Effect of *Escherichia coli* Morphogene *bolA* on Biofilms

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Biofilm physiology is established under a low growth rate. The morphogene *bolA* is mostly expressed under stress conditions or in stationary phase, suggesting that *bolA* could be implicated in biofilm development. In order to verify this hypothesis, we tested the effect of *bolA* on biofilm formation. Overexpression of *bolA* induces biofilm development, while *bolA* deletion decreases biofilms.

In natural environments, most bacteria live attached to surfaces in structures known as biofilms, rather than existing as isolated planktonic cells (14). Biofilm formation is such a common phenomenon that almost any material coming into contact with bacteria present in fluids, e.g., blood or seawater, can become covered with biofilms. Given the important medical and economic consequences of this phenomenon, understanding the colonization process would help in the design of methods to prevent biofilm formation (9). Several properties inherent within bacterial biofilms indicate that gene expression in biofilm-associated bacteria is different from that observed in planktonic bacteria (13). Biofilm-associated cells demonstrate increased resistance to many toxic substances, such as antibiotics, detergents, and host immune defense response products. Furthermore, bacteria within biofilms encounter high-osmolarity conditions, oxygen limitations, and high cell density (9). Slow growth is also an important aspect of bacterial biofilm physiology (1, 10). Altogether, these results suggest that biofilm formation is a response to unfavorable environments, since biofilms are better adapted to different types of stress than are planktonic bacteria.

Stress response genes are induced whenever a cell needs to adapt and survive under adverse growth conditions. The *Escherichia coli* morphogene *bolA* is an example of those genes. It causes round morphology when overexpressed (2). *bolA* was first described as a gene involved in adaptation to stationary-phase growth under the control of a sigma factor (σ^S), and *bolA* mRNA levels are inversely proportional to growth rate (3, 4, 6). However, the function of *bolA* is not confined to stationary phase; *bolA* expression is induced by several forms of stress during early growth phase, such as heat shock, acidic stress, oxidative stress, and sudden carbon starvation (11).

As *bolA* expression and biofilm formation are two events related to stationary phase and stress, we tested the hypothesis that *bolA* could be implicated in biofilm development.

The strains used were *E. coli* MC1061 [F^- *araD139* Δ (*ara-leu*)7697 Δ (*lac*)X74 *galU galK strA*] (3), SK6582 [MC1061 Δ *bolA2::Kan*] (3), and CMA14 [MC1061/pMAK580] (12). Plasmid pMAK580 (2) contains *bolA* under regulation of its own promoters. During the experiments, batch cultures were

launched from overnight growths (performed with Luria broth medium) that were diluted to an optical density measured at 620 nm of 0.08. Cultures were grown aerobically in M9 minimal medium (7) containing 0.4% (wt/vol) glucose (Merck) at 37°C and with shaking at 120 rpm on an orbital shaker. The medium was supplemented as required with 0.8 mM leucine, 20 mg of chloramphenicol ml⁻¹, 50 mg of kanamycin ml⁻¹, and 25 mg of streptomycin ml⁻¹ (all from Sigma).

The determination of biofilm thickness in microtiter plates was carried out as described by O'Toole and Kolter (8). Briefly, cells were allowed to grow in M9 minimal medium for 24 h in 96-well polystyrene microtiter dishes for stationary-phase-growth analysis. Unattached bacteria existing in the culture medium were removed, and the biofilm was stained with 0.2% (wt/vol) crystal violet for 15 min (this dye stains the cells but not the polystyrene). The excess crystal violet dye was washed out, and the samples were washed three times with bidistilled water. Ethanol was added to the wells to release the dye, and the optical density at 600 nm was measured in order to estimate the amount of biofilm formed (8). The results showed that *bolA* overexpression induced biofilm development, since the optical density of crystal violet-stained biofilms was about fourfold higher for the culture of the strain overexpressing *bolA* than for the culture of the wild type (MC1061) (Fig. 1). The deletion of *bolA* decreased biofilm formation (Fig. 1). The same experiments were done with 0.6% (wt/vol) glucose and 0.8% (wt/vol) glucose (data not shown), and the effect of *bolA* on biofilm formation at these glucose concentrations was similar to that obtained with 0.4% (wt/vol) glucose. When the three strains were grown in Luria broth, however, there was practically no biofilm formation. This result was expected, since biofilm formation is a response to unfavorable conditions.

The same results were confirmed by phase-contrast microscopy (Fig. 2). Cells were cultured in a six-well polystyrene microtiter dish at 37°C and with shaking at 120 rpm for 48 h. The supernatant was drained, and the sample was washed repeatedly with bidistilled water in order to eliminate planktonic bacteria. Finally, biofilm development was observed directly on the polystyrene with a phase-contrast microscope (Leica DMRB) and a 100 $\times$ oil objective with a 1.3 aperture. Only in the case of the strain overexpressing *bolA* (CMA14) were biofilm populations observed. After 16 h, microcolonies could already be observed with a microscope in the CMA14 culture (data not shown). However, after 48 h in these growth

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FIG. 1. Positive effect of *bolA* on biofilm development in microtiter plates. The thickness of biofilms in cultures of different strains was measured by determining optical density (O.D.) at 600 nm after staining them with crystal violet. Error bars represent standard deviations. Biofilm can be visualized by crystal violet staining, as shown on the photos inside the graph.

conditions, neither the wild type (MC1061) nor SK6582 (Δ *bolA*) presented any biofilm structures (Fig. 2).

In order to test biofilm formation under stress conditions, stresses were imposed by following the experimental conditions described by Santos et al. (11). For carbon limitation, batch cultures were grown in M9 minimal medium supplemented with 0.4% (wt/vol) glucose until they reached an optical density at 620 nm of 0.3, corresponding to exponential phase. At that moment, the stress was imposed, and the cells were transferred to 96-well polystyrene microtiter dishes for a 48-h culture. Sudden depletion of glucose was achieved by harvesting the cells by centrifugation for 10 min at $7,520 \times g$ at 4°C and washing them twice with 2 volumes of sterile ice-cold M9 medium with no carbon source. The cells were resuspended in the same volume of minimal medium with 0.2% (wt/vol) glucose (corresponding to half the normal level of carbon source). For oxidative stress, H_2O_2 was added to the culture at a final concentration of 15 mM. Under these stress conditions, *bolA* expression induced biofilm formation (Fig. 3). In the case of nutrient limitation (0.2% [wt/vol] glucose) and oxidative stress, the presence of *bolA* in the wild-type strain increased biofilm thickness (measured by crystal violet stain-

ing) about fivefold over that induced by the Δ *bolA* strain. As has been shown previously (11), *bolA* mRNA levels increase fivefold in stationary phase, but under stress conditions these levels can rise 20-fold, which would explain why the wild-type strain presented greater amounts of biofilm under stress conditions, even in the absence of pMAK580.

Overall, these results suggest a new phenotype for the *bolA* gene. In addition to its ability to produce a round morphology, *bolA* is involved in biofilm development. The fact that *bolA* is expressed under unfavorable conditions (i.e., stress and stationary phase) suggests that biofilm formation is a mode of action by which the bacteria protect themselves against the environment. The expression of *bolA* is under the transcriptional control of σ^S (encoded by *rpoS*). The presence or absence of σ^S has an impact on biofilms (5). In *rpoS* deletion strains, the biofilm cell density is reduced by 50%, and there are also differences in biofilm structure (1). Curiously, deletion of *bolA* also reduces biofilm cell density by 50% (Fig. 1). Taking into account that the levels of *bolA* depend on σ^S , we can speculate that σ^S may act via *bolA* in order to facilitate biofilm development. However, the results do not exclude the

FIG. 2. Positive effect of *bolA* on biofilm development as observed by optical microscopy. Phase-contrast microscopy was used to observe biofilm formation directly on polystyrene plastic. The strains used were *E. coli* MC1061 (wild type [wt]), SK6582 (Δ *bolA*), and CMA14 (+*bolA*).

FIG. 3. *bolA* expression and biofilm formation under stress conditions. Under nutrient limitation (0.2% [wt/vol] glucose) and oxidative stress (15 mM of H_2O_2), *bolA* presence enhances biofilm development. Biofilm thickness for wild-type (MC1061) and $\Delta bolA$ (SK6582) strains was measured by crystal violet staining.

possibility that other factors regulated by σ^S might also be involved in biofilm formation.

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