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ORIGINAL RESEARCH PAPER

PotD protein stimulates biofilm formation by *Escherichia coli*

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Abstract In natural environments bacteria often adopt a biofilm-growth mode. PotD is a spermidine/putrescine-binding periplasmic protein belonging to polyamine transport system and we have examined its role during biofilm formation and for planktonic growth in *Escherichia coli* BL21(DE3) strains that either over-express PotD (PotD+), or under-express it (PotDi) and also in a control strain with vector pET26b(+) (PotD0). The three strains displayed similar growth in planktonic growth-mode, but over expression of PotD protein greatly stimulated the formation of biofilms, while less biofilm formed by strain PotDi in comparison to strain PotD0. The expressions of five genes, *recA*, *sfiA*, *groEL*, *groES*, and *gyrA*, were increasingly expressed in PotD+ biofilm cells. Thus, PotD is likely to change the rate of polyamine synthesis, which stimulates the expression of SOS genes and biofilm formation.

Keywords Biofilm · Polyamine transport system · PotD · SOS genes

Introduction

In natural environments bacteria can sense the changes of the environmental factors and transform from planktonic growth to biofilm growth. Biofilms are communities of bacteria embedded with extracellular matrix which consists of polysaccharide, protein and DNA when attached to biotic and abiotic surfaces (Costerton et al. 1995; Matsukawa and Greenberg 2004; Whitchurch et al. 2002). Studies showed that about 65 % of microbial infections were caused by biofilms (Costerton and Stewart 2001). The formation of a biofilm makes the bacteria quite different from their planktonic growth mode. Bacterial cells in biofilm state are 100–1,000 times more resistant to antibiotics (Mah and O'Toole 2001) and the expression of a number of genes or proteins are changed. Compared with the planktonic cells, there may be 1–10 % of total genes or more than half of the proteins differentially expressed in bacterial biofilms, which are associated with adsorption, quorum sensing system, metabolism, molecular chaperone and so on (Jefferson 2004; Zhang et al. 2009). Nearly 2 % of genes are differentially expressed during the *Escherichia coli* K-12 biofilm development, some of which rise and others fall (Schembri et al. 2003). Many proteins of *E. coli* are also differentially expressed

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during biofilm formation as shown by 2-dimensional gel electrophoresis analysis (Tremoulet et al. 2002). The impacts of some proteins during biofilm formation have been studied (Sheldon et al. 2012; Al Safadi et al. 2012).

All organisms have mechanisms to cope with the changing environment with all bacteria responding to DNA damage with stimulation of the SOS system (Manasherob et al. 2012). The system can also affect the cellular activity against changed environments (Kandror et al. 1994, 1999). Many genes are associated with the SOS system, such as *recA*, *lexA*, *sfiA*, *groELS*, *gyrA*, *gyrB* etc., (Fonville et al. 2012; Tattevin et al. 2009). When DNA replication is inhibited, bacteria tend to form bioflims (Gotoh et al. 2008).

PotD is a spermidine/putrescine-binding periplasmic protein with other units PotA/B/C belonging to polyamine transport system, which is a type of the ABC (ATP binding cassette) transporters (Han et al. 2007; Brandt et al. 2010). PotD protein binds spermidine as substrate in the spermidine-preferential uptake system, while PotA protein binds ATP for spermidine uptake in conjunction with transport channels formed by PotB, PotC (Kashiwagi et al. 1993). PotD is involved in proteins secretion during biofilm growth (Zijnge et al. 2012).

In our previous study, PotD protein was differentially expressed significantly in biofilms compared to planktonic cells using 2-dimensional gel electrophoresis analysis, which indicated that PotD plays an important role in the biofilm formation (Liu 2006). We have now analyzed the impact of PotD protein on biofilm formation by over- and under-expression of the protein in *E. coli* BL21 (DE3) and found that PotD is likely to change the rate of polyamine synthesis, which stimulates the expression of SOS genes and biofilm formation.

Materials and methods

Bacterial strains and cultivation conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Eschericha coli* DH5α and BL21(DE3) used for DNA manipulation were grown at 37 °C in LB medium. For planktonic growth, a single colony was inoculated into 100 ml flask with 20 ml modified M9 medium (0.4 % glucose in M9 medium was changed to 10 mM sodium succinate) containing 50 μg kanamycin ml^{-1} with shaking at 170 rpm at 37 °C. IPTG was added into the system at 1 mM, the OD_{600} value was monitored every 1.5 h.

Cloning, over or under expression of PotD

The *potD* gene (GenBank ID: NC_012971) was cloned from the genome of *E. coli* BL21(DE3). The primers for over expression were as follows: fpotD1 (5′-CGCGGATCCGATGAAAAAATGGTCAC-3′) and fpotD2 (5′-CCGCTCGAGTTAACGTCCTGCTTTC-3′); primers for cloning interfere sequence of *potD* gene were: ipotD1 (5′-CGCGGATCCTTAACGTCCTGCTTTC-

Table 1 Bacterial strains used in this study

Strains and plasmids	Description	Use	Source
E. coli DH5α	F−,endA1,recA1,relA1,gyrA96 deoR,nupG,Φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, hsdR17(rK− mK+), λ−	Cloning	Our lab
E. coli BL21(DE3)	F−,ompT,gal,dcm,lon,hsdSB(rB− mB−) λ(DE3 [lacI lacUV5-T7 gene 1 ind1 sam7 nin5])	Expression and forming biofilm	Our laboratory
PotD0	*E. coli* BL21(DE3) strain containing pET26b(+)	Expression and forming biofilm	This study
PotD+	*E. coli* BL21(DE3) strain containing pET26b(+)-f*potD*	Expression and forming biofilm	This study
PotDi	*E. coli* BL21(DE3) strain containing pET26b(+)-i*potD*	Expression and forming biofilm	This study
pET26b(+)	Kanamycin resistance (50 μg ml^{-1})	Vector	Our laboratory
pET26b(+)-f*potD*	pET26b(+)::f*potD*	Cloning and expression	This study
pET26b(+)-i*potD*	pET26b(+)::i*potD*	Cloning and expression	This study

3′) and iPotD2 (5′-CCGCTCGAGATGAAAAAAT GGTCAC-3′), a RNA sequence of 1,047 bp would be expressed as a result to interfere *potD* mRNA expression. The PCR program contained a first denaturation step of 94 °C for 6 min, then 30 cycles of 30 s at 94 °C, 1 min at 58 °C, 1 min at 72 °C, and a last elongation step at 72 °C for 10 min. After double-digestion by *Bam*HI and *Xho*I, the segments were ligated into the vector pET26b(+) using DNA Ligation Kit Ver.2.0 (Takara Bio, China). Ligation products were transformed into *E. coli* DH5α and then *E. coli* BL21(DE3), transformants that would over (PotD+) and under (PotDi) express the PotD protein were screened on the LB agar plates with 50 μg kanamycin ml^{-1} and verified by PCR analysis using the recombinant plasmids as templates.

Protein purification and polyclonal antibody preparation

PotD+ strain was inoculated into 100 ml LB medium. IPTG was added at 1 mM when the OD_{600} reached 0.6–0.8. After being induced for 12 h, cells were collected by centrifugation at 10,000×*g* for 2 min. The bacterial pellets were resuspended by 5 ml of 0.01 M phosphate buffered saline (PBS, pH = 7.4) and ultrasonication was performed to disintegrate the cells. The supernatant was collected by centrifugation at 13,000×*g* for 10 min and loaded onto the Ni–NTA resin (Qiagen, Germany) which was balanced by binding buffer (20 mM phosphate buffer, 500 mM NaCl, 10 mM imidazole, pH = 7.4). After washing with buffer A (20 mM phosphate buffer, 500 mM NaCl, 20 mM imidazole, pH = 7.4), the His-tagged protein was eluted by 20 mM phosphate buffer/500 mM NaCl/250 mM imidazole, (pH = 7.4). The purified protein was examined by SDS-PAGE.

PotD protein (purity > 85 %) was diluted to 0.4 μg $μl^{-1}$ by normal saline and 1:1 mixed with Quick Antibody adjuvant (KBQ, China). Five 8-week old male B6 mice (Shandong University Laboratory Animal Center) were immunized. Each mouse was injected into its hind-leg muscle with 100 μl antigen-adjuvant mixture for two times with 2 weeks interval. Total blood was collected 7 days after final immunization and blood coagulation at 4 °C overnight to collect serum for immune reaction. The titer of the anti-PotD serum was detected by ELISA (Kaneko et al. 2011).

SDS-PAGE and western blotting

Electrophoresis was performed in a mini-gel system in the discontinuous buffer system. 20 μg protein samples were mixed with sample loading buffer, heated at 100 °C for 10 min and allowed to cool to room temperature before loading in the gel (6 % SDS concentration gel; 12 % SDS separation gel). Premixed protein markers (TaKaRa, China) were used. Electrophoresis was at 200 V. One gel was dyed with Coomassie Blue for 2 h and destained overnight. The proteins on the other gel were transferred to a PVDF membrane in transfer buffer (192 mM glycine, 25 mM Tris, 0.1 % SDS, 20 % v/v methanol) at 80 V for 1 h. After being blocked, the membrane was incubated with PotD polyclonal antibody (1:5,000 dilution) or Anti-GAPDH Antibody (Zhongshan Biotechnology, China, 1:500 dilution) in TBST buffer (20 mM Tris/HCl, 150 mM NaCl, 0.1 % Tween 20, pH = 8.0) with 1 % skimmed milk at room temperature for 2 h. After incubating with peroxidase-conjugated AffiniPure Goat Anti-mouse IgG (H+L) secondary antibody (Jackson ImmunoReseach, USA, 1:20,000 dilution) at room temperature for 1 h, signals were detected using eECL Western Blot Kit (KWBio, China) according to the specification with Kodak autoradiographic films exposure.

Formation of biofilms

Biofilms of different strains were formed on glass wool in a chemostat with the modified M9 medium at 37 °C. Each of the 20 portions of the glass wool (0.2 g) was bound to a glass stick fixed in the chemostat. 50 ml overnight cultures of strains PotD0, PotD+ and PotDi were inoculated into the chemostat containing 650 ml sterile modified M9 medium with 50 μg kanamycin ml^{-1}. The aeration rate was 400 l h^{-1} and the fluid inflow rate was set at 0.7 ml min^{-1} to allow biofilm formation. IPTG was added at 1 mM. One portion of the glass wool was taken out every 12 h and subjected to the LIVE/DEAD BacLight Bacterial Viability Kit as instructed by the supplier (invitrogen, USA). Biofilm images were analyzed using a Confocal laser scanning microscope. SYTO 9 images were obtained using the excitation/emission laser wavelengths 485/540 nm, while PI images, 490/639 nm. Live/dead views, 3D surface, and Top/Side views of biofilms were obtained following the manufacturer's guide. The cultivation

was performed in triplicate. Planktonic cells living around the biofilms in the chemostat were also analyzed every 12 h.

RNA extraction and cDNA synthesis

Planktonic cells were enriched by centrifugation at 10,000×*g* for 6 min at 4 °C and washed twice by diethylpyrocarbonate-treated water. Total RNA was extracted by RNAiso Plus (Takara Bio). Biofilms were collected into a 50 ml centrifuge tube at mature phase (96 h). After washing with 0.9 % NaCl and pulse centrifuged at 6,000×*g* to remove the medium, RNAiso Plus was added to cover the glass wool. The tube was vortexed for 3 min and incubated at room temperature for 5 min. The next steps were performed according to the direction of the RNAiso Plus. Residual genome DNA was digested by RNase-free Recombinant DNase I (Takara Bio, China). The RNA purity and concentration were analyzed using Nanodrop 2000 (Thermo, USA). Total RNA was reversely transcribed into cDNA using PrimeScript RT reagent Kit (Takara Bio, China) as guided.

Quantitative real-time PCR

Reactions were set up using SYBR Premix Ex Taq (Takara Bio, China) in a LightCycler (Roche Diagnostics, Switzerland) with three equalities. 16S rRNA was used as internal reference. Reactions were performed according to the following steps: hot starting for 5 min at 95 °C, followed by 40 cycles of 10 s at 95 °C, 20 s at 58 °C, collecting fluorescence signal single at 60 °C. Melt-curve analysis was set up of 15 s at 95 °C, 15 s at 65 °C, then rising to 95 °C and continuously detecting the fluorescence signal at the same time. All the data were analyzed by the software of LightCycler480 (Roche Diagnostics, Switzerland) as instructed. Primers used are listed in Supplementary Table 1.

Results and discussion

Over-expression and purification of PotD His-tagged protein

PotD+ strain was induced by 1 mM IPTG overnight. After ultrasonication treatment, PotD was purified with Ni–NTA resin and detected by SDS-PAGE (Fig. 1). In PotD+, the His tagged protein was highly expressed with an MW ~ 40 kDa. PotD was purified to >85 % homogeneity and used for antibody preparation.

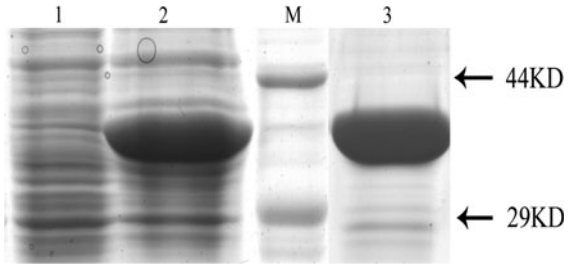


Fig. 1 SDS-PAGE analysis of the expression and purification of His-tagged PotD protein. *Lane 1* total protein from PotD0 strain, *2* total protein from PotD+ strain, *M* protein marker, *3* purified His-tagged PotD protein

Over- and under-expression of PotD in biofilm-growth modes

The *PotD* gene or interfere sequence were cloned and expressed in the cells to form biofilms. SDS-PAGE analysis showed that the expression of PotD protein in the mature biofilms of PotD+, PotD0, PotDi strains were different (Fig. 2a). PotD was obviously over-expressed in PotD+ strain while in PotDi strain it was highly under-expressed compared with that of PotD0 strain. Western blotting analysis using GAPDH protein as internal reference also supported these results (Fig. 2b, c).

Planktonic and biofilm growths of the three strains

Growth of the three strains are shown in Fig. 3. Under planktonic-growth mode in flasks, three strains had similar growth with similar specific growth rates, indicating the PotD protein has little impact on the growth of planktonic cells when not forming biofilms(Fig. 3Aa). In the biofilm-growth mode, however, cells of the wild type strain, PotD0, started to form mature biofilms from microcolonies at 60–96 h. After 96 h, the growth of biofilms and free living cells maintained a balance in the chemostat (Fig. 3Ab). Compared to PotD0, over-expression of PotD protein in PotD + strain greatly stimulated the formation of biofilms, thicker biofilms with less spare space on the glass wool were observed (Fig. 3B), and the free living

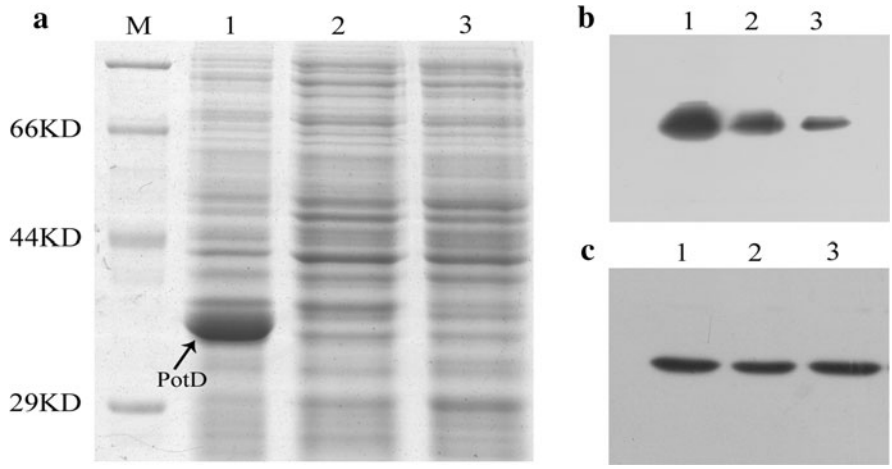


Fig. 2 Over and under expression of PotD in biofilm cells and western blotting detection of the protein expression. **a** SDS-PAGE detection of the expression of PotD. *Lane M* protein marker, *1* total protein of PotD+ iofilm cells, *2* total protein of PotD0 biofilm cells, *3* total protein of PotDi biofilm cells. **b** Western blotting analysis of PotD expression. *1* PotD+ biofilm cells, *2* PotD0 biofilm cells, *3* PotDi biofilm cells. **c** Western blotting of GAPDH expression in biofilms of the three strains as internal reference. *1* PotD+, *2* PotD0, *3* PotDi

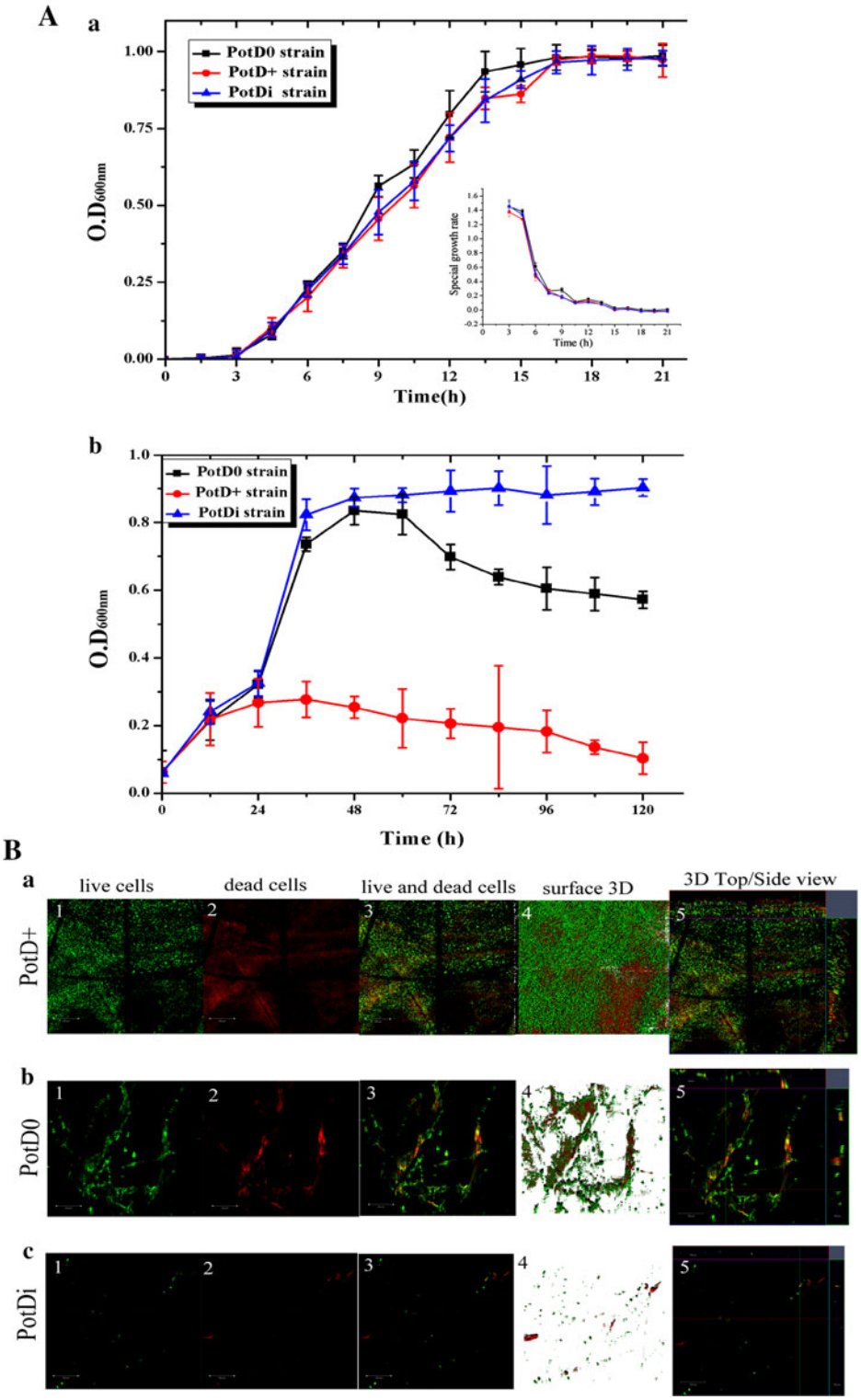
cells in the chemostat around biofilms kept on decreasing slowly as more biofilms accumulated, which meant more cells transferred from free-living growth mode to biofilm growth mode (Fig. 3Ab). With the PotDi strain, only a few microcolonies or relatively much thinner biofilms with large spare spaces on the glass wool were observed, and growth of free-living cells in the chemostat remained similar to the growth when not forming a biofilm. Thus, we can concluded that the PotD protein has little impact on planktonic cells in flasks but is important for the cells to form biofilms. More PotD can stimulate the cells to change from planktonic to biofilm growth mode, and thus more biofilm formed and less planktonic cells existed in PotD+ under biofilm-growth mode.

Quantitative real-time PCR analysis of related genes

The SOS response is related to the rate of polyamine synthesis (Pan et al. 2006). To analyze the functions of PotD and its relationship with the SOS system, we detected the relative expression of five genes related to SOS response including *recA*, *sfiA*, *groEL*, *groES*, and *gyrA* using Real-Time PCR analysis in PotD+ and PotD0 biofilm cells (Fig. 4). Total RNAs of the biofilm cells formed by PotD+ and PotD0 were reverse transcribed to cDNAs which were then used as templates for amplification. The relative expressions of the genes were analyzed using 16 s rRNA as the internal reference.

All of the genes, *recA*, *sfiA*, *groEL*, *groES*, and *gyrA*, participate in SOS responses. RecA protein is involved in multiple biochemical pathways, including recombination processes, SOS induction, and mutagenic lesion bypass (Lusetti and Cox 2002). The *sfiA* gene (also known as *sulA*) is SOS-inducible and its product could inhibit cell division by interacting with FtsZ and inhibiting the GTPase activity (Higashitani et al. 1995; Mukherjee et al. 1998). The chaperonin, GroE, is a molecular chaperone system required for post-translational folding of peptides at later stages of protein folding and protein degradation (Takei et al. 2012). GroE also regulates expression of error-prone DNA polymerase IV which is induced as part of the SOS response (Layton and Foster 2005). GyrA (DNA gyrase) catalyzes the negative supercoiling of DNA which then modulates gene expression and affects bacterial adaptive responses to changed environments (Tse-Dinh et al. 1997; Lopez-Garcia 1999; Prakash et al. 2009).

In the biofilm-growth mode, compared with wild type PotD0 strain, all of the SOS genes were up-regulated in the PotD+ biofilm cells. This indicated that when PotD protein was over-expressed, the polyamine transport system assimilated more polyamine synthesis which induced the SOS responses. Induction of SOS system stimulated the formation of the biofilm (van der Veen and Abee 2010).


A
a
PotD0 strain
PotD+ strain
PotDi strain
O.D600nm
Time(h)
Special growth rate
Time (h)
b
PotD0 strain
PotD+ strain
PotDi strain
O.D600nm
Time (h)
B
a
live cells
dead cells
live and dead cells
surface 3D
3D Top/Side view
PotD+
b
PotD0
c
PotDi

◀ **Fig. 3** Growths of the three strains in planktonic and biofilm modes. **A** Growths curves of the three strains in different growth modes cultured by modified M9 medium. *Error bars* mean the standard deviation. a Planktonic cells of PotD0, PotD+ and PotDi growths curves when not forming biofilms in flasks. The *inset* shows the specific growth rates (SGR) derived from the growth curve. b Growths of free living cells of PotD0, PotD+ and PotDi in chemostat in the process of biofilm formation. For PotD+ and PotD0, planktonic cells tend to form biofilms after 36 h, which caused the falls of the growths curves. The growths curves represent the results of triple independent experiments. **B** CLSM images of biofilms of the three strains PotD+, PotD0 and PotDi formed after 96 h of cultivation in the chemostat. a PotD+, b PotD0, c PotDi. *1* live cells, *2* dead cells, *3* live and dead cells, *4* 3D images demonstrating the surfaces of the biofilms, *5* extended XYZ projection images of the partial biofilms. *Scale bar* in the figure means 100 μm

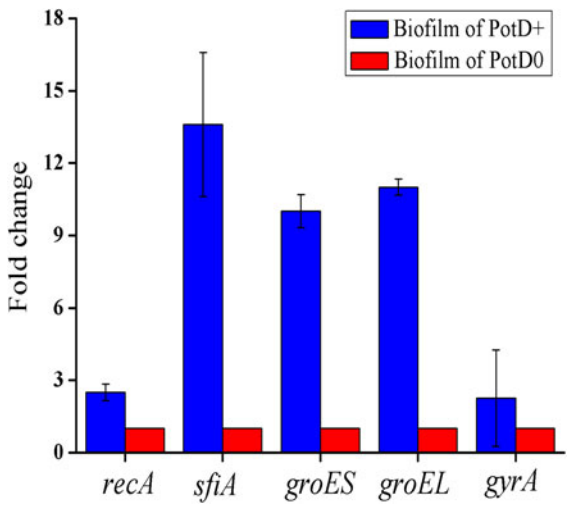


Fig. 4 Real-time PCR of relative mRNA levels of *recA*, *sfiA*, *groES*, *groEL*, and *gyrA* in the bioflim cells formed by PotD+ and PotD0 strains

Conclusions

PotD protein has little impact on the growth of planktonic cells but its over-expression stimulates the transition of bacteria cells from planktonic growth to biofilm growth. Moreover, the over-expression of PotD protein changes the rate of the polyamine synthesis which then induces the over-expression of some genes associated with SOS responses. Then, SOS system stimulated the formation of biofilms. The more PotD that is produced, the higher are the induced SOS responses which then stimulate the generation of biofilms.

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