39 and 40. CFT073 supernatant active fraction, FR2, was purified by using a DEAE-Sepharose column (Amersham Pharmacia, Cleveland, OH) and eluted with 300 mM NaCl in 25% propanol-1/20 mM Tris·HCl, pH 7.5. Molecular weight of the polymer was estimated by gel filtration chromatography on Superdex-200 (Amersham Pharmacia) by using dextran as standard. Polysaccharide degradations were done by total acid hydrolysis (trifluoroacetic acid, 4N, 4H, 100°C) or by aqueous hydrofluoric acid (48%, 2 days on ice water).

Mutagenesis and Molecular Techniques. *mariner* transposon mutagenesis of *E. coli* CFT073 was performed as described in ref. 41. The supernatants of 10,000 transposon mutants incubated for 24 h in M63B1glu at 37°C in 96-well microtiter plates were extracted after centrifugation of plates for 15 min at 5,000 × *g* and their effects on MG1655 F′ biofilm formation were analyzed. Transposon insertion sites were determined as described in ref. 41. Homology searches were performed by using Blast 2.0. Deletion mutants were generated as detailed in ref. 41 by using primers presented in Table 2, which is published as supporting information on the PNAS web site.

Analysis of Physicochemical Properties of Active Fractions. ζ Potential was measured as in ref. 42 after 20 min of incubation of cationic colloids of 10-μm cationic latex particles with dialyzed precipitated supernatants. The latex particles bear a permanent net-positive charge because of their polyethylenimine (PEI) coating. The layer of PEI is a branched 6,400-Da polymer bearing ≈50% of methylated quaternary functions that confer a stable positive charge to the molecule. This polymer was deposited in aqueous phase on the initially carboxylated particles (43). Hydrophilic properties of the supernatants were investigated by determining the contact angle formed by a 2.5-μl ultrapure water droplet with a glass plane surface previously incubated in the supernatants for 20 min. Surface interactions were analyzed by monitoring the adsorption of propidium iodide on supernatant-treated cationic colloids. The affinity of the treated surfaces for the fluorescent probe was tested by using flow cytometry (44) and fluorescence microscopy. All incubations of particles with supernatant were performed at low particle/volume fraction (≈0.2%), likely leading to surface saturation by the active species.

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