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Full Paper

Photomanipulation of antibiotic susceptibility and biofilm formation of *Escherichia coli* heterologously expressing photoactivated adenylyl cyclase

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A *cyaA*-deficient *Escherichia coli* strain was transformed by a plasmid carrying the gene for BsPAC, a photoactivated adenylyl cyclase identified from a *Beggiatoa* sp., and was subjected to an antibiotic susceptibility assay and biofilm formation assay under a light or dark condition. Cells expressing BsPAC that were incubated under blue light (470 nm) were more susceptible to fosfomycin, nalidixic acid and streptomycin than were cells incubated in the dark. Cells expressing BsPAC formed more biofilms when incubated under the light than did cells cultured in the dark. We concluded from these observations that it is possible to determine the importance of cAMP in antibiotic susceptibility and biofilm formation of *E. coli* by photomanipulating the cellular cAMP level by the use of BsPAC. A site-directed mutant of BsPAC in which Tyr^7 was replaced by Phe functioned even in the dark, indicating that Tyr^7 plays an important role in photoactivation of BsPAC. Results of mutational analysis of BsPAC should contribute to an understanding of the molecular basis for photoactivation of the protein.

Key Words——antibiotics; biofilm; cAMP; *Escherichia coli*; photomanipulation

Introduction

cAMP is an important regulator involved in various cellular functions in *Escherichia coli*. In the bacterium, cAMP forms a complex with cAMP receptor protein (Crp encoded by *crpA*) and the cAMP-Crp complex stimulates or inhibits transcription of a large number of genes by binding to the promoter sites (de Crombrugghe et al., 1984). The importance of cAMP in the regulation of gene expression has been analyzed by comparing the phenotype of wild-type cells and that of mutants deficient in adenylyl cyclase (Cya encoded by *cyaA*). Characterization of *E. coli* cells overexpressing Cya in a *crpA*-proficient background is difficult because cAMP is deleterious to the cells if overproduced constitutively.

In order to analyze the effect of increased cellular cAMP levels on *E. coli* cells in the same genetic back-

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ground, we characterized *E. coli* expressing photoactivated adenylyl cyclase BsPAC. BsPAC is the blue-light receptor flavoprotein encoded by gammaproteobacterium *Beggiatoa* sp. PS. It bears a "sensors of blue-light using FAD" domain (BLUF domain) and an adenylyl cyclase domain (AC domain), and it is thought to be an ancestral form of the eukaryotic *Euglena* PAC, a blue-light receptor for photomovement of *Euglena* (Iseki et al., 2002). Bioassays of LacZ activity in BsPAC-expressing *E. coli* cells and photobiochemical characterization of the purified BsPAC have been conducted in previous studies. Results obtained from the experiments have shown that, similar to purified or eukaryotically heterologous-expressed *Euglena* PAC (Iseki et al., 2002; Nagahama et al., 2007; Schroeder-Lang et al., 2007; Yoshikawa et al., 2005), BsPAC is activated by irradiation with blue light and synthesizes cAMP (Ryu et al., 2010; Stierl et al., 2011).

We show in this report that cAMP is important for the antibiotic sensitivity and biofilm formation of *E. coli*. Our results also show that BsPAC is a powerful tool in molecular microbiology for photomanipulating cellular cAMP levels and analyzing the response of bacterial cells.

Materials and Methods

E. coli strains and media. BW25113 (*rrnB* DE *lacZ4787 hsdR514* DE(*araBAD*)*567* DE(*rhaBAD*)*568 rph-1*) and JW3778 (BW25113 *cyaA*::*Km*^{r}) were used as host cells. For liquid culture, *E. coli* cells were inoculated into an LB (0.5% yeast extract, 1% casein hydrolysate and 0.05% NaCl) (Athena Enzyme Systems, MD, USA). For agar plate culture, *E. coli* cells were spread on a Mueller-Hinton agar (0.2% beef extract, 1.75% acid hydrolysate of casein, 0.15% starch and 1.7% agar) (Nissui Pharmaceutical) and LB agar (LB containing 1.5% agar). Ampicillin was added at a final concentration of 50 μg/ml, if necessary.

Plasmids. The coding sequence of BsPAC was synthesized and inserted into *Nde*I − *Sal*I of pColdI (TaKaRa Bio, Shiga, Japan). The resultant plasmid was named pBsPAC4. A gene for the site-directed mutant of BsPAC, in which 5′-TAT for Tyr^7 was replaced with 5′-TTT for Phe, was synthesized and inserted into *Nde*I − *Sal*I of pColdI. The resultant plasmid was named pBsPAC4Y7F.

Sensitivity assays of E. coli cells. The antibiotics used were fosfomycin (Sigma Aldrich, MO, USA), streptomycin (SN disk; Nissui Pharmaceutical, Tokyo, Japan), gentamicin (SN disk; Nissui Pharmaceutical), tobramycin (SN disk; Nissui Pharmaceutical), nalidixic acid (Sigma Aldrich), norfloxacin (SN disk; Nissui Pharmaceutical) and levofloxacin (SN disk; Nissui Pharmaceutical). *E. coli* cells were spread on Mueller-Hinton agar plates and LB agar plates and then paper disks impregnated with antibiotics were placed on the plates. The test cells were incubated at 27°C with or without blue-light irradiation (470 nm; 5 μmol/m^{2}s) using an LED unit, LC-LED470B (Taitec, Tokyo, Japan). After incubation, diameters of inhibition zones formed on the bacterial lawns were measured to determine sensitivity of the test cells.

Biofilm formation assays. Glass rods (3 mm × 30 mm) were incubated with 0.2 ml bacterial suspension, which had been diluted to OD_{600} of 0.1 in fresh LB medium, in 1.5-ml tubes at 27°C for 36 h with or without 470 nm light irradiation. Biofilms formed on the glass rods were washed twice with 1 ml saline and stained with 0.1% crystal violet for 30 min and then washed twice with 1 ml water. To quantify biofilms, the glass rods were soaked in 0.7 ml ethanol and the stained ethanol was removed to read OD_{600}.

Electron microscopic analysis. E. coli cells and biofilms were fixed with 2% glutaraldehyde for 2 h, dehydrated in increasing concentrations of ethanol (50% ethanol for 15 min, 75% ethanol for 15 min, 90% ethanol for 15 min and 99.5% ethanol for 15 min), and treated with TI Blue (Nisshin EM, Tokyo, Japan) for 10 min. The samples were observed with a TM-1000 electron microscope (Hitachi, Tokyo, Japan).

Results and Discussion

BsPAC and BsPACY7F proteins

PAC proteins have been identified from *Beggiatoa* sp. PS, several euglenoids (Iseki et al., 2002; Koumura et al., 2004), and the free-living eukaryotic microorganism *Naegleria gruberi* (Fritz-Laylin et al., 2010). BLUF domains in these proteins conserve Tyr (Fig. 1A). Kraft et al. reported the importance of the conserved Tyr residue in *Rhodobacter sphaeroides* AppA protein. They proposed that photochemical excitation of the flavin resulted in strengthening of a hydrogen bond between the flavin and Tyr, leading to a stable local conformational change in AppA (Kraft et al., 2003). These observations suggest that Tyr^7 in BsPAC plays an important role in the function of the protein. To de-

Fig. 1. Structures of BsPAC and site-directed mutant.

(A) Alignment of BLUF domains. Accession numbers of the proteins are A7BT71 (BsPAC), NAEGRDRAFT_70440 (NgPAC1 encoded by *N. gruberi*), NAEGRDRAFT_72396 (NgPAC2 encoded by *N. gruberi*), NAEGRDRAFT_70439 (NgPAC3 encoded by *N. gruberi*), AB031225 (PACα encoded by *Euglena gracilis*), AB031226 (PACβ encoded by *E. gracilis*) and L42555 (AppA). PACα and PACβ carry two BLUF domains designated F1 and F2. Conserved Tyr residues are highlighted. (B) Schematic structure of BsPAC and that of BsPACY7F.

termine the importance of the residue for the function, we constructed a site-directed mutant of BsPAC in which Tyr^7 was replaced with Phe (Fig. 1B).

Suppression of the morphology of cyaA-deficient E. coli cells

E. coli BW25113 and JW3778 cells were transformed by pColdI, pBsPAC4 and pBsPAC4Y7F and subjected to assays. Cells incubated in the dark and those incubated under blue light were photographed under a microscope (Fig. 2). BW25113(pColdI) cells were rod-shaped, while JW3778(pColdI) cells were short rod-shaped. A short size is the typical morphological phenotype of *cyaA*-deficient cells as described in a previous report (Kumar, 1976). No significant difference was observed in the length of cells incubated in the dark and that of cells incubated under blue light. Change in length depending on the light conditions was observed in JW3778(pBsPAC4) cells. Most of the cells were short rod-shaped when grown in the dark, but almost all of the cells were rod-shaped when grown under blue light, indicating that BsPAC suppressed the morphological phenotype of *cyaA*-deficient cells when the cells were incubated under the light. JW3778(pBsPAC4Y7F) cells were rod-shaped both in the dark and under blue light, indicating that the mutant protein functioned in these conditions. These results indicated that replacement of Tyr^7 by Phe conferred constitutive activation on the enzyme.

Susceptibility of the test cells to antibiotics

E. coli deficient in *cyaA* is known to have decreased susceptibility to some antibiotics such as fosfomycin, streptomycin and nalidixic acid (Holtje, 1978; Kumar, 1976; Sakamoto et al., 2003). We analyzed the sensitivity of BsPAC-expressing *E. coli* cells incubated in the dark and that of cells incubated under blue light to determine whether the cells have increased sensitivity to the antibiotics when cellular cAMP level is increased.

For the assays, test cells were spread on agar plates and subjected to the disk diffusion procedure for determining sensitivity of the cells to fosfomycin, aminoglycosides (streptomycin, gentamicin and tobramycin), quinolone (nalidixic acid) and new quinolones (norfloxacin and levofloxacin). To exclude the possibility that the alteration in susceptibility of the test cells to the antibiotics was medium-specific, we analyzed antibiotic sensitivity with two different media, Mueller-Hinton agar and LB agar. Mueller-Hinton agar is the recommended medium for use in the standardized disk assays for determining susceptibility of bacteria to antibiotics (NCCLS, 2003). LB agar is a medium widely used in molecular microbiology.

Diameters of inhibition zones formed on the bacterial lawns were measured (Fig. 3). JW3778(pColdI) cells incubated under blue light showed almost the same sensitivity to the antibiotics as that of cells incubated in the dark. On the other hand, JW3778(pBsPAC4) cells showed increased sensitivity when incubated under blue light. Light-dependent alteration in antibiotic sensitivity of JW3778(pBsPAC4) cells was observed both on Mueller-Hinton agar plates and LB agar plates. Cells expressing BsPACY7F showed increased sensitivity to the antibiotics even in the dark condition. These results indicated that the sensitivity of *E. coli* cells to the antibiotics increased when cellular cAMP level was increased. The effect of light irradiation observed in cells grown on LB agar plates was more remarkable than that observed in cells on Mueller-Hinton agar plates. However, the reason for the difference in the

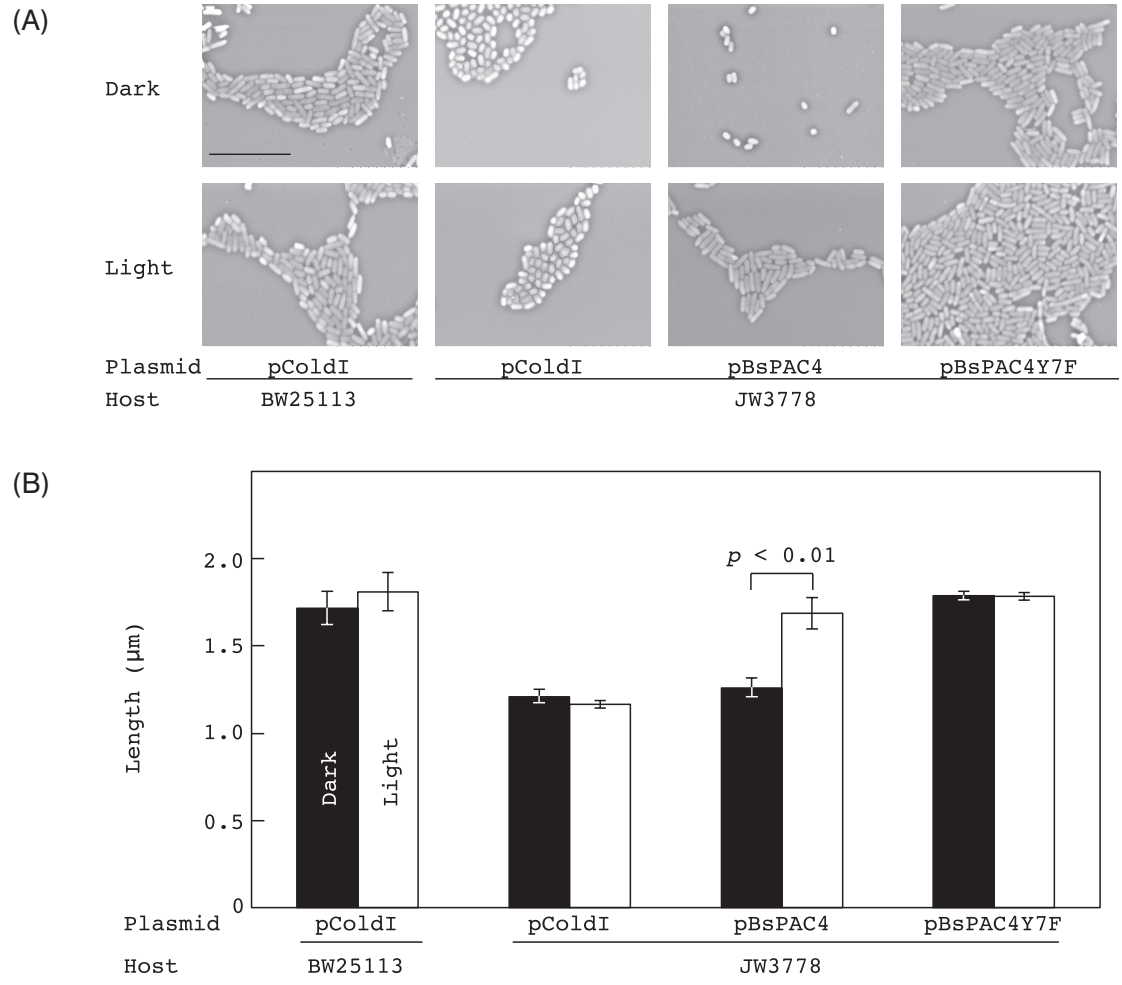

Fig. 2. Morphology of *cyaA*-deficient cells.

(A) Electron microscopic analysis of the cells. Cells incubated in the dark and those incubated under blue light were photographed under an electron microscope. Bar indicates 10 μm. (B) Lengths of the cells. Shaded boxes and white boxes are the lengths of cells incubated in the dark and those incubated under blue light, respectively. Values are means −/+ *SE* obtained from 30 cells.

effect between the media is not clear at present.

Fosfomycin is known to be transported into bacterial cells via GlpT transporter and UhpT transporter (Merkel et al., 1995; Olekhnovich et al., 1999). Quinolones are thought to traverse the outer membrane of *E. coli* through OmpF porins (Hirai et al., 1986). Expression of GlpT, UhpT and OmpF proteins is controlled by cAMP (Merkel et al., 1995; Olekhnovich et al., 1999; Scott and Harwood, 1980), and thus the light-induced increase in sensitivity of BsPAC-expressing cells to fosfomycin and quinolones resulted from an increase in the expression of these proteins. Experiments to determine expression levels of these proteins in BsPAC-expressing cells incubated in the dark and those incubated under blue light are in progress.

The role of porins in uptake of aminoglycosides across the *E. coli* outer membrane has been controversial. Foulds and Chai reported that *E. coli* cells deficient in *ompF* were more resistant to kanamycin and gentamicin (Foulds and Chai, 1978). On the other hand, Hancock et al. reported that porin-deficient mutants show no alteration in susceptibility to these antibiotics (Hancock et al., 1991). In addition to these observations, Kashiwagi et al. reported that aminoglycosides are transported into cells by the oligopeptide transport system (Kashiwagi et al., 1998). We are planning to transform *E. coli* cells deficient in OmpF and those deficient in the protein responsible for the oligopeptide transport system by pBsPAC4 and analyze their sensitivity to aminoglycoside.

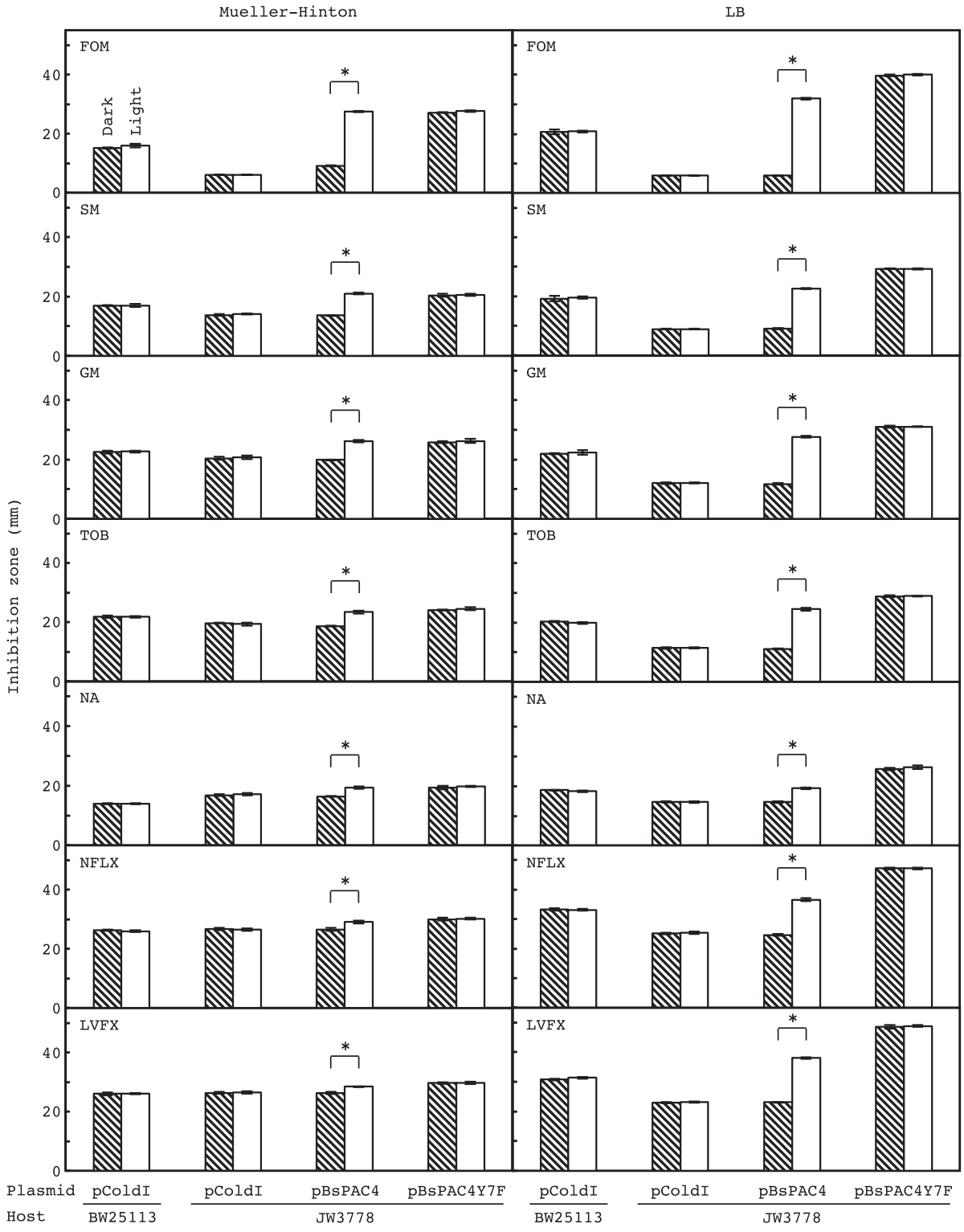

Fig. 3. Sensitivity of *E. coli* cells to fosfomycin, aminoglycosides, quinolone and new quinolones.

Diameters of inhibition zones formed on bacterial lawns were measured to determine sensitivity of the test cells to fosfomycin, aminoglycosides (streptomycin, gentamicin and tobramycin), quinolone (nalidixic acid) and new quinolones (norfloxacin and levofloxacin). Shaded boxes and white boxes are the results obtained from assays conducted in the dark and those obtained from assays conducted under blue light, respectively. FOM: 10 μg fosfomycin, SM: 10 μg streptomycin, GM: 10 μg gentamicin, TOB: 10 μg tobramycin, NA: 10 μg nalidixic acid, NFLX: 10 μg norfloxacin, LVFX: 5 μg levofloxacin. Values are means +/− *SD* obtained from three independent experiments. *, $p < 0.01$.

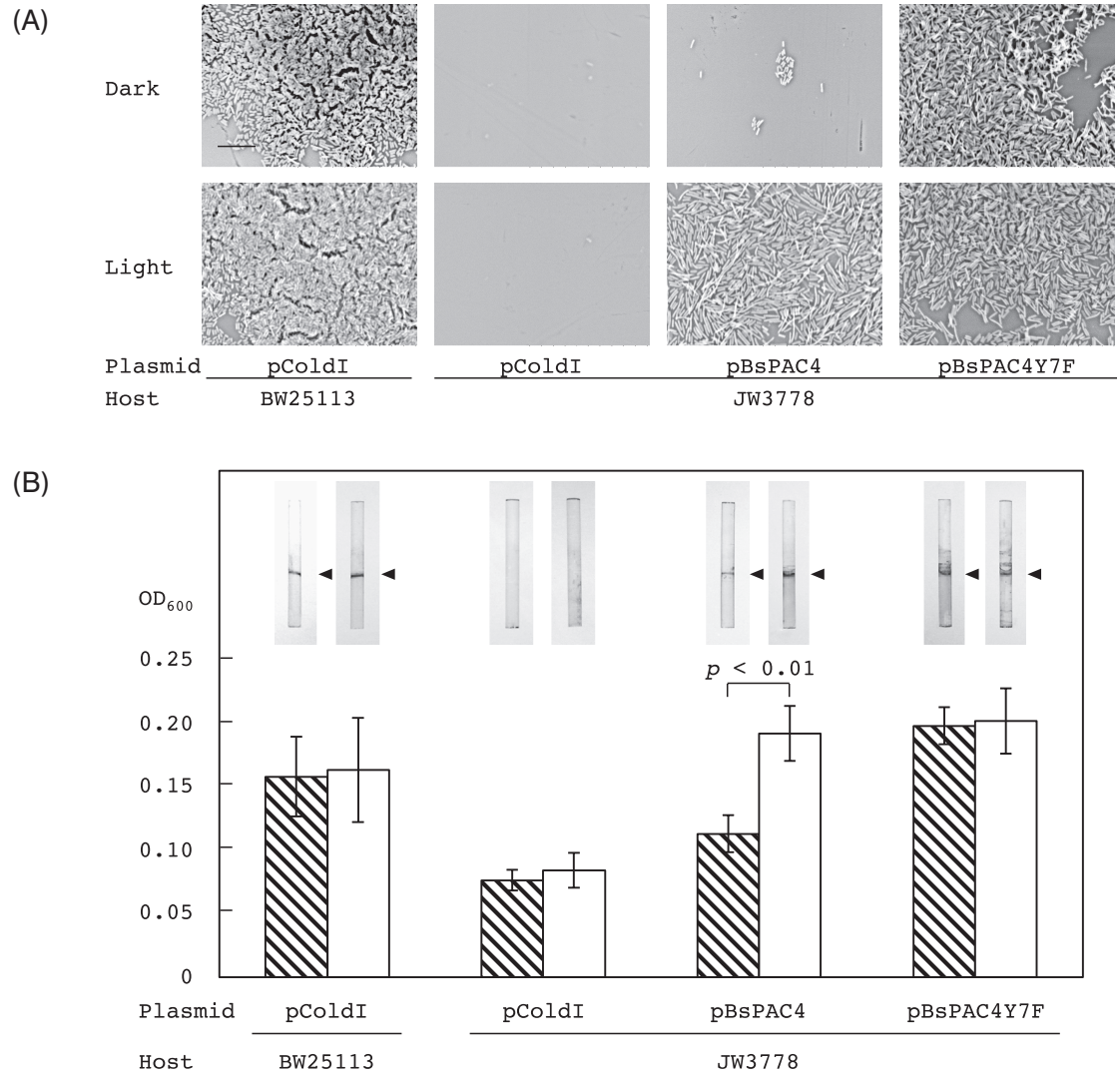

Fig. 4. Biofilms formed by *E. coli* cells.

(A) Electron microscopic analysis of the biofilms. Bar indicates 10 μm. (B) Quantification of crystal violet in the biofilms. Photographs show typical samples stained with crystal violet. Arrowheads indicate the biofilms stained. Shaded boxes and white boxes are the results obtained from assays conducted in the dark and those obtained from assays conducted under blue light, respectively. Values are means +/− *SD* obtained from five samples.

Biofilm formation of the test cells

Biofilms confer decreased susceptibility to antibiotics and chemicals on bacteria in the biofilms and have profound implications for patients. Many genes are associated with the process of biofilm formation, which involves transition from planktonic bacterial cells to biofilm-forming cells and development of a biofilm. Jackson and coworkers reported that biofilm formation is repressed by glucose in *E. coli* and that this effect is mediated by cAMP (Jackson et al., 2002). Niba and coworkers found by systematic genome-wide screening that *cyaA* is one of the genes associated with biofilm formation (Niba et al., 2007). These findings suggest that cAMP up-regulates biofilm formation of *E. coli*.

We analyzed biofilm formation of *E. coli* cells harboring a control vector and those expressing BsPAC. Test cells were inoculated into tubes with glass rods and incubated with or without light irradiation, and then biofilms formed on the glass rods were photographed and stained with crystal violet (Fig. 4). BW25113(pColdI) cells formed biofilms under the experimental conditions. There was no significant difference in the amounts of biofilm formed by cells under dark and

light-irradiated conditions. JW3778(pColdI) cells did not form biofilms under the experimental conditions. On the other hand, JW3778(pBsPAC4) cells initiated biofilm formation under the experimental conditions. Electron microscopic analysis showed that the amount of biofilm formed under blue light was greater than that formed in the dark. JW3778(pBsPAC4Y7F) cells formed almost the same amounts of biofilm in the dark and under blue light.

Our experimental results show that it is possible to photomanipulate biofilm formation by the use of BsPAC. Electron microscopic analysis showed that the biofilms formed by BsPAC-expressing cells were not matured yet under the experimental conditions. Experiments to determine conditions that improve biofilm formation of the cells are in progress.

We are planning to establish a model system to photomanipulate and monitor the process of biofilm development on glass, plastic and metal apparatus. It is expected that the system will contribute to an understanding of the molecular basis for development of biofilms and to prevention of biofilm formation on medical apparatus.

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