Biochemical and Biophysical Research Communications xxx (2018) 1–6

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Two different restriction-modification systems for degrading exogenous DNA in *Paenibacillus polymyxa*

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ARTICLE INFO

Article history:
Received 29 August 2018
Accepted 4 September 2018
Available online xxx

Keywords:
Paenibacillus polymyxa
Restriction-modification
Dam
DNA methylation

ABSTRACT

Accompanied by benefits from horizontally transferred genes, bacteria have to face the risk of the invasion of dangerous genes. Bacteria often use the restriction-modification (R-M) system, which is consisted of methyl transferase (MEase) and restrictase (REase), to protect self-DNA and defend against foreign DNA. *Paenibacillus polymyxa*, widely used as growth promoting rhizobacteria in agriculture, can also produce compounds of medical and industrial interests. It is unclear whether R-M systems exist in *P. polymyxa*. In this study, we used a shuttle plasmid with epigenetic modification from different bacteria to explore R-M systems in *P. polymyxa*. We found that DNA which is methylated by DNA adenine methyltransferase (Dam) in *E. coli* was strongly restricted, indicating the presence of a Dam-methylation-dependent R-M system in *P. polymyxa*. Whereas, DNA from a *dam*⁻ *E. coli* strain was also moderately restricted, indicating the presence of a Dam-methylation-independent R-M system. Degradation of plasmid DNA with Dam methylation by cell-free protein extract of *P. polymyxa* provides additional evidence for the presence of Dam-methylation-dependent R-M system. Taken together, our work showed that there are two different types of R-M system in *P. polymyxa*, providing a foundation for the study of innate immunity in *P. polymyxa* and for the development of genetic engineering tools in *P. polymyxa*.

1. Introduction

Paenibacillus polymyxa, previously named *Bacillus polymyxa*, has been widely used in promoting plant growth. It can produce many useful compounds including peptide antibiotic, such as the last-resort drug polymxyin for combating antibiotic-resistant bacteria [1–4], diverse carbohydrases (e.g. decarboxylase pullulanase, cellulase) [5–7] and the chemical compound 2, 3-butanediol [8–10], which can be converted into the chemical feedstock 1,3-butadiene.

For decades, only a few reports documented plasmid transformation in *P. polymyxa* [11,12]. Most recently, a magnesium aminoclay-based plasmid transformation of *P. polymyxa* was reported and the transformation efficiency of an *Escherichia coli* - *P. polymyxa* shuttle plasmid was 100 transformants/μg [13]. Inefficient transformation of *P. polymyxa* may reflect strong DNA restriction in *P. polymyxa*, probably mediated by the restriction-modification (R-M) system, a prokaryotic innate immunity system [11,13]. There are four types (I-IV) of R-M systems in bacteria [14–16]. Among them, the type I-III system is consisted of methyltransferase (MTase) which methylates the self-DNA and restrictase (REase) which degrades foreign DNA [15,16], while the type IV R-M system contains only a DNA modification-dependent REase which degrades the corresponding DNA with epigenetic modification [14]. For example, the type IV REase *Dpn* I, which has been widely used in site-directed mutagenesis [17], degrades foreign DNA with 5′-GmATC-3' (adenine is methylated) in *Streptococcus pneumonia* [18–21]. Some bacteria and phages have only MTase which is called orphan MTase [22,23]. Recently, a new type of R-M system, which modifies DNA through phosphorothioation, has been found to be widespread in bacteria [24–26]. However, little is known about R-M systems in *P. polymyxa*.

In this study, we used an *E. coli* - *P. polymyxa* shuttle plasmid to explore R-M systems in *P. polymyxa*. Our study showed that *P. polymyxa* is equipped with two different types of R-M systems which can degrade foreign DNA with or without methylation. Our work provides a basis not only for understanding innate immunity of *P. polymyxa* but also for the development of genetic tools for analysis of functional genetics in this species.

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https://doi.org/10.1016/j.bbrc.2018.09.016
0006-291X/

2. Materials and methods

2.1. Bacterial strains, plasmids, primers and growth conditions

All bacterial strains and plasmids used in this study were listed in Table S1. All primers used were listed in Table S2. Bacteria were cultured in Luria-Bertani medium (0.5% yeast extract, 1% tryptone and 1% NaCl) or on LB agar plates supplemented with appropriate antibiotics when necessary at 37 °C or 30 °C. Plasmids were isolated from bacteria with the SanPrep column plasmid mini-preps kit (Sangon Biotech Co., Ltd).

2.2. Construction of the plasmids

To construct pWBUC01-*egfp* for expressing green fluorescent protein, pWBUC01 was linearized by PCR amplification and ligated with the *egfp* gene PCR amplified from pGLO through a One Step Cloning Kit (Vazyme Biotech Co., Ltd). The recombinant plasmid pWBUC01-*egfp* was isolated from the transformants and confirmed by colony PCR and DNA sequencing. Refer to Fig. S1 for details about the construction process.

2.3. Construction of the dam$^-$ mutant in E. coli

Inactivation of *dam* was performed with a CRISPR/Cas9 genome editing system [27]. *E. coli* BW25113 carrying the plasmid pCas [28] was grown overnight in 3 mL LB culture at 30 °C 1 mL of the culture was transferred to 50 mL LB and incubated with shaking (180 rpm). To induce the expression of the λ-Red recombination system and the Cas9 protein in pCas, arabinose (15 mM final concentration) was added to the culture with an OD_{600} of 0.2–0.3. Competent cells were transformed with pTargetF-*dam* which expresses sgRNA and template DNA. The *dam*$^-$ mutant was examined by colony PCR. Refer to Fig. S2 for details about the construction process.

2.4. Plasmid transformation of E. coli, B. subtilis and P. polymyxa

Plasmid transformation of *E. coli* was performed according to the previous procedures [29]. Simply put, plasmid DNA was transferred into chemical competent *E. coli* cells via heat shock for 90 s, followed by resuscitation with preheated recovery medium LB broth and plating on selective plates.

To prepare electrocompetent *B. subtilis*, the culture in LB supplemented with 20 mM sorbitol was grown to an OD_{600} of 2.0, then washed with ice-cold solution A (0.5 M sorbitol, 0.5 M mannitol and 10% glycerol) three times, followed by resuspension in the Solution B (0.5 M trehalose dihydrate, 0.5 M sorbitol, 0.5 M mannitol and 10% glycerol). To prepare electrocompetent *P. polymyxa* cells, the culture in LB supplemented with 20 mM sorbitol was grown to an OD_{600} of 0.5, then washed with ice-cold solution A three times, followed by resuspension in the Solution B.

To examine Dam methylation of the donor plasmid DNA, pWBUC01 isolated from different host bacteria (including *B. subtilis* WB800, *P. polymyxa* ATCC842 and several *E. coli* strains) were digested by *Dpn* I and the digestion products were examined by gel electrophoresis (Refer to supplemental materials for detailed procedures).

To transform *B. subtilis* and *P. polymyxa*, 50 μl aliquots of electrocompetent cells was mixed with plasmid DNA, which had been isolated from *E. coli*, *B. subtilis* or *P. polymyxa*, incubated on ice for 30 min and then transferred to the cuvette for electroporation. Electroporated cells were transferred to 950 μl of the preheated recovery medium (LB liquid medium supplemented with 20 mM sorbitol) followed by incubation at 37 °C for 3–4 h before plating. Colonies were counted after 24 h (*B. subtilis* WB800) or 36 h (*P. polymyxa* ATCC842) of incubation at 37 °C. Transformation frequency and transformation efficiency was calculated by dividing the number of transformants by total number of viable counts and by the amount of donor plasmid respectively.

2.5. DNA degradation in cell-free protein extract of P. polymyxa

Cell-free protein extracts of *P. polymyxa* ATCC842 were isolated with the Gram-positive bacteria protein extraction kit (BestBio Co., Ltd). To test the degradation of plasmid from different host bacteria, 0.5 μg of the plasmid DNA isolated from *E. coli* DH5α (*dam*$^+$), *E. coli* Trans110 (*dam*$^-$) and *B. subtilis* were treated by the cell-free protein extract of *P. polymyxa* ATCC842. At intervals, DNA was extracted by phenol chloroform and examined by gel electrophoresis (Refer to supplemental materials for detailed procedures).

2.6. Fluorescence measurement

Green fluorescence was detected by using either the ultraviolet light or an automatic microplate reader. OD_{600} and relative fluorescence unit (RFU) of the bacterial culture were measured with an automatic microplate reader unless otherwise specified. For green fluorescence, excitation and emission wavelengths were 395 and 509 nm respectively (Refer to supplemental materials for detailed procedures).

3. Results

3.1. Plasmid transformation and expression of exogeneous genes in P. polymyxa

In previous work, we constructed a shuttle plasmid pWBUC01, which can replicate in *E. coli*, *B. subtilis* and *P. polymyxa*. The shuttle plasmid carries a replicon from pUC18, a cloning vector in *E. coli*, and a replicon from pWB980, an expressing vector in *B. subtilis*. Transforming activity of pWBUC01 from different bacterial species was examined in *B. subtilis* WB800 and *P. polymyxa* ATCC842. Interestingly, pWBUC01 isolated from an *E. coli* strain (pWBUC01$_{Eco}$) DH5α transformed *B. subtilis* WB800 with high efficiency, whereas no *P. polymyxa* ATCC842 transformants were detected with the same plasmid (transformation efficiency < 2 CFU/μg). Moreover, neither did pWBUC01$_{Eco}$ from two other *E. coli* strains MC4100 and ZK126 yield any *P. polymyxa* transformants, indicating that DNA from *E. coli* was strongly restricted. However, pWBUC01 isolated from *B. subtilis* WB800 (pWBUC01$_{Bsu}$) transformed *P. polymyxa* ATCC842 with high efficiency ($3.875 \times 10^5 \pm 0.587 \times 10^5$ CFU/μg), indicating that DNA from *B. subtilis* was less restricted. Together, our data strongly support that *P. polymyxa* can differentiate DNA with epigenetic modification from different bacterial species.

To examine whether pWBUC01 can express exogenous genes in *P. polymyxma*, an *egfp* gene was integrated to pWBUC01 under the control of P43, a strong constitutive promoter in *B. subtilis* (Fig. 1A, Fig. S1). The recombinant plasmid pWBUC01-*egfp* was transformed to *P. polymyxa*. Strong green fluorescence was detected from *P. polymyxa* cells which were grown on LB-agar plate (Fig. 1B) or in the liquid cell culture (Fig. 1C). During 14 h of incubation in the liquid culture, the intensity of green fluorescence was increased from 275 ± 13 (RFU) to 2194 ± 231 (RFU) (Fig. 1D), showing strong expression of the exogenous *egfp* gene in *P. polymyxa*.

3.2. Dam methylation is detrimental to plasmid transformation in P. polymyxa

E. coli normally contains two MTases: Dcm which methylates cytosine (5-MeC) at CCATGG and Dam which methylates adenine

Please cite this article in press as: M. Shen, et al., Two different restriction-modification systems for degrading exogenous DNA in *Paenibacillus polymyxa*, Biochemical and Biophysical Research Communications (2018), https://doi.org/10.1016/j.bbrc.2018.09.016