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Elucidation of a carboxylate *O*-methyltransferase NcmP in nocamycin biosynthetic pathway

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ABSTRACT

Nocamycins belong to the tetramic acid family natural products and show potent antimicrobial activity. Recently, the biosynthetic gene cluster of nocamycin was identified from the rare actinomycete *Saccharothrix syringae* and an S-adenosylmethionine (SAM) dependent methyltransferase gene *NcmP* was found to be located within the gene cluster. In this report, the methyltransferase gene *NcmP* was disrupted and a new nocamycin intermediate nocamycin E was isolated from the mutant strain. Meanwhile, NcmP was heterologously expressed in *Escherichia coli* BL21 (DE3) and biochemically characterized as a carboxylate *O*-methyltransferase in nocamycin biosynthetic pathway. Compared to nocamycin I, nocamycin E showed inferior antibacterial activity, indicating the methyl group is essential to antibacterial activity.

The tetramic acid class natural products nocamycin I (**1**) and II (**2**) (Fig. 1) were isolated from the rare actinomycete *Saccharothrix syringae* NRRL B-16468 and they showed potent inhibitory activity toward Gram-positive and Gram-negative bacteria, especially toward some anaerobic bacteria.[1–7] Due to the complex structure, the total synthesis of nocamycin has not been achieved until now and the diversification of nocamycin by chemical synthesis was limited. Thus, biosynthetic pathway engineering provides an alternative approach to generate nocamycin analogs by manipulating biosynthetic gene cluster. Recently, we have identified the nocamycin biosynthetic gene cluster that contained 21 open reading frames (ORFs),[8] including five type I polyketide synthases genes (*NcmAI*, *NcmAII*, *NcmAIII*, *NcmAIV* and *NcmAV*), a non-ribosomal peptide synthetase gene (*NcmB*), a Dieckmann cyclase gene *NcmC*, a short chain dehydrogenase gene (*NcmD*), two cytochrome P450 oxidase genes (*NcmO* and *NcmG*), a glycoside dehydratase gene (*NcmE*), a SAM dependent methyltransferase gene *NcmP*, and five regulators genes (*NcmN*, *NcmJ*, *NcmK*, *NcmI* and *NcmM*). The backbone of nocamycin is assembled by PKS-NRPS and the tailoring enzymes are employed to further modify the structure to yield the nocamycin I. In our previous report, we have isolated two new nocamycin derivatives from the cytochrome P450 gene *NcmG* disruption mutant strain, which provide hints to the nocamycin biosynthetic pathway.[8] In this work, we report (i) isolation of a new intermediate nocamycin E from the methyltransferase gene *NcmP* disruption mutant strain; (ii) biochemically characterization of NcmP as a carboxylate *O*-methyltransferase in nocamycin biosynthesis pathway; (iii) antibacterial activity evaluation of nocamycin E towards a panel of bacteria.

Bioinformatics analysis revealed that *NcmP* is the only methyltransferase gene located within the nocamycin biosynthetic gene cluster, which is accord to the hypothetical nocamyicn biosynthetic pathway. Sequences alignments revealed that NcmP belongs to SAM dependent methyltransferase family and it shares the three common conserved motifs distributed in SAM dependent methyltransferase family (see Supporting information, Fig. S1).[9,10] NcmP shows identity to NokK (44% identity) involved in K-252a pathway from *Nonomuraea longicatena*,[11] NivG (43% identity) involved in nivetetracyclates pathway from *Streptomyces* sp. Ls2151,[12] and AknG (41% identity) involved in aclacinomycins pathway from *Streptomyces galilaeus*.[13] Given that all NcmP homologues are proposed to catalyze methyl esterification reaction, NcmP is most likely to be the candidate involved in the methyl esterification in nocamycin biosynthetic pathway.

To determine the function of NcmP, we inactivate the *NcmP* by replacing partial internal *NcmP* with *aac(3)IV* gene cassette through λ-RED recombination technology to generate the double cross mutant strain *S. syringae* MoS-1004 (see SI). The double-crossover mutant was selected by apramycin resistant kanamycin sensitive

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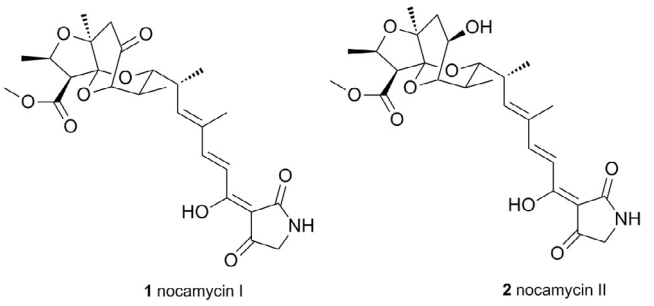

Fig. 1. The structures of nocamycin I (**1**) and nocamycin II (**2**).

phenotype and the genotype was then verified by PCR (Fig. 2). The mutant strain *S. syringae* MoS-1004 was firstly cultured by using the fermentation medium contained 1% soybean, 3% glycerol, 0.2% NaCl, 0.5% mycose, 0.2% $CaCO_3$.[8] After seven days fermentation, the broth was extracted by organic solvent and analyzed by HPLC. HPLC results revealed that the mutant strain *S. syringae* MoS-1004 (*ΔNcmP*) lost the capability to produce nocamycin I and nocamycin II. To our surprise, no other nocamycin derivatives have been detected. Based on the intermediates nocamycin III isolated from the *ΔNcmG* mutant strain,[8] we assumed that the methyl esterification in nocamycin pathway is likely to be a post-tailoring process. Next, *S. syringae* MoS-1004 was cultured with other nine media (see SI). All the fermentation broths were analyzed by HPLC. Results of HPLC revealed that a new more polar peak with characteristic UV absorption to that of nocamycin was detected in four different media (Fig. 2).

To determine the structure of the newly produced nocamycin derivative, 6 L scale fermentation media (1% starch, 1% yeast extract, 0.3% corn flour, 2% glucose, 0.1% beef extract, 0.05% K_2HPO_4, 0.05% $MgSO_4{\cdot}7H_2O$, 0.2% $CaCO_3$, pH 7.0) was prepared to culture *S. syringae* MoS-1004. Ultimately, by using a series of chemical isolation methods, compound **3** (about 10 mg) was purified and subjected to high resolution mass spectrum (HR-MS) and NMR analyses.

Compound **3** was isolated as a yellowish amorphous solid. Its molecular formula was determined as $C_{25}H_{31}NO_9$ on the basis of ion peaks at *m/z* 490.2068 $[M+H]^+$, *m/z* 512.1901 $[M+Na]^+$, and *m/z* 1001.3903 $[2M+Na]^+$ by HR-ESI-MS analysis (Fig. S2), 14 mass units less than that of nocamycin I, corresponding to a demethylation derivate of nocamycin I. Comparisons of the ^1H and ^{13}C NMR spectroscopic data indicate that **3** showed similar structure to that of nocamycin I, except that one methoxy proton

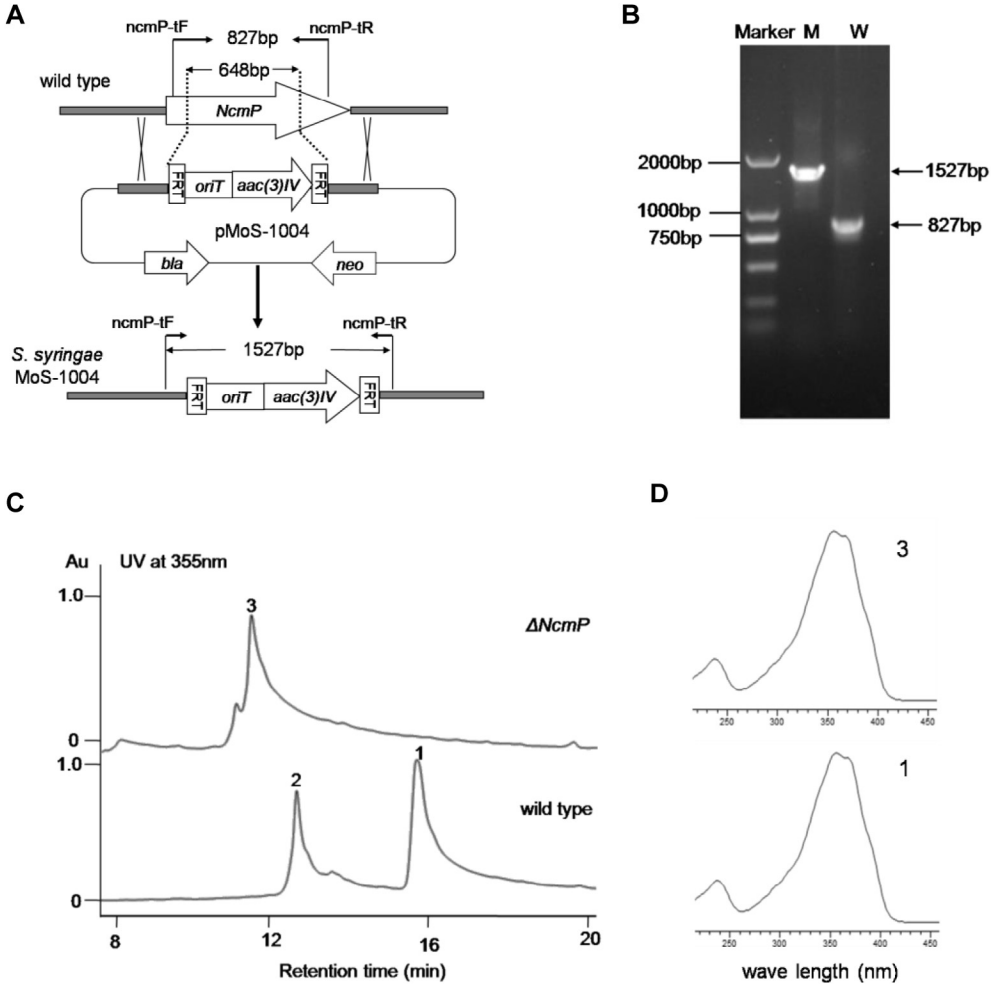

Fig. 2. Construction of Δ*NcmP* gene replacement mutant *S. syringae* MoS-1004 and HPLC analysis of the metabolite profile. (A) Depiction of *NcmP* inactivation. The mutant MoS-1004 was generated by replacing 648-bp internal *NcmP* fragment with the *oriT* and *acc3(IV)* fragment in pMoS-1004, resulting from a double-crossover recombination event. (B) PCR analysis of the double-crossover mutant. W: *S. syringae* wild type (827 bp); M: mutant strain *S. syringae* MoS-1004 (1527 bp); Marker: DNA molecular ladder. (C) HPLC analysis of the metabolite profiles. **1** represents nocamycin I, **2** represents nocamycin II, **3** represents the newly produced nocamycin derivative. (D) The UV absorption of compound **3** and **1**.

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