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A Sporulation Factor Is Involved in the Morphological Change of *Clostridium perfringens* Biofilms in Response to Temperature

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Biofilm formation has been associated with bacterial pathogenesis, such as nosocomial and chronic infections, as the resistance of biofilms to environmental stresses has increased. *Clostridium perfringens* is a Gram-positive spore-forming anaerobic pathogen. This organism survives antibiotic treatment through the formation of biofilms or spores, but the environmental and regulatory factors involved in the biofilm formation remain unclear. Here, we observed that temperature regulates *C. perfringens* biofilm morphology. At 37°C, *C. perfringens* adhered to the substrate surface and formed a flat, thin biofilm, herein referred to as adhered biofilm. However, at 25°C, this bacterium did not adhere and produced a threadlike extracellular matrix, forming a viscous, thick biofilm, herein referred to as pellicle biofilm. Pellicle biofilm formation requires the sporulation master regulator, Spo0A, and the toxin regulator, CtrAB, and is enhanced in the absence of the global repressor, AbrB. These transcriptional regulator genes are regulated by each other and temperature. Adhered-biofilm formation requires AbrB and *pilA2*, which encodes a component of type IV pili (TFP). TFP expression was activated at 37°C and regulated through Spo0A, AbrB, and CtrAB. These results indicate that the morphology of *C. perfringens* biofilm is dependent on temperature through the differential production of extracellular matrix and the activity of TFP. Moreover, pellicle biofilm formation is involved in sporulation and toxin production. Here, we demonstrated that clostridial biofilm formation is closely associated with sporulation and that the morphological change of the biofilms could play an important role in the pathogenesis of this organism.

Clostridium perfringens is a Gram-positive spore-forming anaerobic bacterium that causes gas gangrene, food-borne and non-food-borne poisoning, and antibiotic-associated diarrhea (1–3). Although this organism is an obligate anaerobe, *C. perfringens* is widely distributed throughout many environments, such as soil, sewage, and the intestine of humans and animals, since this organism produces endospores. Bacterial endospores are highly resistant to various stresses, such as oxygen, starvation, UV irradiation, and drying. However, the process of sporulation consumes a lot of energy and does not enable rapid adaption to environmental change. Recently, it has been shown that *C. perfringens* produces biofilms with increasing resistance to oxygen and antibiotics (4). The biofilms can form more quickly than endospores, and the cells in biofilms can respond more quickly to the environment, since endospores are in a highly dormant state, suggesting that biofilm formation, as well as sporulation, is involved in stress resistance and the pathogenesis of this organism.

Biofilm is a multicellular community in which the cells are surrounded by an extracellular matrix and possess phenotypes different from those of planktonic cells, such as resistance to extracellular stresses and specialized gene expression (5). Most bacteria grow in multicellular aggregates and form biofilms. Moreover, due to the resistance of these cells to antibiotics, microbial biofilm formation is involved in the pathogenesis of diseases such as nosocomial and chronic infections by this bacterium (6). *C. perfringens* causes antibiotic-associated diarrhea (AAD) (2) and survives the intestinal environment through biofilm formation and/or sporulation even when antibiotics are administered to the patient.

The regulatory cascade of sporulation and biofilm formation is shared among spore-forming bacilli, and the biofilm is an optimal environment for sporulation (7, 8). *Bacillus* spp. and *Clostridium* spp. share a regulatory cascade for sporulation. The sporulation master regulator Spo0A is required for sporulation and efficient biofilm formation in some *Bacillus* spp. and *Clostridium* spp. (9–11). Therefore, sporulation and biofilm formation would be relevant in *Clostridium* and probably regulated through a common regulatory cascade. However, in other clostridia, except *C. difficile*, this possibility has not been confirmed (12).

Varga et al. observed *C. perfringens* biofilm formation, showing that type IV pili and glucose concentration are involved in maximal biofilm formation on the substrate surface (4). However, most regulatory and environmental factors involved in *C. perfringens* biofilm growth remain unknown, and knowledge of these factors is important to understand the regulation of biofilm formation in *Clostridium* species. In the present study, we observed that *C. perfringens* forms biofilms with different structures in response to different temperatures. Specifically, at 37°C, cells adhered densely to the substrate surface, forming a flat, thin biofilm. However, at 25°C, most of the cells did not adhere to the surface, and they formed a viscous, thick, and pelliclelike biofilm. Moreover, the sporulation transcription factors Spo0A and AbrB and the toxin regulator CtrAB are required for the regulation of type IV pili and the production of an extracellular matrix, which contribute to the differing morphologies of biofilms in response to temperature. Therefore, the structural changes of *C. perfringens*

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TABLE 1 Bacterial strains used in this study

Strain	Relevant characteristic(s)	Reference or source
13	Wild type	13
HN13	*galKT* in-frame deletion mutant of strain 13	15
NO16	*ctrAB* operon null mutant of strain 13; Em^r	14
NO23	*spo0A* mutant of strain 13; Em^r	This study
NO24	*abrB* null mutant of strain 13; Em^r	This study
NO25	*pilA2* in-frame deletion mutant of HN13	This study
NO33	*ctrAB* mutant of NO25; Em^r	This study

biofilms are involved in sporulation and toxin production and are likely associated with the pathogenesis of this organism.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The strains of *C. perfringens* used in this study are listed in Table 1. All strains were cultured under anaerobic conditions using an Anaeropack (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) at 25 or 37°C in Gifu anaerobic medium (GAM) broth (Nissui Co. Japan) with or without 50 μg/ml of erythromycin. Plasmid vector-harboring strains were cultured in GAM broth containing 10 μg/ml chloramphenicol.

Oligonucleotides. Table S1 in the supplemental material lists the oligonucleotide primers used in this study.

Mutant strain construction. The *spo0A* and *abrB* mutant strains (NO23 and NO24, respectively) were constructed using double-crossover homologous recombination as previously described (14). The upstream and downstream regions of the disrupting genes were PCR amplified using primers NOB-0428/NOB-0429 or NOB-0436/NOB-0437 and NOB-0495/NOB-0496 or NOB-0438NOB-0439 from the genomic DNA of *C. perfringens* and subsequently digested with EcoRI and BamHI, respectively. The *ermBP* gene, amplified from pJIR418 using primers NOB-0234/NOB-0240, was digested with EcoRI and BamHI. To construct the mutant strains NO23 and NO24, the DNA fragments amplified from the ligation product of the upstream and downstream regions and the *ermBP* fragments, amplified using primers NOB-0428/NOB-0496 or NOB-0436/NOB0439, were introduced into *C. perfringens* strain 13 through electroporation. The transformants were selected on brain heart infusion (BHI) sheep blood agar containing 50 μg/ml of erythromycin. To construct the *pilA2* mutant strain, we used an in-frame deletion system as previously described (15). The upstream and downstream regions of the *pilA2* gene were amplified using primers NOB-0576/NOB-0577 and NOB-0578/NOB-0579 and digested with SalI/EcoRI and EcoRI/BamHI, respectively. These fragments were cloned into the SalI/BamHI site of pCM-GALK, and the resulting plasmid was introduced into *C. perfringens* HN13 through electroporation. Each mutant was confirmed through PCR and DNA sequencing.

Northern blot analysis. Total RNA was extracted from *C. perfringens* derivatives grown at 37°C in GAM broth and used for Northern blot analysis as previously described (16), with digoxigenin (DIG)-labeled DNA probes generated by using DIG-High Prime according to the manufacturer's instructions (17). The template DNA for the toxin gene-specific probes was PCR amplified from strain 13 genomic DNA using the primers described in Abe et al. (18).

Western blot analysis. *C. perfringens* strain 13, harboring the CtrA and CtrB-His_6 expression vector pCPE6 (14), was cultured at 37°C in GAM broth for 2, 3, or 4 h. The cells were harvested through centrifugation and washed with sterile phosphate-buffered saline (PBS). Whole-cell lysate was extracted using glass beads, and the CtrB-His_6 proteins in the lysate were detected through Western blotting as previously described (14).

Biofilm assay. *C. perfringens* was cultured overnight in PGY medium (3% proteose peptone no. 3, 2% glucose, 1% yeast extract, and 0.1% sodium thioglycolate) and diluted 1:50 into 1 or 3 ml of GAM broth or TY-G1 medium (3% tryptone, 2% yeast extract, 1% glucose, and 0.1% sodium thioglycolate) in 24- or 6-well polystyrene plates, and the cells were subsequently incubated under anaerobic conditions for 1 to 2 days. After incubation, the supernatants were removed, and the wells were washed once with sterile phosphate-buffered saline (PBS). The surface-associated cells were stained with 0.1% crystal violet for 10 min, followed by being washed once with sterile PBS. To quantify the amount of surface-adhered cells, each well was incubated with 1 ml of 99.5% ethanol for 10 min, and subsequently, the liquid was transferred to fresh 96-well plates. The optical density at 570 nm (OD_{570}) of each well was measured using an Ultramark microplate reader (Bio-Rad).

Visualization of biofilms. *C. perfringens* biofilms were formed in 35-mm glass-based dishes (Iwaki) and monitored using confocal reflection microscopy (CRM) (19, 20). A Carl Zeiss LSM710 laser scanning microscope equipped with a 63×/1.4 numerical aperture Plan-Apochromat objective (Carl Zeiss, Jena, Germany) was used to capture the confocal microscopy images. The cells were irradiated with a 514-nm argon laser, and 500- to 530-nm reflected light was used to visualize the cell localization and attachment to the substrate surface in biofilms. The images were processed using ZEN and Imaris software (Carl Zeiss, Jena, Germany).

SEM of biofilms. A total of 4 ml of GAM broth in 6-well plates (Iwaki) containing 12-mm coverslips (Fisher) precoated with polylysine was inoculated with 80 μl of overnight culture. After incubation for 2 days at 25 or 37°C, the coverslips were transferred to a fresh 24-well plate, and the cells attached to the coverslips were fixed in 2.5% glutaraldehyde–10 mM sodium phosphate (pH 7.5) overnight. The samples were washed with 10 mM sodium phosphate (pH 7.5) twice and dehydrated in 50%, 70%, 90%, and 99.5% ethanol. Subsequently, the ethanol was replaced with 50% ethanol–50% isoamyl acetate and 100% isoamyl acetate. The coverslips were mounted onto aluminum stubs, dried using a critical-point dryer (HCP-2; Hitachi Ltd., Japan), and subsequently sputter coated with platinum using an E-1030 ion sputtering machine (Hitachi Ltd., Japan). The biofilms were observed using a scanning electron microscope (SEM) (HITACH-S-4200) (Hitachi Ltd., Japan).

RESULTS

***C. perfringens* forms differently structured biofilms in response to temperature.** Most pathogens regulate virulence factors in response to temperature change for adaptation to the conditions in the host tissue and for efficient infection (21, 22). Here, we compared *C. perfringens* biofilms grown at 25 and 37°C. These temperatures resemble the environments outside or inside the host, such as in soil or in the intestine, respectively. *C. perfringens* formed biofilms that adhered to the substrate surface at 37°C, as previously reported (Fig. 1A, left) (4). The adhered cells were stained with crystal violet and easily visualized (Fig. 1B, left). However, *C. perfringens* formed thick, pelliclelike, viscous biofilms on the bottom of the well at 25°C, although under this condition, the cells did not adhere to the surface and were not detected through crystal violet staining because the biofilm structure was strongly connected and easily removed through pipette aspiration (Fig. 1A and B, right). This result suggests that at 25°C, the adherence of *C. perfringens* to the surface decreases and the biofilm morphology is dramatically changed. Hereinafter, we refer to the biofilms formed at 37°C as "adhered biofilm" and those formed at 25°C as "pellicle biofilm." Confocal laser microscopy was used to visualize the cells in the biofilms, revealing that at 37°C, the surface of the glass-based dishes were densely covered with adhered biofilms, with many cells attached to the surface (Fig. 1C and D, left). This observation is consistent with the results of a previous report (4). However, the pellicle biofilm formed at 25°C did not contact the surface, and the density of the cells in this biofilm was lower

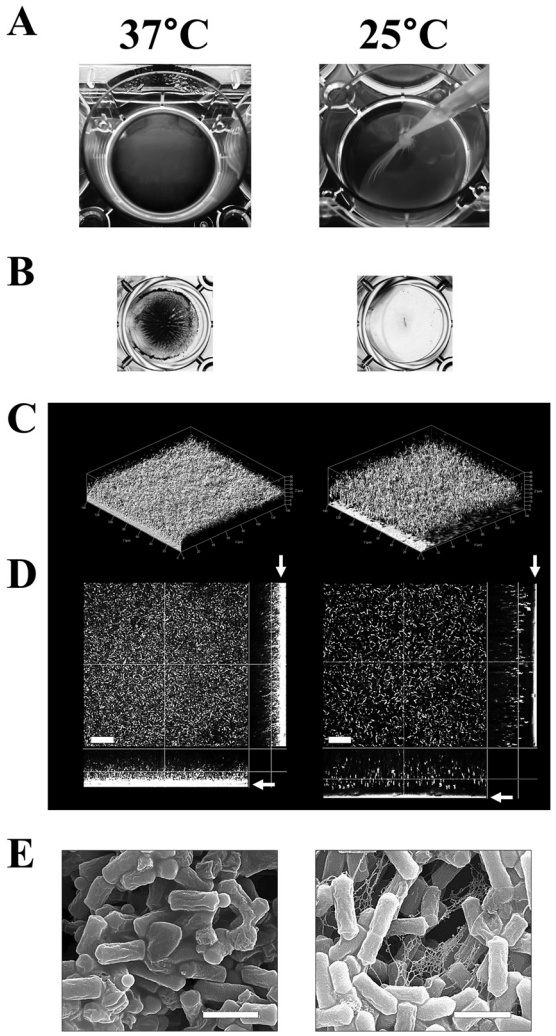

FIG 1 *C. perfringens* forms biofilms with different structures in response to temperature. (A) Biofilms were formed at 37°C (left) or 25°C (right). The edges of pellicle biofilms, formed at the bottom of wells, were picked through gentle pipette aspiration. (B to E) The biofilms were visualized through crystal violet staining (B), confocal reflection microscopy (C and D), and scanning electron microscopy (E). Three-dimensional projection (C) and orthometric images (D) show a field of 140 by 140 μm. The arrows indicate the substrate surface (D). The bars indicate 20 μm (D) or 2 μm (E).

than that in the adhered biofilm (Fig. 1C and D, right). These observations suggest that the cells at 25°C do not adhere to the surface and that they form biofilms with a structure different than that of biofilms at 37°C, indicating that pellicle biofilms predominantly contain extracellular matrix. Indeed, SEM imaging revealed threadlike extracellular materials between the cells in the pellicle biofilm but not in the adhered biofilm. Threadlike structures are detected in biofilms of various organisms, such as *Bacillus subtilis* and *Pseudomonas aeruginosa* (23, 24), suggesting that the threadlike material is an extracellular polymeric substance (EPS) of the pellicle biofilm and that temperature affects the specific EPS production (Fig. 1E).

DNA and protein are components of the extracellular matrix in pellicle biofilm. Next, we focused on the formation of a novel biofilm structure, the pellicle biofilm that is primarily comprised of extracellular matrix. To identify the extracellular matrix components of pellicle biofilm, we added enzymes that degrade extracellular matrices to the culture for pellicle biofilm formation. Figure 2A shows the pellicle biofilm after gentle agitation. The addition of DNase I or proteinase K at the start of the culture made the pellicle biofilm structure fragile, but RNase A addition had no apparent effect (Fig. 2A). Therefore, extracellular DNA and proteins are the components of pellicle biofilms. Biofilms of various organisms contain extracellular DNA (eDNA), and eDNA could be the primary structural component of biofilms and used as an adhesion factor (5, 25, 26). We isolated the extracellular nucleic acids from pellicle biofilm and analyzed these components using agarose gel electrophoresis. A 23-kb DNA fragment was observed in the biofilm fraction but not in the planktonic cell sample, indicating that pellicle biofilm contains DNA as a matrix component (Fig. 2B, lanes 2 to 5). Because 23S and 16S rRNA were detected, magnesium sulfate, which is required for DNase I activity, was added to the culture because magnesium divalent cations could stabilize extracellular RNAs (eRNAs) (Fig. 2B, lanes 6 to 10). However, the addition of magnesium sulfate did not affect the structure of pellicle biofilm (Fig. 2A; also data not shown). Under this condition, the addition of DNase I and RNase A completely degraded eDNA and eRNA, respectively. Furthermore, proteinase K treatment decreased both eDNA and eRNA. These results indicate that long eDNA is a component of pellicle biofilm and important for the formation of the viscous film structure. However, although SEM imaging showed that DNase I or proteinase K treatment decreased the amount of extracellular materials around the cells, threadlike strands remained (Fig. 2C).

Transcriptional regulators necessary for biofilm formation. The Gram-positive aerobic spore-forming bacterium *B. subtilis* forms robust biofilms, and the regulatory cascade of this biofilm formation has been well studied (27). The master regulator of sporulation, Spo0A, and the global repressor, AbrB, control the biofilm formation in *B. subtilis* positively and negatively, respectively (8, 28). *C. perfringens* contains homologs of the *spo0A* and *abrB* genes, and Spo0A is also necessary for efficient sporulation in *C. perfringens*, similar to *B. subtilis* (10). In addition, we recently reported a protein complex of CtrA and CtrB (clostridial toxin regulator; formerly CPE1447-CPE1446) as a novel toxin regulator (14). The *ctrAB* complex is widely conserved in clostridia and regulates many genes, including those encoding toxins, suggesting that *ctrAB* is important for clostridial metabolism. Therefore, we examined biofilm formation in *spo0A*, *abrB*, and *ctrAB* mutant strains. As shown in Fig. 3A, *spo0A* and *ctrAB* mutants did not form pellicle biofilms, and instead, these mutants formed adhered biofilms at 25°C (Fig. 3B). However, the adhered-biofilm formation at 37°C was not significantly affected in these mutants, as demonstrated through microtiter plate crystal violet assay (Fig. 3B and C). The *abrB* mutant forms thick pellicle biofilms at 25°C (Fig. 3A), and the efficiency of the adhered-biofilm formation in this mutant was significantly lower than that in wild-type strains (Fig. 3B and C). Confocal laser microscopy also indicated that the *ctrAB* and *spo0A* mutant cells adhered to the surface and formed dense populations at 25°C (Fig. 3D). We used SEM to observe the extracellular matrix of the biofilms formed by *spo0A*, *abrB*, and *ctrAB* mutants at 25°C. We observed that *spo0A* and *ctrAB* mutants showed no production of threadlike EPS, but the *abrB* mutant featured robust production of EPS (Fig. 3E). Complementation of *spo0A* or *ctrAB* restored pellicle biofilm formation (see Fig. S1 in

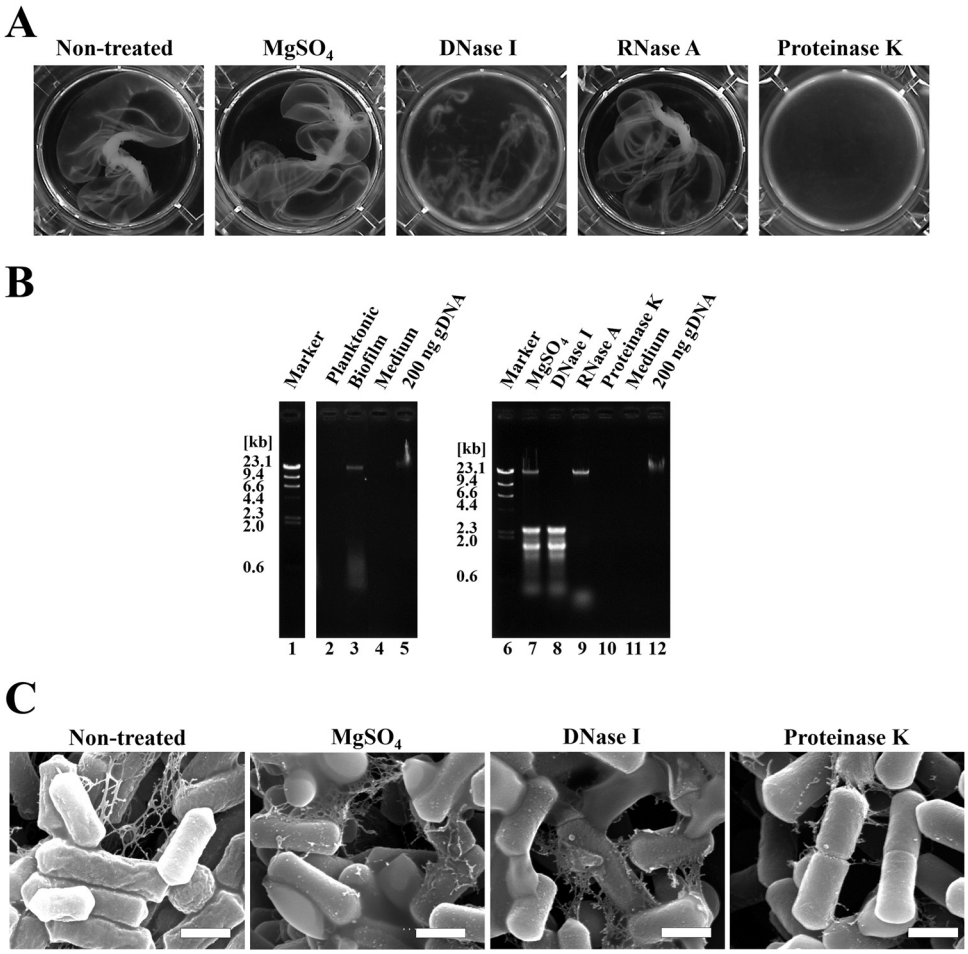

FIG 2 Treatment with DNase I or proteinase K affects the pellicle biofilm structure. (A) A concentration of 10 mM MgSO4, 1 mg/ml DNase I, 1 mg/ml RNase A, or 0.2 mg/ml proteinase K was added to the medium prior to culturing for 2 days. The biofilm formed in the medium was gently agitated to make pellicle biofilm more visible. (B) Extracellular nucleic acids extracted from the biofilms were dissolved in 0.8% agarose. Each lane contains equivalent amounts of the culture obtained at an OD_{600} of 0.2. gDNA, genomic DNA. (C) Scanning electron microscopy images of the biofilm treated with DNase I or proteinase K. The bars indicate 1 μm.

the supplemental material). These results suggest that *spo0A*, *abrB*, and *ctrAB* are involved in cell adherence and EPS production. Thus, Spo0A and CtrAB are required for pellicle biofilm formation and AbrB is required for adhered-biofilm formation.

The morphological change of biofilms in response to temperature requires the transcriptional regulators Spo0A, AbrB, and CtrAB. Thus, we compared the expression of genes encoding these transcriptional regulators at 25°C and 37°C and performed a Northern blot analysis using wild-type *C. perfringens* (Fig. 4A). The *plc* gene encoding the alpha-toxin, phospholipase C, is highly expressed at the exponential phase and activated at a lower temperature, and this toxin was used as an intrinsic control (18, 29). The expression of *spo0A* and *ctrAB* also peaked at the exponential phase, and these genes were more highly activated at 25°C than at 37°C, implying that transcription of *spo0A* and *ctrAB* is activated at low temperatures and, subsequently, morphological changes of the biofilm are induced (Fig. 4A). In contrast, temperature did not affect the expression of *abrB* (Fig. 4A). We also examined whether these transcriptional regulators control each other's expression. Northern blot analysis of the mutant strains showed that *spo0A* expression was derepressed in the *abrB* mutant but was not affected in the *ctrAB* mutant (Fig. 4B). *abrB* expression was derepressed in both *spo0A* and *ctrAB* mutants (Fig. 4C). These results suggest that the expression of *spo0A* and *abrB* is repressed by AbrB and Spo0A, respectively, as has been observed in *B. subtilis* (28, 30). Moreover, CtrAB could repress *abrB* transcription in *C. perfringens*. We also tested whether *ctrAB* expression is affected by *spo0A* and *abrB* mutations by using Western blot analysis of CtrB-His_6 expressed from the pCPE6 vector, in which *ctrA* and *ctrB*-His_6 genes are transcribed from the *ctrAB* native promoter. However, the amount of CtrB-His_6 in *spo0A* or *abrB* mutants was not changed compared with the amount in the wild-type strain (Fig. 4D). Moreover, we analyzed chromosomally encoded *ctrAB* expression by Northern blotting, and the amount of *ctrAB* mRNA was not significantly different in the mutants (data not shown). These data suggest that *ctrAB* expression is not regulated through Spo0A and AbrB. Therefore, these transcriptional regulators control the expression of the other genes and could affect biofilm formation.

TFP component PilA2 is activated at 37°C. As shown by the results described above, the adhesive property of *C. perfringens* was controlled through temperature. It had been reported that

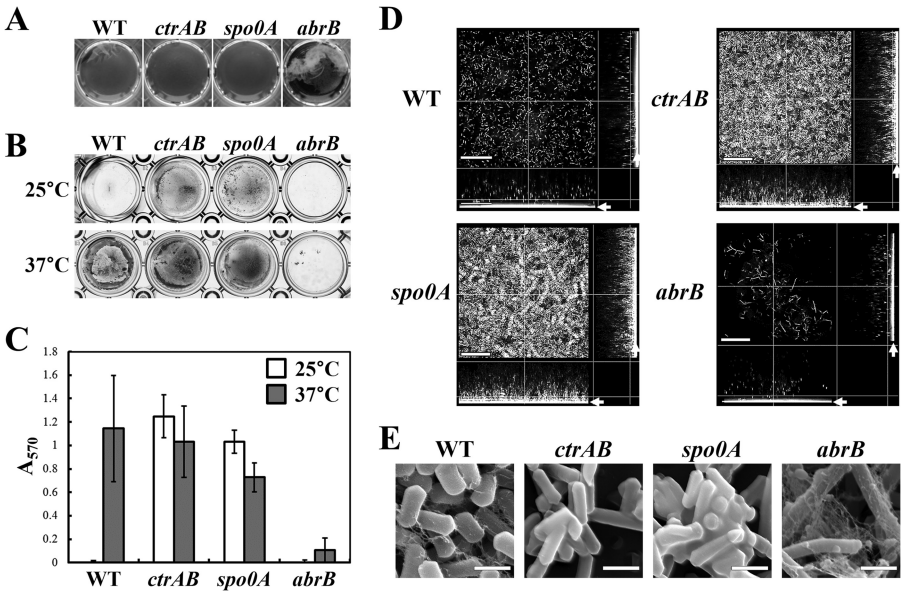

FIG 3 Multiple transcriptional regulators affect biofilm formation in *C. perfringens*. (A) Pellicle biofilm formation at 25°C by *ctrAB*, *spo0A*, or *abrB* mutant strains. The cells were incubated at 25°C for 2 days. The edges of pellicle biofilms formed at the bottom of wells were picked and flipped through gentle pipette aspiration. (B) Adhered-biofilm formation of the mutant strains. The cells were cultured at 25 or 37°C for 2 days and stained with crystal violet. (C) Quantification of the adhered-biofilm biomass. The means and standard deviations of the OD_{570} values of three independent experiments are shown. (D) Confocal microscopy images of the mutant strain biofilms formed at 25°C. The arrows indicate the substrate surface. (E) Scanning electron microscopy of the mutant cells in the biofilms formed at 25°C. The bars indicate 30 μm (D) or 2 μm (E).

type IV pili (TFP) are required for the maturation of adhered biofilm and adherence to myoblast cells (4, 31, 32), suggesting that the temperature-dependent regulation of the adhesive property is associated with the TFP machinery. First, we analyzed the TFP biosynthesis gene expression at different temperatures. Three gene clusters encoding putative TFP biosynthesis proteins were identified in the *C. perfringens* genome (31). We performed Northern blot analysis using DNA probes specific for these genes. The *pilT-ftsA-ftsZ* mRNA expression was not significantly changed at different temperatures, and *pilB-pilC-CPE1842-CPE1841* mRNA was undetectable on Northern blots due to low expression levels (data not shown). Although PilT and PilC were required for gliding motility and adherence to eukaryotic cells in *C. perfringens*, neither gene was drastically regulated through temperature. The 4.4- and 0.6-kb transcripts were detected on the Northern blot using the DNA probe specific for the *pilA2* gene that is located in the residual gene cluster encoding TFP biosynthesis proteins (Fig. 5A and B). Because putative intrinsic transcriptional terminators were predicted downstream from *pilA1* and *pilA2* ($\Delta G = -14.7$ and -4, respectively) (33), these 4.4- and 0.6-kb transcripts would, respectively, represent *pilD-pilB2-pilC2-pilA2* polycistronic and *pilA2* monocistronic mRNA. The expression peaked at the exponential phase and was activated at 37°C, suggesting that *pilA2* expression was regulated through temperature (Fig. 5C). Moreover, the *pilA2* expression was higher in the *ctrAB* and *spo0A* mutant strains at 25°C and was repressed in the *abrB* mutant strain compared with its level in the wild-type strain (Fig. 5D). Thus, the amount of the *pilA2* mRNA corresponded to the efficiency of adherence to the substrate surface and the biomass of the adhered biofilm (Fig. 3B and 5D). These results suggest that temperature-dependent *pilA2* regulation requires *ctrAB* and *spo0A* and basal *pilA2* expression requires *abrB*.

PilA2 is required for cell adherence to the substrate surface and adhered-biofilm formation. The reduction of *pilA2* expression and cell adherence to the substrate surface at 25°C suggest that changes in the adhesive property and biofilm morphology of *C. perfringens* occur through the regulation of *pilA2* expression in response to temperature. To examine this idea, we constructed a *pilA2* mutant strain and analyzed whether *pilA2* is required for adherence to the substrate surface and adhered-biofilm formation. We used an in-frame deletion system to construct the mutant according to the method of Nariya et al. (15). Subsequently, the strains were cultured in GAM or TY-G1 at 37°C. The wild-type and HN13 (parent strain of the *pilA2* mutant; a *galKT* disruptant) strains showed comparable formation of adhered biofilms, but the *pilA2* mutant could not form adhered biofilms in GAM and showed poor formation of adhered biofilms in TY-G1 compared with the adhered-biofilm formation of the parent strain (Fig. 6A and B). In addition, confocal microscopy revealed that the *pilA2* mutant strain did not adhere to the glass surface in GAM broth (Fig. 6C). The *ctrAB* mutant could not form pellicle biofilm but did form adhered biofilm at 25°C. We constructed a *ctrAB pilA2* double mutant and analyzed biofilm formation using this mutant. The *ctrAB* mutant of HN13 also formed adhered biofilm at 25°C, and a reduction in the biofilm biomass was observed for the *ctrAB pilA2* double mutant compared with the biofilm mass of the *ctrAB* mutant (Fig. 7A). The *ctrAB pilA2* mutant could not form pellicle biofilm, and the number of cells adhered to the substrate surface was drastically decreased in the confocal microscopy image (Fig. 7B and C). These results indicate that the *pilA2* gene, encoding the main component of TFP, pilin, is necessary for adherence to the substrate surface and the formation of adhered biofilm in *C. perfringens*.

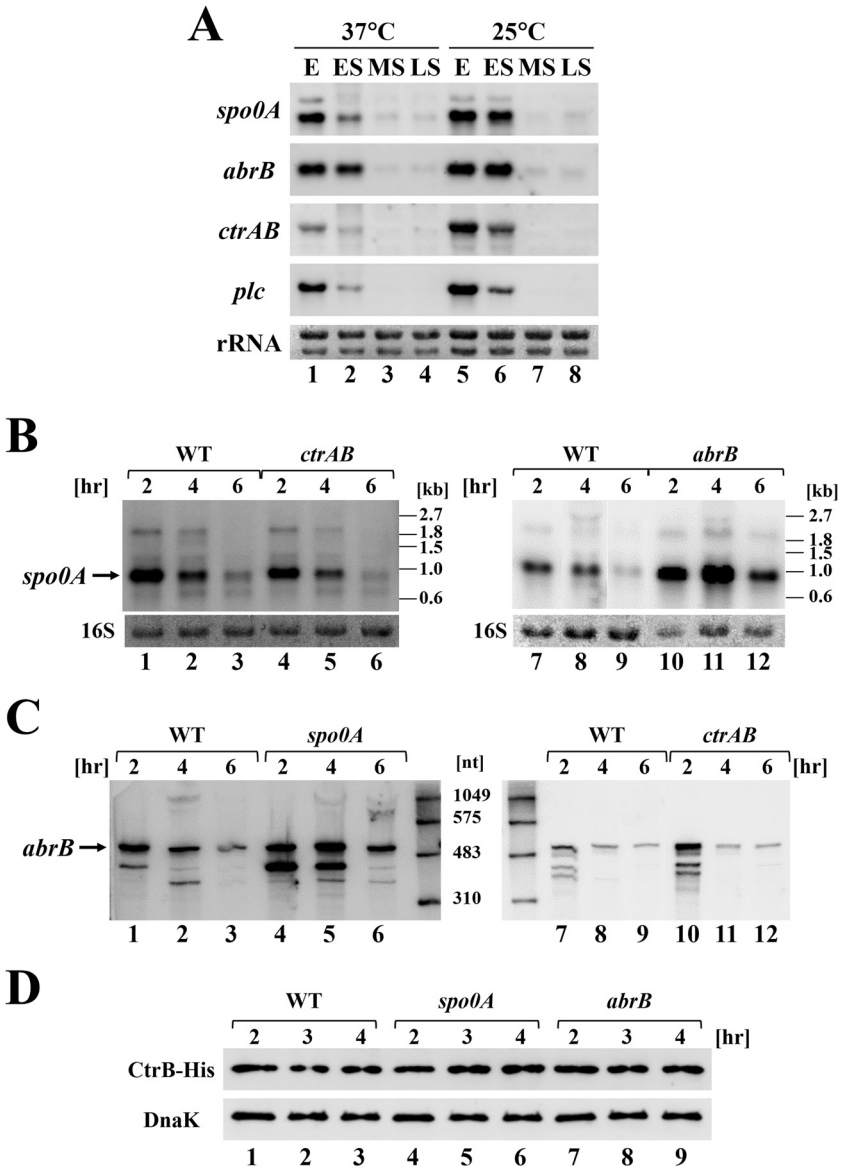

FIG 4 Expression of transcriptional regulator genes involved in biofilm formation. (A to C) Northern blot analyses of *spo0A*, *abrB*, and *ctrAB*. 23S and 16S rRNAs stained with methylene blue are presented as loading controls at the bottom as indicated. In each lane, 2 μg of total RNA was loaded. (A) Wild-type *C. perfringens* was cultured at 37 or 25°C and harvested at the exponential (E; OD_{600} of 1.5 to 2.0), early stationary (ES; OD_{600} of 4.5 to 5.0), mid-stationary (MS; 2 and 8 h after OD_{600} reached 4.5 at 37 and 25°C, respectively), and late-stationary (LS; 4 and 16 h after OD_{600} reached 4.5 at 37 and 25°C, respectively) phases. (B) *spo0A* expression in wild-type and *abrB* mutant cells cultured at 37°C for 2, 4, or 6 h, corresponding to the exponential, early stationary, and mid-stationary phase, respectively. (C) *abrB* expression in wild-type and *spo0A* mutant cells cultured at 37°C for 2, 4, or 6 h, corresponding to the exponential, early stationary, and mid-stationary phase, respectively. nt, nucleotides. (D) Western blot analysis of CtrB-His_6 proteins using the anti-His tag antibody. Wild-type *C. perfringens* and *spo0A* and *abrB* mutant strains harboring the CtrA and His-tagged CtrB expression vector were cultured at 37°C for 2, 3, and 4 h, corresponding to the mid-exponential, late-exponential, and early stationary phase, respectively. DnaK proteins were detected using the anti-DnaK antibody and are indicated as a loading control at the bottom.

DISCUSSION

We observed that the *C. perfringens* biofilm structure is drastically different at 25°C than at 37°C. At 37°C, *C. perfringens* adhered to the substrate surface and formed a flat, thin, adhered biofilm in which the cells were densely packed. However, at 25°C, the cells did not adhere to the surface and precipitated to the bottom of the well, forming a thick, viscous, pellicle biofilm in which the density of the cells was low. These results suggest that the pellicle biofilm comprises extracellular matrices. Indeed, SEM imaging of adhered and pellicle biofilms indicated that the threadlike extracellular structures were only observed in pellicle biofilms and were comprised of a low-temperature-specific EPS produced in the process of *C. perfringens* biofilm formation. *C. perfringens* grows more rapidly at 37°C than 25°C, and it might be possible that biofilm morphology is caused by differences in cell density and/or physiological status. However, we observed no pellicle biofilm formation when cells were cultured at 37°C for 12 h or 24 h, which are shorter incubation times than used for the biofilm formation assay

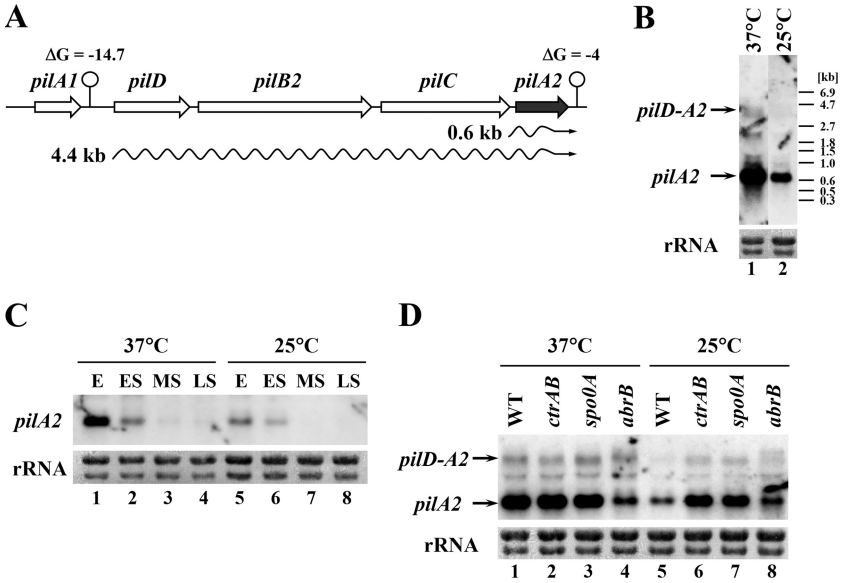

FIG 5 *pilA2* expression is regulated through temperature and multiple transcriptional factors. (A) Schematic of the type IV pilus biosynthesis operon containing the *pilA2* gene. The predicted intrinsic transcriptional terminators and Δ*G* values are indicated. The arrows represent transcriptional units. (B to D) Northern blot analyses of the *pilA2* gene. 23S and 16S rRNAs stained with methylene blue are represented as loading controls at the bottom of the gels. In each lane, 2 μg of total RNA was loaded. (B) Expression of *pilD-pilA2* polycistronic and *pilA2* monocistronic mRNA at 25 or 37°C. (C) *pilA2* expression in wild-type *C. perfringens* grown to the exponential (E; OD_{600} of 1.5 to 2.0), early stationary (ES; OD_{600} of 4.5 to 5.0), mid-stationary (MS; 2 and 8 h after OD_{600} reached 4.5 at 25 and 37°C, respectively), and late-stationary (LS; 4 and 16 h after OD_{600} reached 4.5 at 25 and 37°C, respectively) phases. (D) *pilA2* expression in *ctrAB*, *spo0A*, and *abrB* mutants grown to the mid-exponential phase at 25 or 37°C.

whose results are shown in Fig. 1 (data not shown). In addition, adhered biofilm never formed at 25°C even if the incubation was prolonged for 8 days (data not shown). Thus, we concluded that pellicle biofilm formation is not an early stage of adhered, dense biofilm formation and that the differences in the morphology of biofilms formed by *C. perfringens* are dependent on temperature. We therefore suggest that *C. perfringens* regulates adherence and EPS production through changes in the biofilm structure in response to temperature.

The biofilm component EPS is important for biofilm architecture, protection, and energy storage (5). Treatment with DNase I or proteinase K destabilized the pellicle biofilm structure, suggesting that extracellular DNA or proteins are components of pellicle biofilms. It has been reported that *C. difficile* biofilm formation was reduced through DNase I and proteinase K treatment and that the biofilm contained extracellular DNA and proteins (34). Thus, extracellular nucleic acids and proteins could be important for biofilm formation in *Clostridium* species, similar to other bacterial species. However, although DNase I or proteinase K treatment makes the *C. perfringens* pellicle biofilm fragile, it does not completely inhibit biofilm formation. SEM revealed that DNase I or proteinase K could not digest the threadlike EPS, which is specifically produced at lower temperatures and would also be a main component of the pellicle biofilm. Therefore, threadlike EPS could not be protein or DNA. Threadlike EPS in the biofilms formed by other species have also been analyzed using SEM. Extracellular polysaccharide and poly-γ-glutamate in *B. subtilis* and Pel extracellular polysaccharide in *P. aeruginosa* have been detected as threadlike structures and are the components of the biofilms formed by these organisms (23, 24). These observations imply that the EPS in the pellicle biofilm also contains extracellular polysaccharide.

In general, adherence to the substrate surface is an important step in surface-associated biofilm formation, and TFP is involved in cell attachment and biofilm formation in various species (35–37). It has been reported that the maturation of *C. perfringens* surface-associated biofilm formation requires type IV pili (TFP) and the transcriptional regulator CcpA (4). TFP is involved in gliding motility and cell attachment of *C. perfringens* and is an important factor for biofilm formation and pathogenesis in many bacteria (31, 32, 38). We observed that the expression of *pilA2*, which encodes pilin, a major component of TFP, is activated at 37°C but not at 25°C. This result suggested that *pilA2* regulation is thermodependent and TFP activity is controlled through temperature. In addition, adhered-biofilm formation requires *pilA2*, indicating that the thermoregulation of *pilA2* expression is important to control the biofilm morphology. We detected *pilD-pilC2-pilB2-pilA2* polycistronic and *pilA2* monocistronic mRNA using Northern blot analysis, and the amount of the latter transcript was markedly affected by temperature. This effect suggests that the transcription from a promoter upstream from the *pilA2* gene is regulated through temperature, but no sequence corresponding to a promoter has been identified. In addition, we cloned a DNA fragment containing the *pilA2* gene and 200 bp of its upstream sequence into the *Escherichia coli-C. perfringens* shuttle vector and introduced the resultant plasmid into *C. perfringens*. No *pilA2* transcript or PilA2 protein was derived from the plasmid, suggesting that *pilA2* monocistronic mRNA was processed from the *pilD-pilC2-pilB2-pilA2* polycistronic mRNA and posttranscriptional regulation was involved in the expression of *pilA2* (data not shown).

We identified the transcriptional regulators Spo0A, AbrB, and CtrAB as essential factors for biofilm formation in *C. perfringens*. In addition, we revealed that threadlike-EPS production and cell

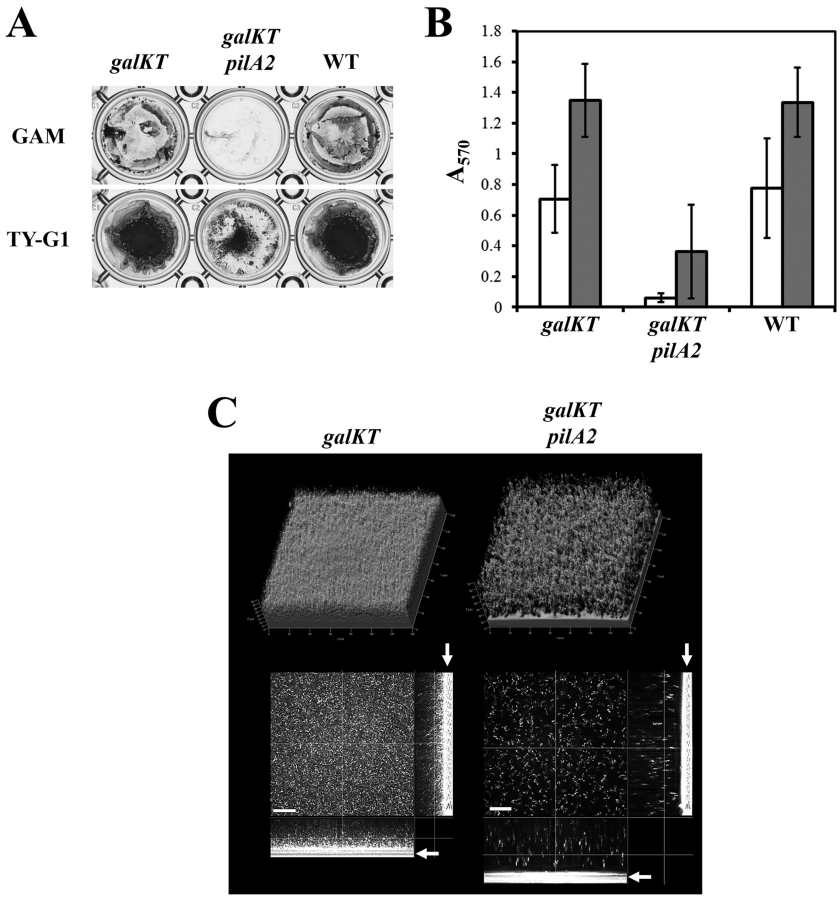

FIG 6 PilA2 is required for adhered-biofilm formation. (A) Adhered-biofilm formation of the *pilA2* mutant strain. *C. perfringens* was cultured at 37°C in GAM or TY-G1 broth for 2 days, and the adhered cells were stained with crystal violet. (B) Quantification of the adhered-biofilm biomass. The means and standard deviations of the OD_{570} values of three independent experiments are shown. (C) Confocal laser microscopy images of the *galKT* or *galKT pilA2* mutant strain biofilms formed at 37°C in GAM broth. The bars indicate 20 μm. The arrows indicate the substrate surface.

adherence to the surface were regulated by these global transcriptional factors. In bacilli, the regulatory cascade of sporulation is involved in biofilm development (8). Spo0A is an essential factor for biofilm formation and sporulation in bacilli. In *C. perfringens*, *spo0A* is also required for sporulation (10), and the *spo0A* mutant could form adhered biofilm but not pellicle biofilm (Fig. 3). Therefore, it was suggested that pellicle biofilm formation is associated with sporulation in *C. perfringens*. In *C. difficile*, the biofilm formation of the *spo0A* mutant was decreased compared with that of the wild-type strain (12, 34). Thus, there might be a deep connection between biofilm formation and sporulation in clostridia.

AbrB is a global repressor involved in myriad biological processes, such as antibiotic production, sporulation, and biofilm formation in *B. subtilis* (28). The *abrB* gene is conserved in *Bacillus* and *Clostridium* species and is widely investigated in *Bacillus* but not in *Clostridium* species. In the present study, we observed that AbrB is also important for cell adherence to the substrate surface and adhered-biofilm formation (Fig. 3). In addition, AbrB negatively regulates *spo0A* expression and vice versa (Fig. 4B and C). The expression of *abrB* is directly regulated through Spo0A in *B. subtilis*, and we identified the 0A box, the consensus sequence of the Spo0A binding site, in the *C. perfringens abrB* promoter region (39; also data not shown). In addition, *abrB* regulates *spo0A* expression through the repression of *sigH*, which encodes the stationary-phase sigma factor in *B. subtilis* (28, 30). We confirmed that *sigH* expression is derepressed in the *C. perfringens abrB* mutant (data not shown). Therefore, Spo0A and AbrB directly or indirectly reciprocally regulate each other in *C. perfringens*. The *abrB* mutant produces abundant threadlike EPS and forms a robust pellicle biofilm (Fig. 3). We also observed cell elongation and aggregation of the *abrB* mutant, and the exogenous expression of *abrB* from the plasmid vector restored the phenotype (see Fig. S2 in the supplemental material). The dramatic phenotype of the *abrB* mutant suggested that the AbrB repressor possesses a pivotal biological role in *C. perfringens*.

We previously identified a CtrAB protein complex as a global toxin regulator and observed that *ctrAB* genes are widely conserved in *Clostridium* species (14). The results obtained in the present study revealed that the protein complex regulator also regulates the formation and morphology of the biofilm. The deletion of the *ctrAB* genes reduced *abrB* expression, and *ctrAB* mRNA accumulated to a greater extent at 25°C than at 37°C (Fig. 4A). Therefore, the CtrAB protein complex might be a critical determinant of biofilm morphology, and the homologous proteins identified in other *Clostridium* species might also be important for biofilm formation.

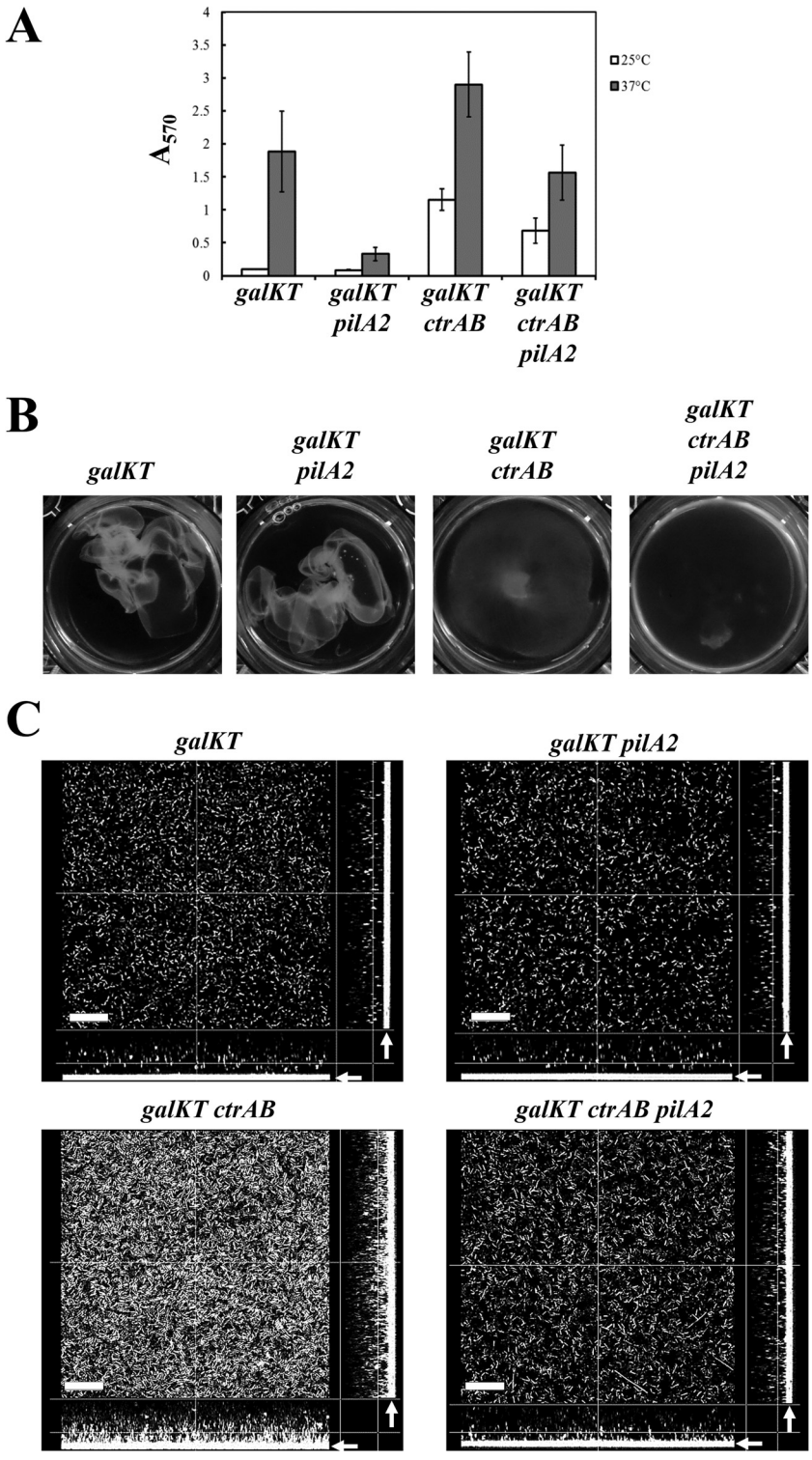

FIG 7 PilA2 affects cell adherence in the *ctrAB* mutant. (A) Adhered-biofilm formation of the mutant strains. *C. perfringens* was cultured at 25 or 37°C in GAM broth for 2 days, and the biomass of the adhered biofilm was quantified through staining with crystal violet. The means and standard deviations of the OD_{570} values of a representative experiment are shown. The experiments were repeated at least three times. (B) Pellicle biofilm formation of the mutant strains at 25°C. The cells were incubated at 25°C for 2 days. The photographs show the pellicle biofilm after picking by gentle pipette aspiration. (C) Confocal laser microscopy images of the *galKT* or *galKT pilA2* mutant strain biofilms formed at 37°C in GAM broth. The bars indicate 30 μm. The arrows indicate the substrate surface.

It has been reported that toxin gene expression was altered in the *ctrAB* mutant (14). Moreover, we analyzed the toxin gene expression in the *spo0A* or *abrB* mutant and observed altered expression of several toxin genes through Northern blot analysis (see Fig. S3 in the supplemental material). These results suggest that the morphological changes of the biofilm in response to temperature were associated with toxin gene regulation. Indeed, it has been reported that the expression of the phospholipase C gene, *plc*, was activated at lower temperatures through changes in the affinity of RNA polymerase for poly(A) tracts upstream from the *plc* promoter (40). Moreover, we observed that the sporulation factors Spo0A and AbrB are involved in biofilm morphology and that the CtrAB toxin regulator also controls biofilm formation, suggesting that the morphological changes of the biofilm could be involved in the toxin production and virulence of this organism.

Temperature is an important factor for biofilm formation, and numerous studies have indicated that temperature affects the amount of biofilm in *Escherichia coli*, *Legionella pneumophila*, *Listeria monocytogenes*, and *B. cereus* (41–44). In *C. perfringens*, temperature drastically affects biofilm morphology, potentially reflecting the temperature-dependent regulation of EPS production and cell adhesion. We suggest that temperature-regulated biofilm formation is a strategy for adaptation to the environment and appropriate infection, as temperature is an environmental signal that alternates between the outside and inside of the mammalian host. After infection, *C. perfringens* spores are germinated and grown at 37°C, and these cells produce TFP and adhere to the epithelial cells. Subsequently, they secrete a number of toxins that degrade the host cell. However, when the host dies or *C. perfringens* cells are secreted from the host, these organisms might sense the shift to lower temperatures and produce extracellular matrices to form the pellicle biofilm. Under this condition, these cells are more likely to be exposed to oxygen. Thus, the pellicle biofilm, which predominantly contains the extracellular matrix, might provide increased resistance to external environments, thereby facilitating survival. Therefore, the morphological changes of biofilms in *C. perfringens* could be involved in the pathogenesis of these organisms. However, the mechanisms underlying the regulatory cascade in *C. perfringens* biofilm formation remain unknown, and further investigation is needed.

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