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MUTATION OF *RSMC* GENE FROM *SERRATIA MARCESCENS* QBN USING CRISPR-CAS9 TO ENHANCE PRODIGIOSIN SYNTHESIS

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SUMMARY

Prodigiosin is a bright red pigment, which is produced by various gram-negative and gram positive bacteria include *S. marcescens, Hahella chejuensis, Vibrio psychroerythrus, Streptomyces coelicolor* and many marine bacteria such as *Pseudomonas sp, Vibrio sp*. Prodigiosin has garnered considerable interest among the scientific community due to its immunosuppressive and anticancer properties against various drug-resistant cancer cell lines. In this study, we utilized the biosynthetic pathway of prodigiosin (Pg), using CRISPR/Cas9 gene editing techniques to eliminate the gene inhibiting Pg synthesis, known as *rsmC*, aiming to enhance the ability to synthesize Pg from *S. marcescens* strains. The *rsmC* gene of *S. marcescens* QBN was randomly mutated using the CRISPR-Cas9 system with the pCasPA vector (containing Cas9) and pCRISPR (carrying the spacer segment and sgRNA synthesis). A 20-nucleotide spacer segment was selected on *rsmC* preceding the TGG triplet at the 3' end (PAM, the recognition site of Cas9) and inserted into pCRISPR between two *Bsa*I sites. Following the binding of sgRNA to Cas9, mutations occurred in the nucleotides of the *rsmC* gene, resulting in the cleavage of the double-stranded DNA at the targeted site. A sequence homologous to the *rsmC* gene, lacking 100 nucleotides, was introduced into the cell and will involve the cleaved DNA's repair mechanism. The mutated *rsmC* strain of S. *marcescens* QBN synthesized Pg at 216 - 231 mg/L, higher than the original strain (180 mg/L). Furthermore, HPLC results and ^{1}H spectral analysis showed that the synthesized red pigment from the mutated strain *S. marcescens* QBN was prodigiosin. These findings provide a basis for further improving the productivity of Pg synthesis in the *S. marcescens* QBN strain and serve as a basis for editing genes in other microorganisms using the CRISPR-Cas9 system.

Keywords: CRISPR-Cas9, pCasPA, pCRISPR, Prodigiosin, RsmC, *Serratia marcescens*

INTRODUCTION

Prodigiosin, a naturally occurring red pigment synthesized from specific microbial strains, particularly from the strain *Serratia marcescens*, has gained attention in recent years for its valuable properties such as antibacterial, antifungal,

antioxidant, and notably, anticancer abilities. This compound holds promise as a novel and effective ingredient in anticancer drug synthesis. The exact targets of Pg and its derivatives are not clearly defined, but their anticancer activity is believed to be closely related to the apoptosis process of cancer cells (Khanafari *et al.,* 2006; Montaner *et al.,* 2000; Pandey *et al.,* 2007). Two derivatives of Pg, metacycloprodigiosin and undecylprodigiosin, have also been studied and shown to exhibit toxic activity against five human cancer cell lines including P388, HL-60, A-549, BEL-7402, and SPCA4. Research on the anticancer activity of Pg is increasingly expanding and investigating into its mechanisms of action on cancer cells, opening up various avenues for experimentation with different types of cancer cells. The significant potential of Pg to become an effective cancer treatment requires a large source of raw material for extracting this Pg compound. In addition to screening and selecting *S. marcescens* strains with high Pg synthesis capabilities and optimizing culture environments and conditions, a stronger approach to enhancing gene expression at the transcriptional level is currently of interest, such as promoter techniques or modifying certain unfavorable factors for the Pg synthesis pathway. In this regard, Pan *et al.* (2022) enhanced the Pg synthesis capability of *S. marcescens* JNB5-1 by mutating the regulatory factor OmpR that affects Pg synthesis. The authors created a mutation library by randomly inserting transposons and screened for a mutant strain with a 1.6-fold increase in Pg synthesis yield. Another study demonstrated that RsmC is a multifunctional post-transcriptional and translational regulator in *S. marcescens*. Mutations in the *rsmC* gene increased Pg synthesis capability, surface-active compound production, and cell aggregation (Williamson *et al.,* 2008). Hampton et al. (2016) also created insertional mutations in *rsmC* using the I-F CRISPR endogenous system to enhance Pg synthesis capability.

One of our aims is to establish procedures to yield pure prodigiosin abundantly. *S. marcescens strains*, which produces prodigiosin, will be renovated for higher-yield prodigiosin production using CRISPR/Cas9. Knock down *rsmC* (regulator of secondary metabolism C) gene, which is strongly reduced sdhEygfX expression by acting through FlhDC. A post-transcriptional mRNA-binding protein, RsmA (regulator of secondary metabolism A), also reduced sdhEygfX levels, but through both FlhDC-dependent and -independent routes. In addition to their role in sdhEygfX regulation, RsmA, RsmC and FlhDC exhibited co-ordinate control of motility and prodigiosin production.

MATERIALS AND METHODS

The chemicals used in the research were purchased from reputable chemical companies: Thermor, Invitrogen, Bioneer. Plasmids pCasPA and pCRISPR were obtained from Addgene. The strain *S. marcescens* QBN was provided by the Enzyme Biotechnology Department, Institute of Biotechnology, Vietnam Academy of Science and Technology.

Design of vector for gene mutation

The dual vector system pCasPA and pACRISPR were used to mutate genes in the genome of *S. marcescens* QBN. The spacer sequence was designed and inserted into the

pACRISR vector according to Chen et al. (2018). (1) The spacer sequence is a 20-nucleotide DNA segment on the target gene *rsmC* for mutation. The oligonucleotide sequence was designed as follows: 5'-GTGGatcgacagcgcgcgccgctg-3', 3'-tagctgtcgcgcgcggcgagCAAA-5'. (2) Next, the oligos were phosphorylated in a reaction containing: 5 µL 10× T4 DNA ligase buffer, 2 µL 50 µM Oligo F, 2 µL 50 µM Oligo R, 1 µL T4 polynucleotide kinase, 40 µL H_2O (chemical from Thermo). The reaction was incubated at 37°C for 1 hour. (3) The annealed oligos after phosphorylation were paired by adding 2.5 µL of 1 M NaCl to the reaction mixture after phosphorylation at 95°C for 3 minutes. The paired oligos were diluted 20-fold with H_2O to a final concentration of approximately 100 nM. (4) The spacer was ligated into the pACRISPR vector at the *Bsa*I (Thermo) cutting site. The reaction mixture consisted of 1 µL 10× T4 DNA ligase buffer, 1 µL annealed oligos (100 nM), 1 µL plasmid pACRISPR (20 nM), 0.5 µL T4 DNA ligase, 0.5 µL *Bsa*I, and 6.0 µL H_2O. The reaction was carried out for 25 cycles (37°C for 3 minutes, 16°C for 4 minutes), followed by 80°C for 15 minutes, then maintained at 10°C. (5) The ligated reaction mixture was then electroporated into *E. coli* DH5α cells, selected on LB-agar plates supplemented with 50 µg/mL carbenicillin, and incubated at 37°C overnight. The structure of the pACRISPR-spacer vector was verified by PCR and sequencing of the DNA segment at the inserted spacer site.

Preparation of electrocompetent *S. marcescens* QBN cells

An overnight culture of the *S. marcescens* QBN strain was inoculated at a ratio of 1 mL into 100 mL of LB medium and shaken at 200 rpm at 37°C. When the cell density of the culture at 600 nm (OD600) reached approximately 1.0, the cells were harvested by centrifugation at 6000 rpm for 5 minutes. The cell pellet was washed twice with 20 mL of 10% (v/v) sterile glycerol solution and finally resuspended in 1 mL of 10% (v/v) glycerol solution, aliquoted into tubes, with each tube containing 50 µL for use in plasmid transformation.

Creation of *S. marcescens* QBN strain with *rsmC* gene mutation using the pCasPA/pACRISPR system

The plasmid pCasPA was electroporated into electrocompetent *S. marcescens* QBN cells prepared using a 1 mm cuvette (Bio-Rad) under the conditions of 2100 V, 100 Ω, 25 µF. The bacteria containing the pCasPA plasmid were selected on LB-agar plates supplemented with 100 µg/mL tetracycline. A colony containing the pCasPA plasmid was picked and inoculated into 3 mL of LB medium at 37°C with shaking at 200 rpm overnight. 1 mL culture was then inoculated into 100 mL of LB medium and shaken under the same conditions until the OD_{600} reached approximately 1.0~1.5. L-arabinose was then added to a final concentration of 2 mg/mL and incubated for 2 hours to induce the expression of Cas9 and λ-Red system. The cells were then harvested and made electrocompetent following the steps described above. The pACRISPR-spacer plasmid was electroporated into cells containing the pCasPA plasmid, incubated for 1-2 hours in LB at 37°C, and then spread on LB-agar plates supplemented with 100 µg/mL tetracycline and 150 µg/mL carbenicillin. The DNA donor fragment is generated using the fusion PCR technique from two fragments of the rmsC gene,

resulting in a fragment that is 100 nt shorter than the original gene. It is then co-transformed with a plasmid carrying the cas9 gene.

Screening of mutant strains

The *S. marcescens* QBN strains after transformation with the pCasPA/pACRISPR-spacer vector system was grown on selective media supplemented with tetracycline and carbenicillin, and then shaken to extract total DNA. A segment of *rsmC* gene DNA (550 bp in size) was amplified from these DNA templates along with DNA from the original *S. marcescens* QBN strain as a control sample using specific primers. The PCR products were electrophoresed on a 15% acrylamide gel and sequenced.

***S. marcescens* culture**

To produce Pg, the *S. marcescens* was cultivated on a medium containing 2% peanut seed powder and 2% agar, followed by incubation on a tray (20 x 30 cm) at 28°C for 48 hours. Cells grown on the surface were collected for Pg extraction and purification.

Purification and identification of prodigiosin

The shaken culture was extracted with ethyl acetate at a ratio of 1 solvent volume:1 sample volume. After shaking the solvent-sample mixture at 200 rpm for 3 hours, it was centrifuged at 4000 rpm for 10 minutes at 4°C or allowed to settle for approximately 30 minutes. The upper layer of the resulting orange-red solution containing the Pg-dissolving solvent was measured for OD at 535 nm to determine the Pg content. The identified segments were collected in groups to continue passing through the column or to dry for further studies. Thin layer chromatography was performed on a silica gel plate with 0.25 mm thick silica gel 60 F254. The solvent system was n-hexane:ethyl acetate (1:1) as the mobile phase. The compounds were visible by iodine staining. Prodigiosin purity was determined by HPLC. A sample (10 µL) was applied to the system. The purification experimental design was determined by LC/MS 1100 Agilent Ion sources ESL, column ODS C18, 3.0 x 150 mm, 3.5 µm, mobile phase MeOH:H_2O (80:20; v/v). NMR 1D and 2D spectra were measure by Bruker AV 500 MHz in CDCl3 solvent. 1H NMR were determined at 500 MHz. Tetramethylsilane (TMS) was used as the internal standard. The signals were obtained as singlet (s), doublet (d), double doublet (dd), triplet (t), quintet (quint), and multiplet (m). MS spectra was measured by LC-MS Agilent 1100 (USA). The culture broth was analyzed by HPLC mobile phase in MeOH:H_2O = 20:80 for 2 min, MeOH:H_2O = 20 – 100/80 – 0 for 17 min, MeOH:H_2O = 100:0 for 8 min, MeOH:H_2O = 20:80 for 5 min. The quantity of Pg in the culture broth was determined on HPLC based on standard Pg. Standard prodigiosin (Sigma) was diluted at various concentrations. Then, use a spectrophotometer to measure the absorbance at 535 nm.

Thin layer chromatography (TLC)

The Pg-extracted solution was tested by TLC on silica gel 60F254 thin-layer plates, 0.25 mm thick. The solvent used as the mobile phase was a mixture of ethyl acetate and toluene in an 8:2 ratio. The chromatogram was visualized using the iodine staining method for about 5-10 minutes.

RESULTS AND DISSCUSSION

Construction of *rsmC* gene mutation vector

The vector pCasPA is responsible for synthesizing the Cas9 nuclease and the λ-Red protein system (which enhances the ability to pair similar DNA segments) under the control of the ParaB promoter induced by L-arabinose. On the other hand, pACRISPR synthesizes sgRNA under the control of a strong promoter, *trc*. Additionally, pACRISPR provides a site for inserting a DNA spacer segment between two *Bsa*I cleavage points (Figure 1). When the spacer segment is inserted at this site, the two *Bsa*I cleavage points will lose their recognition sequences. However, pACRISPR still contains another *Bsa*I site in the plasmid, which can be used to cleave and select plasmids carrying the spacer.

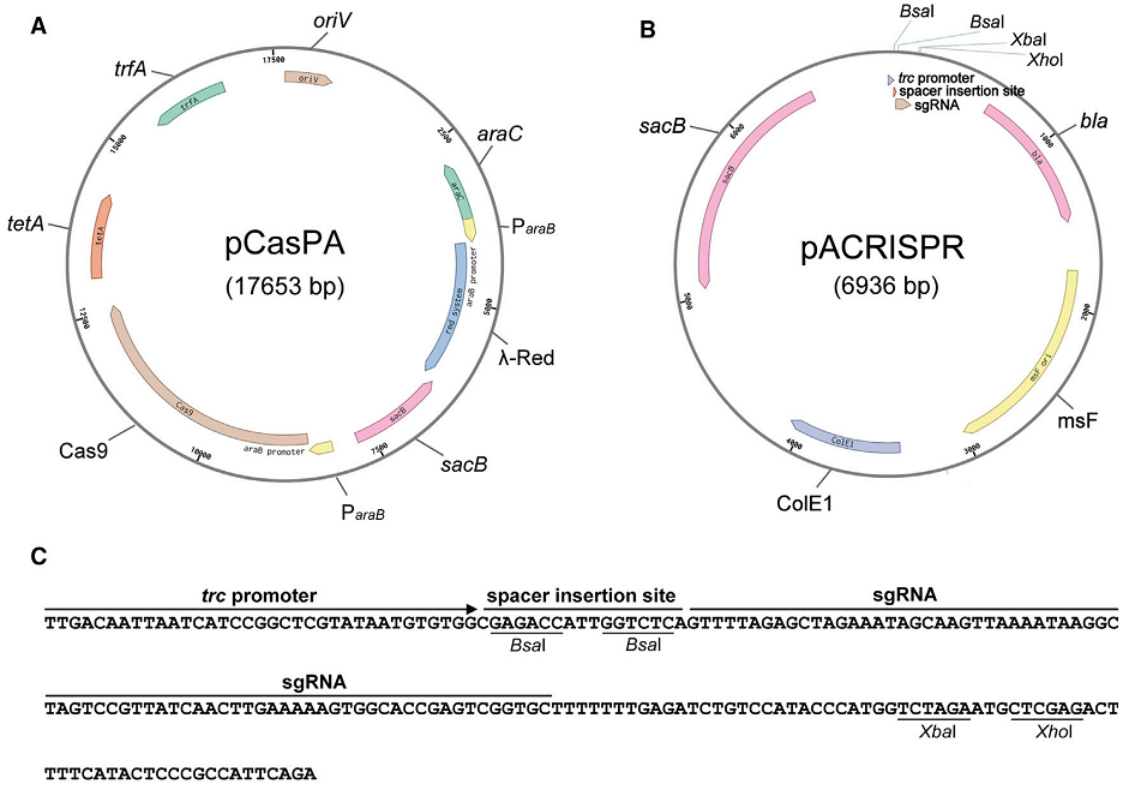

Figure 1. Vector pCasPA/pACRISPR

The spacer segment is a 20-nucleotide DNA segment on the target gene *rsmC* to be mutated. The spacer segment is chosen between the *rsmC* gene and the nucleotide triplet NGG (PAM site) in the 5'-3' sequence (Figure 2A). The spacer segment is inserted between two *Bsa*I sites as described in the schematic diagram of the pACRISPR vector in Figure 1 and selected on media supplemented with carbenicillin. The selected strains are then separated from the plasmid, cleaved and checked by *Bsa*I, and

electrophoresed on agarose gel. The gel shows two strains when cleaved with *Bsa*I, resulting in an open circular plasmid band of approximately 7kb (Figure 2B, lanes 3 and 4), while plasmids from other strains show two corresponding bands of approximately 3 kb and 4 kb. This result indicates that strains 1 and 2 have correctly inserted the spacer segment between the two *Bsa*I sites as calculated, so when cleaved by *Bsa*I, the plasmid remains open circular (due to the presence of another *Bsa*I cleavage site in the plasmid), while other strains did not insert the spacer segment, so the two *Bsa*I sites remain intact, resulting in plasmid cleavage into two fragments.

Thus, the pACRISPR-spacer vector carrying the DNA segment recognizing the target gene *rsmC* has been designed to further transfect into *S. marcescens* QBN along with the pCasPA plasmid to mutate the *rsmC* gene in the genome. The spacer segment has also been verified by sequencing with the primers in pACRISPR before transfection into *S. marcescens* QBN.

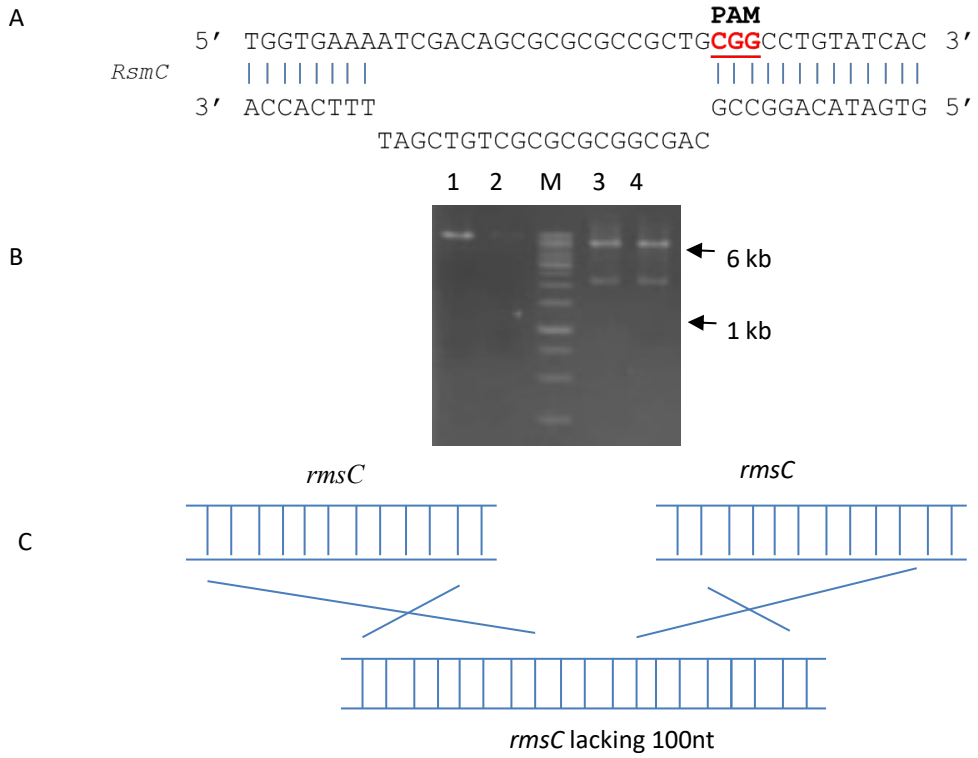

Figure 2. Designing the PAM site and the DNA donor fragment.The position of the spacer sequence in *rsmC* (A), electrophoresis of the product from the digestion of the pACRISPR-spacer plasmid line with *Bsa*I (B) and cells repair processes (C).

Random mutation on the *rsmC* gene and evaluation of Pg synthesis ability of mutant strains

The *S. marcescens* QBN strains after transfection with the pCasPA/pACRISPR-spacer vector system, the Cas9 nuclease DNA cleaves the target locus, creating a double-stranded break in the DNA sequence of the gene set. A DNA fragment, which had 100 nucleotides removed and was homologous to the *rsmC* gene, will participate in the DNA repair process (Figure 2C). Cells will undergo repair processes, and only those cells that have undergone homologous recombination can survive after the double-stranded DNA of the gene set is cleaved. Therefore, bacteria growing on selective media supplemented with tetracycline and carbenicillin have the ability to mutate and have their gene set repaired. Darker red colonies are selected, cultured, and total DNA is extracted to check the size of the *rsmC* segment.

The *S. marcescens* QBN strains after transfection with the pCasPA/pACRISPR-spacer vector system growing on selective media supplemented with tetracycline and carbenicillin with darker red color are selected, cultured, and total DNA is extracted. The PCR products amplifed from a segment of the *rsmC* gene (size) from the strains were electrophoresed on a polyacrylamide gel. With a 15% acrylamide gel concentration, different-sized DNA segments can be separated in the range of 25-150 bp. The DNA-PAGE electrophoresis results in Figure 3 showed the amplified bands of the *rsmC* gene segment from the strains (lanes 2 and 5) are smaller in size compared to the *rsmC* gene segment amplified from total DNA from the *S. marcescens* QBN strain (lane 8). With this result, it can be initially inferred that the *rsmC* gene in the strains 2 and 5 may be loss of nucleotides after the DNA template is cut.

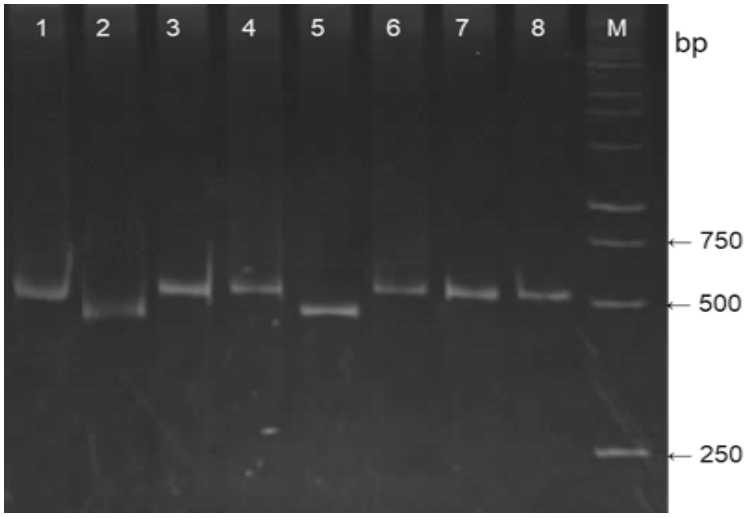

Figure 3. DNA-PAGE electrophoresis profile of *rsmC* gene amplified from the genomes of mutated *S. marcescens* QBN strains. 1-7: Selected strains were cultured on media supplemented with tetracycline and carbenicillin; 8: Original *S. marcescens* QBN strain; M: DNA standard ladder (SM0311, Thermo).

The mutant strains 2 and 5, which were obtained on selective media, along with the original *S. marcescens* QBN strain, exhibited the deepest red color among all the strains. These strains were cultured in NB medium supplemented with 2% peanut, then the culture was harvested and extracted to initially assess their prodigiosin synthesis ability. After determining the prodigiosin content in the extract at a wavelength of 535 nm, the synthesis yields of strains 2 and 5 were found to be 216 and 231 mg/L of medium, respectively, which were higher than that of the original strain (180 mg/L) (Figure 4).

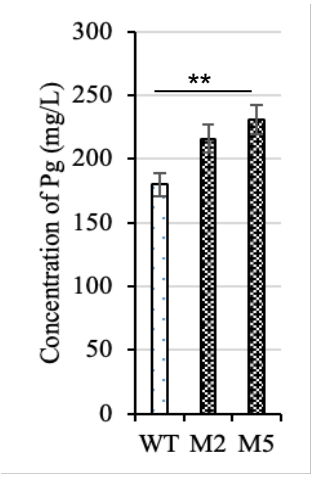

Figure 4. The concentration of Pg (mg/L) in mutant strains M2, M5 and the wild-type strain (**p<0.05).

The results of the Pg concentration test in the two mutant strains indicate that the Pg content in mutant strain QBN M5 was 28.3% higher than that in the wild-type strain. Therefore, we chose this strain for purification and identification of Pg. Thus, the CRISPR-Cas9 system can be utilized for genome editing in *S. marcescens*. Currently, there is no publication employing CRISPR-Cas9 in *Serratia* sp. However, CRISPR-Cas9 has been employed for genome editing in some other Gram-negative bacteria such as *Pseudomonas aeruginosa* (Chen *et al*., 2018) and *E. coli* (Jiang *et al*., 2013). The *rsmC* gene plays a significant role in the regulation of prodigiosin production in bacteria. Research has shown that the RsmA and RsmC proteins act through the FlhDC complex to repress the SdhE flavinylation factor, which in turn controls motility and prodigiosin production in *Serratia* species (Hampton *et al*., 2016). Further studies have identified essential genes associated with prodigiosin production, highlighting the complex regulatory mechanisms that govern this process. For instance, a mutagenesis library revealed that certain genes outside the prodigiosin biosynthetic cluster are involved in its production, including transcription-regulating genes, membrane protein-encoding genes, and metabolism enzyme-encoding genes (Jia et al., 2021). These findings suggest that manipulating the *rsmC* gene and other related regulatory genes could potentially enhance prodigiosin production, which is of great interest for its promising applications in medicine and industry due to its anticancer and antimicrobial properties. These findings support with the studies by Wiliam et al. (2008) and Hampton *et al.* (2016), where inserting additional segments into *rsmC* to inactivate the RsmC regulatory factor using the CRISPR system enhanced prodigiosin synthesis in *Serratia* 39006.

Purification and identification of prodigiosin from *S. marcescens*

S. marcescens QBN M5 strain will be cultured on NA medium to biosynthesize Pg. The determination of an effective solvent system for Pg extraction from *S. marcescens* culture was previously established (Vu *et al*., 2021). In our present investigation, we employed a solvent mixture of ethyl acetate

and acetone (1:1) for extracting prodigiosin from *S. marcescens*. The resultant cell-free extract underwent chromatographic separation on a silica gel column. The prodigiosin-enriched fractions were selectively collected and subjected to a second purification step through another silica gel column. Two fractions were obtained, and subsequent analysis revealed a singular band representing the putative compound. The purified Pg, after two rounds of purification, was analyzed using thin layer chromatography (TLC) (Figure 5). After two rounds of purification, the Pg on the TLC plate (lane 2 and 3) showed a single band with a size equivalent to the standard Pg (C).

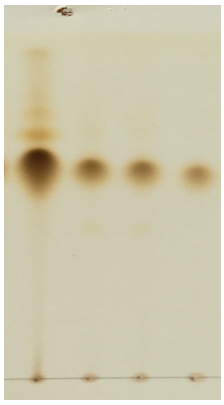

Figure 5. Thin layer chromatography of Pg purified from *S. marcescens* QBN strain. The crude extraction of Pg (1), purified Pg (2, 3), Pg standard from Sigma (C).

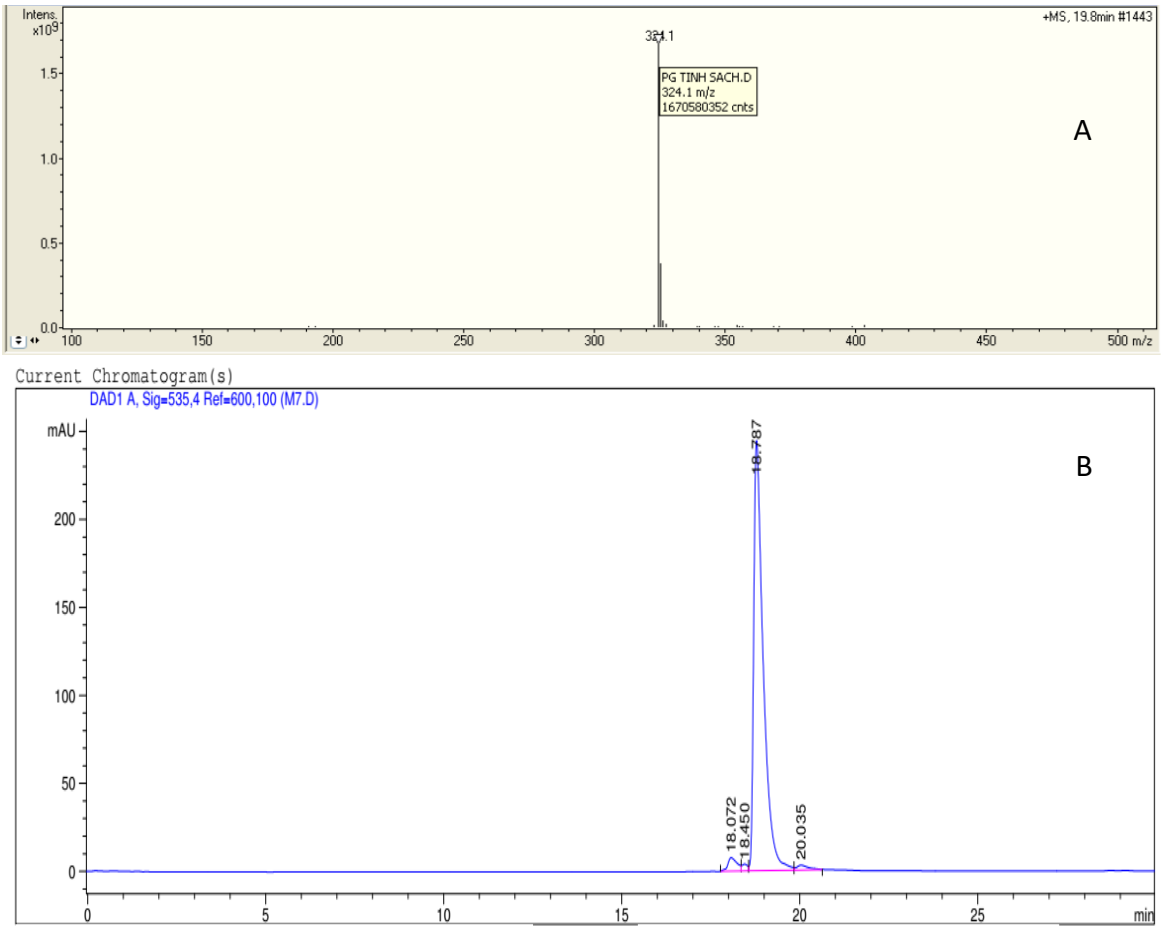

Figure 6. Mass spectrometry (A) HPLC (B) of purified prodigiosin.

The compound was isolated as a red pigment powder with a melting point of 151-152°C. Mass spectrometry for peak m/z [M + H] +324,1 corresponds to molecular formula $C_{20}H_{25}N_3O$. The HPLC result indicated that the compound purified harbored 1 single peak of pure prodigiosin with a purity of 98% (Figure 6). According to Song and colleagues in 2006, *Serratia* sp. KH-95 was cultured in a casein-rich medium, and the prodigiosin mass was determined to be 323.0 Da corresponding to 324.0 (M+H)+ with the formula $C_{20}H_{25}N_3O$ (Song *et al*., 2006). Additionally, Lapenda and colleagues (2015) determined the prodigiosin absorption wavelength to be 534 nm and identified the molecular mass as 323 m/z using GC/MS (Lapenda *et al.,* 2015). The purified Pg reach 98% was similar with the results from Kamble's study (Kamble *et al*., 2012). Hence, prodigiosin was successfully purified from the crude. *Serratia* sp. KH-95 was cultured in a casein-rich medium and purified using TLC, revealing a prodigiosin content of approximately 120 mg/L with a purity of 95% (Song *et al.,* 2006). Patil and co-workers in 2011 identified prodigiosin with strong absorption at a wavelength of 536 nm and purity exceeding 95% using HPLC from the strain *S. marcescens* NMCC46 (Patil *et al*., 2011). Danyuo and colleagues purified and determined the prodigiosin content by HPLC with a purity of 92.8% from the strain *S. marcescens*.

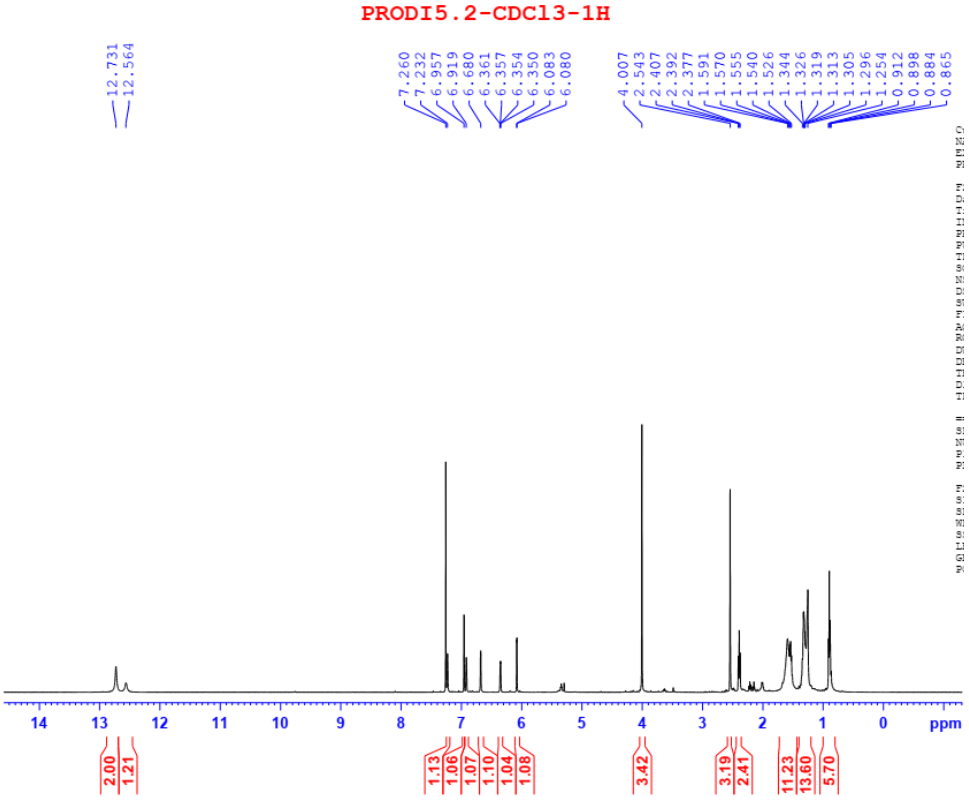

Figure 7. ^{1}H NMR proton spectrum.

The purified compound from *S. mercescens*, as showed in Figure 7, exhibits characteristic ^{1}H NMR spectral features. Broad singlet signals, binding 2 hydrogen atoms to a nitrogen atom at 12.564 ppm (^{1}H) and 12.731 ppm, specifically correspond to H-1 and H-1'. In the aromatic hydrogen and olefin region, six resonant signals manifest, accounting for 6 protons. Within this set, three singlet signals at δH 6.957, 6.680, and 6.080 ppm are discernible, while the remaining three signals pertain to 3 protons within the same spin system. Observable signals include those of an OMe group (singlet at 4.007), a methyl group at δH 2.543 (singlet), a terminal methyl carbon group (triplet at 0.898), two methylene groups (triplets at 2.377 and 1.54), and two overlapping sets of methylene groups presenting as multiple signals at δH 1.254. From the above NMR spectra, we can conclude the purified compound was Pg.

CONCLUSION

The preliminary study was designed using the CRISPR-Cas9 system from the pCasPA/pACRISPR vector system to create gene mutations in the genome of *S. marcescens*. These findings establish a basis for future studies to employ the CRISPR-Cas9 mechanism for genomic modifications in *Serratia marcescens*, *Pseudomonas*, and various other microbes.

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REFERENCES

Chen W, Zhang Y, Zhang Y, Song L, Wang Y, Ji Q (2018) CRISPR/Cas9-based genome editing in *Pseudomonas aeruginosa* and cytidine deaminase-mediated base editing in *Pseudomonas* species. *iScience* 6: 222-231.

Hampton HG, McNeil MB, Paterson TJ, Ney B, Williamson NR, Easingwood RA, Bostina M, Salmond GPC, Fineran PC (2016) CRISPR-Cas gene-editing reveals RsmC and RsmC act through FlhDC to repress the SdhE flavinylation factor and control motility and prodigiosin production in *Serratia*. *Microbiology* 162: 1047-1058.

Jia X, Liu F, Zhao K, Lin J, Fang Y, Cai S, Lin C, Zhang H, Chen L, Chen J (2021) Identification of essential genes associated with prodigiosin production in *Serratia marcescens* FZSF02. *Front Microbiol* 12.

Khanafari A, Assadi MM, Fakhr FA (2006) Review of prodigiosin, pigmentation in *Serratia marcescens*. *J Biol Sci* 6: 1-13.

Lapenda JC, Silva PA, Vicalvi MC, Sena KX, Nascimento SC (2015) Antimicrobial activity of prodigiosin isolated from *Serratia marcescens* UFPEDA 398. *World J Microbiol Biotechnol* 31(2): 399-406 doi:10.1007/s11274-014-1793-y.

Montaner B, Navarro S, Piqué M, Vilaseca M, Martinell M, Giralt E, Gil J, Pe'rez-Toma's R(2000) Prodigiosin from the supernatant of *Serratia marcescens* induces apoptosis in haematopoietic cancer cell lines. *Br J Pharmacol* 131: 585-593.

Pan X, You J, Tang M, Zhang X, Xu M, Yang T, Rao Z (2022) Improving prodigiosin production by transcription factor engineering and promoter engineering in *Serratia marcescens*. *Front Microbiol* 13.

Pandey R, Chander R, Sainis KB (2007) Role of interleukin-2 regulated proteins in immunosuppression by the N-alkylated prodigiosin analogue. *Int Immunopharmacol* 7: 1695-1703.

Patil CD, Patil SV, Salunke BK, Salunkhe RB. (2011) Prodigiosin produced by *Serratia marcescens* NMCC46 as a mosquito larvicidal agent against *Aedes aegypti* and Anopheles stephensi. *Parasitol Res* Oct;109(4): 1179-87.

Song MJ et al. (2006) Purification and characterization of prodigiosin produced by integrated bioreactor from *Serratia* sp. KH-95. *J Biosci Bioeng* 101: 157-161.

Vu TL, Nguyen SLT, Do TT, Cuc DT, Do TT (2021) Prodigiosin purification from *Serratia marcescens* M10 and its antitumor activities. *Vietnam J Biotechnol* 19, 289–299. https://doi.org/10.15625/1811-4989/15722.

Williamson NR, Fineran PC, Ogawa W, Woodley LR, Salmond GP (2008) Integrated regulation involving quorum sensing, a two-component system, a GGDEF/EAL domain protein and a post-transcriptional regulator controls swarming and RhlA-dependent surfactant biosynthesis in *Serratia*. *Environ Microbiol* 10:1202-1217.