Exploring the pathogenic function of an endogenous plasmid of Pantoea ananatis by a simple and efficient plasmid elimination strategy

Xiaozhen Zhao
Nanjing Agricultural University
Lu Gao
Nanjing Agricultural University
Hai Huang
Nanjing Agricultural University
Yi Zhao
Nanjing Agricultural University
Alvina Hanif
Nanjing Agricultural University
Huijun Wu
Nanjing Agricultural University
Qin Gu
Nanjing Agricultural University
Liming Wu
Nanjing Agricultural University
Xuewen Gao (gaoxw@njau.edu.cn)

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Abstract

Background: The bacterium Pantoea ananatis is associated with devastating diseases in many crops that cause serious economic losses. We previously isolated strain DZ-12 from maize brown rot leaves and, genome sequencing revealed that it belongs to P. ananatis and contains a large, endogenous plasmid, pDZ-12. Virulence plasmids are essential for pathogenesis in many bacterial pathogens. However, nothing was known regarding the role of this plasmid in P. ananatis pathogenicity in maize. Results: Here, we eliminated the endogenous plasmid from P. ananatis by substituting its native replicon with a temperature-sensitive replicon. The resulting temperature-sensitive plasmid could be cured by growing cells at high temperature (37 °C). Loss of pDZ-12 from P. ananatis DZ-12 led to decreased disease severity in maize plants, suggesting the endogenous plasmid was important for pathogenesis. Meanwhile, loss of pDZ-12 also affected the ability of the bacterium to form biofilms. The method described here, which is efficient and needs only two steps to cure the endogenous plasmid without antibiotic resistance, was also shown to work in Bacillus subtilis, and may be generally applicable in bacteria. Conclusions: This study provides the first evidence that the endogenous plasmid of P. ananatis DZ-12 is important for pathogenesis in maize plants and in the ability of this species to form biofilms. It also presents the first report on curing plasmid DNA from P. ananatis.

Background

The genus *Pantoea* are ubiquitous Gram-negative bacteria with peritrichous flagella and belong to the family Enterobacteriaceae [1]. They are notable plant pathogens as well as opportunistic pathogens of humans [2]. *P. ananatis* causes disease in crops including eucalyptus, honeydew melon, maize, onion, pineapple and sudangrass [3, 4], with symptoms including stalk rot, brown rot, grain discoloration and leaf blight [1]. These diseases may result in serious economic losses in agriculture [5].

Successful infection and colonization of *Pantoea* in host tissue involves virulence factors including cell wall-degrading enzymes, extracellular polysaccharides (EPSs), quorum sensing, biofilms, swimming, twitching motility, endoglucanase, and the type VI secretion system [6]. EPS is a major virulence factor since an EPS-deficient *P. stewartii* mutant failed to induce wilt [7]. Virulence determinants are frequently carried on endogenous plasmids. In *Bacillus anthracis*, two large plasmids are required for full pathogenicity and elimination of either markedly attenuates virulence [8]. Therefore, numerous approaches have been used to cure virulence plasmids, including microwave treatment, chemical treatment (sodium dodecyl sulfate and ethidium bromide) [9], single square electric pulse [10], and the use of incompatibility plasmids [11]. However, these methods can be fastidious, slow, have low efficiency, and may induce unexpected mutations, so there is a need to develop a curing strategy that is simple, rapid and accurate.

We previously isolated a bacterial strain, DZ-12, from maize brown rot leaves and it contained a large, endogenous plasmid, named pDZ-12 [12]. In the present study, we first identified strain DZ-12 as *P. ananatis* by genome sequencing and phylogenetic analysis. However, nothing was known regarding the

role of pDZ-12 in pathogenicity of *P. ananatis* in maize. So, to explore the function of pDZ-12 in pathogenesis, we developed a new strategy based on the temperature-sensitive replicon *rep101* to eliminate the endogenous plasmid from *P. ananatis* DZ-12. We investigated pathogenesis of, and biofilm formation by, the cured *P. ananatis* strain. Furthermore, the curing method can be applied to the Gram-positive bacterium *Bacillus subtilis*.

Results

Genome and phylogenetic analysis of strain DZ-12

We sequenced the genome of the maize bacterial pathogen, strain DZ-12. The genome consisted of a chromosome of 4.85 Mb (average G+C content 54.72%), and a large plasmid, pDZ-12, of 304 kb (average G+C content) 53.61% (Fig. 1a). There are 4,367 predicted coding sequences (CDSs) with an average length of 901 nucleotides in the chromosome, and 297 predicted CDSs with an average length of 871 nucleotides in the plasmid. Putative functions have been assigned to 72% of the encoded proteins in the chromosome, and 58% in the plasmid.

A phylogenetic dendrogram based on GyrB amino acid sequences indicated that strain DZ-12 was most closely related to *P. ananatis* strain LMG 20103 (Fig. 1b), suggesting that strain DZ-12 belongs to *Pantoea ananatis.*

Construction and verification of *P. ananatis* DZ-12 endogenous plasmid-curing mutant and complemented strains

Many pathogens, including *P. ananatis*, harbor plasmids that may contain virulence determinants [8, 13]. Hence, we used the λ-Red-recombineering technique [14] to cure the endogenous plasmid of *P. ananatis* strain DZ-12. The *repA* (WP_013028019.1) gene encodes a replication protein located on pDZ-12, the endogenous plasmid of strain DZ-12. We substituted it with the temperature-sensitive *repA101*(Ts) gene, amplified from pCas (15) using primers Frep101-F/Frep101-R (Table 2). The resulting strain was grown at high temperature (37 °C), yielding mutant Δp lacking pDZ-12 (Fig. 2). The pDZ-12 deletion was complemented by electroplating pDZ-12 into the Δp strain, yielding strain Δp-c.

P.ananatis produces a yellow pigment via phytoene synthase, the product of the *crtB* gene [16, 17]. The *ctrB* gene is present on pDZ-12. We detected a yellow pigment in the wild-type (WT) strain. However, the Δp mutant no longer produced this pigment (its colonies appeared white) (Fig. 3a). Furthermore, Southern blot analysis showed that pDZ-12 was cured (Fig. 3b). Loss of the plasmid did not affect the growth of *P. ananatis* (Fig. 3c).

The endogenous plasmid of *P. ananatis* DZ-12 is important for pathogenesis in maize

We determined the ability of WT DZ-12, Δp and Δp-c strains of *P. ananatis* to infect maize plants by monitoring disease symptoms. The WT and complemented strains showed similar pathogenicity,

whereas the Δp strain was significantly reduced in pathogenicity compared with the WT ($p \leq 0.05$) (Figs. 4a, 4b). The results suggest that pDZ-12 is important for pathogenesis in maize.

The endogenous plasmid of *P. ananatis* DZ-12 is important for biofilm formation

Biofilms are considered virulence determinants of many bacterial pathogens and are important for colonization of plants [18]. Therefore, we investigated *P. ananatis* biofilm assembly by confocal scanning laser microscopy (CSLM). The Δp mutant had impaired ability to form biofilms, while the complemented strain performed similarly to the WT (Figs. 4c, 4d). We conclude that pDZ-12 is important for biofilm formation in *P. ananatis* DZ-12.

The endogenous plasmid elimination strategy is also applicable to *Bacillus subtilis*

To evaluate the broader applicability of our protocol for curing endogenous plasmids, Gram-positive *Bacillus subtilis* strain NCIB 3610, harboring the endogenous plasmid pBS32 [19], was selected. RepN (WP_020846123.1) is a replication protein located on pBS32, thus we replaced the native *repN* with *repPE194*(Ts) from pJOE8999 [20]. The endogenous plasmid pBS32 was cured by growing at high temperature (50 °C), yielding strain ΔpBS32. The observed efficient curing of the endogenous plasmid indicated a possible broader applicability of this methodology in both Gram-negative and -positive bacteria.

Discussion

P.ananatis causes disease in a wide range of economically important plants [2, 5], and the number of reported hosts is increasing [1]. We previously isolated strain DZ-12 from maize brown rot leaves, and genome analysis and a neighbor-joining phylogenetic tree revealed that DZ-12 belongs to *P. ananatis.* Sequencing also revealed that it contains a large endogenous plasmid, named pDZ-12.

Virulence plasmids are essential for pathogenesis in many bacterial pathogens, including *Salmonella enterica* and *B. anthracis* [8, 21]. However, nothing was known regarding whether pDZ-12 is involved in pathogenicity of *P. ananatis*. Conventional plasmid-curing strategies either have low efficiency, or the process is complex, fastidious, and time-consuming [9, 10, 11]. Here, we eliminated pDZ-12 from *P. ananatis* DZ-12 by substituting its native replicon with a temperature-sensitive replicon, yielding a temperature-sensitive plasmid that could be destroyed by growing cells at high temperature (in this case, 37 °C). In addition, the endogenous plasmid pBS32 of Gram-positive *B. subtilis* NCIB 3610 was cured by the same strategy. The approach described is fast, efficient, safe, reliable, and needs only two steps to cure the endogenous plasmid. The strategy may be broadly applicable in bacteria.

The *P. ananatis* DZ-12 plasmid-cured mutant strain Δp showed significantly decreased pathogenicity in maize plants relative to the WT. The type VI secretion system (T6SS) is important for virulence in some Gram-negative bacteria [22]. There is a T6SS-2 gene cluster located on pDZ-12. Previous research found that T6SS-2 was a host range or virulence determinant of *P. ananatis* [13]. Thus, loss of the T6SS-2 gene

cluster may be related to the reduced pathogenicity of the cured strain. Further studies are needed to delete the T6SS-2 gene cluster from pDZ-12 to verify its involvement in pathogenicity of *P. ananatis*.

Biofilms are highly-organized bacterial communities enclosed in EPSs. They confer resistance to harsh environmental conditions. Moreover, biofilm growth is a strategy used by pathogens to overcome the host immune response and it is therefore critical in the establishment of most bacterial infections [23]. In this work we found that pDZ-12 was important for the ability of *P. ananatis* DZ-12 to form biofilms. The decreased pathogenicity of the cured strain may also be related to the impairment in biofilm formation.

Conclusions

The endogenous plasmid of *P. ananatis* DZ-12 is important for pathogenesis in maize plants and in the ability of this species to form biofilms. The results further our understanding of the pathogenicity of *P. ananatis.* Meanwhile we present the first report on curing plasmid DNA from *P. ananatis*. We substituted the native replicon of the endogenous plasmid with a temperature-sensitive replicon and cured the plasmid without antibiotic resistance. The strategy was also shown to work in Gram-positive *Bacillus subtilis*, and may be generally applicable in bacteria.

Materials And Methods

Strains, plasmids and growth conditions

Table 1 lists the bacterial strains and plasmids used in this work. All *P. ananatis* mutants were derived from strain DZ-12 (referred to here as wild-type [WT]). Bacteria were grown in Luria-Bertani (LB) broth or on LB plates supplemented with 1.5% agar at 30 °C (*P. ananatis*) or 37 °C (*Escherichia coli*) The medium was supplemented with 50 μg/ml kanamycin and 50 μg/ml spectinomycin as required.

Generation of mutants of *P. ananatis* DZ-12

First, *P. ananatis* DZ-12 was transformed with the broad-host-range plasmid pCas (Table 1) expressing the λ-Red recombinase system from the P_{araB} promoter and encoding a temperature-sensitive *rep101* gene which can be cured at high temperature (37 °C) [15]. A starter culture of *P. ananatis* DZ-12 (pCas) was diluted 100-fold in LB broth containing kanamycin and arabinose (10 mM) to induce expression of the λ recombinase system. When the optical density of the culture at 600 nm (OD_{600}) reached 0.6, the cells were made electrocompetent and transformed with 500 ng of PCR product containing the temperature-sensitive replicon *repA101*(Ts) and the selectable spectinomycin resistance gene *speR* (Table 1) with flanking regions homologous to those upstream and downstream of the *repA* gene of pDZ-12. The resulting strain was selected by resistance to kanamycin and spectinomycin. The selected strain was grown at high temperature (37 °C), which cured it. Loss of the plasmid was confirmed by simultaneously streaking the strain on LB-agar and on LB-agar containing kanamycin and spectinomycin. This cured strain was called Δp. Further, pDZ-12 was electroplated into *P. ananatis* strain Δp to create complementation strain Δp-c.

Southern blotting

Total DNA of *P. ananatis* WT and Δp strains was used for Southern blotting as described by Kimura et al. [24]. The DNA was digested with *Hin*dIII.

Growth curve determination

Growth curves were obtained by measuring OD_{600} values of cultures hourly for 24 h (n = 3).

Pathogenicity assays

The pathogenicity of *P. ananatis* strains was determined in maize plants according to [25]. In each test, all leaves of 7-day-old B73 maize (cultivar B73) seedings were inoculated with 1×10^7 colony-forming units/ml at one site per leaf. Negative controls used sterile water. The plants were maintained in a greenhouse at 30 °C during the evaluation period with natural light. The development of disease symptoms was monitored and pictures were taken 7 days post-inoculation.

Biofilm assays

The plasmid pBBR-GFP (Table 1), from which green fluorescent protein (GFP) is constitutively expressed [26], was introduced into the WT, Δp and Δp-c strains of *P. ananatis*. Cells were cultured to OD_{600} = 1.0 and 150 μl were transferred to Nunc Lab-Tek™ II chamber slides then incubated statically for 4 days at 28 °C. We used CSLM to image the surface and thickness of the biofilms formed [27].

Phylogenetic analysis

GyrB amino acid sequences from *Pantoea* and *E. coli* were searched in the NCBI database. Sequences were aligned using ClustalW2 software. MEGA 5 software was used to construct a neighbor-joining phylogenetic tree using the maximum likelihood method [28].

Genome sequencing

Genomic DNA was extracted using a TIANamp Bacteria DNA kit, and the genome was sequenced using the Illumina HiSeq™ 2000/MiSeq platform, and submitted to NCBI (accession no. PRJNA506327).

Statistical analysis

Each experiment was repeated at least three times. Data were evaluated using analysis of variance, then Fisher's least significant difference test (p = 0.05).

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Competing interests

The authors declare that they have no competing interests.

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Author's contributions

XZZ conceived and performed experiments, analyzed data, and wrote the paper; XWG designed experiments and reviewed the manuscript; LG performed experiments; HH analyzed data; AH, HJW, QG, LMW discussed the results. All authors read and approved the final manuscript.

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Author details

College of Plant Protection, Nanjing Agricultural University, Key Laboratory of Monitoring and Management of Crop Disease and Pest Insects, Ministry of Education, Nanjing 210095, China

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Tables

Due to technical limitations, the tables have been placed in the Supplementary Files section.

Figures

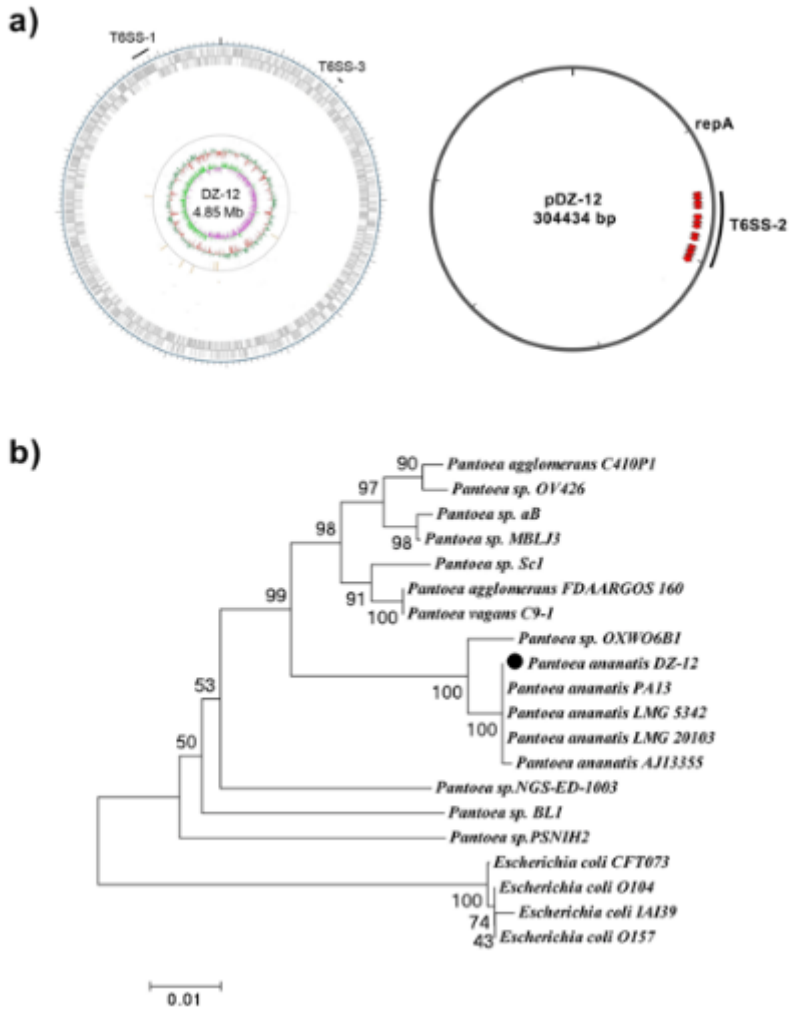

Figure 1

Genome and phylogenetic analysis of Pantoea ananatis strain DZ-12. a The genome was sequenced using the Illumina HiSeq™ 2000/MiSeq platform; b A phylogenetic dendrogram (bootstrap n = 1,000) based on a ClustalW alignment of GyrB amino acid sequences indicates the phylogenetic relationships of strains.

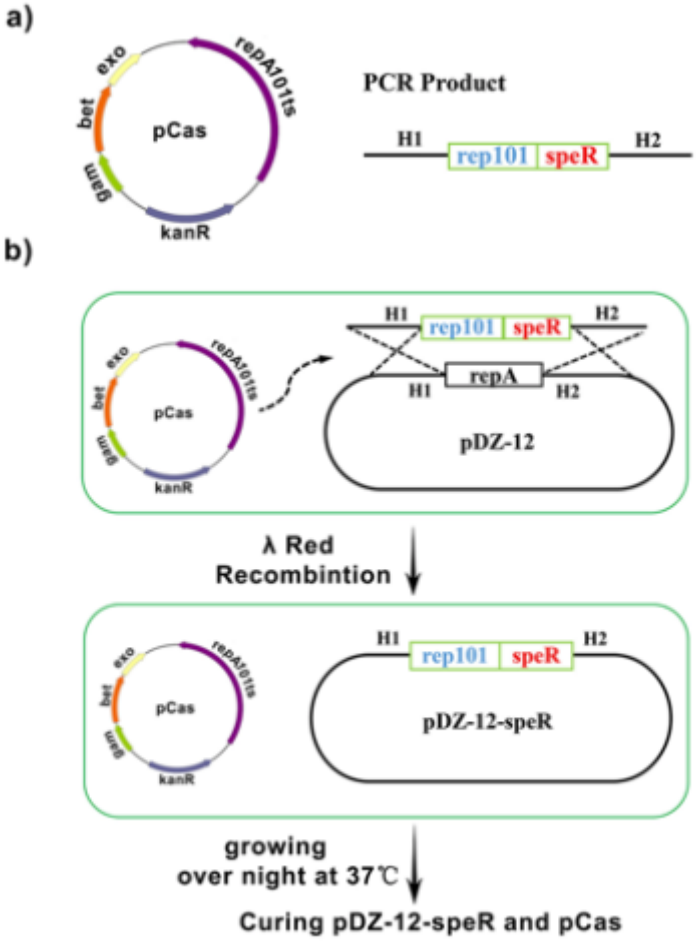

Figure 2

Steps involved in plasmid curing. a Plasmid pCas contains a repA101(Ts) cassette, and phage λ gam, bet and exo genes under the control of ParaB; it confers kanamycin resistance. The PCR product contains the repA101(Ts) gene and the selectable spectinomycin resistance gene speR with flanking regions homologous to those upstream and downstream of the repA gene of pDZ-12 (H1 and H2); b Using the λ-Red-recombineering technique to delete the repA gene of the endogenous plasmid, it was substituted with the repA101 (Ts)-speR fragment. The endogenous plasmid harboring temperature-sensitive (Ts) replicon repA101 was then cured by growing cells overnight at 37 °C nonselectively.

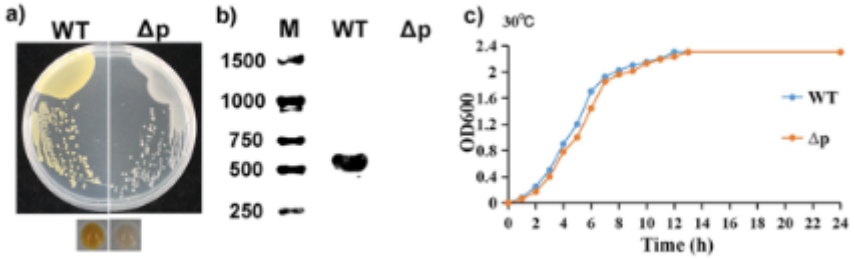

Figure 3

Confirmation and growth of P. ananatis wild-type (strain DZ-12) and the loss of endogenous plasmid to create strain Δp. a Luria-Bertani (LB)-agar plates streaked with P. ananatis DZ-12 (left) and the mutant strain Δp (right) grown for 24 h, showing loss of pigmentation from the mutant strain; b Southern blot to confirm curing of the endogenous plasmid. Lane 1, DZ-12; lane 2, Δp; c Growth of P. ananatis strains DZ-12 and Δp in LB broth at 30 °C.

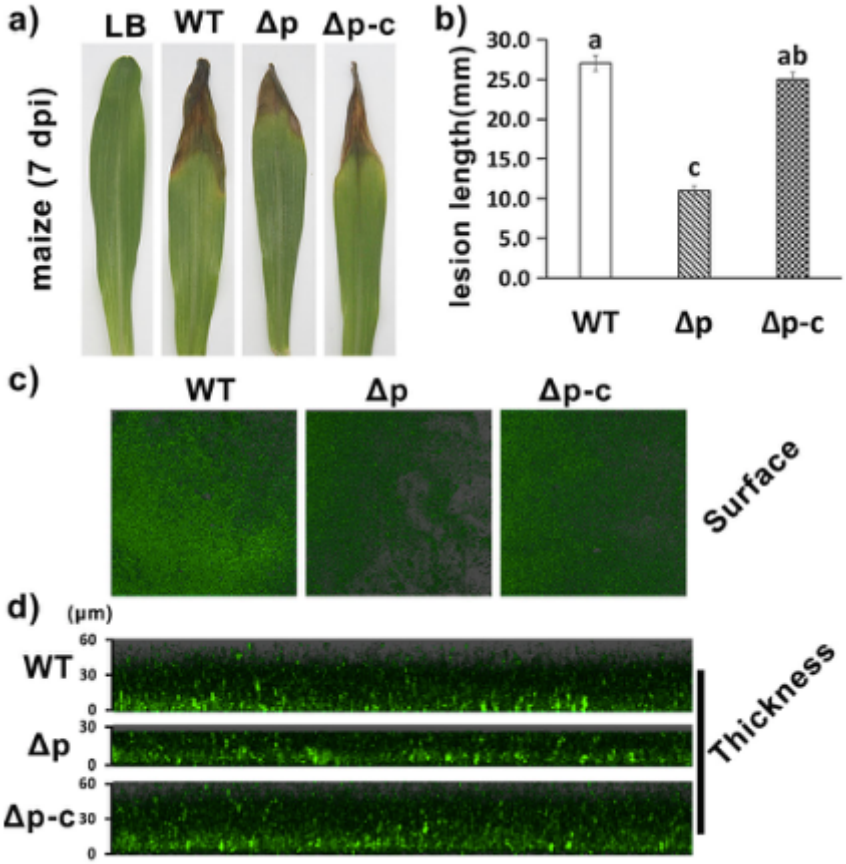

Figure 4

Pathogenicity of and biofilm formation by P. ananatis wild-type (strain DZ-12) and Δp mutant lacking the endogenous plasmid. a Disease symptoms of maize plants inoculated with the P. ananatis strains. Pictures were taken 7 days post-inoculation (dpi) and indicate representative results; b at 7 dpi, the lesion length was measured. Error bars represent the standard error of the mean. Different letters indicate significant differences among strains (p = 0.05) according to Fisher's honest significant difference test; c and d Confocal scanning laser microscopy analysis of biofilm formation by GFP-labeled strains DZ-12, Δp, and the plasmid complemented cured strain Δp-c. Images are of biofilms formed for 4 days.

Supplementary Files

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- Tables.pdf