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Exopolysaccharide Production Is Required for Development of *Escherichia coli* K-12 Biofilm Architecture

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Although exopolysaccharides (EPSs) are a large component of bacterial biofilms, their contribution to biofilm structure and function has been examined for only a few organisms. In each of these cases EPS has been shown to be required for cellular attachment to abiotic surfaces. Here, we undertook a genetic approach to examine the potential role of colanic acid, an EPS of *Escherichia coli* K-12, in biofilm formation. Strains either proficient or deficient in colanic acid production were grown and allowed to adhere to abiotic surfaces and were then examined both macroscopically and microscopically. Surprisingly, we found that colanic acid production is not required for surface attachment. Rather, colanic acid is critical for the formation of the complex three-dimensional structure and depth of *E. coli* biofilms.

Bacterial biofilms have been described as sessile bacterial communities that live attached to each other and to surfaces (2, 3, 4, 5, 10). In natural settings, many bacterial species live predominantly in these communities, with a smaller portion of the bacterial population subsisting as free-swimming (planktonic) organisms (10). In addition to their abundance in natural environments, biofilms also impinge significantly upon our industrialized world. For example, bacterial biofilms can form on catheters and prostheses and thereby cause persistent, antibiotic-resistant infections (5, 12). Biofilms can also clog pipes (1) and contaminate food in industrial settings (21). However, biofilms can also have beneficial functions, for example, by acting as biocontrol agents by preventing fungal infections in certain plants (9). Given the preponderance of biofilm communities in nature as well as their medical and industrial impact, it is clearly important to understand the molecular mechanisms that govern both the formation and dissolution of these sessile communities.

The three-dimensional architecture of a number of single-species bacterial biofilms has been previously described (5, 8). The two most generalizable features of these biofilms are microcolonies, composed of cells surrounded by large amounts of exopolysaccharide (EPS), and water-filled channels, which have been hypothesized to promote the influx of nutrients and the efflux of waste products.

Previous work with *Pseudomonas aeruginosa* and with *Escherichia coli* has shown that EPS (alginate and colanic acid, respectively) synthesis is induced upon attachment of the bacteria to a surface (6, 7, 17). However, these results have not revealed the role(s) that EPS plays in biofilm formation. Studies with the gram-negative organisms *Shewanella putrefaciens* and *Vibrio cholerae* and the gram-positive organism *Staphylococcus epidermidis* revealed that EPS is required for initial attachment to surfaces (15, 20; D. Newman and R. Kolter, unpublished data). Here, we describe the role of EPS in *E. coli* biofilm formation and note that this role is dramatically different than that described for *S. putrefaciens*, *V. cholerae*, and *S. epidermidis*.

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Isolation of an *E. coli* strain defective in colanic acid production. We performed mini-Tn*10cam* transposon mutagenesis (14; P. N. Danese, unpublished observation) on *E. coli* K-12 in an effort to find mutations that rendered *E. coli* defective in swarming along a hard agar surface (13). Because of the parallels between the movement of bacteria along surfaces during swarming and the formation of communities of cells attached to surfaces (biofilms), we were interested in examining the effects of certain swarming defect mutations upon biofilm formation.

One of the insertion mutations isolated in the surface-swarming screen (*wcaF31*::*cam*) disrupted the *wcaF* open reading frame via insertion into the 107th codon of the *wcaF* gene, which normally encodes a protein comprised of 182 residues. Based on sequence similarity and its chromosomal location within the *cps* (capsule) gene cluster, *wcaF* has been proposed to be required for production of colanic acid (Fig. 1), an EPS produced by *E. coli* K-12 (18).

Consistent with this hypothesis, overproduction of RcsF, a positive regulator of colanic acid synthesis (11), in strain ZK2686 [W3110 Δ(*argF-lac*)*U169*] resulted in a mucoid colony phenotype, whereas overproduction of RcsF in strain ZK2687 (ZK2686 *wcaF31*::*cam*) had no such effect. In addition, in vitro quantification of EPS production (19) demonstrated that our *wcaF* mutant had severely reduced EPS production (data not shown).

CV analysis of cell attachment to PVC. Cultures of strains ZK2686 (colanic acid-positive [CA^+]) and ZK2687 (CA^-) were grown in Luria-Bertani broth (LB) in polyvinylchloride (PVC) wells. After various periods of growth (Fig. 1), the planktonic cells were removed by vigorous rinsing with water, and the extent of biofilm formation of both strains was analyzed macroscopically by staining with crystal violet (CV), a dye which stains attached cells but not PVC (16). As illustrated in Fig. 2, during the early time points (17 h or less) the CV staining observed for the strain defective in colanic acid production was significantly less intense than that observed for the wild-type strain. However, the CV staining increased over time, and ultimately it more closely approximated that of the wild-type parent (Fig. 2). These initial observations indicated that the production of colanic acid affects *E. coli* biofilm formation but its absence does not completely abolish surface attachment. Qualitatively equivalent results were obtained

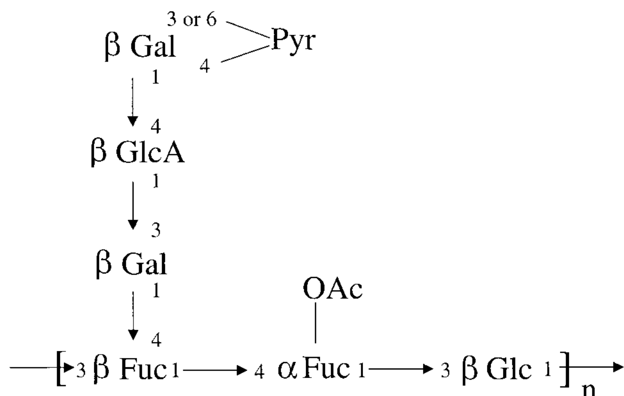

FIG. 1. Chemical structure of the colanic acid monomer. Fuc, L-fucose; Gal, D-galactose; GlcA, D-glucuronic acid; Glc, D-glucose; OAc, *O*-acetyl; Pyr, pyruvate. This figure was adapted from reference 18.

when the CA^+ and CA^- strains were allowed to form biofilms in minimal glucose medium (data not shown).

Fluorescence microscopy of CA^+ and CA^- cells. In order to more precisely understand the effects of colanic acid production (or the lack thereof) on *E. coli* biofilm formation, CA^+ (ZK2686) and CA^- (ZK2686 containing *cpsC*::Tn*10* [19], *cpsE*::Tn*10* [19], or *wcaF*::*cam* mutations) strains were transformed with a green fluorescent protein expression plasmid and analyzed by fluorescence microscopy. The cells (either CA^+ or CA^-) were allowed to grow in rich medium for 72 h in the presence of a glass coverslip. Planktonic cells were removed from the coverslip by rinsing with water, and the remaining attached cells were examined by fluorescence microscopy.

The three-dimensional complexity of the wild-type *E. coli* biofilm can be clearly observed by examining a sequential Z-series scan of all *xy* focal planes of the biofilm. Importantly, a similar collection of Z-series scans of a colanic acid-defective biofilm illustrates the tightly packed cells and the absence of both significant depth and complex architecture. Video clips of these Z-series scans can be viewed at http://gasp.med.harvard.edu/biofilms/ecoli/colanic.html.

A different perspective of the structures formed on an abiotic surface (by CA^+ and CA^- strains) can also be obtained by viewing a sagittal section of the attached cells (Fig. 3). As illustrated in Fig. 3c, the wild-type strain forms pillars of cells that are approximately 26 μm high. In addition, it is clear that the cell bodies within microcolonies often do not physically interact but instead appear to be suspended above the surface (Fig. 3c). This complex structure is in stark contrast to that observed in the sagittal section of the CA^- attached cells (Fig. 3d). This CA^- strain forms densely packed structures with extensive cell-surface and cell-cell interactions. Importantly, there is no evidence of the extensive depth seen with the wild-type parent (Fig. 3c). Video clips showing the rotation from the overhead (*xy*) to the sagittal (*xz*) perspective (both shown in Fig. 3) of the CA^+ and CA^- attached cells can be viewed at http://gasp.med.harvard.edu/biofilms/ecoli/colanic.html. These video clips provide additional perspectives of the CA^+ and the CA^- attached cells and emphasize the differences between their respective structures. We also note that this collapsed phenotype can be complemented by a plasmid-carried copy of *wcaF*, indicating that the phenotypes observed are due solely to the disruption of *wcaF* (data not shown).

Concluding remarks. A previous report based on sequence similarity led to the hypothesis that *wcaF* is involved in colanic acid production (18). Specifically, based on its similarity to other genes and its chromosomal position within the *cps* (capsule) gene cluster, it was hypothesized that the *wcaF* gene product might function as an acetyltransferase during the synthesis of colanic acid (18). The evidence presented here provides genetic data demonstrating that the *wcaF* product is indeed required for colanic acid production.

However, the most striking finding reported here is that colanic acid is required not for initial attachment to an abiotic surface but rather for establishing the complex three-dimensional structure of an *E. coli* biofilm. Indeed, we have examined the initial attachment of both the wild type and the *wcaF* mutant to PVC via phase-contrast microscopy at times of <2 h, and we observe no difference in initial cellular attachment (data not shown), consistent with the view that colanic acid is required not for surface attachment but for biofilm architecture. This result is dramatically different from the phenotypes

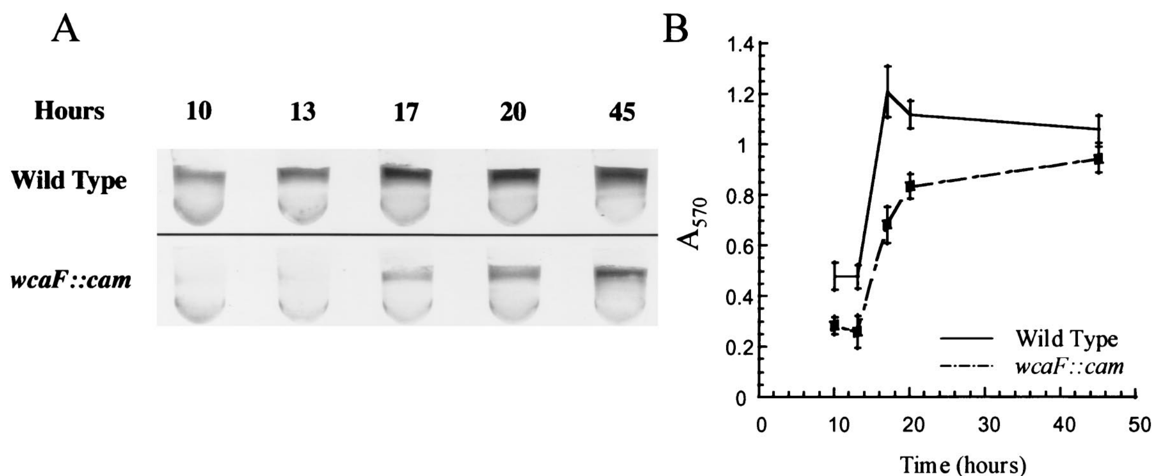

FIG. 2. Colanic acid is important for biofilm formation in *E. coli* K-12. The wild type {ZK2686 [W3110 Δ(*argF-lac*)*U169*]} and colanic acid mutant (ZK2687 [ZK2686 *wcaF31*::*cam*]) strains were grown in PVC microtiter dishes in LB at 30°C without shaking for 24 h and then subcultured (1:100) into PVC microtiter dishes containing LB. These cultures were then grown at 30°C in LB without shaking for the indicated times. (A) The dishes were then rinsed and stained with CV as previously described (16). (B) Quantification of CV staining. The amount of CV staining at each time point was determined as previously described (16). Note that the exponential growth rates of ZK2686 and ZK2687 were indistinguishable under the conditions tested (doubling times of ~60 min).

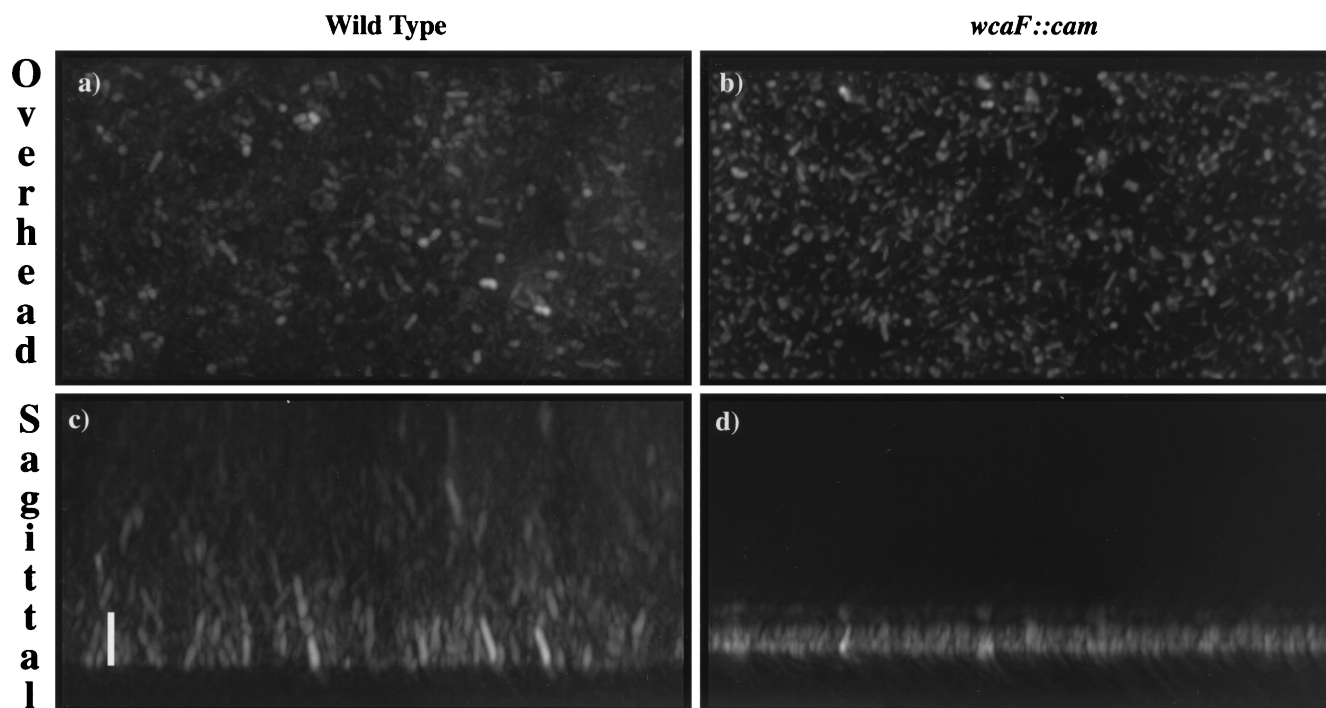

FIG. 3. Overhead (*xy*) and sagittal (*xz*) images of wild-type and colanic acid-defective biofilms. Strains ZK2686 [W3110 Δ(*argF-lac*)*U169*] and ZK2687 [ZK2686 *wcaF31*::*cam*] were transformed with pND103 (which encodes the green fluorescent protein) and grown at 30°C in the presence of a borosilicate glass coverslip. Cultures were grown in LB with ampicillin (100 μg/ml) for 72 h without shaking. Saturated medium was replaced with fresh LB and ampicillin every 24 h. After 72 h, the coverslip was rinsed to remove nonattached cells, and the remaining attached cells were examined via epifluorescence microscopy using a 40× oil immersion objective (numerical aperture, 1.4) on an Olympus optical-sectioning microscope equipped with deconvolution software (Applied Precision). Sagittal images (c and d) were created from a collection of 130 consecutive Z-series scans of the wild-type and colanic acid-defective biofilms. The step size between each Z section was 0.2 μm. Sagittal images were generated using the Volume View program of the Deltavision software package (Applied Precision). Bar, 5 μm.

observed with other bacterial species. Mutations that abolish EPS production in either *V. cholerae* (20), *S. putrefaciens* (Newman and Kolter, unpublished), or *S. epidermidis* (15) render these strains severely defective in the very initial stages of attachment to abiotic surfaces.

In *E. coli* K-12, mutations that prevent EPS (colanic acid) production do not block the ability of the cells to initially attach to abiotic surfaces. In fact, *E. coli* K-12 strains defective in colanic acid production form bacterial films that are one to two cells in depth (Fig. 2B and 3d). These results highlight the fact that the role(s) EPS plays in the formation of *E. coli* biofilms is, in at least one respect, different from the role (i.e., adhesion) it plays in other organisms. However, it is important to emphasize that despite the successful attachment of *E. coli* CA^- mutants, such mutants do not display the pillars of cells that are typical of biofilms even at times at which the CV staining of the *wcaF* mutant equals that of the wild type (100 h). We should emphasize that we routinely rinse our biofilms before microscopic examination. Thus, it is possible that colanic acid is not required specifically for establishing biofilm architecture. Rather, it may be required to maintain biofilm architecture in the face of environmental vicissitudes, such as alterations in medium bulk flow.

Regardless of this possibility, our results demonstrate that it is not simply pili, flagella, or other cellular appendages that contribute to the strength of this complex three-dimensional biofilm structure. Rather, colanic acid is an integral part of this elaborate structure.

It is worth noting that the phenotype displayed by the CA^- *E. coli* K-12 strain is analogous to that observed with *P. aeruginosa* strains that are unable to produce the intercellular signaling compound *N*-(3-oxododecanoyl)-L-homoserine lactone (8). In both instances, the three-dimensional, complex structure of the biofilm is completely absent. Instead, the cells appear collapsed and tightly packed close to the surface. The similarity of these phenotypes suggests the intriguing possibility that intercellular signaling is an integral part of the induction and/or arrangement of colanic acid within the microcolonies. We are currently investigating this possibility.

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REFERENCES

1. **Carr, J. H., R. L. Anderson, and M. S. Favero.** 1996. Comparison of chemical dehydration and critical point drying for the stabilization and visualization of aging biofilm present on interior surfaces of PVC distribution pipe. J. Appl. Bacteriol. **80:**225–232.
2. **Costerton, J. W.** 1995. Overview of microbial biofilms. J. Ind. Microbiol. **15:** 137–140.
3. **Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott.** 1995. Microbial biofilms. Annu. Rev. Microbiol. **49:**711–745.
4. **Costerton, J. W., Z. Lewandowski, D. DeBeer, D. Caldwell, D. Korber, and G. James.** 1994. Biofilms, the customized microniche. J. Bacteriol. **176:**2137–2142.
5. **Costerton, J. W., P. S. Stewart, and E. P. Greenberg.** 1999. Bacterial biofilms: a common cause of persistent infections. Science **284:**1318–1322.
6. **Davies, D. G., A. M. Chakrabarty, and G. G. Geesey.** 1993. Exopolysaccharide production in biofilms: substratum activation of alginate gene expression

by *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **59:**1181–1186.
7. **Davies, D. G., and G. G. Geesey.** 1995. Regulation of the alginate biosynthesis gene *algC* in *Pseudomonas aeruginosa* during biofilm development in continuous culture. Appl. Environ. Microbiol. **61:**860–867.
8. **Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg.** 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science **280:**295–298.
9. **Geels, F. P., and B. Schippers.** 1983. Reduction of yield depressions in high frequency potato cropping soil after seed tuber treatments with antagonistic fluorescent *Pseudomonas* spp. Phytopathol. Z. **108:**207–214.
10. **Geesey, G. G., R. Mutch, J. W. Costerton, and R. B. Green.** 1978. Sessile bacteria: an important component of the microbial population in small mountain streams. Limnol. Oceanogr. **23:**1214–1223.
11. **Gervais, F. G., and G. R. Drapeau.** 1992. Identification, cloning, and characterization of *rcsF*, a new regulator gene for exopolysaccharide synthesis that suppresses the division mutation *ftsZ84* in *Escherichia coli* K-12. J. Bacteriol. **174:**8016–8022.
12. **Goto, T., Y. Nakame, M. Nishida, and Y. Ohi.** 1999. Bacterial biofilms and catheters in experimental urinary tract infection. Int. J. Antimicrob. Agents **11:**227–231.
13. **Harshey, R. M., and T. Matsuyama.** 1994. Dimorphic transition in *Escherichia coli* and *Salmonella typhimurium*: surface-induced differentiation into hyperflagellate swarmer cells. Proc. Natl. Acad. Sci. USA **91:**8631–8635.
14. **Kleckner, N., J. Bender, and S. Gottesman.** 1991. Uses of transposons with emphasis on Tn*10*. Methods Enzymol. **204:**139–180.
15. **McKenney, D., J. Hubner, E. Muller, Y. Wang, D. A. Goldmann, and G. B. Pier.** 1998. The *ica* locus of *Staphylococcus epidermidis* encodes production of the capsular polysaccharide/adhesin. Infect. Immun. **66:**4711–4720.
16. **Pratt, L. A., and R. Kolter.** 1998. Genetic analysis of *Escherichia coli* biofilm formation: defining the roles of flagella, motility, chemotaxis and type I pili. Mol. Microbiol. **30:**285–294.
17. **Prigent-Combaret, C., O. Vidal, C. Dorel, and P. Lejeune.** 1999. Abiotic surface sensing and biofilm-dependent regulation of gene expression in *Escherichia coli*. J. Bacteriol. **181:**5993–6002.
18. **Stevenson, G., K. Andrianopoulos, M. Hobbs, and P. R. Reeves.** 1996. Organization of the *Escherichia coli* K-12 gene cluster responsible for production of the extracellular polysaccharide colanic acid. J. Bacteriol. **178:**4885–4893.
19. **Trisler, P., and S. Gottesman.** 1984. *lon* transcriptional regulation of genes necessary for capsular polysaccharide synthesis in *Escherichia coli* K-12. J. Bacteriol. **160:**184–191.
20. **Watnick, P. I., and R. Kolter.** 1999. Steps in the development of a *Vibrio cholerae* El Tor biofilm. Mol. Microbiol. **34:**586–595.
21. **Wong, A. C.** 1998. Biofilms in food processing environments. J. Dairy Sci. **81:**2765–2770.