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COMMUNICATION TO THE EDITOR

Identification of the biosynthetic gene cluster for the herbicide phosphonothrixin in *Saccharothrix* sp. ST-888

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Natural products containing carbon–phosphorus (C–P) bonds are widely used in medicine, agriculture and research.[1,2] These C–P compounds (phosphonates) include fosfomycin, bialaphos, fosmidomycin and FR-900098, which have been isolated from the fermentation broths of actinomycetes (Supplementary Figure S1). Phosphonothrixin is also a natural phosphonate, which has been isolated from the fermentation broth of the soil actinomycete *Saccharothrix* sp. ST-888 (Figure 1).[3] In addition to a C–P bond, phosphonothrixin possesses a characteristic tertiary carbon with a chiral center.[4] This compound exhibits herbicidal activity by suppressing the germination of gramineous and broadleaf weeds, which induces chlorosis in leaves.[3] To date, although the enantioselective chemical synthesis of phosphonothrixin has been reported,[5] neither the biosynthesis nor the mode of action have been elucidated. In this communication, we describe the identification of the biosynthetic gene cluster of phosphonothrixin in *Saccharothrix* sp. ST-888. This study lays the foundation for the elucidation of the novel bacterial biosynthetic machinery for natural phosphonates.

We first *de novo* sequenced the genome of *Saccharothrix* sp. ST-888. An assembly of the sequence reads yielded 2822 contigs with 8605814 total base pairs. In the biosynthesis of fosfomycin, bialaphos and FR-900098, the initial reactions begin with a conversion of phosphoenolpyruvate (PEP) to phosphonopyruvate (Supplementary Figure S1).[1,2] This conversion is catalyzed by PEP phosphomutase (PEPPM), which is encoded by the *fom1*, *bcpB* or *frbD* genes, for the biosynthesis of fosfomycin, bialaphos or FR-900098, respectively.[1,2] Therefore, we hypothesized that the initial reaction in phosphonothrixin biosynthesis is also catalyzed by PEPPM. Thus, we retrieved a PEPPM homolog from the genomic DNA sequence of *Saccharothrix* sp. ST-888 and identified a homologous open reading frame (ORF) with 34%, 40%, and 49% identities to Fom1, BcpB and FrbD, respectively (Supplementary Figure S2). Moreover, an analysis of the regions flanking the PEPPM homologous gene revealed that 14 additional genes were located in these regions in the same direction as the PEPPM homologous gene, suggesting that these 15 genes form a biosynthetic gene cluster for phosphonothrixin.

We constructed a cosmid library for the ST-888 genome using the cosmid vector pOJ446[6] and screened the library for cosmids that contained the regions including the PEPPM homologous gene using the gene itself as a probe. Subsequently, three positive cosmids (cos-1, cos-11 and cos-12) were introduced into *Streptomyces albus* J1074, which has previously been used as a heterologous host.[7] The resultant transformants were cultured for heterologous expression under the identical conditions used for ST-888.[3] The metabolites, which were partially purified by anion exchange chromatography using AG 1-X8 resin (Cl^- form; Bio-Rad, Tokyo, Japan), were analyzed using ^{31}P NMR spectroscopy (Figure 1a). The heterologous production of phosphonothrixin was confirmed in the transformants harboring cos-11 or cos-12. The titers of phosphonothrixin in both the transformants were ~8.7 mg l^{-1} broth, which were one-half that of the wild-type ST-888 strain (17.3 mg l^{-1}). This result indicates that both cos-11 and cos-12 contain all the genes required for phosphonothrixin biosynthesis. Some unidentified phosphonates were also produced by the transformants. The production of multiple phosphonates upon the heterologous expression of phosphonate biosynthetic gene clusters has been previously reported.[8] We suspect that these unknown metabolites may be the biosynthetic intermediates of phosphonothrixin or phosphonates that are detoxified by the heterologous host.

We sequenced and analyzed the insert DNA of the cos-12 cosmid. Cos-12 contains 35 ORFs, including a PEPPM homolog. The length of the inserted DNA spans ~43 kb (Supplementary Table S1 and Supplementary Figure S3). To identify the ORFs required for phosphonothrixin biosynthesis, we constructed a series of cosmids (cos-121–cos-125) in which several genes were deleted from the parental cos-12 using the lambda-Red recombination system[9] and individually introduced the deletion cosmids into *S. albus* (Supplementary Figure S3). Each transformant was cultured, and the metabolites in the culture broth were analyzed using ^{31}P NMR spectroscopy (Figure 1a) and LC/MS (Supplementary Figure S4). The transformants harboring the cosmid containing ORF13–34 (cos-121), the cosmid containing ORF20–34 (cos-122) or the cosmid containing ORF21–34 (cos-123) continued to produce phosphonothrixin, although the yields clearly decreased (Figure 1a). The titers of phosphonothrixin in each transformant were ~2.7, 1.5 and 1.5 mg l^{-1} broth, respectively. In contrast, phosphonothrixin production was completely abolished in the transformants harboring the cosmid containing ORF22–34 (cos-124) or the cosmid containing ORF1–27 (cos-125). Therefore, at most, the region from ORF21 to ORF34 is sufficient to confer phosphonothrixin production in *S. albus*. Thus, we named these ORFs *ptxB1–14*

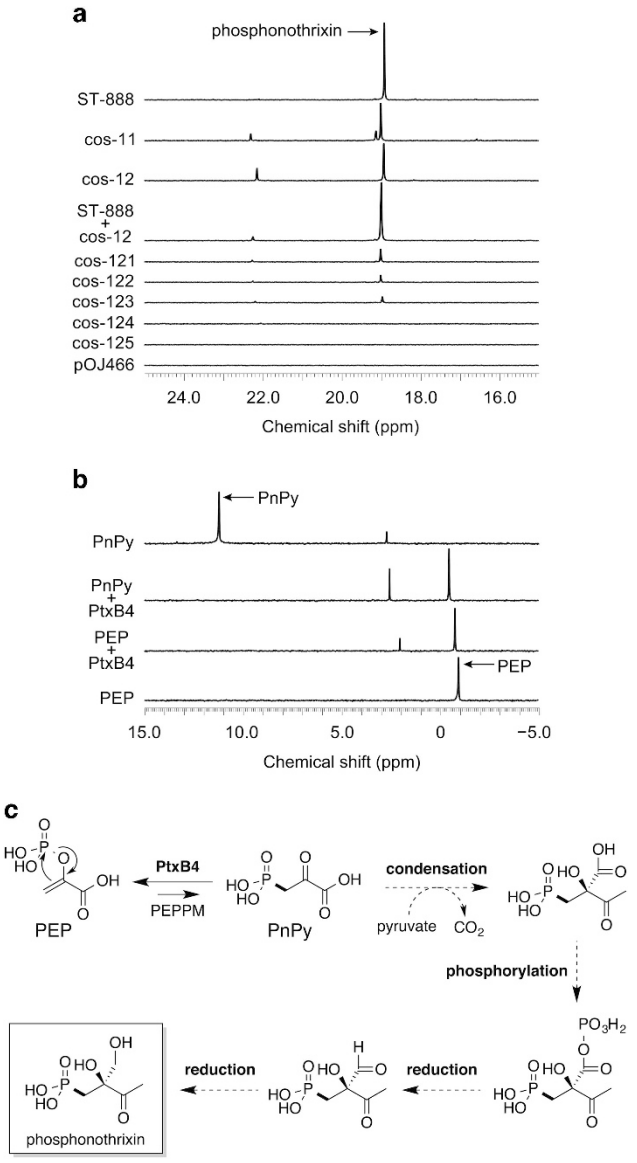

Figure 1 (**a**) Heterologous production of phosphonothrixin in *Streptomyces albus* transformants. ^{31}P NMR spectra (ECA 600, JEOL; 242 Hz) of partially purified phosphonates are shown. The transformants harboring cos-11, cos-12, cos-121, cos-122, cos-123, cos-124 or cos-125 (Supplementary Figure S3) were investigated. The phosphonothrixin-producing strain ST-888 and the transformant harboring the pOJ446 vector were used as positive and negative controls, respectively. Phosphonothrixin was detected at ~19 p.p.m. A 1:1 ST-888: cos-12 sample (ST-888+cos-12) exhibited a single peak at 19 p.p.m., unequivocally indicating the heterologous production of phosphonothrixin in the *S. albus* transformant harboring cos-12. (**b**) *In vitro* analysis of the PtxB4 reaction. ^{31}P NMR spectra (242 Hz) of the assay mixtures for PEPPM activity are shown. Recombinant PtxB4 completely converted PnPy to PEP over a 2-h incubation, as confirmed by ^{31}P NMR spectroscopy and comparison with commercial PEP (Sigma-Aldrich, Tokyo, Japan). In contrast, PEP was not converted to PnPy, when incubated with PtxB4. This result is consistent with the observation that the equilibrium between PEP and PnPy favors PEP by >500-fold.[11] The signals at 2–3 p.p.m. are due to impurities. (**c**) Proposed biosynthesis of phosphonothrixin. The first step that is confirmed by the experimental data with the purified recombinant PtxB4 enzyme is shown with solid arrows, whereas putative transformations are indicated with dashed arrows. Bold lines represent carbon–phosphorus bonds. PEP, phosphoenolpyruvate; PEPPM, PEP phosphomutase; PnPy, phosphonopyruvate.

for phosphonothrixin biosynthesis (Supplementary Table S1). However, several ORFs (for example, transporters) upstream of ORF21 could be necessary for the full production of phosphonothrixin. Curiously, putative non-ribosomal peptide synthetase-related ORFs (ORF21, ORF28 and ORF30) are likely involved in phosphonothrixin production. In addition, ORF14–34 have high sequence identities with the uncharacterized phosphonate biosynthetic gene clusters (accession number: KF386859 from *Streptomyces* sp. XY431 and accession number: KF386880 from *Streptomyces* sp. WM4235) (Supplementary Table S1).[10] Therefore, we suspect that *Streptomyces* sp. XY431 and *Streptomyces* sp. WM4235 are also phosphonothrixin producers.

PEPPM, which catalyzes the first step in phosphonate biosynthesis, greatly favors the reaction toward the carbon–phosphorus bond cleavage over the carbon–phosphorus bond formation (Supplementary Figure S1).[1,2] Similarly, the purified recombinant ORF24 (PtxB4) protein indeed exhibits such PEPPM activity (Figure 1b). Therefore, to ensure the carbon–phosphorus bond formation during phosphonate biosynthesis, a second enzyme following PEPPM is crucial.[1,2] To date, two biosynthetic reactions following the PEPPM reaction have been identified (Supplementary Figure S1).[1,2] The first reaction involves phosphonopyruvate decarboxylase, which catalyzes the decarboxylation of phosphonopyruvate to produce phosphonoacetoaldehyde in the biosynthesis of bialaphos and fosfomycin. The second reaction involves phosphonomethylmalate synthase, which is a homocitrate synthase homolog that is observed in FR-900098 biosynthesis. Both reactions are irreversible and exergonic. However, the phosphonothrixin biosynthetic gene cluster does not contain homologs of these two enzymes (Supplementary Table S1). We hypothesize that ORF25 and ORF26, which encode the transketolase N-terminal subunit and the transketolase C-terminal subunit, respectively, or ORF27, which encodes thiamine pyrophosphate enzyme, may catalyze the thiamine pyrophosphate-dependent condensation of phosphonopyruvate and pyruvate with concomitant loss of CO_2 (Figure 1).

In summary, we identified the *ptxB* genes required for phosphonothrixin biosynthesis using genome sequencing and heterologous expression. On the basis of bioinformatics analysis of the *ptxB* gene cluster (Supplementary Table S1), we propose the biosynthesis of phosphonothrixin as shown in Figure 1c, although it remains highly speculative. Further studies including the identification of the biosynthetic intermediates and *in vitro* assays of the *ptxB* gene products will enable us to clarify the entire phosphonothrixin biosynthetic pathway, leading to the elucidation of the novel biosynthetic machinery for natural phosphonates.

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Supplementary Information accompanies the paper on The Journal of Antibiotics website (http://www.nature.com/ja)