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Impact of *rpoS* Deletion on *Escherichia coli* Biofilms

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Slow growth has been hypothesized to be an essential aspect of bacterial physiology within biofilms. In order to test this hypothesis, we employed two strains of *Escherichia coli*, ZK126 (Δ*lacZ rpoS*$^+$) and its isogenic Δ*rpoS* derivative, ZK1000. These strains were grown at two rates (0.033 and 0.0083 h^{-1}) in a glucose-limited chemostat which was coupled either to a modified Robbins device containing plugs of silicone rubber urinary catheter material or to a glass flow cell. The presence or absence of *rpoS* did not significantly affect planktonic growth of *E. coli*. In contrast, biofilm cell density in the *rpoS* mutant strain (ZK1000), as measured by determining the number of CFU per square centimeter, was reduced by 50% ($P < 0.05$). Deletion of *rpoS* caused differences in biofilm cell arrangement, as seen by scanning confocal laser microscopy. In reporter gene experiments, similar levels of *rpoS* expression were seen in chemostat-grown planktonic and biofilm populations at a growth rate of 0.033 h^{-1}. Overall, these studies suggest that *rpoS* is important for biofilm physiology.

In their natural environments, bacteria often adhere to surfaces on which they form biofilm communities that may be several millimeters thick. Within biofilms, individual bacteria are encased in a polysaccharide matrix, which functions to bind cells together and facilitates adhesion to the underlying surface. Bacteria are not distributed uniformly throughout a biofilm but rather aggregate into microcolonies, which are typically a few micrometers in diameter (6). Studies employing scanning confocal laser microscopy (SCLM) have shown a wide range of bacterial growth rates throughout a biofilm. The fastest growth was observed at the biofilm-liquid interface. Bacteria in the biofilm interior, particularly those inside microcolonies, grew much more slowly, presumably due to limited access to nutrients (10, 13). These and other studies have led to the hypothesis that slow growth is a major aspect of bacterial biofilm physiology (4). In order to test this hypothesis, we investigated whether the absence of a slow-growth-activated gene, *rpoS* (7), could affect the biofilm formation of *Escherichia coli* under defined growth conditions (15). Here we report that deletion of *rpoS* greatly reduces the ability of *E. coli* to grow in biofilms yet has little effect on the growth of planktonic (i.e., unattached) bacteria.

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Strains and culturing conditions. The strains of *E. coli* used in this study are ZK126 (Δ*lacZ*), ZK1000 (ZK126 Δ*rpoS*) (1), and DS526 (ZK126 λRZ5 *rpoS742*::*lacZ*) (13a). Cultures were stored frozen at −80°C in Luria-Bertani (LB) broth containing 15% (vol/vol) glycerol as described elsewhere (14). Prior to each experiment, the appropriate *E. coli* strain was streaked from a frozen stock culture onto LB agar, checked for purity, and grown overnight in 5 ml of glucose-limited, defined medium (GDM) (15) containing 0.25 g of glucose per liter.

Biofilm chemostat experiments. Chemostats were coupled to a modified Robbins device (MRD; Tyler Research, Edmonton, Alberta, Canada) as described by Whiteley et al. (15). Briefly, this consisted of filling a chemostat with sterile GDM and inoculating it with 1 ml of an overnight *E. coli* culture in GDM. This culture was allowed to grow overnight under batch conditions, after which continuous culture was commenced at a dilution rate (DR) of either 0.033 or 0.0083 h^{-1}. The chemostat cultures were allowed to equilibrate for 1 generation time (121 h at a DR of 0.0083 h^{-1} and 30 h at a DR of 0.033 h^{-1}), after which time the chemostat was connected to an MRD containing 7-mm-diameter silicone rubber plugs. A peristaltic pump was used to circulate the chemostat culture through the MRD at a flow rate of 100 ml min^{-1}. After 48 h, the experiment was stopped and nine plugs were removed from the MRD, sonicated, serially diluted in phosphate-buffered saline, and plated onto LB agar (Difco Laboratories, Detroit, Mich.) as previously described (8, 15). Each chemostat-MRD culture experiment was replicated a minimum of three times. Within each chemostat-MRD replicate, a minimum of five measurements were taken.

When biofilm cultures were to be examined by SCLM, the chemostat was established as described previously and attached by capillary tubing to a flow cell (2) (Water Technologies, Bozeman, Mont.) to which was attached a glass microscope slide. A Pharmacia peristaltic pump (Amersham Pharmacia Biotech, Inc., Piscataway, N.J.) was used to circulate the chemostat culture through the flow cell at a rate of 8.3 ml min^{-1}. For SCLM examination, the glass slide was removed and stained with BacLite Live/Dead viability stain (Molecular Probes, Eugene, Oreg.) in order to estimate the viability of individual cells. Biofilm formation in flow cells was examined by SCLM with an Olympus IX-70 inverted microscope (Olympus America Inc., Melville, N.Y.) coupled with a Bio-Rad 1024 SCLM System (Bio-Rad Laboratories, Hercules, Calif.). The slides were placed with the biofilm side facing the 60× Uplan Apo (Olympus) oil immersion objective lens.

***rpoS* expression assay.** In order to compare the levels of *rpoS* expression in biofilm cells and planktonic cells, reporter strain DS526, containing an *rpoS*::*lacZ* fusion on a λ phage, was constructed by D.A. Siegele, Texas A&M University, as previously described (5). This strain was cultured in the chemostat-MRD apparatus at a DR of 0.033 h^{-1} as described above. After 48 h of biofilm growth, biofilm and planktonic samples were removed and frozen at −80°C for 2 weeks until analyzed. We permeabilized *E. coli* cells with chloroform and sodium dodecyl sulfate and quantified the β-galactosidase activity with

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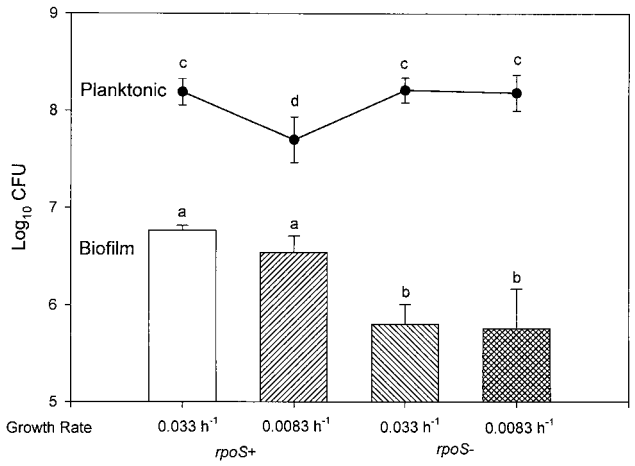

FIG. 1. Graph showing effects of *rpoS* deletion and growth rate on planktonic (expressed as $\log_{10}$ CFU per milliliter) and biofilm (expressed as $\log_{10}$ CFU per square centimeter) cultures. Error bars represent standard deviations. Values with the same letter are not significantly different ($P = 0.05$).

o-nitrophenyl-β-D-galactopyranoside (ONPG) as described by Miller (9). The cell number was determined on the basis of direct cell counts of 4′,6-diamidino-2-phenylindole (DAPI)-stained biofilm and planktonic cell suspensions. β-Galactosidase activity was expressed as nanomoles of ONPG cleaved per cell per minute.

Data analysis. Biofilm cell densities, expressed as $\log_{10}$ CFU per square centimeter, and planktonic cell densities, expressed as $\log_{10}$ CFU per milliliter, were analyzed by one-way analysis of variance.

The influences of *rpoS* deletion and growth rate on *E. coli* biofilms and planktonic cultures are shown in Fig. 1. As can be seen, deletion of *rpoS* had a major impact on biofilm populations and less of an impact on planktonic populations. SCLM examinations (Fig. 2) showed differences in *E. coli* biofilm structures in the presence (Fig. 2A) and absence (Fig. 2B) of *rpoS*. When bacteria were grown at a DR of 0.033 h^{-1}, similar levels of β-galactosidase activity were seen in biofilm (3.04×10^{-6} nmol of ONPG min^{-1} $cell^{-1}$) and planktonic (3.08×10^{-6} nmol of ONPG min^{-1} $cell^{-1}$) populations of *E. coli* DS526 containing an *rpoS*::*lacZ* fusion.

Several lines of evidence support the role of slow growth in biofilm physiology. Due to their enhanced access to nutrients, bacteria on the periphery of biofilm microcolonies grow much more quickly than do the nutrient-limited organisms in the interior (10). One striking feature of biofilm growth is that bacteria are significantly more resistant to antimicrobial agents than they are during planktonic growth (11). To investigate this finding, Evans et al. (3) compared the antibiotic resistance of planktonic chemostat cultures at various growth rates. They found antibiotic susceptibility to be correlated with growth rate and thus attributed biofilm antimicrobial resistance to a reduced growth rate. The study of Evans et al. (3) provides additional impetus for studying biofilms at reduced growth rates.

Several notable effects of *rpoS* deletion were observed in the present study. These include significant differences in biofilm cell density (Fig. 1) and differences in biofilm structure (Fig. 2). The influence of *rpoS* deletion on planktonic cells was minimal at either DR (Fig. 1). One possible explanation for this phenomenon was that *rpoS* was expressed only during biofilm growth. We measured patterns of *rpoS* expression in *E. coli* DS526, which contains an *rpoS-lacZ* fusion, at a DR of 0.033 h^{-1}. In this experiment, *rpoS* expression, as indicated by the amount of β-galactosidase activity per cell, was equivalent in both planktonic (3.08×10^{-6} nmol of ONPG min^{-1} $cell^{-1}$) and biofilm (3.04×10^{-6} nmol of ONPG min^{-1} $cell^{-1}$) populations. Our observations are consistent with those of Notley and Ferenci (12), who observed *rpoS* expression in chemostat cultures of *E. coli* at a DR of ≤ 0.2 h^{-1}. Deletion of *rpoS* had an impact on biofilm cell density (Fig. 1) and cell arrangement

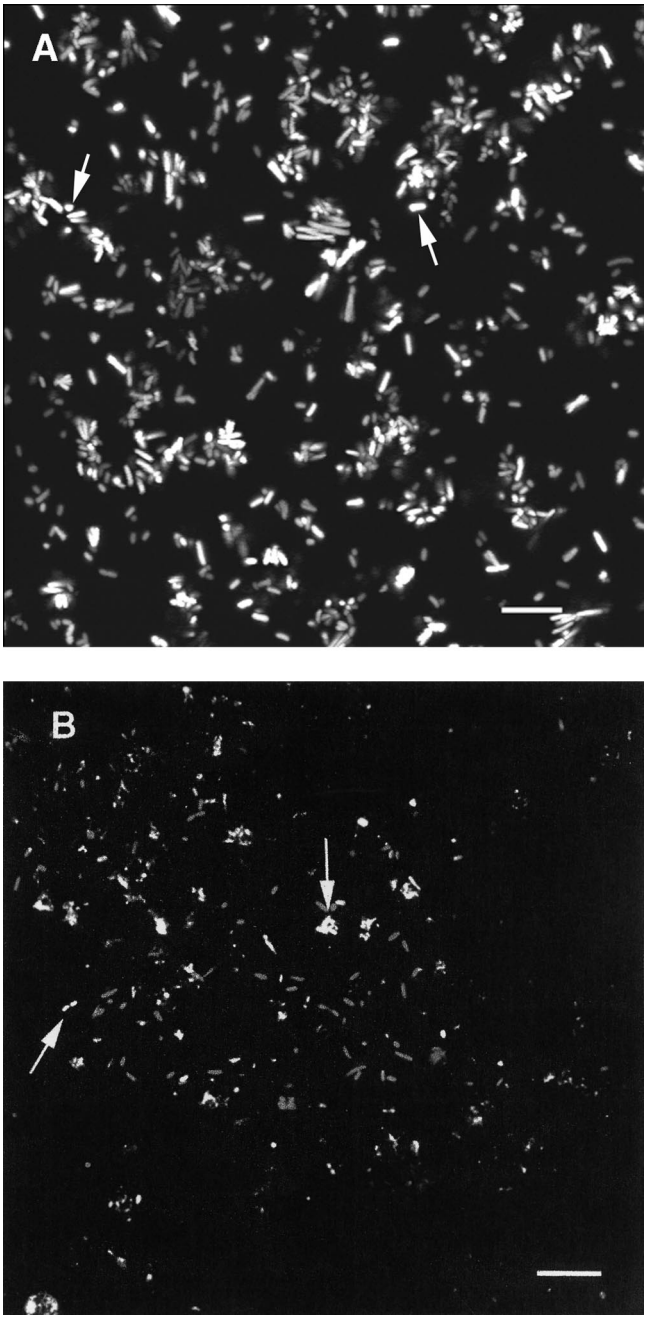

FIG. 2. SCLM micrographs of *E. coli* biofilms stained with Live/Dead viability stain in the presence (A) and absence (B) of *rpoS*. The viable (brightly stained) cells are indicated by an arrow. Bars, 2 μm in panel A and 3 μm in panel B.

(Fig. 2), so it is likely that *rpoS* expression is more important to biofilm populations than to planktonic populations.

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