Biofilm Formation by Bacillus cereus Is Influenced by PlcR, a Pleiotropic Regulator

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Bacillus cereus is a pathogen that causes two distinct types of food poisoning, the diarrheal and emetic syndromes, as well as a variety of local and systemic infections such as endophthalmitis, endocarditis, meningitis, periodontitis, osteomyelitis, wound infections, and septicemia (12, 32). B. cereus can be readily isolated from food and agricultural products, as well as soil, vegetation, dust, and natural waters. It is regarded as one of the common organisms that impair the quality of dairy products (23, 25, 30, 34). Its ubiquitous nature, combined with its ability to sporulate and grow at refrigeration temperature, make it difficult to control. B. cereus has been shown to be able to form biofilms on plastic, glass wool, and stainless steel (2, 26, 28), and the biofilm cells were more resistant than planktonic cells to chemical sanitizers (29). Biofilm accumulation in food processing environments can lead to decreased food quality and safety (20, 35, 36), which can lead to decreased food quality and safety (20, 35, 36), which impacts public health as well as the economy.

In the present study, the role of PlcR in biofilm formation by B. cereus strain ATCC 14579 was investigated. PlcR, a pleiotropic regulator, is activated by a small diffusible peptide (PapR) that acts as a quorum-sensing effector (33). It controls the expression of a variety of genes, many of which encode potential virulence factors, including enterotoxins, hemolysins, phospholipases C, and proteases (1, 7, 15). We found that biofilm formation was enhanced under low nutrient conditions and was dependent on biosurfactant production, which was directly or indirectly repressed by PlcR.

Biofilm formation. B. cereus ATCC 14579 and its ΔplcR mutant (31) were grown in Luria-Bertani (LB) broth (Difco/Becton Dickinson, Sparks, Md.) at 32°C and 200 rpm overnight to generate inoculum cultures. For the ΔplcR mutant, kanamycin was added at a final concentration of 150 µg/ml. Since nutrient availability is one of the major factors affecting biofilm formation, we compared the ability of the wild-type and mutant strains to develop biofilms in rich and low-nutrient media. Overnight cultures were adjusted to an optical density at 620 nm (OD620) of 0.01 in LB or EPS, a low nutrient medium that contained 7 g of K2HPO4, 3 g of KH2PO4, 0.1 g of MgSO4 · 7H2O, 0.01 g of CaCl2, 0.001 g of FeSO4, 0.1 g of NaCl, 1 g of glucose, and 0.125 g of yeast extract (Difco) per liter (11). Then, 2 ml was added to wells of polystyrene 24-well plates (Falcon/Becton Dickinson, Franklin Lakes, NJ), followed by incubation at 32°C and 50 rpm for 8 h. The total growth (OD630) in each well was measured; planktonic bacteria were removed, and the wells were washed with distilled water and air dried. Biofilm cells were stained with 2 ml of 0.3% crystal violet for 10 min, washed with distilled water, and air dried. The crystal violet in the biofilm cells was solubilized with 2 ml of 70% ethanol, and the optical density at 590 nm (OD590) was measured (13). The total growth of the wild type and the ΔplcR mutant was similar in EPS and LB; however, biofilm formation in EPS by the ΔplcR mutant was about four times higher (P < 0.05) than that by the wild-type strain (Fig. 1). A dramatic decrease in biofilm development was observed in a rich medium such as LB. Recently, Auger et al. (2) also observed that LB did not support biofilm formation by B. cereus ATCC 14579. We therefore used EPS for subsequent experiments.

To monitor biofilm development over time, overnight cultures were adjusted to an OD620 of 0.01 in EPS, and 10 ml was added to 60- by 15-mm polystyrene petri dishes (Falcon), followed by incubation at 32°C and 50 rpm. At specific times, planktonic cells were removed, and biofilm cells were rinsed and air dried. For enhanced visualization of biofilm cell morphology and structure, cells were fixed with 2 ml of 1% glutaraldehyde, stained with 10 ml of acridine orange (0.025% in 0.026 M citric acid buffer [pH 6.6]; Sigma, St. Louis, Mo.), washed, and air dried. Biofilm cells were observed with an Olympus BH2 microscope equipped for epifluorescence with an HBO100W mercury burner lamp, a 490-nm excitation filter, and a 515-nm barrier filter. At 6 h, very few cells of the wild type had attached to the bottom of the plate, whereas the ΔplcR mutant started to develop a biofilm (Fig. 2). The ΔplcR mutant biofilm reached maximum density at 12 h, after which the cells started to detach. At 36 h, few cells remained on the plate. A modest increase in attachment was observed for the
wild type by 12 h and, as with the mutant, the cells started to detach with prolonged incubation.

**Determination of biosurfactant production.** In a separate study to examine motility (unpublished data), we observed that when grown on EPS plates containing 0.7% agar, the ΔplcR mutant but not the wild type formed dendritic colonies similar to those observed in *Bacillus subtilis* (17). In addition, a delimiting ring was visible around the *B. cereus* ΔplcR mutant colony but not the wild-type strain at 60 h (data not shown). A delimiting ring became visible around the wild-type colony at 72 h (Fig. 3A); however, the ring was much smaller than that of the ΔplcR mutant. Delimiting rings were first described by Julkowska et al. (17, 18), who observed that a transparent zone, delimited by a narrow ring, preceded the advancing fronts of dendritic colonies of *B. subtilis*. The rings were due to the secretion of surfactin, a lipopeptide biosurfactant. When a drop (5 μl) containing 0.05 to 50 μg of surfactin (Sigma) was allowed to spread on the EPS agar surface, a delimiting ring was visible, and the ring diameter increased with increasing surfactin concentration. A representative ring formed by 1.25 μg of surfactin is shown in Fig. 3A.

Biosurfactant compounds are produced by many bacteria and can have a variety of structures, such as neutral lipids, phospholipids, glycolipids, and lipopeptides (14). One of their properties is the ability to reduce surface tension (4, 27). To visualize any surface tension change that may be associated with the delimiting ring, a drop of trypan blue (3 μl of 1.5 mg/ml) was spotted within the delimiting ring and on an uninoculated area as a control. The drop spread wider in the ring area of the ΔplcR mutant colony and surfactin-coated plate than in the wild type (Fig. 3B). This suggests that the delimiting ring could be due to biosurfactant production and that the ΔplcR mutant produced more biosurfactant than the wild type.

After a BLAST search of the whole genome sequence of *B. cereus* ATCC 14579, no srf operon, which harbors the surfactin synthetase genes, was found. This suggests that the biosurfactant produced by *B. cereus* ATCC 14579 is not surfactin. There are a very limited number of studies on biosurfactant production in *B. cereus*. A monoglyceride biosurfactant (9) and plipastatin (24), a lipopeptide that potentially may have biosurfactant activity, have been reported.

We partially purified biosurfactants from the wild type and its ΔplcR mutant and also from *B. subtilis* strain 3A1 (*Bacillus* Genetic Stock Center; same as ATCC 6051), which produces surfactin. Cultures were grown in 500 ml of EPS and incubated at 32°C and 50 rpm for 72 h. Biosurfactant was purified as described by Kim et al. (19) and quantitated by using the modified drop-collapse method (5). Biosurfactant concentrations in the samples were determined by using a standard curve generated with different concentrations of surfactin. A linear correlation was found between surfactin concentration and drop diameter, in the range of 0 to 0.0375 mg/ml ($r^2 = 0.912$). The ΔplcR mutant produced more biosurfactant (6 ng/ml) than did the wild type (0.5 ng/ml). *B. subtilis* 3A1 produced the highest amount (40 ng/ml).

**Surfactin-coated plates promote biofilm formation.** The higher amount of biosurfactant produced by the ΔplcR mutant may play a role in its increased ability to a biofilm compared to the wild type. To test this, we used surfactin, which is commer-
cially available and easily quantified. Portions (2 ml) of increasing amounts of surfactin (0.1 to 1 mg/ml) were added to 60-by-15-mm polystyrene petri dishes and aspirated after 10 min, and the dishes were air dried for 30 min. These surfactin-coated dishes were used to generate biofilms by the wild type as described above. For dishes coated with 0.1 mg of surfactin/ml, biofilm formation was evident by 12 h, and the biofilm remained as robust up to 24 h, while as noted previously minimal biofilm formed on the uncoated surface (Fig. 4). After 40 h, most of the biofilm cells had detached. Similar results were obtained when surfactin concentrations of 0.2 or 0.5 mg/ml were used (data not shown). Biofilms formed by the ΔplcR mutant on surfactin-coated dishes (0.1 to 0.5 mg/ml) were more abundant than on uncoated dishes at 12 and 24 h. However, when the concentration was increased to 1 mg/ml, no biofilm was developed by the wild type or the mutant (data not shown). Growth was not affected by any of the surfactin concentrations we tested. It appears that biofilm formation by B. cereus is supported by an optimal concentration range of biosurfactant.

Biosurfactants have been reported to play various roles in biofilm formation. Pellicle or surface biofilm formation in B. subtilis also required surfactin (8). It has been reported that the addition of about 0.1 mg of surfactin/ml could rescue pellicle formation by surfactin-deficient mutants of B. subtilis A1/3 (16). Surfactin produced by B. subtilis 6051 was required for biofilm formation in microtiter plates and on Arabidopsis root surfaces (3). In contrast, the addition of surfactin inhibited biofilm formation by Salmonella enterica serovar Typhimurium, Escherichia coli, and Proteus mirabilis but not by Pseudomonas aeruginosa (22). Rhamnolipid, a biosurfactant produced by P. aeruginosa, was reported to be involved in the development (21) and maintenance of biofilm architecture (10, 21). However, Boles et al. (6) observed that rhamnolipids mediated the detachment of P. aeruginosa from biofilms.

In conclusion, biofilm formation by B. cereus ATCC 14579 on polystyrene is promoted by low-nutrient conditions and is related to the production of a biosurfactant, which appears to be negatively regulated by PlcR. The involvement of a biosurfactant and PlcR in biofilm formation by B. cereus has not been reported previously. All of the genes belonging to the PlcR regulon are positively regulated by PlcR, suggesting that the negative role of PlcR on biosurfactant production is likely indirect. Whether biosurfactant biosynthetic genes are con-
trolled by PlcR or by other genes under PlcR regulation re-
mains to be determined.

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Volume 72, no. 7, p. 5089–5092, 2006. Page 5089, column 2, lines 1 through 4: The formulation for EPS medium should include 0.1 g of (NH₄)₂SO₄ per liter.