AN EFFICIENT METHOD FOR GENERATING GENE DELETION MUTANTS OF THE DIMORPHIC PROSTHECATE BACTERIA MARICAULALES

By

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Maricaulales, the taxonomic Latin nomenclature for a particular bacterial order, have emerged as a prominent group among marine dimorphic prosthecate bacteria, primarily due to their distinctive cellular morphology. This study selected four representative strains of Maricaulales to devise a standardized gene knockout methodology. Through a systematic optimization of conjugation transfer factors, a dependable conjugation transfer technique was successfully established. Following this, the utilization of the optimized conjugation transfer protocol facilitated the successful knockout of the genes encoding the holdfast anchor protein in all four chosen Maricaulales strains. In conclusion, this research introduces a novel and efficient gene knockout tool specifically developed for engineering Maricaulales.

Keywords*: CCUS, Dimorphic prosthecate bacteria, Maricaulales, Conjugation, Gene knockout*

Introduction

Carbon capture, utilization, and storage (CCUS) represents a pivotal technology in mitigating the environmental repercussions of carbon dioxide (CO_2) emissions. The bioconversion of CO_2 by microorganisms for energy recovery holds tremendous potential for practical applications. Among these microorganisms, Dimorphic prosthecate bacteria (DPB) stand out due to their distinct cell morphology and widespread occurrence in various environments. Nonetheless, comprehensive investigations have predominantly centered on *Caulobacter crescentus*, a freshwater member of the DPB family, primarily owing to the absence of robust gene knockout methodologies [1]. Consequently, the molecular mechanisms that govern the life activities of other DPB remain enigmatic. Within the marine DPB, *Maricaulales* serves as a prominent representative, encompassing twenty-eight recognized species. However, the lack of an efficient gene knockout technique has hindered

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comparative molecular-level studies of *Maricaulales*.Allelic exchange emerges as an effective gene knockout approach, finding utility across a broad spectrum of bacterial species [2]. The introduction of suicide vectors into bacterial cells constitutes a vital step in allelic exchange, typically achieved via transformation or conjugation. Nevertheless, the suitability of these methods for *Maricaulales* species remains uncertain. Consequently, it becomes imperative to systematically establish optimal parameters tailored specifically for *Maricaulales* species. In this study, we introduce a conjugation-based gene knockout method tailored for *Maricaulales*. This approach was subsequently applied to four exemplar *Maricaulales* strains. The advent of this gene knockout method is expected to enhance our comprehension of functional genomics and cellular processes within *Maricaulales* and other DPB, paving the way for deeper insights into their biology.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

*Glycocaulisalkaliphilus*6B-8^T, henceforth denoted as *G. alkaliphilus*,wascultivated in lysogeny broth, hereindesignated asLBor on LB agar,under optimal conditions of pH 8.0 at 30°C.*Maricaulismaris* CM11^T(hereafter referred to as*M.maris*),*Oceanicaulisalexandrii* C116-18^T(hereafter referred to as*O. alexandrii*), and *Woodsholeamaritima* CM243^T(hereafter referred to as*W. maritima*) werecultivated in Zobellmarine broth,designated as 2216E or on 2216E agar,under optimal conditions of pH 7.8 at 30°C.*Escherichia coli*WM3064(hereafter referred to as*E. coli*)was cultured in LB or LBA at a pH of 7.0 and a temperature of 37°C,withthe culture medium enriched with diaminopimelic acidat a concentration of 300 μmol/L.

Antibiotics sensitivity assay

Kanamycin, streptomycin, gentamycin, tetracycline,and chloramphenicol were used in the antibioticsensitivity assay of the four *Maricaulales* strains. Briefly, strains were pre-cultured to the mid-logarithmic phase. Cells were then washed three times with sterile phosphate-buffered solution(PBS) and then diluted to an optical density at 600 nm(OD_{600}) of 1.0 in sterile PBS.A volume of 5 μL of the cell suspension was individually placed onto the surface of LB agar or 2216E agar plates containing each antibiotic. These plates were then placed in incubation at 30°C for 7 days.

Development of a conjugal transfer system

A shuttle vector named pMR20-egfpwas used for optimizing the conjugation protocol of *G. alkaliphilus*. The conjugation between *G. alkaliphilus* and *E. coli* was carried out as described previously with some modifications[3].Briefly, *E. coli* harboring the pMR20-egfp underwent incubation in LB supplemented with tetracycline until reaching an OD_{600} of 0.5. Simultaneously, *G. alkaliphilus* was cultured in LB until attaining an OD_{600} of 0.5. Subsequently, 2 mLof the *E. coli* donor cells and 2mLof the *G. alkaliphilus*recipient cells were mixed and washed twice with LBby centrifugation. The supernatantwas discarded and the cell pelletresuspended in 30 μL LB. The suspension was spotted onto an LB agarcontaining diaminopimelic acid. After overnight incubation at 30°C, the cells were scraped off the plate, washed twice with LB, and finally resuspended in 100μLLB.The suspension was plated onLB agar containingtetracyclineand incubated at 30°C for 7 days until the transconjugants appeared.The conjugative frequency was calculated based on the number

of transconjugants divided by the number of recipient cells.

Construction of suicide vector

The construction of the suicide vector used for gene knockoutof *G. alkaliphilus*was based on the suicide plasmid named pNPTS138.A tetracycline resistance gene was required because no proper selection marker for *G. alkaliphilus*was available in pNPTS138. Thus, a fragment of the tetracycline resistance gene was amplified fromthe expression plasmidnamed pFLP3. Subsequently, two gel-purified DNA fragments were ligated, resulting in the plasmid namedpNPTS138-Tet.To build a suicide vector forthe deletion of the geneencoding the pilus assembly protein (hereafter referred to as*cpaAB*),distinct sequences for the upstream and downstream regions of the target genes were individually amplified from the genomic DNA of *G. alkaliphilus*.Then, the pNPTS138-Tet plasmid backbone was amplified.The three gel-purified DNA fragmentswere ligated, resulting in the plasmidnamed pNPTS138-TetΔ*cpaAB*. Next, plasmids were extracted and subsequently introduced into *E. coli* via electroporation[5].

Construction of the mutant strain

To delete the *cpaAB* gene in the *G. alkaliphilus* genome, pNPTS138-TetΔ*cpaAB*was introduced from*E. coli* into *G. alkaliphilus* using the optimal conjugal transfer method. A schematic overview of the process is given in Fig. 1.A single homologous recombination event of tetracycline-resistant colonies was verified by polymerase chain reaction (PCR). The resulting strain was grownto the mid-exponential phase in LB. Cells were then streaked on NaCl-free LB agar supplemented with 20% (wt/vol) sucrose and incubated at 30 °C for 5 days. Single colonies werepicked and transferred onto LB agar plates with and without tetracycline.

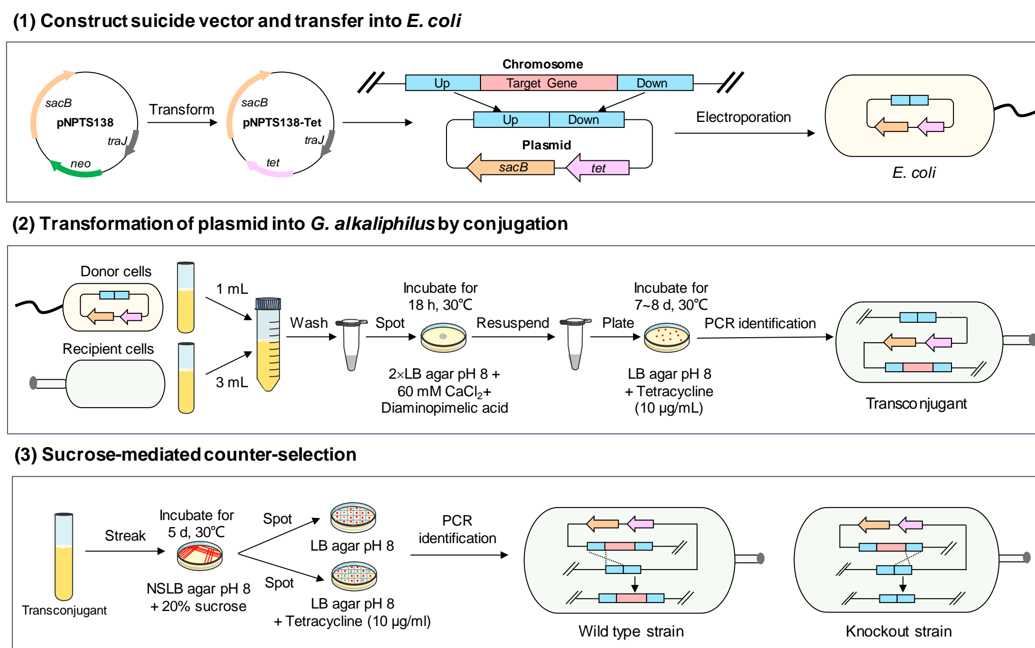

Fig.1 Overview of the generation of *G. alkaliphilus* gene deletion strain via conjugation.

These plates were then incubated at 30°C for 4 days. Subsequent identification of tetracycline-sensitive cells was achieved through PCR analysis to confirm disruption of the target gene. Consequently, the knockout strain Δ*cpaAB* was generated.Similarly, to excise the gene encoding the holdfast anchor protein (hereafter referred to as*hfaB*)in *G. alkaliphilus*, *M.*

maris, O. alexandrii,and*W. maritima*, the suicide plasmids namedpNPTS138-TetΔ$hfaB_{Ga}$, pNPTS138-CmΔ$hfaB_{Mm}$, pNPTS138-CmΔ$hfaB_{Oa}$, and pNPTS138-CmΔ$hfaB_{Wm}$were constructed, respectively. Then, the *hfaB* genes were knocked out according to the above method.As a result, the knockout strains Δ$hfaB_{Ga}$,Δ$hfaB_{Mm}$,Δ$hfaB_{Oa}$,andΔ$hfaB_{Wm}$ were obtained.

Results and Discussion

Antibiotic resistance in different Maricaulales strains

To find the most suitableresistance markers for maintaining the vectors in their properhosts, we conducted a comprehensive assessment of antibiotic resistance across four strains of *Maricaulales*.The findings revealed an inherent resistance among all four strains to kanamycin, streptomycin, and gentamycin. Notably, *G. alkaliphilus* exhibited sensitivity to tetracycline at a concentration of 10 μg/mL, while *M. maris*, *O. alexandrii*, and *W. maritima* displayed sensitivity to chloramphenicol at a concentration of 25 μg/mL.Taken together, tetracycline and chloramphenicol resistance genes serve as good candidates ofconstructing vectors for universal gene knockout in *Maricaulales*.

Optimization of a conjugation protocol for G. alkaliphilus

To enhance the frequency of conjugation, we conducted single-factor pre-experiments and orthogonal experiments. Optimization factors included: the growth phase of the recipient (early-exponential phase, mid-exponential phase, and late-exponential phase), the ratio of donor to recipient (5:1, 4:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:4, and 1:5), the duration of conjugation (6, 12, 18, and 24 h), the concentration of $CaCl_2$ in the conjugation medium (0, 10, 20, 40, and 60 mmol/L),the nutrient concentration in the conjugation medium (LB, 2×LB, 3×LB, and 4×LB), and the concentration of sucrose in the medium for pre-culturing the recipient (0, 1, 5, 10, and 20% (wt/vol)).The result of single-factor pre-experimentsrevealed that optimal conjugative frequency was achieved during the mid-exponential phase, with a donor-to-recipient ratio of 1:3, a conjugation duration of 18 h, a $CaCl_2$ concentration of 40 mmol/L, nutrient concentration of 3×LB, and a sucrose concentration of 1%, respectively (Fig. 2a-f).

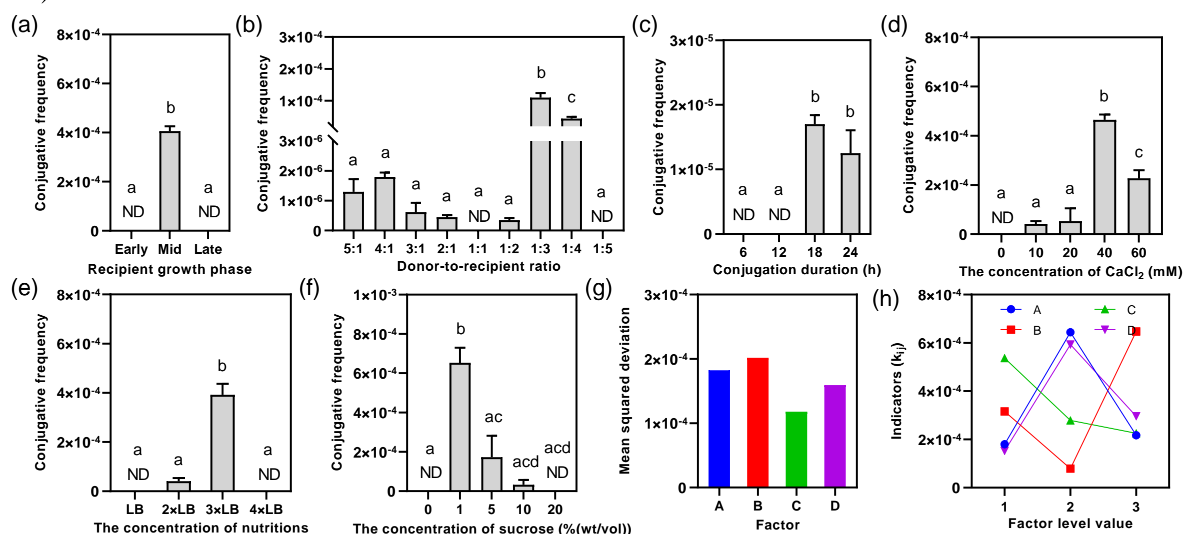

Fig.2 Effects of each factor on the conjugative frequency.

(a) Recipient growth phase. (b) Donor-to-recipient ratio. (c) Conjugation duration. (d) The concentration of $CaCl_2$. (e) The concentration of nutrients. (f) The concentration of sucrose (g)

Effect of each factor on the mean squared deviation of the conjugative frequency. (h) Influence of different factor levels on the conjugative frequency.Data represent mean ± SD of three independent replicates.ND representsno transconjugants were observed.

To further optimize the conjugative frequency of *G. alkaliphilus*, we conductedorthogonal experiments to assess the influence of donor-to-recipient ratio (A), $CaCl_2$concentration (B), nutrient concentration (C), and sucrose concentration (D) on the conjugative frequency. Following the orthogonal experimental design table, we tested a total of 9 mixtures (Table 1). Given that the single-factor experiments suggested a negligible impact of *G. alkaliphilus* growth phase and conjugation duration on other factors, we employed cells in the mid-exponential phase and conducted conjugation for 18 h in all orthogonal experiments. The sensitivity of each factor to the orthogonal test outcome was assessed using the range analysis method. Results indicated that $CaCl_2$ concentration exerted the most significant influence on conjugative frequency, followed by donor-to-recipient ratio, while sucrose concentration and nutrient concentration exhibited relatively weaker effects. Moreover, mean square deviations of the indicators were computed to further corroborate the range analysis results (Fig. 2g).

Varied levels of each factor demonstrated distinct effects on conjugative frequency (Fig. 2h). The order of factor level effects on conjugative frequency was as follows: $A_2>A_3>A_1$, $B_3>B_1>B_2$, $C_1>C_2> C_3$, and $D_2>D_3>D_1$.Consequently, the optimal combination of tested factors was determined as $A_2B_3C_1D_2$. With this refined protocol, plasmid conjugative frequency reached1.38×10^{-3} transconjugants per recipient cell, proving sufficient for conducting the gene knockout assay.In this study, we found that modifications of each factor were required, especially for the concentration of $CaCl_2$, which had the strongest impact on conjugative frequency. Ca^{2+} is commonly added to the conjugation medium to improve the frequency of conjugation.The survival rate of recipient cells during conjugation is potentially increased possibly because thatCa^{2+} is an activator of a variety of enzymes and is involved in regulating the permeability of cell membranes.Similarly,one study found that $CaCl_2$ produced higher conjugative frequency in *Streptomyces*[4].Additionally, we identified the donor-to-recipient ratio as another influential determinant shaping conjugative efficiency. The majority of studies on developing conjugation protocols for target strains have optimized donor-to-recipient cell numbers [5].

Table 1. Orthogonal experiments design, results, and analyses.

No.	Factor				Conjugative frequency
	A	B	C	D	
1	1:2	20	2×LB	0	1.44×10^{-4}
2	1:2	40	4×LB	1	3.60×10^{-5}
3	1:2	60	3×LB	5	3.60×10^{-4}
4	1:3	20	4×LB	5	4.40×10^{-4}
5	1:3	40	3×LB	0	1.12×10^{-4}
6	1:3	60	2×LB	1	1.38×10^{-3}
7	1:4	20	3×LB	1	3.64×10^{-4}
8	1:4	40	2×LB	5	8.80×10^{-5}
9	1:4	60	4×LB	0	2.00×10^{-4}
R	4.64×10^{-4}	5.68×10^{-4}	3.12×10^{-4}	4.41×10^{-4}	

Generation of the mutant strain

To assess the applicability of the optimized conjugal transfer system in *G. alkaliphilus*, we targeted the*capAB*gene for deletion.Subsequently, the pNPTS138-TetΔcpaAB construct was developed and introduced into *G. alkaliphilus* through conjugation.PCR identification revealed a conjugative frequency of 5.2×10^{-9} transconjugants per recipient cell, with a 42% positivity rate for colonies.To evaluate the impact of *capAB* gene deletion on *G. alkaliphilus*, we conducted a twitching motility assay[6]. Results indicated a significant 78% reduction in the twitching zone diameter of Δ*cpaAB* (2.0 ± 0.07 mm) compared to wild-type *G. alkaliphilus* (9.0 ± 0.1 mm), indicating the loss of twitching motility mediated by pilus.These outcomes underscore the efficiency of the conjugation-based gene knockout method for *G. alkaliphilus*.

To further verify thegenerality of this method, we targeted the hfaB gene for deletion in the four *Maricaulales* strains. Employing pNPTS138-Tet for *G. alkaliphilus* and pNPTS138-Cm for *M. maris*, *O. alexandrii*, and *W. maritima*, we successfully generated unmarked mutants for each. Conjugative efficiencies of the suicide plasmids in the *Maricaulales* strains ranged between 4.0×10^{-9} and 2.0×10^{-8} transconjugants per recipient cell, with positivity rates ranging from 67% to 100%.Further confirmation of *hfaB* gene deletion in the *Maricaulales* strains was attained through biofilm formation assays[7]. These assays demonstrated a 21.0% reduction in biofilm formation upon *hfaB* gene deletion in *G. alkaliphilus*, with similar reductions observed in $\Delta hfaB_{Mm}$ (23.5%), $\Delta hfaB_{Oa}$ (21.0%), and $\Delta hfaB_{Wm}$ (19.5%).

Currently, electroporation and conjugation are the predominant gene knockoutmethods in DPB research.In this study, after numerous unsuccessful attempts to deliver the vector into *G. alkaliphilus* cells through electroporation, we attained success by employing conjugation as a gene knockout method for this microorganism.Electroporation allows the transfer of all DNA types suspended in water, unlike conjugation, which is limited to DNA transfer between bacterial cells. However, electroporation conditions are harsh, resulting in greater cell damage (between 50% and 70% of cells exposed to high electric field strengths are killed). In contrast, conjugation is gentler, avoiding DNA degradation by extracellular nucleases, making it suitable for *Maricaulales*.

Conclusions

In this study, we developed an efficient gene knockout method for *Maricaulales* strains.The genome editing tool was successfully utilized to delete genes. It is expected that through some modifications, the system described abovewill be extended to other DPB strains.This tool opens the door to a range of powerful genetic approachesthat can be used to interrogate the biology of DPB.The devised methodology may expedite the application of *Maricaulales* in CCUS.

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