Electronic Journal of Biotechnology 59 (2022) 74–82

Contents lists available at ScienceDirect

Electronic Journal of Biotechnology

www.elsevier.com/locate/ejbt

Research Article

Plasmid pND6-1 enhances the stability and conjugative transfer of co-resident companion plasmid pND6-2 in the naphthalene-degradative *Pseudomonas putida* strain ND6

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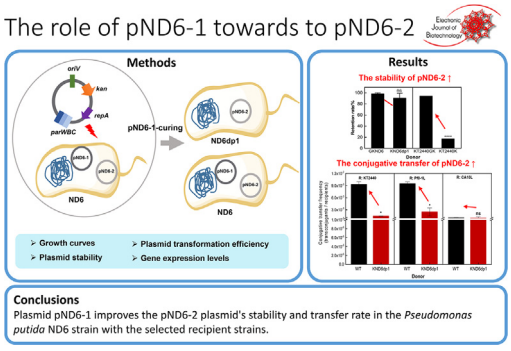
GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 21 January 2022
Accepted 4 August 2022
Available online 8 August 2022

Keywords:
Conjugative plasmid
Conjugative transfer
Co-resident plasmid
Naphthalene degrading bacteria
Plasmid
Plasmid curing
Plasmid interaction
Plasmid stability
Plasmid transfer rate
Pseudomonas putida.

ABSTRACT

Background: Bacterial cells often harbor multiple plasmids, but the interaction between these plasmids is poorly understood. *Pseudomonas putida* ND6, one of the typical naphthalene-degrading bacteria, contains two annotated plasmids, pND6-1 and pND6-2. The conjugative plasmid pND6-2 can be used as a helper to mobilize the naphthalene-degrading plasmid pND6-1. The role of pND6-1 in functions other than naphthalene degradation has never been explored. Therefore, it is of great significance to study the possible effect of pND6-1 on the transfer rate or maintenance of pND6-2.
Results: To investigate the interaction between pND6-1 and pND6-2, we successfully cured plasmid pND6-1 using the incompatibility method, generated the pND6-1-null strain ND6dp1, and first classified it into the IncP-7 group by curing experimental evidence. Subsequently, the pND6-1-cured mutant showed an insignificant increase in cell growth compared with the wild ND6, indicating that a lower cost was imposed on the host by pND6-1. The influences of pND6-1 on the stability and conjugation of plasmid pND6-2 were subsequently explored. The results showed that the presence of pND6-1 enhanced the stability of pND6-2 in natural host ND6 and heterologous host *P. putida* KT2440. In addition, the presence of pND6-1 in wild-type hosts promoted intra-specific and inter-specific conjugative transfer efficiency of pND6-2 by 3.9–6.3-fold with *P. putida*, *P. resinovans,* and *P. fluorescens* as the recipients, respectively.
Conclusions: Collectively, these results suggest that the plasmid pND6-1 improves the pND6-2 plasmid's stability and transfer rate in the ND6 strain with the selected recipient strains.

Peer review under responsibility of Pontificia Universidad Católica de Valparaíso
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https://doi.org/10.1016/j.ejbt.2022.08.002

How to cite: Wang S, Liu M, Wang D, et al. Plasmid pND6-1 enhances the stability and conjugative transfer of co-resident companion plasmid pND6-2 in the naphthalene-degradative *Pseudomonas putida* strain ND6. Electron J Biotechnol 2022;59. https://doi.org/10.1016/j.ejbt.2022.08.002.

1. Introduction

Plasmids provide their hosts with extra accessory genes that promote bacterial populations for bacterial adaptation and evolution in novel environments [1]. Many bacteria simultaneously harbor multiple plasmids. The maintenance of plasmids and their accessory genes in bacterial populations can be explained by coadaptation [2,3]. Most megaplasmids could be capable of horizontal transfer by conjugation, which may result in the dispersion of many beneficial genes. This should increase the relative frequency of the plasmid genes in the population, including antibiotic genes and xenobiotic degrading genes [4,5]. However, the plasmid conjugation process involving type IV secretion system is energetically costly, and sufficient metabolic activity is required [6]. The metabolic burden (fitness cost) can also be produced by the plasmid when they arrive at a new host. This cost is responsible for one of the main limits to plasmid spreading in bacterial populations [7].

Much effort has been devoted to elucidating the influence of multiple plasmids on microbial population dynamics. *Bacillus anthracis* contains multiple plasmids that are all required for exerting maximum virulence in its hosts, which suggests the presence of increasing fitness of the multiple plasmids on *B. anthracis* [8]. A similar phenomenon is also observed in *Rhizobium leguminosarum* strains, which contain four plasmids [9]. However, despite the research findings in bacterial populations, studies that focus on the interactions of co-resident plasmids are limited [10,11]. It is commonly accepted that the coexistence between plasmids belonging to the same incompatibility group is unstable because they compete for the common replication and partitioning systems [12]. In this case, interactions between co-occurring plasmids impair plasmid stability. Nevertheless, the interactions between compatible plasmids can enhance plasmid stability by either increasing the benefits from plasmids or by reducing their costs. Recently, a study on chromosomal mutations and conjugative plasmids carrying resistance genes demonstrated that positive interactions could improve bacterial fitness [13,14]. San Millan et al. [13] reported that positive epistasis decreases the cost of multiple plasmids in short term and enhances the stability of the small plasmid in the long term. The complex interactions of multiple plasmids on the influence of plasmid profiles still need further research.

Plasmid pND6-2 is a self-transmissible megaplasmid harbored by the naphthalene degrading bacterium *Pseudomonas putida* ND6. This plasmid is responsible for the mobilization of another endogenous plasmid, pND6-1, a plasmid that encodes for naphthalene degradation [5,15,16]. In our previous report [5], the transfer of pND6-1 promoted the dissemination of degradative genes on the mobilizable plasmid and accelerated the elimination of naphthalene in culture. Plasmid-mediated gene bioaugmentation endows environmental indigenous microorganisms with pollutant degradation genes, effectively improving the degradation ability of indigenous microorganisms and thereby improving the *in-situ* remediation efficiency of polluted sites. Therefore, studying the characteristics of degradation plasmids such as pND6-1 can achieve more effective treatment of naphthalene pollution. Our previous research indicated that the two transmissible plasmids pND6-1 and pND6-2 could be transferred from the ND6 strain through the single transfer system encoded by pND6-2 in a particular order; that is, self-transmission of pND6-2 may occur first, and pND6-1 is subsequently mobilized through the channels encoded by pND6-2 [5]. Thus, additional studies are needed to further investigate the role of plasmid pND6-1 on the effect of pND6-2 plasmid stability and conjugative transfer ability, which are the most concerning aspects.

Various techniques have been applied previously for plasmid curing in microorganisms. Heat-shock treatment is an efficient method that is usually combined with other chemical reagents, such as sodium dodecyl sulfate (SDS), acridine orange, ethidium bromide, and N-methyl-N'-nitro-N-nitrosoguanidine [17]. Undesirably, most traditional curing agents could potentially bring unknown mutations in the chromosome with high frequency [18]. In contrast, curing the plasmid based on the strategy of incompatibility is an efficient method that may cause little damage to the DNA sequences [19,20]. In this study, we employed the incompatibility method to eliminate the endogenous megaplasmid pND6-1 in the ND6 strain and first classified it into the IncP-7 group by curing experimental evidence. Furthermore, through the characterization of the pND6-1-null derivative and comparing it to the wild ND6 strain, we investigated the influence of pND6-1 on the plasmid stability and transfer efficiency of pND6-2.

2. Materials and methods

2.1. Strains, plasmid, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. All the bacterial strains were cultivated in Luria-Bertani (LB) liquid medium or LB agar. The *P. putida* ND6 and its derivatives were grown in mineral salt medium (MSM) containing 2 g/L naphthalene as a sole source of carbon and energy at 30°C with shaking at 150 rpm [5]. *Escherichia coli* DH5α was the host for the plasmid pUCARori004ΔparA and was cultivated at 37°C with shaking at 150 rpm. When needed, ampicillin (Ap), kanamycin (Km), gentamycin (Gm), and tetracycline (Tc) were added at final concentrations of 30, 50, 25, and 50 μg/mL, respectively.

2.2. DNA manipulation

The PCR assay was performed with Takara HS PrimeSTAR Polymerase (Takara Bio, Japan) at a final volume of 50 μL containing 10 μL 5 × PrimeSTAR buffer, 4 μL dNTP mixture (2.5 mmol each), 1.5 μL of each primer (10 μM), 1 μL of the template (<200 ng), 0.5 μL PrimeSTAR HS DNA Polymerase (2.5 U/μL), and 32.5 μL double distilled water. The PCR protocol was used in the assay with the following thermal cycling parameters: pre-denaturation at 98°C for 5 min, followed by 30 cycles of 98°C for 10 s, 55°C for 30 s, 72°C for 1 kb/min, and final elongation at 72°C for 5 min.

2.3. Curing plasmid pND6-1 by plasmid incompatibility

2.3.1. Preparation of competent ND6 cells for electroporation

The *P. putida* ND6 strains were cultivated in 5 mL LB medium at 30°C for 12 h. Then, 1.0 mL culture was transferred into a 1.5 mL EP tube and incubated on ice for 10 min. After centrifugation at 8000 *g* for 1 min, the cell pellets were washed with 1.0 mL electroporation buffer (1.03 g/L sucrose and 0.0068 g/L monobasic potassium phos-

Table 1
Strains and plasmids used in this study.

Strains and plasmid	Feature	Source
Strains		
P. putida		
ND6	Wild-type strain; Nah$^+$; Ap^r	[15]
GKND6	ND6 derivative harboring pND6-1::Gm and pND6-2::Km; Nah$^+$; Ap^r, Gm^r, Km^r	[5]
ND6dp1	Elimination of pND6-1 in ND6 by plasmid incompatibility method	This work
KND6dp1	ND6dp1 (pND6-2::Km)	This work
KT2440	Wild-type strain; pCA$^+$	[21]
KT2440GK	KT2440 derivative harboring pND6-1::Gm and pND6-2::Km; pCA$^+$; Gm^r, Km^r	[5]
KT2440K	KT2440 derivative harboring pND6-2::Km; pCA$^+$; Km^r	[5]
P. fluorescens Pf0-1L	Pf0-1 with *lacI*^{q}; Tc^r	The University of Tokyo
P. resinovans CA10L	CA10dm4 with *lacI*^{q}; Tc^r	[22]
E. coli DH5α	F$^-$, φ80*lacZ*ΔM1, Δ(*lacZYA-arg*F)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(r_k^-, m_k^+), *phoA*, *supE*44, λ$^-$ *thi*–1, *gyrA*96, *relA*1	Takara Bio, Japan
Plasmids		
pND6-1	Endogenous plasmid of ND6; Nah$^+$; IncP-7	[23]
pND6-2	Endogenous plasmid of ND6;	[16]
pEX-ABC	pEX-18Tc with *kan* gene; Tc^r; Km^r	[5]
pUCARori004ΔparA	pUC19 containing 7-kb *Nhe*I-*Hin*dIII fragment of pCAR1 with mutated *parA* gene; Km^r	The University of Tokyo

*Nah$^+$: the ability to degrade naphthalene; pCA$^+$: the ability to degrade *p*-coumaric acid; Ap^r: resistance to ampicillin; Km^r: resistance to kanamycin; Gm^r: resistance to gentamicin; Tc^r: resistance to tetracycline.

phate) three times. Finally, the pellets were re-suspended in equal volumes of electroporation buffer.

2.3.2. Introduction of the incompatible plasmid into ND6 by electroporation

The incompatible plasmid pUCARori004ΔparA (2.0 μg) was mixed with competent ND6 cells and incubated on ice for 20 min. The mixture was then transferred into a 1-mm electroporation cuvette, and electroporation was performed at a voltage of 1.50 kV for 3.0 ms using a MicroPulser system (Bio-Rad, USA). After pulsing, the cells were transferred into a 1.0 mL LB medium and cultured at 30°C (150 rpm) for 3 h. Then, the culture was plated onto the Km-selective agar plates and incubated at 30°C for 24 h.

Subsequently, the single clones were randomly selected, and the genotypes of the clones were confirmed by PCR amplification with primers repA-F: TTGGGATTTACGGGACTGCT/repA-R: TCGGATGCCTATCAACGATT to verify the elimination of pND6-1. The verified derivative ND6dp1(pUCARori004ΔparA) was subcultured in LB liquid medium for 20 generations to cure the pUCARori004ΔparA plasmid. The final obtained mutant without pND6-1 nor pUCARori004ΔparA was named ND6dp1.

2.4. Assessment of growth and plasmid stability

Growth curves were determined using LB medium. The spore suspension was inoculated into fresh LB medium and incubated overnight in a rotary shaker at 150 rpm and 30°C. Then, 0.5 mL of seed culture was transferred into another 250 mL flask containing 50 mL of LB medium and cultivated at 30°C with shaking at 150 rpm. One milliliter of the culture was taken from three replicates every 1 h up to 12 h for the cell growth analysis, which was monitored by measuring optical density at 595 nm using a Model 680 Microplate Reader (BioRad, USA).

Plasmid stability experiments were performed as described previously [24]. Briefly, different strains carrying plasmid pND6-2 were subcultured in LB liquid medium without antibiotic pressure six times, respectively. Subsequently, the culture was collected and spread on an LB agar plate in the presence or absence of Km to count the number of cells that host the plasmid pND6-2. The stability assays were performed in triplicate. The plasmid retention rate was calculated by Equation (1):

$$R_n = \frac{N_n}{N} \times 100\% \quad (1)$$

where R_n is retention rate; N is the total number of host strains (*P. putida* ND6, ND6dp1, KT2440GK, or KT2440K) (CFU/mL); n represents pND6-2; and N_n is the number of related strains containing pND6-2 (CFU/mL).

2.5. Filter mating assay

To add a selective label, the *kan* gene was tagged into pND6-2 in ND6dp1 using homologous recombination with the recombinant plasmid pEX-ABC containing the resistance gene flanked by homology arms as described previously [5].

The donor and recipient strains were cultivated separately in LB liquid medium at 30°C overnight. After harvesting and washing, the donor and recipient strains (10^8 CFU/mL) were mixed in 10 μM $MgSO_4$ solution (total volume of 200 μL) and spotted on a 0.22 μm membrane filter that was placed on an LB agar plate without antibiotics. All mating systems were evaluated in triplicate. After incubating at 30°C for 24 h, cells on the filter were serially suspended in sterile water, and appropriate dilutions of each sample were spread onto transconjugant selection plates containing ampicillin and kanamycin. Intraspecific transfer frequency was determined by calculating the ratio of transconjugants to recipient cells (T/R, transconjugant/recipient).

2.6. Analysis of gene transcription by real time quantitative PCR (RT-qPCR)

Total RNA was extracted using Total RNA Kit (TIANGEN, China) according to the manufacturer's instructions. The quality of the obtained RNA samples was assessed via electrophoresis, after which the samples were reverse-transcribed using the FastKing RT Kit (TIANGEN, China). RT-qPCR was conducted in triplicate in a LightCycler® 96 Instrument (Roche, Germany) using the SuperReal preMix Plus kit (SYBR Green) (TIANGEN, China). The transcription of target genes was internally normalized to the 16S rRNA gene and quantified using the $2^{-\Delta\Delta CT}$ method [25]. All primer pairs involved in this study were summarized in Table 2.

2.7. Statistical analysis

The data analysis was performed with SPSS 21 (IBM, USA). The Shapiro–Wilk test was used to assess data normality. Where appropriate, significance differences were conservatively evaluated using Student *t*-test or Kruskal–Wallis test between the means of

Table 2
Primers used in RT-qPCR.

Primers	DNA sequences (5′-3′)	Genes
Q16S-1	CGGGAATCTGACACAGGTGCT	16S rRNA
Q16S-2	GATCCGGACTACGATCGTTTTGT	
QparA_P2-F	ATCGGCCTGTGGATCGTTGT	*parA*(pND6-2)
QparA_P2-R	CATTCCCTCGGCCTATCACAC	
QrepB_P2-F	CCTGTTCGCGACCTTATGGA	*repB*
QrepB_P2-R	TGATCGAGGCGCTGGCAA	
QDnaA_ND6-F	GATCCGTCCGCTACAGGTCGA	*dnaA*
QDnaA_ND6-R	CCGCTACCGTTCTCACCCAA	
QparA_ND6-F	GGGCAGTCGATGAGGATGTA	*parA*(chromosome)
QparA_ND6-R	CAGGCTATGCACTACTCCGA	
QpilQ_P2-F	CCAAGCCGGTCGGTCTGAT	*pilQ*
QpilQ_P2-R	ATGCCGTGTCTTGTCGATGGT	
QdotG-F	GCCTTGATCGCCTCGTCATTA	*dotG*
QdotG-R	CCGAACTGGCGTAACCGAGA	
QdotB-F	AGTGAAGGTCAACCGCGAGAA	*dotB*
QdotB-R	CAGAGATCCGCGTGGTACTCC	

two groups. A two-sided value of $P < 0.05$ was considered significant. Standard deviation was determined and presented as error bars. The bars represent the mean and standard deviation values obtained from at least three biological replicates.

3. Results and discussion

It is very common for multiple plasmids to be harbored in the same host [26,27,28]. The diverse functional genes in co-occurring plasmids endow diverse beneficial phenotypes and help the host survive in multiple environmental niches. Many reports have revealed the additive costs of plasmids to their hosts, and elimination of the plasmid induces a faster growth of the host [29,30]. However, there is limited research that has focused on the interactions of co-resident plasmids. In this study, the pND6-1 plasmid was eliminated using the incompatibility-based plasmid curing method and its influence on the stability and conjugative transfer of the other co-occurring plasmid pND6-2 was studied.

Among the various methods for plasmid curing, plasmid incompatibility is widely used for its high efficiency and reduced risk of chromosomal mutations [30]. Although no experimental results confirm the incompatible group, some studies have reported that plasmid pND6-1 may belong to the IncP-7 group due to the high homology of its *repA* and putative *oriV* regions with those of pCAR1 [31,32].

In this study, the pCAR1 derivative plasmid pUCARori004ΔparA was used as a minireplicon of the IncP-7 plasmid. After introducing pUCARori004ΔparA into the ND6 strain, a mutant harboring pUCARori004ΔparA but without pND6-1 was obtained, which was named ND6dp1 (pUCARori004ΔparA). Due to the mutated *parA* gene, the introduced heterogeneous plasmid pUCARori004ΔϖparA was not stable in ND6dp1 (pUCARori004ΔparA). After subculturing several times without kanamycin, the ND6 derivative with neither pND6-1 nor pUCARori004ΔparA was obtained, designated as ND6dp1. The obtained transformant ND6dp1 did not grow on MSM with naphthalene, indicating that the naphthalene-degrading plasmid pND6-1 was lost (Fig. 1). To further corroborate the trait of ND6dp1, PCR analysis was performed targeting the *repA* gene of the plasmid (Fig. 1). The positive results were detected in the wild-type ND6 and ND6 (pUCARori004ΔparA) strains with either pND6-1 or pUCARori004ΔparA plasmid. This indicates that pND6-1 belongs to the IncP-7 group of plasmids. To the best of our knowledge, it is the first report confirming the Inc. group of pND6-1 as IncP-7 by experimental results. Nevertheless, our previous research found that plasmid pND6-1 could transfer from *P. putida* ND6 to KT2440 in both membrane and liquid matrices with a lower transfer efficiency (3.12×10^{-9}–10^{-10}), though rare genes involved in the conjugation system have been detected in plasmid pND6-1. One of the reasonable interpretations is that the two transmissible plasmids in the ND6 strain share the single transfer system, and pND6-1 could transfer through the channels encoded by pND6-2 [5]. Therefore, it is necessary to investigate the influence on the stability and conjugative transfer of pND6-2 after curing the plasmid pND6-1.

3.1. Curing plasmid pND6-1 increased cellular growth

The growth curves of ND6 and the cured pND6-1 derivative ND6dp1 were determined in LB medium (Fig. 2). After subculturing for 2 h, the OD_{595} of ND6 began to increase and reached 1.19 at around 12 h, while the OD_{595} of ND6dp1 reached 1.25 at the same time. Furthermore, pND6-1-eliminated strain ND6dp1 exhibited a slightly higher growth rate (around 0.113 a.u./h) than the wild-type ND6 strain (around 0.107 a.u./h) after 2 h. The result showed that ND6dp1 grew slightly faster than the wild ND6, but it is not obvious which implies that the presence of plasmid pND6-1 in the host ND6 imposes merely low fitness costs. Normally, there is a fitness disadvantage for megaplasmid derivatives [13,33,34]. Here, the compensatory adaptation of pND6-1 in ND6 may be produced through compensatory mutations in the plasmid or the chromosome over time [2,35,36].

In addition to the potential survival benefits endowed by plasmids, they also produce a burden (fitness cost) from multiple origins to the host, such as competing the replication and expression system with chromosomes, resulting in a decreased growth efficiency and reduced stability of plasmid-harboring strains under the conditions without selective pressure [7,37]. Therefore, curing endogenous megaplasmids may help reduce the burden of redundant metabolic networks and provide a more efficient system than plasmid-bearing strains. Feng et al. cured the endogenous plasmid pMC1 by incompatibility in a γ-PGA-producing strain *Bacillus amyloliquefaciens* LL3. By comparing the cell growth and γ-PGA production of pMC1-cured strain with wild-type LL3 strain, the growth rate and γ-PGA production of plasmid-cured strain were increased [30]. Furthermore, plasmids can also produce a fitness cost when they transfer to a new host [38]. Dourado-Morales et al. found that plasmid pUR2940 imposed a strong fitness cost on both its natural host *Staphylococcus aureus* ST398 and new host *S. aureus* MW2 PF_{t0} [34]. Ma et al. evaluated the fitness cost of the plasmid p3R-IncX3 in different *Enterobacteriaceae* strains by growth kinetics assays of the strains with and without plasmid p3R-IncX3. After harboring plasmid p3R-IncX3, 75.9% of the transformants did not show any fitness cost, while 6.9% of strains showed improved growth compared with their corresponding wild strains, which is in contrast to traditional plasmid burden [39]. Similar results have also been obtained in *Clostridium saccharoperbutylacetonicum* N1-4 strain. With the elimination of the endogenous megaplasmid from the natural host N1-4, the obtained transformant N1-4-C exhibited a lower growth rate than the wild strain under the same culture conditions [10,11,13,14,24]. One of the reasonable interpretations is that the elimination of megaplasmids may have disturbed the original metabolic balance in host strains, resulting in delayed or reduced cell growth. The various effects of the same plasmid on the growth of different strains indicated that the associations between plasmid and its host strain are complex, which need further study.

3.2. Curing plasmid pND6-1 decreased the stability of pND6-2

To evaluate the possible relationship between pND6-1 and the stability of pND6-2 in the host, the wild-type ND6 strain and pND6-1 elimination derivative were subcultured without antibiotics six times. Then, the plate counting method was employed

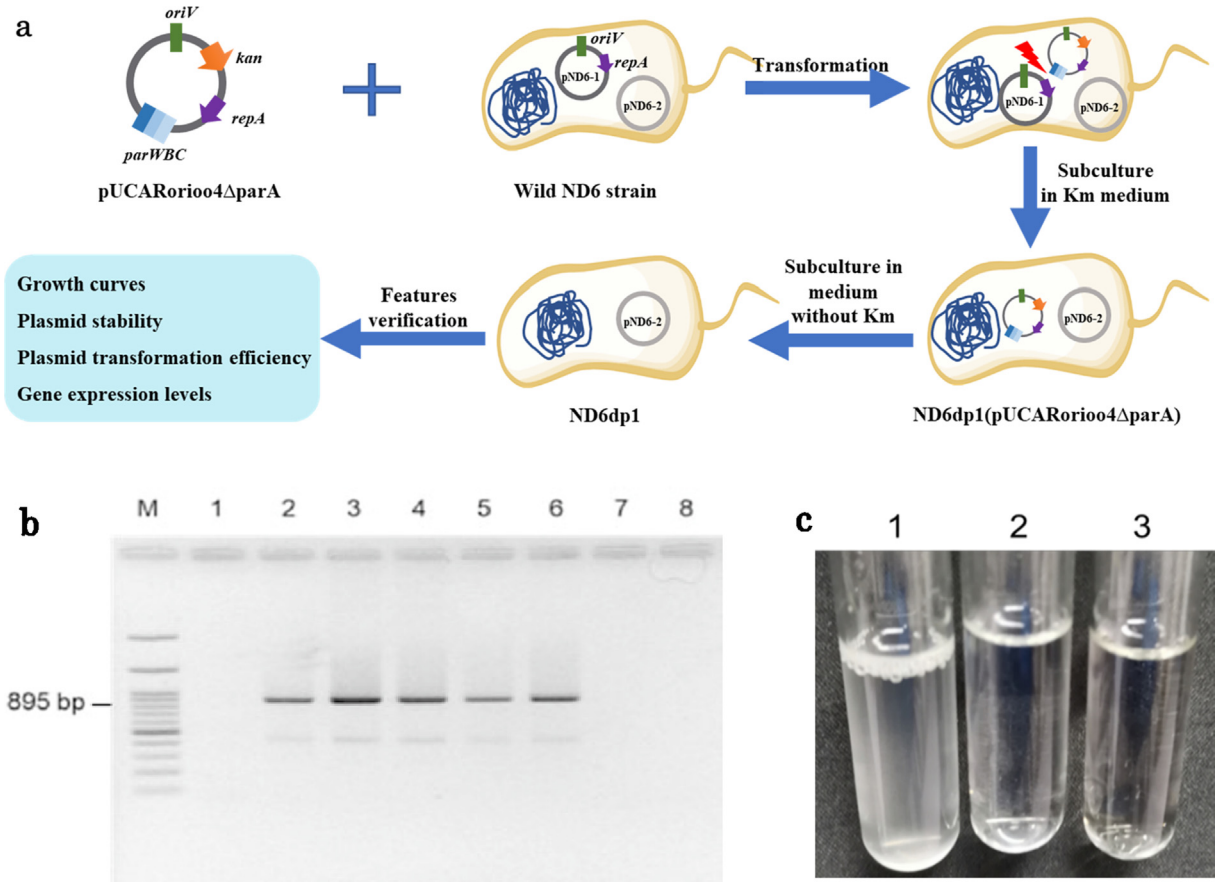

Fig. 1. Curing plasmid pND6-1 from *Pseudomonas putida* ND6 by incompatibility. (a) The elimination of the endogenous pND6-1 plasmid from *P. putida* ND6 using IncP-7 plasmid pUCARori004ΔparA. *oriV*: the origin of replication; *repA*: initiation protein-coding gene; *parWBC*: partition protein-coding genes; *kan*: kanamycin resistant marker. (b) Confirmation of the pND6-1 plasmid elimination by PCR of partial *repA* gene in pND6-1. M, DNA Marker; the PCR results of partial *repA* gene in pND6-1 with templates of ddH2O (1), plasmid pUCARori004ΔparA (2), wild ND6 strain (3–4), ND6(pUCARori004ΔparA) (5–6), ND6dp1 (7–8). (c) The growth of ND6 (1), ND6(pUCARori004ΔparA) (2) and ND6dp1 (3) on naphthalene.

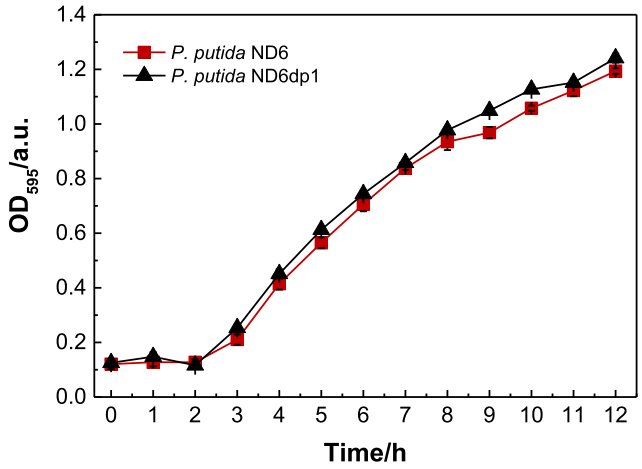

Fig. 2. The growth curves of wild-type ND6 strain and pND6-1 elimination derivative ND6dp1 in Luria-Bertani medium. Error bars represent the standard deviation of triplicate trials. *P*-value = 0.826.

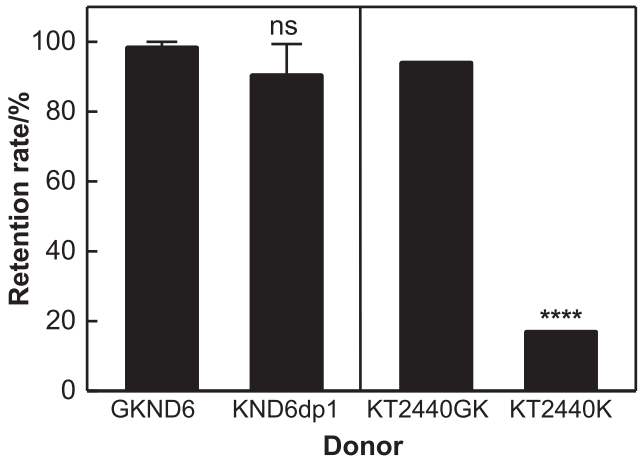

Fig. 3. Evaluation of the plasmid pND6-2 stability in ND6, KT2440GK, and their pND6-1 curing derivative ND6dp1 and KT2440K. The cultivation of KT2440GK in Luria-Bertani medium was conducted without any selection stress at 30°C with shaking at 150 rpm. Error bars represent the standard deviation of triplicate trials. Statistical significance is indicated as follows: ****P-value < 0.0001; ns, not significant.

to count the number of cells that host the plasmid pND6-2 (Fig. 3). Though there was no antibiotic pressure in culture, more than 98% of ND6 still carried plasmid pND6-2, indicating that pND6-2 was stable in its native host ND6. However, the retention rate of pND6-2 in ND6dp1 decreased, which suggested that the curing plasmid pND6-1 decreased the stability of pND6-2. We inferred that plasmid pND6-1 may contribute to the stability of the co-occurring plasmid pND6-2. To test this hypothesis, we further detected the stability of pND6-2 in another host, *P. putida* KT2440, in the presence or absence of pND6-1 (Fig. 3). As expected,

plasmid pND6-2 was much more stable in KT2440GK, which also harbored the pND6-1 plasmid, than in KT2440K, where pND6-1 is not present. After six subcultures, the retention rate of pND6-2 in KT2440GK with the co-occurrence of pND6-2 was around 96%, which was slightly lower than that in its native host ND6. Strikingly, only 17% of KT2440K still harbored plasmid pND6-2 in the absence of pND6-1, indicating that the stability of pND6-2 was lower without the co-occurrence of pND6-1.

Furthermore, we detected the expression level of genes for replication and partition of plasmid pND6-2 and the chromosome in wild-type ND6 strain and pND6-1 elimination derivative (Fig. 4). These results supported the hypothesis that the presence of pND6-1 helps increase the stability of plasmid pND6-2. Plasmid pND6-2 contained one putative replicase-encoding gene *repB*. According to the report [40], the RepB (iteron-type) replication system has unique features among plasmid-encoded proteins involved in rolling-circle replication or conjugative mobilization. After curing the pND6-1 plasmid, the mRNA level of *repB* gene decreased by 33% in ND6dp1 compared with that in wild ND6, although the transcription level of *parA* remained basically unchanged. The decreased expression level of key genes in replication and partition systems may contribute to the decreased stability of plasmid pND6-2 in ND6dp1. These results emphasized our inference that the two co-resident plasmids pND6-1 and pND6-2 interact to influence the stability of plasmid pND6-2. Currently, research on the interactions of co-resident plasmids is still limited [26]. *Agrobacterium tumefaciens* harbors two distinct megaplasmids, which confer virulence and competitiveness in the rhizosphere. The measured costs of two plasmids in the same host were not equal to the sum of single plasmid costs, which were significantly lower than expected. This phenomenon suggested that the complex epistatic interactions between two naturally co-existing megaplasmids could significantly influence the fitness of different plasmids. Furthermore, the presence of a potential synergistic relationship between co-occurring plasmids leads to improve plasmid stability. However, considering the observed influence of environmental factors on the plasmid's costs and benefits to the hosts, this synergistic relationship between co-resident plasmids may not be stable [41].

3.3. Curing plasmid pND6-1 diminished conjugative transfer of pND6-2

As per our previous report, both the co-resident plasmids pND6-1 and pND6-2 in ND6 strain could transfer to the recipient KT2440 through the single conjugative system encoded by pND6-2, though the transfer efficiency of pND6-1 plasmid was much lower than that of pND6-2 in both filter and liquid mating tests [5]. Gama et al. studied the interaction between two plasmids co-resident in the same host. One plasmid usually has a negative impact on the other co-existing plasmid, including reducing the conjugative efficiency of each plasmid, which may be due to the higher fitness cost imposed on host cells [11]. However, the related experiments were carried out to explore the influence of pND6-1 on a transfer profile of pND6-2. Here, we compared the transfer efficiency of plasmid pND6-2 in ND6 and ND6dp1 with different recipients (Fig. 5). The transfer efficiencies of pND6-2 in wild ND6 strain with the co-resident of pND6-1 were significantly higher than those in ND6dp1 in the absence of pND6-1 in all three recipients. Especially with *P. putida* KT2440 and *P. fluorescens* Pf0-1L as the recipient, the conjugative frequency of pND6-2 in ND6 was 6.3- and 3.9-fold that in ND6dp1, respectively. These results indicated that the elimination of pND6-1 markedly decreased the plasmid transfer efficiency of pND6-2 under the experimental conditions of the conjugation of the selected recipients. The consistent results were also observed from the transcript level analyses of three core coding genes (*dotG*, *dotB*, and *pilQ*) of conjugation system of pND6-2 (Fig. 6). As expected, the mRNA levels of all three conjugative genes in wild ND6 strain were higher than those in ND6dp1 by 24–70.4-fold.

Contrary to our results, it has been reported that the presence of the megaplasmid Csp_135p in N1-4 (HMT) impaired the plasmid transfer efficiency of pMTL82151, pMTL83151, pMTL85151, pMTL82151-Hox, pMTL82151-GLE, and pMTL82151-icmAB [24]. Thus, they proposed that the elimination of the endogenous megaplasmid may be an efficient strategy to promote plasmid transfer. However, in our study, both the analysis of conjugation efficiency and the transcription levels of conjugative genes indicated that the presence of pND6-1 improved the conjugative behaviors of plasmid pND6-2. One of the potential explanations for this phenomenon is that the potential regulators encoded in plasmid pND6-1 could increase the expression of conjugative system of pND6-2. The transcription and translation factors in both co-resident plasmids could influence the expression of genes on the other plasmid. Similar research has been reported on the interaction between At and Ti plasmids in *Agrobacterium tumefaciens*. Lang et al. illustrated that an opine-dependent transcriptional repressor encoded in the Ti plasmid could affect the conjugation profile of the co-occurring At plasmid [42]. Another obvious piece of evidence revealing the interaction regulation between At and Ti

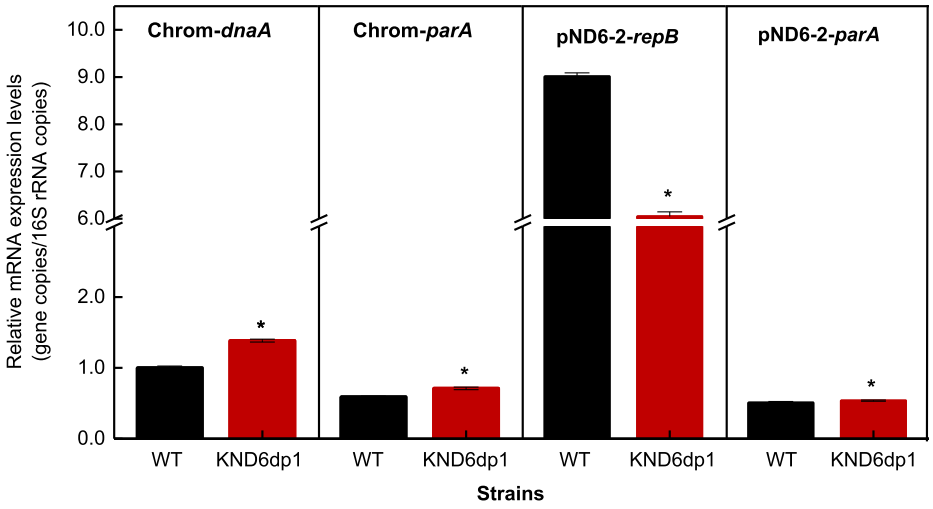

Fig. 4. The transcription level of genes for replication and partition systems of chromosome and plasmid pND6-2 in ND6 (1) and ND6dp1 (2). The relative amounts of mRNA for each gene are internally normalized to the 16S rRNA gene of each strain. Each value represents three replicates. Statistical significance is indicated as follows: * *P*-value < 0.05.

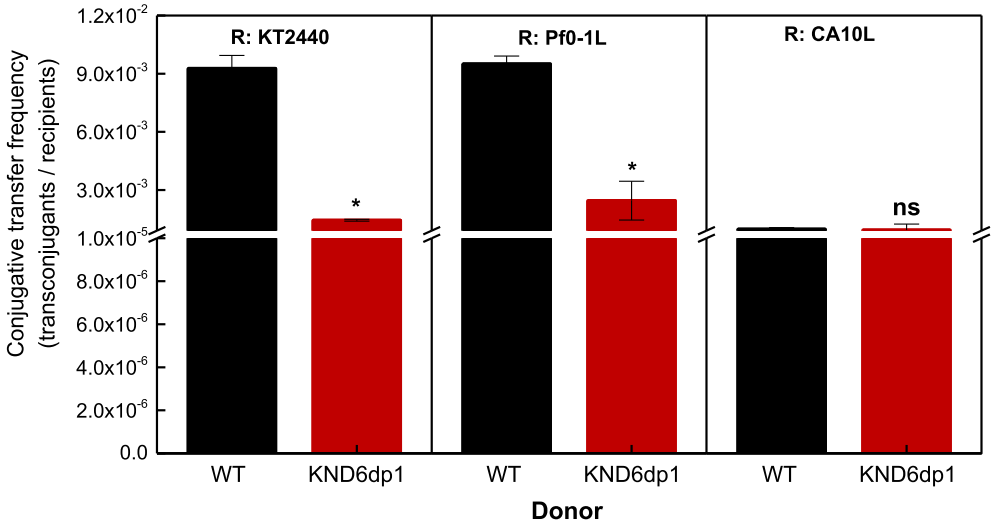

Fig. 5. The conjugative frequency of plasmid pND6-2 with different recipients. Plasmid-harboring strains of ND6 (1) or ND6dp1 (2) were used as donors. *Pseudomonas putida* KT2440, *P. fluorescens* Pf0-1L and *P. resinovorans* CA10L was used as the recipient strain. Cell mixtures were incubated for mating on a solid-agar LB plate surface for 24 h at 30°C. Error bars represent the standard deviation of triplicate trials. Statistical significance is indicated as follows: * *P*-value < 0.05; ns, not significant.

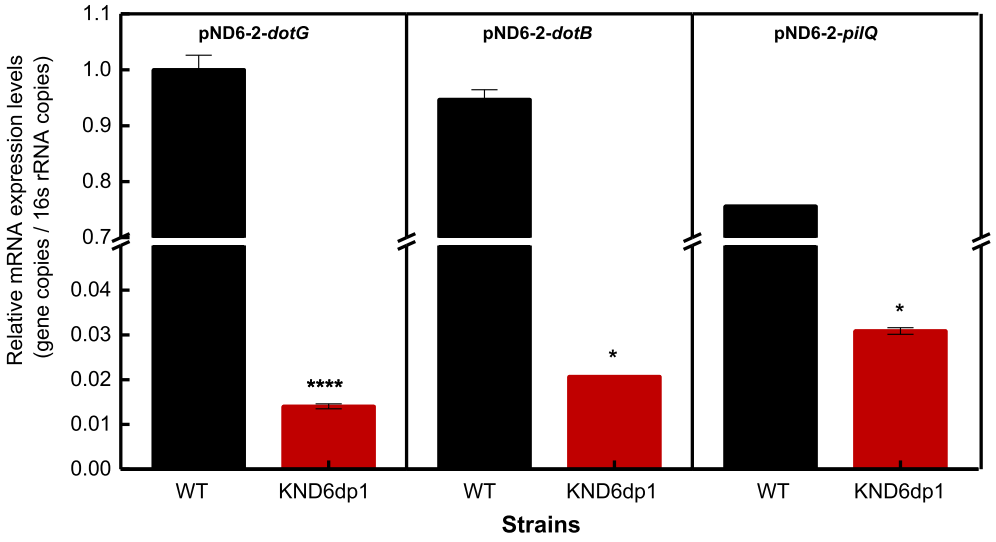

Fig. 6. The transcription level of key conjugation genes *dotG*, *dotB* and *pilQ* in ND6 (1) and ND6dp1 (2). The relative amounts of mRNA for each gene are internally normalized to the 16S rRNA gene of each strain. Each value represents three replicates. Statistical significance is indicated as follows: ****P-value < 0.0001; * *P*-value < 0.05.

plasmids is that the presence of plasmid At has decreased the expression of *vir* genes on Ti plasmid, leading to decreased costs for hosts harboring both plasmids in the presence of selective pressure [26]. Therefore, co-evolution of co-occurring plasmids could generate complex interactions between plasmids, resulting in the influence of one plasmid on the other.

It is noteworthy that the transfer efficiencies of pND6-2 from *P. putida* ND6 to *P. putida* KT2440 and *P. fluorescens* Pf0-1L were much higher than that to *P. resinovorans* CA10L by 9.7- and 9.5-fold, respectively (Fig. 5). Furthermore, a similar tendency was observed with ND6dp1 as the donor. These results suggested that plasmid pND6-2 could be transferred from *P. putida* ND6 to different *Pseudomonas* spp. with different frequencies. Sakuda et al. revealed the conjugative selectivity of plasmids R388 (IncW), pB10 (IncP-1β), pCAR1 (IncP-7), and NAH7 (IncP-9) with *P. putida* KT2440 and *P. resinovorans* CA10dm4 as the recipients [43]. This may be attributed to the interactions between the plasmid and the recipient chromosome during transcriptional regulation of transfer genes [44,45]. The plasmid stability and genotype might be affected by chromosome modification system of the host, such as methylation, restriction endonuclease system, and clustered regularly interspaced short palindromic repeat (CRISPR)-Cas system. The CRISPR-Cas system provides prokaryotic organisms with an efficient defense mechanism to degrade heterogeneous DNA [46,47]. Richter et al. proposed that increasing a new spacer in the type I-F CRISPR-Cas system would lead to the elimination of the plasmid and that increasing the number of spacers would diminish the conjugative transfer efficiency [48]. Furthermore, CA10L is a derivative of *P. resinovorans* CA10 that eliminates the IncP-7 plasmid pCAR1. CA10L, as a natural host of IncP-7 pCAR1 plasmid, may have better compatibility with IncP-7 plasmid than pND6-2, which does not belong to IncP-7 group. This could result in the degradation of pND6-2 in CA10L and diminish the conjugative efficiency.

In this study, the pND6-1 plasmid was successfully eliminated from *P. putida* ND6 by an incompatible method, the result of which confirmed for the first time that pND6-1 belonged to IncP-7 plasmid. Furthermore, the results demonstrated that the elimination of plasmid pND6-1 slightly enhanced the cell growth rate in

wild-type host but decreased the plasmid stability in both the host strains ND6 and KT2440. In addition, the transfer efficiency of plasmid in different recipients decreased in different extents. The results showed a clear demonstration of intricate interactions between two naturally co-resident megaplasmids, pND6-1 and pND6-2, that significantly affect plasmid behaviors, including plasmid stability and conjugation. By exploring the conjugation and stability of plasmid pND6-1 to pND6-2, the study on the relationship between the two endogenous plasmids in ND6 was promoted. In addition, it also indicates the importance of interactions between co-occurring plasmids, although genomics and transcriptomics should be complemented with metabolomic and proteomic analyses to reveal the potential interaction mechanisms.

Author contributions

-Study conception and design: S Li; W Yan.
-Data collection: S Wang.
-Analysis and interpretation of results: S Wang; M Liu.
-Draft manuscript preparation: S Wang; D Wang.
-Revision of the results and approved the final version of the manuscript: S Wang; S Li.

Financial support

This work was supported by the National Natural Science Foundation of China (Grant NO. 31670512), Natural Science Basic Research Plan in Shaanxi Province of China (Program No. 2018JM3039).

Conflict of interest

None.

Acknowledgments

We thank Prof. Hideaki Nojiri and Chiho Suzuki-Minakuchi for providing plasmid pUCARori004ΔparA, *P. fluorescens* Pf0-1L, *P. resinovans* CA10L strain and their guidance in curing plasmid pND6-1.

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