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RESEARCH ARTICLE

# Genetic insights into biofilm enhancement: The impact of *yicC* gene disruption in *Pseudomonas plecoglossicida* NAN2

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## Abstract

Biofilm formation plays a pivotal role in mediating plant-microbial interactions. However, despite its significance, the molecular mechanisms underlying biofilm formation by *Pseudomonas plecoglossicida* remain largely unexplored. Thus, this study aimed to identify genetic determinants involved in biofilm regulation by constructing a transposon mutant library of *P. plecoglossicida* NAN2. From the 2000 mutants screened, one mutant (M770) exhibited significantly superior biofilm production and distinct colony morphology and pellicle formation compared with the wild-type strain. Arbitrary PCR analysis revealed that the disrupted gene in M770 encoded a YicC family protein. Interestingly, despite the enhanced biofilm formation, root colonization assays indicated that the mutation in the *yicC* did not impair the ability of M770 to colonize plant roots. To the best of our knowledge, this is the first report on a transposon mutant with enhanced biofilm formation linked to a mutation in the *yicC* gene in *P. plecoglossicida* NAN2. These findings offer new insights into the regulatory role of the *yicC* in biofilm formation, providing potential targets for modulating biofilm dynamics in plant-microbe systems.

**Keywords:** biofilm formation; *Pseudomonas plecoglossicida*; SEM; transposon mutagenesis; YicC protein family

## Introduction

Excessive use of agrochemicals, such as fertilizers and pesticides, poses significant challenges for both the agricultural sector and the environment. Prolonged use of these chemicals leads to a decline in soil water-holding capacity, reduced fertility and imbalances in soil nutrient composition (1,2). Sustainable agriculture aims to maintain soil health and microbiomes, facilitating nutrient cycling, organic matter decomposition and soil integrity without compromising ecological balance. Certain bacterial species within the soil microbiome can be formulated into biofertilizers, commonly referred to as Plant Growth-Promoting Rhizobacteria (PGPR) (3,4). PGPR are widely applied to improve plant growth and agricultural productivity, with commercialized strains belonging to various genera including *Agrobacterium, Azospirillum, Azotobacter, Bacillus, Burkholderia, Delftia, Pseudomonas, Rhizobium* and *Serratia* (5). However, despite their potential role in plant growth, the effective use of PGPR in sustainable agriculture remains challenging. Factors such as abiotic stress, climate change and competitive growth against native microbiomes hinder the survival and growth promoting effects of biofertilizer strains (6). *Pseudomonas* species are well-known, ubiquitous Gram-negative gammaproteobacteria, which are recognized for their remarkable diversity and potent Plant Growth Promoting (PGP) traits. These bacteria are widely distributed in soil, water and other environmental niches, where they coexist with various host organisms and engage in diverse interactions (7,8). *Pseudomonas* species play a crucial role in promoting plant growth and biological control against plant pathogens (9,10). *P. plecoglossicida*, a PGPR, has been shown to significantly enhance plant growth (11). These are ideal candidates for application as plant and soil inoculants for growth promotion and disease management (11,12).

Biofilm formation serves an essential function in plant bacterial interactions, allowing bacteria to adhere to root surfaces and establish stable communities (13–16). Mature biofilms can enhance PGP functions, protection from adverse conditions such as antibiotics, desiccation or protozoan predation, improve nutrient uptake and increase bacterial populations, thereby facilitating the synthesis of secondary metabolites that combat pathogens during plant-microbe interactions (17). Secondary metabolites produced by rhizosphere microbes significantly influence biofilm formation at the root. Biofilms maintain a critical cell mass at the root surface, benefiting the host plant (18). Major extracellular components of biofilms, such as polysaccharides and proteins, enhance root colonization, thereby supporting plant immunity modulation and efficient nutrient cycling in the rhizosphere (19). Additionally, biofilms help to protect plants from abiotic stress. Due to this versatile properties, biofilm-forming PGPR are subjected to active investigation for sustainable agriculture (20). Genetic modifications, such as mutations, can enhance biofilm production in bacteria. A study on hyper-biofilm forming mutants of *B. amyloliquefaciens* demonstrated their ability to withstand higher drought stress in tomato plants (21). Similar genetic modifications in various bacterial species have influenced excessive biofilm production. Previous research has shown that in *P. aeruginosa* B18 and *P. chlororaphis* subsp. *aurantiaca* JD37 strains, the *efp, flgBCDEFGHI, hfq* and *motAB* genes are associated

with biofilm formation (22, 23).

However, most regulatory genes involved in biofilm formation remain unclear and they may influence biofilm formation to varying extents. In this study, we demonstrate a hyper-biofilm producing *P. plecoglossicida* through Tn5 transposon mutagenesis. Additionally, we provide novel insights into the genetic basis of biofilm formation in *P. plecoglossicida* NAN2 and contribute to the understanding of its potential as a plant-beneficial microbe.

We utilized Tn5 transposon random mutagenesis to generate a mutant library and screened approximately 2000 mutants to identify strains exhibiting enhanced biofilm formation. Further, we investigated the mutated genes and their effectiveness in root colonization. Biofilm formation by *Pseudomonas* enhances its colonization of plant roots. Therefore, hyper-biofilm forming mutants have extraordinary applications in sustainable agriculture.

## Materials and Methods

### Bacterial strains

*P. plecoglossicida* NAN2 was previously isolated from the rice rhizosphere and utilized in this transposon mutagenesis study to investigate biofilm formation (24). *E. coil* MFDλpir (CRBIP 19.334) carrying the pIT2 plasmid with a mini-Tn5 transposon was adopted as the Tn donor. Strains were cultured in Luria-Bertani (LB) medium supplemented with relevant antibiotics: ampicillin (100 µg/mL) and diaminopimelic acid (DAP) (60 µg/mL) for the donor strain and tetracycline (15 µg/mL) for the selection of transposon mutants.

### Construction of transposon insertion mutant library

Equal volumes of overnight cultures of the donor and recipient strains were mixed, centrifuged and the pellet was resuspended in 20 µL of LB broth. The suspension was placed onto LB medium and incubated at 37 °C for 1 hr to enable conjugation. Cells were then dissolved in LB broth, plated onto LB agar containing tetracycline (15 µg/mL) and incubated at 37 °C for 24 hr (25).

### Biofilm formation assay

#### Microtitre plate assay for biofilm quantification

Tn5 mutants were screened for biofilm formation using the crystal violet staining assay, following the method described by previous study (26). Briefly, 96-well microtiter plates were inoculated with 160 µL of overnight grown LB broth culture to an optical density OD600 of 0.1. The plates were incubated at 37 °C for 24 hr to allow biofilm development. After incubation, the liquid contents of each well were gently removed using a pipette. To eliminate non-adherent cells, the wells were washed three times with sterile water. The attached biofilms were stained with 200 µL of 0.1 % (w/v) crystal violet for 30 min at room temperature. Excess stains were removed by washing the wells three times with sterile water. The retained crystal violet was then solubilized in 70 % ethanol and the solution was transferred to a 96-well microplate. Absorbance was measured at 595 nm using a microplate reader (N10588 - Rev 1.0 - Thermo Scientific Multiskan GO). The mean ± Standard Deviation (SD) was calculated from triplicate measurements. Statistical significance was determined using one-way ANOVA, followed by Tukey's post-hoc test.

#### Test tube method

Biofilm formation was also assessed using the tube method as described in a previous study (27). Overnight cultures of each mutant were grown in LB broth with shaking at 150 rpm at 37 °C and the OD was adjusted to 0.1 at 600 nm. Subsequently, 30 µL of cultures were inoculated into 3 mL of LB broth and incubated under static conditions at 37 °C for 24 hr. After incubation, the cultures were discarded and the tubes were washed slowly with distilled water. Adherent bacterial cells were stained with 0.1 % crystal violet for 30 min at room temperature and rinsed with distilled water to remove excess stain. Biofilm formation in mutants was compared with the wild-type strain.

#### Colony morphology and pellicle formation assay

Wild-type and mutant strains were patched onto Congo red agar and incubated for 48 hr to observe morphological differences (28). Pellicle formation was assessed using a standard protocol described previously (29).

#### Root colonization assay

Dehusked rice (*Oryza sativa* L.) seeds were surface sterilized with 0.1 % mercuric chloride for 3 min, followed by thorough washing with sterile water to remove chemical residues (30). After air-drying, the seeds were placed in Murashige and Skoog (MS) medi um (31) and grown under an 8 hr light and 16 hr dark photoperiod for 14 days. The seedlings were carefully removed and their roots were immersed in wild-type and mutant bacterial suspensions ($10^7$ CFU/mL) for 1 hr. Subsequently, the seedlings were transferred to MS broth and incubated for 10 days. After incubation, the root samples were treated with 2 % glutaraldehyde for 4 hr. Samples were then rinsed with phosphate buffer and progressively dehydrated twice using 20 %, 40 %, 60 %, 70 %, 80 % and anhydrous ethanol for 20 min each. Samples were sputter-coated with chromium and then analyzed using a LEO 1450 VP Scanning Electron Microscope (SEM) (32).

### Transposon insertion confirmation

#### Amplification of transposon specific internal sequence

Polymerase Chain Reaction (PCR) was employed to confirm the presence of the Tn5 transposon in the mutant strain. A single bacterial colony from the mutant, donor and wild-type strains was diluted in 10 µL of distilled water and used as a template for the PCR. The PCR was conducted in a total reaction volume of 20 µL, comprising 1 µL each of the primers pIT2 INFP and pIT2 INRP, 10 µL of Ampliqon 2X master mix, 1 µL of template and 7 µL of nuclease-free water. Amplification of the Tn5 transposon-specific region (836 bp) was carried out using the primers pIT2 INFP and pIT2 INRP (Table 1) under the following conditions: initial denaturation at 95 °C for 5 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec, extension at 72 °C for 1 min and final extension at 72 °C for 4 min.

The PCR-amplified products were evaluated by 1 % agarose gel. This PCR based confirmation strategy is commonly used to verify the stable insertion of the Tn5 transposon into bacterial genomes (33).

#### Amplification of transposon vector junction to confirm no plasmid replication

The lack of plasmid replication was confirmed by amplifying a

508 bp vector junction region using the primers Ext pIT2FP and pIT2 OUTRP under the following PCR conditions: an initial denaturation at 95 ℃ for 5 min, followed by 30 cycles of denaturation at 95 ℃ for 30 sec, annealing at 58 ℃ for 45 sec, extension at 72 ℃ for 1 min and final extension at 72 ℃ for 4 min. The PCR products were analyzed on a 1 % agarose gel. The absence of amplification of the transposase gene in the Tn5 mutants confirmed successful transposon integration and loss of the plasmid backbone, as described in previous study (34).

#### Identification of transposon insertion site

Arbitrary PCR was performed to identify the transposon insertion site using the primers pIT2 RP1 and pIT2 RP2, along with the arbitrary primers AB1 and AB2 (35) (Table 1). The PCR amplified products were purified, evaluated on 1 % agarose gel and subsequently subjected to Sanger sequencing. The obtained nucleotide sequences were analyzed using the BLASTn program in NCBI database to identify the disrupted gene (36).

**Table 1.** Primer sequences used in this study

| Sl.No | Primer name | Sequence |
|---|---|---|
| 1 | Ext PIT2 FP | GTGGCGATAACTCAAAGAGGTGGTG |
| 2 | PIT2 OUT RP | AAGCATCACCATCACCATCACTACC |
| 3 | PIT2 InFP | ACCGCCCAGTCTAGCTATCGCCATG |
| 4 | PIT2 INRP | GGGTCGTTAAATAGCCGCTTATGTC |
| 5 | PIT2 RP1 | AAGCATCACCATCACCATCAC |
| 6 | PIT2 RP2 | CCATCACCATCACTACCCG |
| 7 | AB1 | GGCCACGCGTCGACTAGTACNNNNNNN NNNGATAT |
| 8 | AB2 | GGCCACGCGTCGACTAGTAC |

## Results

### Screening for biofilm-enhanced mutant

Tn5 mutagenesis of *P. plecoglossicida* NAN2 produced approximately 2000 mutant colonies. An overview of the transposon mutagenesis workflow is presented in Fig. 1. Among the screened mutants, one isolate, designated M770, exhibited a marked increase in biofilm production compared with the wild-type strain. Biofilm assays using a 96-well microtiter plate revealed that M770 consistently produced significantly higher OD 595 values following crystal violet staining, indicating an increased accumulation of biofilm biomass relative to the wild type (Fig. 2A). This finding was further corroborated by qualitative visualization using the borosilicate glass tube assay. The M770 mutant exhibited visibly denser and more intense biofilm staining on the inner surface of the glass tube than the wild type, suggesting stronger surface attachment and extracellular matrix production (Fig. 2B, C). These results demonstrate that the transposon insertion in M770 led to a significant enhancement of biofilm formation, prompting further investigation into the genetic basis of this phenotype.

### Pellicle development and altered colony morphology in M770

Pellicle formation at the air liquid interface signifies mature biofilm as well as substantial extracellular matrix production and intracellular interactions. Under static growth conditions, the M770 mutant produced a prominent pellicle layer at the air-liquid interface, in sharp contrast to the wild type of strain, which displayed only a thin or poorly developed pellicle (Fig. 3A, B). The pellicle formed by the M770 strain appeared thicker and stronger, indicating that the mutant significantly impacted biofilm-related pathways promoting increased aggregation and extracellular matrix secretion.

Characterization of phenotype alterations linked to M770 was assessed with Congo red staining, which selectively binds to Extracellular Polymeric Substances (EPS), notably curli fibers and other polysaccharides in the biofilm matrix. It was observed that the M770 mutant displayed distinct colony morphology compared with the wild type when supplemented with Congo red. The mutant colonies were darker in color and had greater

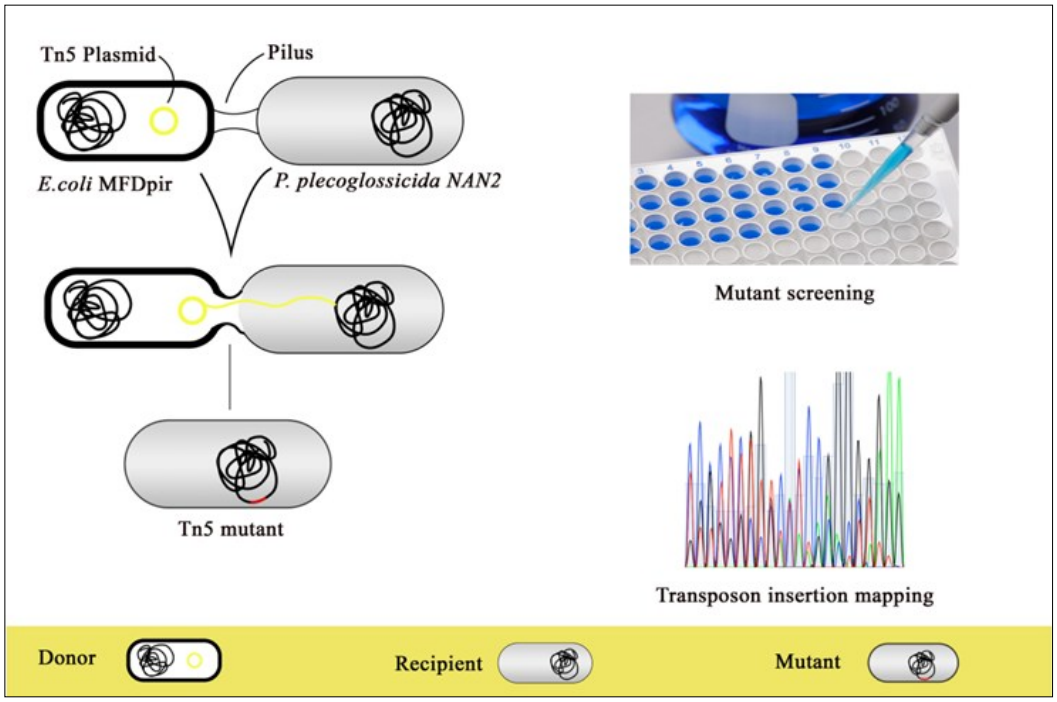


**Fig. 1.** Workflow of transposon mutagenesis in *P. plecoglossicida* NAN2.

*P. plecoglossicida* NAN2 was subjected to biparental mating with *E. coil* MFDλpir carrying the Tn5 transposon plasmid. Transconjugants were selected on tetracycline (Tetra$^r$) plates. Mutants were screened for enhanced biofilm formation using a microtiter plate assay, followed by identification of transposon insertion site through arbitrary PCR and sequencing analysis.

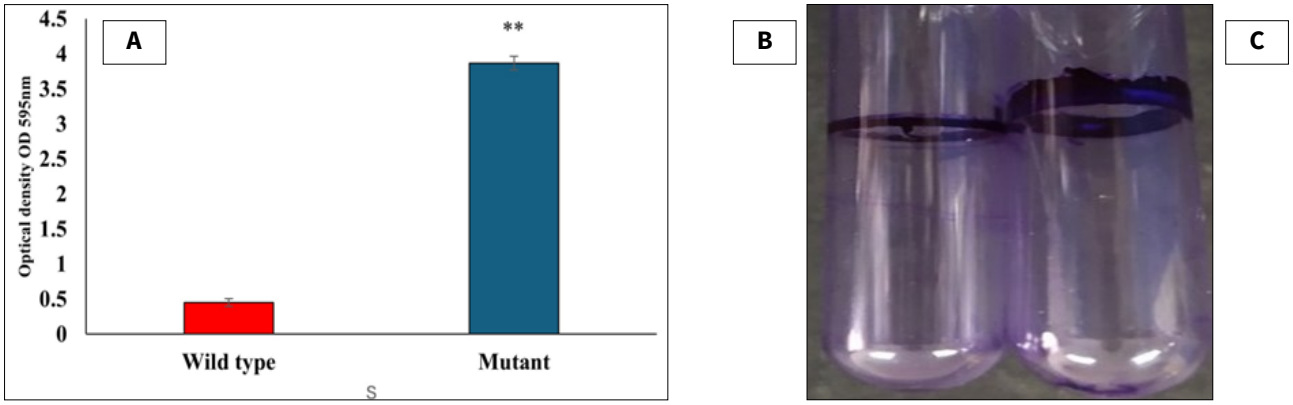


**Fig. 2.** Biofilm formation assay.

A) The microtiter plate assay evaluates biofilm formation via a plate reader. Statistical significance was evaluated using a one-way ANOVA, followed by Tukey's post-hoc test. A p-value < 0.0001 was considered highly significant (**); Ring-shaped biofilm formation in test tubes B) Wild type; C) M770

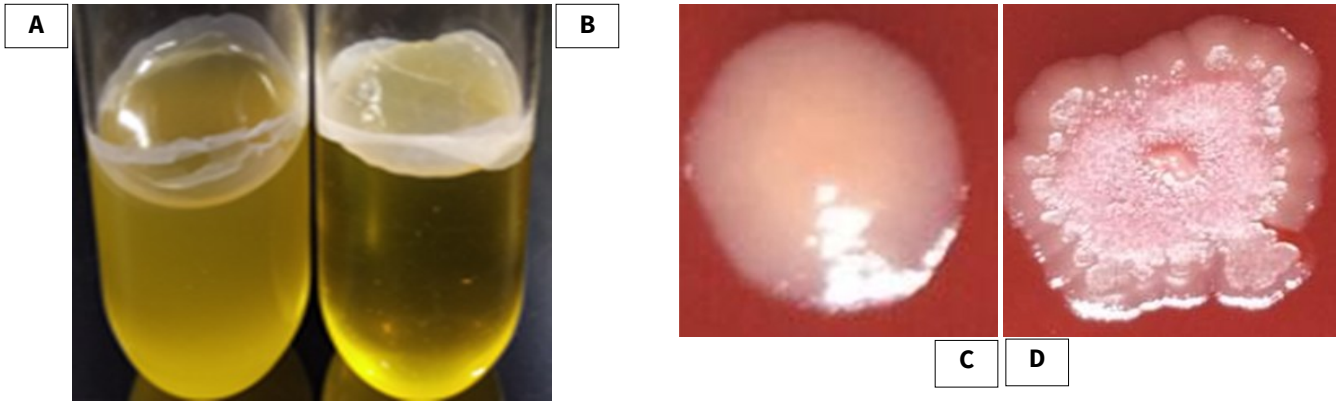


**Fig. 3.** Pellicle formation and colony morphology assay.

Pellicle formation in LB broth A) Wild type; B) M770; Colony morphology on Congo red agar plates; C) Wild type; D) M770

wrinkling. The enhanced dye binding showcases higher EPS production (Fig. 3C, D). Morphological modifications in M770 indicate a hyper-biofilm phenotype and further substantiate the hypothesis that Tn5 insertion in M770 results in genetic alterations that augment biofilm-associated characteristics.

These findings imply that Tn5 insertion in M770 improves both structural (pellicle) and surface-associated (colony morphology) aspects of the biofilm, suggesting that a strong regulatory gene in biofilm development was mutated.

## Comparative assessment of root colonization by wild type and M770 mutant strains

Since biofilm formation is a key aspect of successful root colonization, the root colonization of the mutant and wild type strains was assessed through SEM. Under controlled conditions, the wild-type *P. plecoglossicida* NAN2 and the M770 mutant were inoculated onto the roots of rice seedlings and the colonization patterns were imaged.

The results demonstrated that the M770 mutant effectively adhered to and colonized the root surface, forming dense bacterial aggregates like those observed in the wild type strain (Fig. 4). No significant variations were observed in the attachment of bacterial cells between the wild-type and mutant strains. This indicates that Tn5 insertion in the M770 mutant did not negatively impact or enhance the critical characteristics necessary for root colonization.

Additionally, these results indicate that the mutation influencing the hyper-biofilm phenotype in M770 did not impair its capacity to form plant-microbe relationships. The mutant maintained its rhizosphere competence, underscoring its future potential for plant growth-promoting applications despite the modified regulatory route influencing biofilm formation.

## Confirmation of transposon insertion and absence of vector junction

PCR amplification was conducted with plasmid-specific primers to study the retention of the plasmid and the insertion of the Tn5 transposon in the recipient strain. Among the three strains (donor, wild type and M770), a 508-bp fragment was detected only in the donor strain, confirming the absence of the donor plasmid in the wild type and M770 mutant (Fig. 5A). This established that the observed features could not be plasmid-mediated and caused by the integration of the transposon into the genome of the recipient bacteria.

Transposon insertion was validated by PCR amplification using Tn5 transposon-specific primers and genomic DNA from the donor, wild type and mutant strains was analyzed. An 863-bp fragment was observed in the donor and the M770 mutant, whereas no products were detected in the wild-type strain (Fig. 5B), validating the existence of the Tn5 region within the mutant genome and its consistent incorporation.

The results collectively demonstrate that the M770 mutant harbors a stably integrated Tn5 transposon and lacks the delivery vector backbone, confirming the successful generation of a stable, plasmid-free mutant suitable for subsequent functional studies.

## Identification of transposon insertion site

The specific genomic site of Tn5 transposon insertion in the

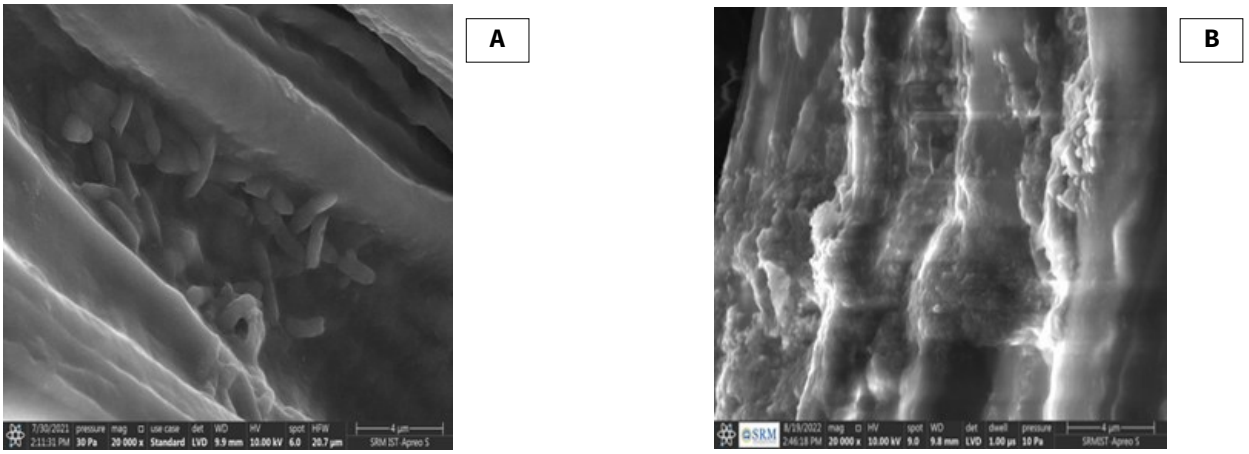


**Fig. 4.** Scanning electron micrograph of paddy roots colonized by *P. plecoglossicida* NAN2 and M770.

A) Root colonization by NAN2; B) Presence of M770 strain on root surface

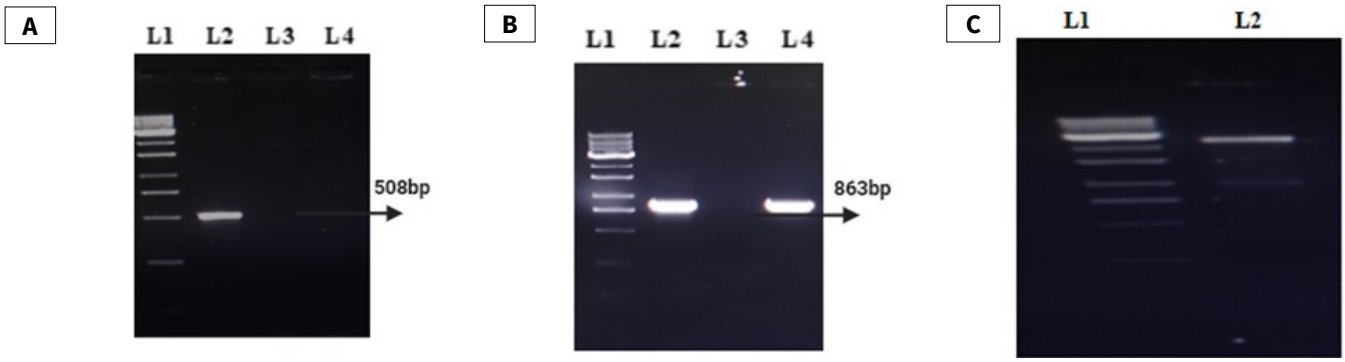


**Fig. 5.** Screening of Tn5 Insertion.

A) Amplification of plasmid sequence. Lane1: M,1kb DNA marker (Gene Directx), Lane 2: Donor (Positive control), Lane3: Wild type (Negative control), Lane 4: M770 (Transposon mutant); B) Confirmation of Tn5 insertion in recipient genome. Lane 1: M,1kb marker, Lane 2: Donor, Lane 3: Wild type, Lane 4: M770; C) Arbitrary PCR Lane 1: M,1kb DNA marker, Lane 2: M770

hyper-biofilm forming mutant M770 was identified using arbitrary PCR (Fig. 5C). Sequencing and BLAST analysis of the amplified product showed that the transposon was integrated into a gene exhibiting significant sequence similarity to the *yicC* gene. This gene is predicted to encode a conserved protein implicated in regulatory processes, although its specific role in *Pseudomonas plecoglossicida* remains largely uncharacterized.

The insertion of Tn5 at this locus likely disrupted normal gene function, coinciding with the hyper-biofilm phenotype reported in the M770 mutant.

This study indicates that the YicC family protein may function as a negative regulator of biofilm formation and its disruption might prevent the suppression of biofilm-related pathways, particularly those associated with pellicle development and extracellular matrix production.

## Discussion

Mutational approaches, such as transposon mutagenesis, have demonstrated its efficacy in identifying essential genes associated with biofilm dynamics across several bacterial species (37). A similar study reported that Tn5 mutagenesis in the intergenic region of a DNA-binding protein and the Aer aerotaxis receptor negatively regulated biofilm formation in *P. protegens* Pf-5 (38). In another investigation, biofilm production was significantly enhanced by the Tn5-45 mutant of *Burkholderia seminalis* strain R456, where Tn5 insertion disrupted a gene encoding a histone H1-like protein, resulting in increased production compared with the wild type (34). However, no study has thus far explored plant-microbe interactions in *Pseudomonas plecoglossicida* using transposon mutagenesis. To the best of our knowledge, this is the first report focusing on Tn5 insertion in a gene encoding a YicC family protein in *Pseudomonas plecoglossicida* NAN2, which enhanced biofilm formation. While the exact mechanism of increased biofilm formation requires further investigation, available data suggest that the disruption of the *yicC* gene leads to this effect. The YicC belongs to a stress-induced protein family and studies on this gene are limited. One study described a *Clostridioides difficile* CD25890 mutant, belonging to a YicC-like protein family, which exhibited enhanced sporulation (39). In another study, mutated *yicC* in *Salmonella* was shown to suppress persistence (40).

In the M770 strain, Tn5 transposon insertion in the *yicC* region resulted in a similar stress-induced response event, namely hyper-biofilm formation. However, hyper-biofilm production was observed even under non-stress conditions, notably increasing the response. In *Pseudomonads*, the YicC family protein may play a regulatory role in biofilm formation under normal conditions, suggesting a constitutive activation of stress-related pathways. Moreover, *yicC* may serve a broader role in modulating cellular adaptation beyond persistence and sporulation, possibly acting as a checkpoint between major stress response pathways. While the specific molecular mechanism remains to be elucidated, the observed investigation highlights a potential link between the *yicC* family protein and pathways controlling stress response behavior in this strain. The regulation of colony morphology and biofilm

development is a complex process that frequently involves multiple signaling cascades (41). Further investigation on *yicC* will reveal the exact mechanism behind hyper-biofilm formation by the M770 strain.

Rhizobacteria establish microcolonies or clusters on root surfaces, which are infrequent and non-uniform in patterns (42). Colonization of the M770 strain on the root surface remains like that of the wild type, despite the disruption of the *yicC* gene. These results suggest that the gene disrupted in the M770 mutant does not alter root surface colonization; the enhanced biofilm formation may even support stable root association. Such traits are crucial for the potential application of this mutant as a bioinoculant in agricultural settings.

These findings contribute to our understanding of genetic factors influencing biofilm dynamics in plant associated *Pseudomonas* sp., with implications for both microbial ecology and biocontrol applications. Moreover, YicC proteins, including YloC, are known to regulate small RNAs and function as endoribonucleases. These proteins belong to a highly conserved YicC family protein commonly distributed across bacterial species (43).

This study shows that the YicC family protein may play a key role in modulating biofilm development by impacting the expression of regulatory pathways associated with colony morphology and extracellular matrix production.

## Conclusion

The generated random transposon mutagenesis library of *Pseudomonas plecoglossicida* NAN2 identifies genes involved in biofilm regulation via biparental conjugation. A mutant with Tn5 insertion in the *yicC* gene showed a significant increase in biofilm formation with observable alterations in colony morphology. Our findings indicate that the YicC family protein is essential for regulating biofilm formation in *P. plecoglossicida* NAN2, presumably by influencing cellular signaling networks or stress response mechanisms. While the exact molecular mechanisms require further study, the significant phenotypic consequences resulting from gene disruption suggest that YicC may function as a regulator of biofilm development.

The finding of this regulatory protein paves the way for enhanced comprehension of the genetic and physiological regulation of biofilm dynamics in beneficial rhizobacteria. This understanding can be leveraged to improve the efficacy of bioinoculants in sustainable agriculture by augmenting their root colonization efficiency and durability. Furthermore, these findings may have applications in industrial biotechnology, where manipulating biofilm formation is advantageous for optimizing microbial activities. Further research focusing on the proteomic analysis of the mutant strain, alongside the functional identification of *yicC* associated pathways, will yield a deeper understanding of its role in bacterial behavioral changes.

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## Authors' contributions

NK conducted the experiments and contributed to writing the original draft. GG participated in the investigation, writing of the original draft, as well as reviewing and editing the manuscript. IS conducted the experiments, participated in the investigation, contributed to writing the original draft, conceptualization the study, supervision the work and participated in manuscript review and editing. All authors read and approved the manuscript.

## Compliance with ethical standards

**Conflict of interest:** The authors do not have any conflict of interest to declare.

**Ethical issues:** None

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