

## Emergence of Biofilm-Forming Subpopulations upon Exposure of *Escherichia coli* to Environmental Bacteriophages

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**Exposure of *Escherichia coli* MG1655 to environmental bacteriophages results in rapid selection for phage-tolerant subpopulations displaying increased biofilm formation. Analysis of one phage-tolerant strain revealed large amounts of the DNA-binding Dps protein in the outer membrane protein and production of fimbria-like structures. In *dps* and *fimA* mutant derivatives of MG1655, no selection of phage-tolerant bacteria upon exposure to bacteriophages occurred, suggesting a role for Dps and type I pili in bacteriophage tolerance.**

In natural environments, bacteria can often be found as sessile communities, usually referred to as biofilms (3, 17). Most biofilms produce an extracellular matrix (extracellular polymeric structure or EPS), composed of both polysaccharides and proteins, which can constitute a relevant part of their total dry weight (4). This extensive EPS production takes place even if biofilms are growing in oligotrophic environments, despite the high energy consumption required by EPS biosynthesis, suggesting that growth of the EPS matrix confers important advantages on the microorganisms. EPS production can also protect bacteria against predation by bacteriophages (24), which can be present at an up to 10:1 ratio to bacteria in natural environments such as seawater (25). Finally, the EPS is likely to contribute, together with specific gene expression and lower growth rates, to biofilm resistance to a number of environmental stresses, such as treatment with biocides (5, 7).

In order to investigate interaction between bacteria and environmental bacteriophages, we isolated bacteriophages from an urban sewage water treatment plan in Opfikon, Switzerland. After water filtration, bacteriophages were propagated as previously described (8) on liquid cultures of the laboratory strain *Escherichia coli* MG1655 (1). The phages obtained were specific for *E. coli* and showed no activity against any other bacterial species tested (*Bacillus subtilis*, *Staphylococcus epidermidis*, *Pseudomonas putida*, and *Citrobacter freundii*) or the yeast *Saccharomyces cerevisiae* (data not shown). Two bacteriophages, OP7061 and OP10081, were purified from single plaques after infection of *E. coli* on agar plates and used to reinfect liquid cultures of *E. coli* MG1655. The phage lysates were cleared with chloroform and used in further experiments; both lysates showed a phage titer of roughly  $10^9$  PFU/ml. Although OP7061 and OP10081 were very similar with respect to their spectra of bacterial activity (Table 1) and plaque morphology (data not shown), bacteria tolerant to OP7061 could

still be reinfected with OP10081, but not vice versa, suggesting that the two bacteriophages are not identical. In standard infection experiments with liquid cultures, such as those shown in Table 1, bacteria from overnight cultures (usually grown in Luria-Bertani [LB] medium at 37°C with full aeration) were diluted into fresh medium at a final concentration of roughly  $10^6$  CFU/ml. After 30 min of incubation, bacteriophages were added at different concentrations, ranging from  $10^5$  to  $10^8$  PFU/ml, i.e., at MOIs (multiplicities of infection; i.e., phage/bacterium ratios used in the infection experiments) ranging from 0.1 to 100. Antibiotic-resistant strains were pregrown in the presence of the corresponding antibiotic but diluted in antibiotic-free medium for the infection experiments. The endpoint of phage susceptibility by bacteria was determined by visual analysis of culture turbidity after 3.5, 24, and 48 h. Both the OP7061 and OP10081 phages were highly virulent against *E. coli* laboratory strains, with infection resulting in complete lysis of bacterial cultures in 3 to 4 h of incubation at 37°C even at an MOI of 0.1; however, they proved to be ineffective against environmental isolates. Table 1 shows the phage sensitivity of liquid cultures of MG1655 (a standard laboratory strain) and WK1 (an *E. coli* strain isolated from river waters in Canton Zurich, Switzerland); these values are representative of other laboratory and environmental strains, respectively (data not shown). Growth in different media (LB and M9-glucose) and at different temperatures (30 and 37°C) did not significantly affect phage sensitivity (data not shown).

Although *E. coli* laboratory strains were sensitive to environmental phages, tolerant bacterial subpopulations developed after ca. 24 h of incubation (Table 1). Bacterial growth in the presence of phages was not due to loss of virulence, since chloroform-extracted phage particles from cultures showing bacterial growth were able to successfully infect a fresh MG1655 culture. Bacterial regrowth after initial lysis is thus likely to be due either to mutations leading to phage resistance in MG1655 or to phage lysogeny. The phage-tolerant bacteria displayed a stable phenotype; i.e., they could not be reinfected by phages OP7061 and OP10081 even after several passages on

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TABLE 1. Sensitivity of bacterial strains to bacteriophages<sup>a</sup>

Bacterial strain	Sensitivity (PFU/ml) <sup>b</sup> to:			
	OP7061		OP10081	
	3.5 h	48 h	3.5 h	48 h
MG1655	$2.5 \times 10^5$	$>10^8$	$2.5 \times 10^5$	$>10^8$
WK1	$>10^8$	$>10^8$	$5 \times 10^7$	$>10^8$
AL7061	$>10^8$	$>10^8$	$>10^8$	$>10^8$
AL10081	$>10^8$	$>10^8$	$>10^8$	$>10^8$
AL126 ( <i>dps::kan</i> )	$\leq 10^5$	$\leq 10^5$	$\leq 10^5$	$\leq 10^5$
AL132 <i>dps::kan</i> pBAD18- <i>dps</i> <sup>c</sup>	$\leq 10^5$	$>10^8$	$\leq 10^5$	$10^7$
AL127 ( <i>fimA::kan</i> )	$2.5 \times 10^5$	$\leq 10^5$	$\leq 10^5$	$\leq 10^5$
AL128 ( <i>csxA::kan</i> )	$\leq 10^5$	$>10^8$	$\leq 10^5$	$>10^8$
AL129 ( <i>fliC::kan</i> )	$2.5 \times 10^5$	$>10^8$	$2.5 \times 10^5$	$>10^8$

<sup>a</sup> Bacterial strains were challenged with phage suspensions ranging from  $10^5$  to  $10^8$  PFU/ml, corresponding to MOIs ranging from 0.1 to 100.

<sup>b</sup> Indicated as the lowest phage concentration at which total bacterial lysis was detectable at the given times.

<sup>c</sup> LB medium was supplemented with 0.1% arabinose to induce *dps* expression from the pBAD18-*dps* plasmid.

phage-free medium. No prophage induction could be achieved through exposure to heat (43°C) or to DNA-damaging agents (UV light or methyl methane sulfonate), suggesting that they might be phage-resistant mutants. However, since we have not characterized any mutation responsible for phage resistance, we will refer to them as phage-tolerant bacteria in our report.

Interestingly, tolerant bacteria grown in the presence of phages displayed a different phenotype, such as increased

clumping in liquid cultures (Fig. 1A). We isolated single colonies from OP7061 and OP10081 phage-tolerant cultures (named strains AL7061 and AL10081, respectively); liquid cultures of both isolates showed cell clumping. We tested the adhesion properties of AL7061 and AL10081 as the ability to form biofilms in microtiter plates (18). Cultures were grown in LB medium overnight at 37°C; the liquid medium was removed and the attached cells washed with sterile phosphate-buffered saline (pH 7.0). The bacterial biofilm was visualized by staining with 1% crystal violet for 20 min at room temperature, followed by rinsing with water and air drying. Quantification of biofilm was achieved by dissolving the crystal violet-stained biofilm in 95% ethanol, followed by spectrophotometric determination of optical density at 600 nm. The absorbance of the crystal violet solution was divided by the optical density at 600 nm of the corresponding planktonic culture to adjust for total bacterial growth; the adjusted value obtained for strain MG1655 was arbitrarily set as 1 (Fig. 1B). As shown in Fig. 1, biofilm formation was dramatically increased in both strains. MG1655 derivatives resistant to either wild-type  $\lambda$  or P1vir (12) phage were also tested in adhesion experiments: the P1-resistant AL10082 strain, but not the  $\lambda$ -resistant AL10083 strain, also displayed significantly increased adhesion properties (Fig. 1B). Negative-staining transmission electron microscopy analysis (9, 20) of liquid cultures of the phage-tolerant, adhering AL7061 strain revealed production of fimbria-like extracellular structures totally absent in the MG1655 parental strain (Fig. 2). AL10081 cells underwent extensive breakage during preparation of the samples for electron microscopy analysis, suggesting that mutations that occurred in this strain might have resulted in gross alterations of its membrane. For this reason and due to the possible instability of the mutations, as judged by the presence of different colony morphologies on L agar plates, the AL10081 strain was not investigated any further.

In order to understand what determinants might be responsible for increased adhesion and cell aggregation, we analyzed the lipopolysaccharide and outer membrane protein (OMP) patterns of the AL7061 strain and compared them to those of its parent, MG1655. No significant differences were found in the core oligosaccharides (the part of lipopolysaccharide produced by K-12 strains of *E. coli* such as MG1655) (data not shown). In contrast, AL7061 displayed significant differences in the OMP pattern compared to MG1655 (Fig. 3); proteins corresponding to differently expressed bands were excised from the gel, digested with trypsin, and identified through matrix-assisted laser desorption/ionization-time of flight analysis (MALDI-TOF) (2). In AL7061, the expression of the two main porins, OmpC and OmpF, as well as of the OmpX protein, was significantly reduced; in contrast, the OmpA porin was present in greater relative amounts in the phage-tolerant strain. This drastic change in the OMP expression pattern of AL7061 might be due to regulatory mutations affecting OMP production; alternatively, it might depend upon the different physiological conditions due to growth as cell aggregates in AL7061 (Fig. 1A). Either way, a decrease in OMP production might account at least partially for the increased resistance to bacteriophages, since OmpF can act as a phage receptor protein (13). Interestingly, the prominent AL7061 OMP corresponded to a band with an apparent molecular mass of 20 kDa; this protein was identified as Dps, a bacterial ferritin capable of

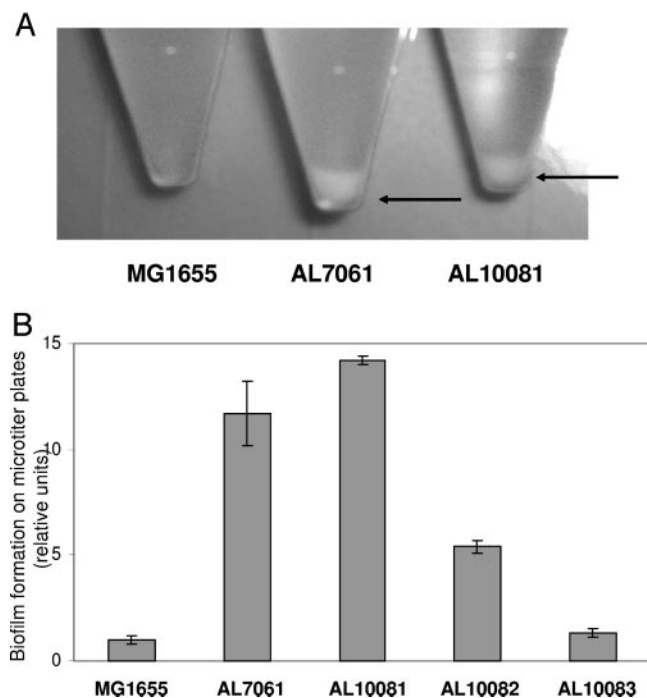


FIG. 1. (A) Cell sedimentation in liquid cultures of MG1655, AL7061, and AL10081. Overnight cultures in LB medium were left standing at room temperature for 1 h; arrows indicate cell pellets. (B) Adhesion to microtiter plates by phage-sensitive and phage-tolerant strains. The experiments were repeated four times, and standard deviations are shown.

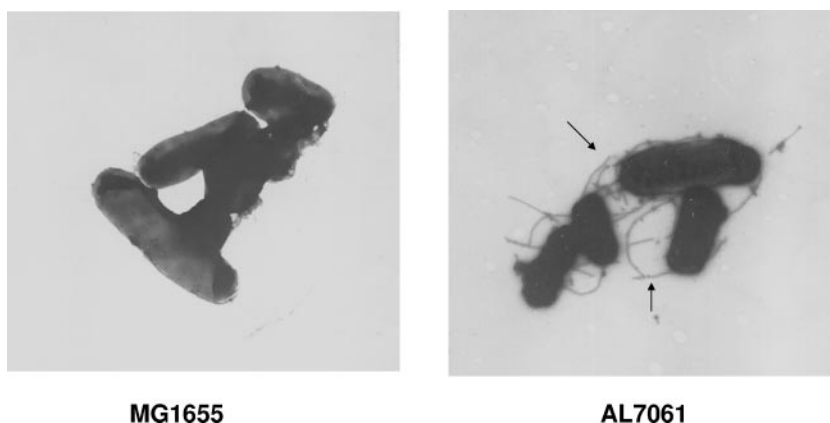


FIG. 2. Negative-staining transmission electron microscopic picture of MG1655 (wild type) and the phage-tolerant AL7061 strain. The samples were prepared as previously described (8, 18). Magnification,  $\times 12,000$ .

nonspecific DNA binding (11, 26). Although the OmpX and Dps proteins have almost identical molecular masses and migrate to similar positions on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, MALDI-TOF analysis clearly shows neither the presence of Dps in the MG1655 strain nor contamination of the Dps band by OmpX in AL7061, suggesting that these proteins are indeed differentially expressed in the two strains. Dps is a cytoplasmic protein mainly expressed in the stationary phase of growth under the control of the stress-responding *rpoS* gene, which is involved in cell protection from a variety of physiological and environmental stresses (11, 14); we are not aware of any reports suggesting its possible location

in the outer membrane compartment of the cell. However, the lack of any other major cytoplasmic proteins in the outer membrane fraction of AL7061 seems to rule out the possibility that the presence of Dps is due to general leakage of cytoplasmic components in AL7061.

We investigated the possible relevance in phage resistance and in cell adhesion of the apparently increased Dps expression and/or its unusual localization in the outer membrane. To this purpose, we transduced a *dps::kan* mutation (11) into our MG1655 strain and exposed the AL126 strain obtained to bacteriophages; interestingly, in the *dps* mutant AL126 strain, no selection of any phage-tolerant subpopulations could be observed at up to 48 h of growth. Expression of the Dps protein from the pBAD18-*dps* plasmid (11) in the AL126 strain restored the phage tolerance phenotype, strongly suggesting that the *dps* gene is indeed directly involved in this process (Table 1). In contrast, overexpression of the Dps protein in MG1655 did not result in increased adhesion, nor did it protect MG1655 from phage infection, suggesting that neither stimulation of biofilm production nor phage tolerance depends on Dps expression alone (data not shown). A direct test of the contribution of a functional *dps* gene to these processes in AL7061 could not be done, since neither P1 transduction nor other standard genetic techniques aimed to gene inactivation have so far been effective for this strain (data not shown).

Increased ability to form biofilm and cell aggregation by the phage-tolerant isolates obtained in our study in comparison to the MG1655 parental strain (Fig. 1) could be linked to the ability to produce factors involved in adhesion and cell-cell aggregation, such as the fiber-like structures observed in AL7061 by transmission electron microscopy (Fig. 2). In the absence of conjugative plasmids, which allow extensive biofilm formation via expression of conjugative pili (6, 21), *E. coli* can produce several extracellular fiber-like structures: the flagellum (encoded by the *fli* genes) (10), type I pili or fimbriae (encoded by the *fim* genes) (16), and curli (encoded by the *csg* genes) (15). Interestingly, these factors can all play a role in biofilm formation by *E. coli* (17, 18, 22, 23), and the corresponding genes are often cryptic or not expressed under standard laboratory growth conditions (19). To determine if factors known to be involved in biofilm formation forming fiber-like

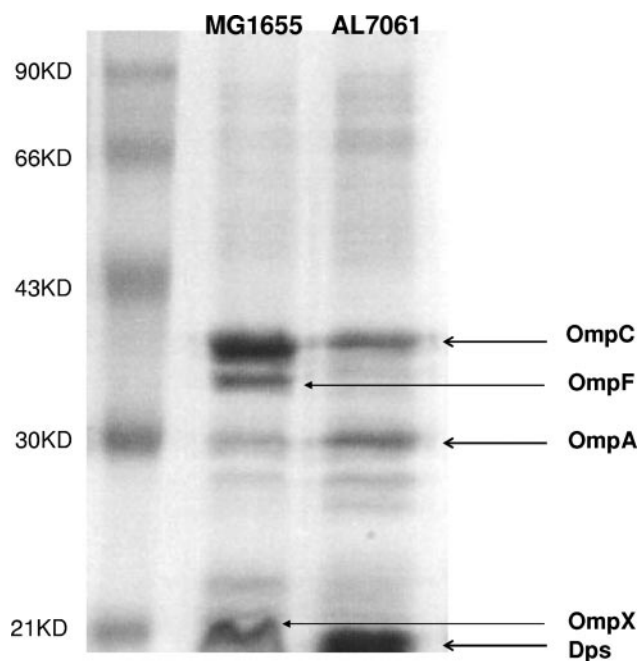


FIG. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (10% acrylamide, 32.5:1 acrylamide/*N,N'*-bisacrylamide ratio) analysis of OMP patterns of MG1655 (wild type, lane 2) and AL7061 (phage tolerant, lane 3). Molecular size markers (KD, kilodaltons) are shown in lane 1. Proteins identified by mass spectrometry whose expression differs in the two strains are indicated.

structures are needed for phage tolerance in MG1655, we inactivated the *csgA*, *fimA*, and *fliC* genes, respectively encoding the major subunit of the curli fibers, type I pili, and flagella. Inactivation was carried out by transduction of null alleles from strains PHL856 (*csgA::kan*) (23), FB22974 (*fimA::kan*), and FB20528 (*fliC::kan*), respectively. Both FB22974 and FB20528 were obtained from the Systematic Mutagenesis Strain Collection at the University of Wisconsin (<http://www.genome.wisc.edu/functional/tmmutagenesis.htm#Progress>). Inactivation of *fimA*, but not of the *csgA* or *fliC* gene, totally abolished selection for tolerant bacteria upon exposure to bacteriophages OP7061 and OP10081 (Table 1). The null alleles tested all carry the same kanamycin resistance cassette, suggesting that the effects observed do not depend on indirect effects of the antibiotic resistance genes on phage susceptibility (Table 1). Thus, our observations indicate that fimbria-mediated biofilm formation might indeed be a strategy to escape bacteriophage attack in *E. coli* MG1655. We are currently investigating the possibility that increased fimbria production and localization of the Dps protein in the outer membrane are part of a coordinated response to attack by bacteriophages. Our results suggest that growth as a biofilm might be an additional mechanism of resistance to bacteriophages complementary to or synergistic with more specific processes such as loss of specific phage receptors and expression of DNA restriction enzymes, thus pointing to a close evolutionary relationship between the ability to form a biofilm and resistance to phages.

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