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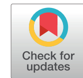
Engineering the bacterium *Comamonas testosteroni* CNB-1: Plasmid curing and genetic manipulation



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ABSTRACT

Comamonas species are attractive microbial hosts for bioremediation engineering due to their versatile metabolic capacity and diverse niches inhabited. To establish *Comamonas testosteroni* CNB-1—a common *Comamonas* strain—as a programmable cellular chassis, its indigenous 91-kb-long plasmid, pCNB1, must be eliminated; in addition, the organism has to exhibit feasiblity for effective genetic manipulation. Here we present a novel plasmid curing strategy involving rare-cutting homing endonuclease and a selection and counter-selection system. With a self-eliminating helper plasmid, we successfully removed the target plasmid pCNB1, along with the helper plasmid itself, in a single step at an efficiency of 64%. By enhancing the homing endonuclease expression, we were able to further improve the efficiency to almost 100%. The curing of pCNB1 provided a plasmid-free strain for hosting an IncP-type replicon based shuttle vehicle which can be utilized for gene circuit engineering. Additionally, using a PCR-based *Cre-loxP* system, we demonstrated an increased ability to genetically manipulate the chromosome of *C. testosteroni* CNB-1. This study provides a rapid and effective solution to eliminate indigenous plasmids, and also facilitates the development of *C. testosteroni* as a promising chassis for future remediation applications.

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1. Introduction

Comamonas is a class of Gram-negative, strictly aerobic species ubiquitously found in natural environments, such as soil, mud, water and marshes, that are often contaminated with organic pollutants and heavy metals [1]. It is also characterized by its versatile metabolism. Many *Comamonas* species can sense and pursue xenobiotic compounds through chemotaxis [2,3], and further mineralize them as sole carbon and energy sources [4–8], thereby making an important contribution to bioremediation and material cycling. Recent studies have further showed that their capacity to adapt their metabolism to changing environments and xenobiotic compounds lies in their genome sequences [9–11]. Thus, *Comamonas* has received increasing attention as attractive microbial biotechnological hosts [12,13]. Since 2000, synthetic biology has emerged into an interdisciplinary research field for cellular functionality programming [14–17], offering tremendous potential for a variety of novel applications including environmental remediation. Toward this end, *C. testosteroni* CNB-1—a common *Comamonas* strain—becomes a highly promising candidate as a versatile and programmable cellular chassis, due to the existing knowledge on its genome and proteomics as well as its genetic and biochemical backgrounds [3,18–22].

Synthetic biotechnological engineering typically relies on plasmids to deliver and carry gene circuits [23]. When introduced into a host containing an indigenous plasmid, the exogenous plasmid often faces the issue of stability, especially when the plasmids share a common replicon group. In addition, native plasmids can interfere with the replication of an expression vector, reducing its transformation efficiency [27]. Moreover, curing of indigenous plasmids can reduce the host's burden and improves its metabolic

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performance [28,29]. Thus, eliminating indigenous plasmids often represents a first step toward the creation of cellular chassis for various applications [26]. For the case of *C. testosteroni* CNB-1, it contains a 91-kb-long indigenous plasmid, named pCNB1, which is shown extremely stable according to our ten years of experience in handing and culturing the strain. Thus, our first step is to eliminate the plasmid pCNB1 to create a plasmid-free host.

Due to the intrinsic stability of native indigenous plasmids, plasmid curing is often tedious. Traditional methods for plasmid curing are based on prolonged growth under stressful conditions, such as elevated temperature or the addition of DNA intercalating agents (e.g., sodium dodecyl sulfate (SDS) and ethidium bromide), to interfere with plasmid replication [28,30–32]. Their colony screening process is also time-consuming. Additionally, many agents used are toxic to the host and can induce mutations in the chromosome. To address these issues, a few new methods have been recently developed. One approach utilizes plasmid incompatibility by introducing a high-copy-number plasmid from the same incompatibility group into the target strain [33]. However, it is subject to the prediction of plasmid incompatibility and limitation of available replication origins [34]. Another promising approach is to use the CRISPR-Cas system to induce double strand DNA (dsDNA) breaks in the plasmid DNA [35]. However, the off-target effect of the system leads to the concern over unwanted cleavages and mutations in chromosomal DNA [36].

In this study, we developed a novel strategy for plasmid curing in *C. testosteroni* by exploring the rare-cutting restriction feature of the homing endonuclease and selection-counter selection systems [37,38]. We first designed and constructed a one-step plasmid curing strategy involving an inducible self-eliminating helper plasmid. Then, using a strong constitutive promoter to drive the endonuclease expression, we further improved the efficiency of plasmid curing. We examined the resulting plasmid-free strain, which has no growth differences with the wild type, and explored the IncP type plasmid as a delivery system for gene circuit cargo. Furthermore, using a PCR-based Cre-*loxP* system, we finally demonstrated the increased feasibility of genome editing in plasmid-free *C. testosteroni*.

2. Material and methods

2.1. Strains, plasmids, and cultivation conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* NEB10β and *C. testosteroni* strains were cultured in LB (Luria-Bertani) broth or on LB agar aerobically at 37 °C and 30 °C, respectively. When necessary, kanamycin was used at a final concentration of 50 μg/mL in *E. coli* and 220 μg/mL in *C. testosteroni*. Chloramphenicol was used at 20 μg/mL in *E. coli* and 25 μg/mL in *C. testosteroni.* Sucrose (20% w/v) was used as a counter-selector in LB agar plate. L-arabinose was added to the media as inducers at concentrations indicated. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) was plated on LB agar for blue/white selection.

2.2. DNA analysis, manipulation and transformation

The sequence of the indigenous plasmid pCNB1 is available on GenBank (Accession no. NC_010935.1). The promoter sequence analysis was performed with NNPP program of BDGP [39].

Plasmid DNA was isolated from *E. coli* and *C. testosteroni* using OMEGA Plasmid Mini Kit (Omega Genetics, Beijing, China). The 91-kb plasmid pCNB1 was isolated as described previously [40]. Total DNA of *C. testosteroni* was isolated using OMEGA DNA Kit (Omega Genetics, Beijing, China). Enzymes for restriction digestion, PCR, and Gibson mix were all purchased from New England BioLabs (NEB). Q5 High-Fidelity DNA polymerase was used for amplifying fragments for plasmid construction. OneTaq 2X master mix was used for PCR verification. DNA sequencing was performed by Genewiz (Beijing, China).

Both of *E. coli* and *C. testosteroni* were prepared for electroporation by washing twice with ice-cold 10% glycerol and concentrating 100-fold. Electroporation was performed with pre-chilled 2 mm gap electroporation cuvette (Bio-Rad) and electroporated at 2.5 kV with a Bio-Rad MicroPulser. One mL of LB was added to shocked cells and recovered for 1 (*E. coli*) and 2 h (*C. testosteroni*), respectively, before plating on LB agar with appropriate antibiotics.

2.3. Plasmid construction

The primers used in this study were summarized in Table S1. Plasmid construction was performed with Gibson assembly method [41] in *E. coli* NEB10β electro-competent cells, and then transformed into *C. testosteroni* through electroporation. The constructed plasmids were confirmed by restriction digestion and sequencing. As shown in Fig. S1, the original kanamycin marker cassette in plasmid pBBR1-MCS2 [42] was replaced by the chloramphenicol marker cassette from plasmid pKTTrfp [43] to generate the intermediate plasmid pCN001. The P_{TEF1}-*lacZ*-T1T2 cassette (amplified from plasmid pCM1001), the *araC*-P_{BAD} (amplified from plasmid pKD46) [44], and I-*Sce*I sequence was synthesized (Genewiz. Beijing), and was amplified from template synthesized, two I-*Sce*I cleavage sites were introduced on the primers ISCEI-PCN001 F and ISCEI-PBAD R, the three fragments were inserted into plasmid pCN001 in an appropriate order to generate plasmid pCN1001. The cassette P_{CN}-GFP*mut3b*-T1T2 was amplified from plasmid pCP202 and assembled to plasmid pCN001 to generate plasmid pCN002. The I-*Sce*I was used to replace GFP*mut3b* of plasmid pCN002 to generate plasmid pCN003. The *sacB* cassette was amplified from pK18mobSacB [45] and inserted into plasmid pCN003 to generate plasmid pCN2001. The I-*Sce*I-GFP*mut3b* fusion plasmid pCN2002 was constructed by amplifying GFP*mut3b* from pCP202 and inserted it downstream of I-*Sce*I of pCN2001. The L-arabinose operon (*araC*-P_{BAD}) was amplified from pKD46 and used to replace promoter P_{CN} of plasmid pCN2002 to generate plasmid pCN1002.

The pEC01 plasmid was linearized by inverse amplification with primer pairs Blut-PEC01 F/R, the P_{CN}-GFP*mut3b*-T1T2 cassette was amplified from plasmid pCP202 and inserted into linearized pEC01 to generate plasmid pEC01GFP. The GFP CDS was replaced by *atzA* CDS to generate plasmid pEC01ATZA.

2.4. Integration of I-*Sce*I cleavage site into pCNB1

Amplicons spanning 1-kb upstream and 1-kb downstream of the *cnbF* locus were separately amplified from *C. testosteroni* CNB-1 total DNA. The kanamycin cassette was amplified from plasmid pBBR1-MCS2, and two I-*Sce*I recognition sites were introduced to flank the kanamycin cassette. The three fragments were assembled together through SOE (Splicing by overlap extension) PCR, and electroporated into *C. testosteroni* CNB-1. It was then plated on LB agar supplemented with kanamycin for selection. Two pairs of specific primers CON.P F/CON.KAN R and CON.KAN F/CON.P R were used to confirm the positive clones. As pCNB1 is a multi-copy plasmid, there were false positive colonies within which the native plasmid pCNB1 and pCNB1:(I-*Sce*I-kan^R-I-*Sce*I) coexisted. Primer CON.CNBF R specific to *cnbF* locus being replaced and primer CON.P F and was used to screen out false positive colonies. PCR product was sequenced for verification.

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