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In Vivo Characterization of Phosphotransferase-Encoding Genes *istP* and *forP* as Interchangeable Launchers of the C3′,4′-Dideoxygenation Biosynthetic Pathway of 1,4-Diaminocyclitol Antibiotics S

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Deactivation of aminoglycosides by their modifying enzymes, including a number of aminoglycoside *O*-phosphotransferases, is the most ubiquitous resistance mechanism in aminoglycoside-resistant pathogens. Nonetheless, in a couple of biosynthetic pathways for gentamicins, fortimicins, and istamycins, phosphorylation of aminoglycosides seems to be a unique and initial step for the creation of a natural defensive structural feature such as a 3′,4′-dideoxy scaffold. Our aim was to elucidate the biochemical details on the beginning of these C3′,4′-dideoxygenation biosynthetic steps for aminoglycosides. The biosynthesis of istamycins must surely involve these 3′,4′-didehydroxylation steps, but much less has been reported in terms of characterization of istamycin biosynthetic genes, especially about the phosphotransferase-encoding gene. In the disruption and complementation experiments pointing to a putative gene, *istP*, in the genome of wild-type *Streptomyces tenjimariensis*, the function of the *istP* gene was proved here to be a phosphotransferase. Next, an in-frame deletion of a known phosphotransferase-encoding gene *forP* from the genome of wild-type *Micromonospora olivasterospora* resulted in the appearance of a hitherto unidentified fortimicin shunt product, namely 3-*O*-methyl-FOR-KK1, whereas complementation of *forP* restored the natural fortimicin metabolite profiles. The bilateral complementation of an *istP* gene (or *forP*) in the ΔforP mutant (or ΔistP mutant strain) successfully restored the biosynthesis of 3′,4′-dideoxy fortimicins and istamycins, thus clearly indicating that they are interchangeable launchers of the biosynthesis of 3′,4′-dideoxy types of 1,4-diaminocyclitol antibiotics.

Keywords: Aminoglycoside, istamycin, fortimicin, *istP*, 3′,4′-didehydroxylation, 1-*O*-methyl-FOR-KK1

Aminoglycosides (AGs) are the first and foremost classical antibiotics that are biosynthesized by attachment of amino-sugar units via glycosidic bonds to aminocyclitol derivatives [1]. They are recognized to inhibit prokaryotic protein synthesis by binding to the 16S rRNA subunit of the 30S bacterial ribosome, thus causing irreversible bactericidal effects against the pathogens [2]. According to the chemical features of the aminocyclitol moiety and the core aglycone unit, AGs have been categorized into well-known types containing 2-deoxystreptamine (DOS), (including 4,5-disubstituted butirosins and neomycins or 4,6-disubstituted gentamicins and kanamycins) and 1,4-diaminocyclitol-containing AGs [3]. In the late 1970s, the above 1,4-diaminocyclitol-containing AGs, namely fortimicins (FORs) and istamycins (ISTs), were isolated from *Micromonospora olivasterospora* and *Streptomyces tenjimariensis*, respectively [4, 5]. The two share an unusual pseudo-disaccharide moiety that is different from a common

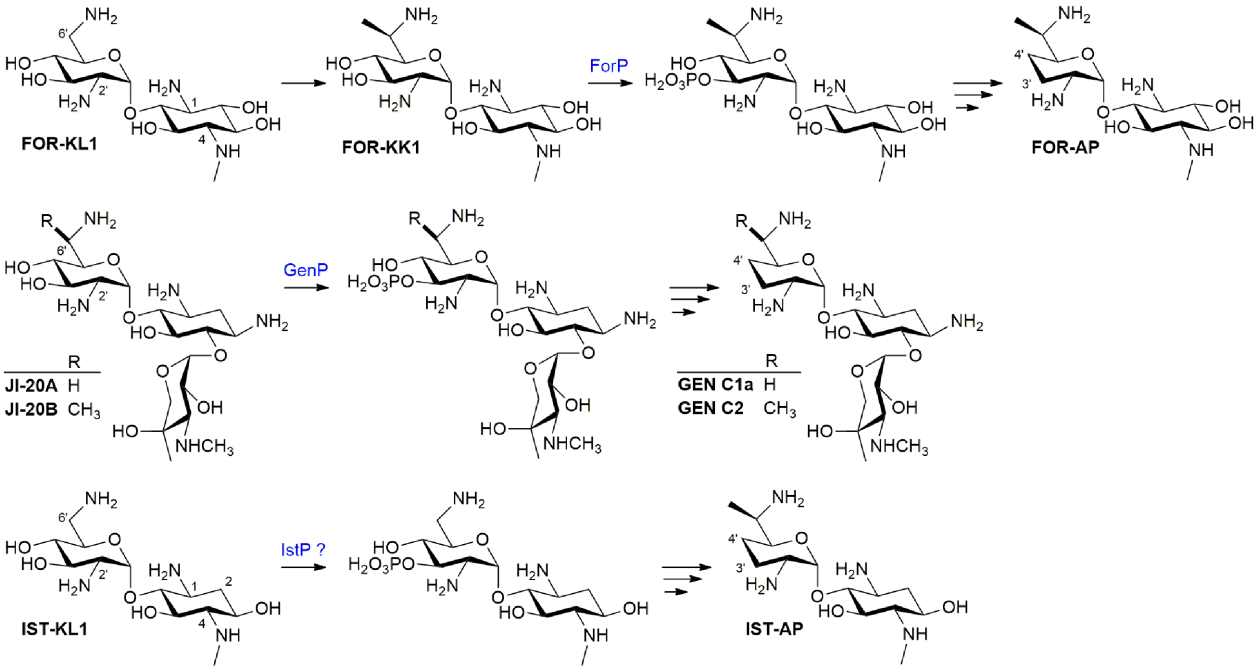

Fig. 1. The biosynthetic route to 3′,4′-dideoxy types of fortimicins, gentamicins, and istamycins.
Three different phosphotransferases involved in the above aminoglycosides' biosynthesis are depicted as ForP, GenP and IstP, respectively.

pseudotrisaccharide nature of the DOS-containing gentamicins (Fig. 1); however, they show not only decreased ototoxicity and nephrotoxicity compared to the DOS-containing AGs but also refractoriness toward AG-resistance mechanisms [6]. Deactivation of AGs by AG-modifying enzymes (AMEs) in pathogens is the most ubiquitous resistance mechanism and causes severe clinical problems [7]. Fortunately, unlike most AGs, some AGs possess a natural defensive structural feature effective against these AMEs, *e.g.*, 3′,4′-dideoxygenation, which is unique to gentamicins (GENs), FORs, and ISTs [8].

In 1992, while organizing a FOR biosynthetic gene cluster, Dairi and Japanese coworkers aligned *fms8* (also known as *forP*) as a homologue of a neomycin resistance gene [9]. In the complementation studies here using a cell-free extract of the ΔforP mutant and its wild-type strain, the gene product ForP was proven to perform phosphorylation of FOR-KK1 in the presence of ATP, thus demonstrating that the 3′,4′-dideoxygenation reaction is likely to occur via the phosphorylation of the 3′-hydroxyl group (Fig. 1). Recently, a putative biosynthetic gene, *gntI* (also known as *genP*), isolated from the gentamicin gene cluster, was expressed in *Escherichia coli*, and its protein product GenP was also characterized as a regio-specific phosphotransferase in in vitro enzymatic reactions [10]. Therefore, the above-mentioned reports suggest not only that these kinds of phosphotransferase-encoding genes exist in the gene clusters responsible for 3′,4′-dideoxy AG biosynthesis but also that phosphorylation of the 3′-hydroxyl functional group should be the initial catalytic step of the 3′,4′-dideoxygenation AG biosynthetic pathways [9, 10]. Most recently, as the last tailoring step for the diversification of 3′,4′-dideoxy-GENs C, including the biosynthesis of GENs C1 and C2b from GENs C2 and C1a, respectively, a new 6′-*N*-methyltransferase, GenL, was discovered and functionally characterized [11]. Nonetheless, the biochemical details on C3′,4′-dideoxygenation sequential steps for AGs still await to be elucidated. Of note, the biosynthesis of ISTs must surely involve the 3′,4′-didehydroxylation steps that are equivalently shared by GEN and FOR biosynthetic pathways. To our knowledge, however, much less has been learned regarding the characterization of IST biosynthetic genes, especially about the phosphotransferase-encoding gene.

Herein, after being chosen by *in silico* analysis as a homolog of two known phosphotransferase-encoding genes, *genP* and *forP*, the gene *istP* was disrupted from the genome of wild-type *S. tenjimariensis* by targeted in-frame deletion. Liquid chromatography (LC) with electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) analyses of the IST metabolites obtained from the resulting ΔistP mutant strain clearly indicated the complete abrogation of the

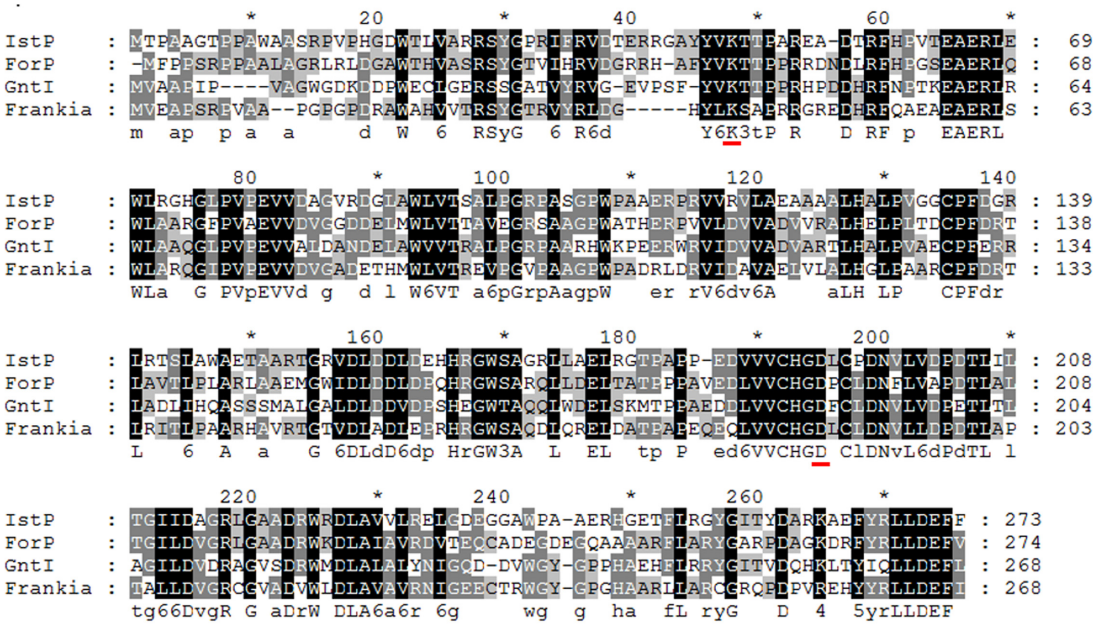

Fig. 2. *In silico* amino acid sequence alignment of IstP and its homologs such as ForP, GntI (also known as GenP) and a gene product from *Frankia* species involved in aminoglycoside biosynthesis.
Two red underlined residues are associated with ATP-binding sites.

synthesis of 3′,4′-dideoxy ISTs, with accumulation of IST-KL1 as compared to the wild-type strain. Complementation of this ΔistP strain restored the production of 3′,4′-dideoxy ISTs, including IST-A and IST-B, as in the wild type. Next, instrumental analyses of FOR congeners produced in the ΔforP mutant strain of *M. olivasterospora* revealed emergence of a new FOR shunt product, namely 3-*O*-methyl-FOR-KK1, along with the expected FOR-KL1. Moreover, the bilateral complementation of the *istP* gene (or *forP*) in the ΔforP mutant (or ΔistP mutant strain) successfully restored the biosynthesis of 3′,4′-dideoxy FORs and ISTs.

Based on *in silico* analysis, gene product IstP could be a member of the AphA/APH(3′) subfamily of AG kinases and was also predicted to be a unique phosphotransferase found in the IST biosynthetic gene cluster (GenBank Accession No. AJ845083). In addition, according to a multiple amino acid sequence alignment, this deduced protein is highly homologous to known ForP and GenP; the identity of these proteins is approximately 67–71% and similarity 65–93% (Fig. 2). To investigate the biochemical properties of the product of the putative *istP* gene, it was disrupted by targeted in-frame deletion using a homologous recombination approach; DNA fragments flanking upstream and downstream parts of this gene were amplified from the genomic DNA of *S. tenjimariensis* ATCC31603 (Table S1). All the bacterial strains and plasmids used in this study were also summarized in Table S2. A conserved 423 bp sequence of the *istP* gene was removed by means of primer pairs (istP-L1/L2 and istP-R1/R2) in PCR. The PCR products were each cloned into a pGEM-T-easy vector and were verified by sequencing. After that, the right-hand PCR fragments were cut out with EcoRI and BamHI and ligated into a derivative of *Streptomyces–E. coli* shuttle vector pKCE, into which erythromycin-resistance gene *ermE* was additionally introduced via the BglII site of the original pKC1139 vector [12]. Next, left-hand PCR fragments were digested with XbaI and HindIII and ligated into the vector at the same restriction sites, yielding the disruption plasmid pIP501. This plasmid was transfected into a strain of *E. coli* ET12567/pUZ8002 [12], and subsequently introduced into the wild-type strain of *S. tenjimariensis* ATCC31603 by intergeneric conjugation on R6 agar plates. After overlaying with apramycin (25 μg/ml), nalidixic acid (25 μg/ml), and erythromycin (50 μg/ml), incubation was continued at 28°C for a week. The genomic DNA of the resulting exconjugant was isolated and PCR was carried out using the oligonucleotide primers designed for *istP*; the PCR product (675 bp) was sequenced and compared with that from the wild-type strain of *S. tenjimariensis* (1,098 bp), thus, the ΔistP mutant strain was confirmed (Fig. S1). The authentic mutant and its wild-type strains of *S. tenjimariensis* were cultivated in the ISP1 liquid medium at 28°C for

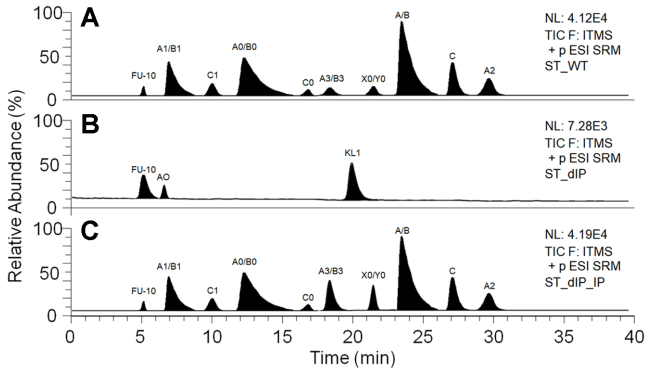

Fig. 3. LC-ESI-MS/MS analysis of the istamycin congener profiles produced by (**A**) the wild-type strain, (**B**) the ΔistP mutant strain, and (**C**) the complemented (ΔistP::*istP*) strain of *Streptomyces tenjimariensis* ATCC31603.

Tracing was performed using MS/MS operated in the SRM mode by choosing mass pairs specific to the istamycin congeners to detect the transition of the protonated parent ion to typical product ion; IST-FU-10 (*m/z* 325.3 > 145.1), IST-A1(or IST-B1) (*m/z* 418.3 > 126.2), IST-C1 (*m/z* 432.3 > 140.2), IST-A0(or IST-B0) (*m/z* 333.3 > 126.2), IST-C0 (*m/z* 347.3 > 140.2), IST-A3(or IST-B3) (*m/z* 417.3 > 126.2), IST-X0(or IST-Y0) (*m/z* 319.3 > 112.1), IST-A(or IST-B) (*m/z* 390.3 > 126.2), IST-C (*m/z* 404.3 > 140.2), IST-A2 (*m/z* 433.3 > 126.2), IST-AO (*m/z* 338.3 > 145.1) and IST-KL1 (*m/z* 337.3 > 144.1).

5 days, and then the resultant IST metabolite profiles were analyzed by LC-ESI-MS/MS as described before [13]. As shown in Fig. 3, the wild-type strain produced diverse kinds of IST congeners, in good agreement with our previous publication (Fig. 3A) [13]. Nonetheless, the ΔistP mutant did not produce any kinds of 3′,4′-dideoxy ISTs, instead accumulating IST-KL1 as the main one (Fig. 3B). A complementation plasmid was prepared by cloning *istP* into pNPBE1 (a derivative of the pSET152 plasmid [14], into which a chloramphenicol resistance gene, *cmr*, was inserted under the control of the P*erm*E* promoter). The PCR products obtained for the cloning of *istP* were inserted into pNPBE1 between the BamHI and XbaI sites to produce pISTP (Table S1). After sequencing confirmation, this plasmid was introduced into the ΔistP mutant strain of *S. tenjimariensis* by conjugation, and then the complemented exconjugants (ΔistP::*istP*) were verified on the basis of chloramphenicol resistance and confirmed by PCR (data not shown). The genetic complementation of the ΔistP mutant with an *istP* gene induced the corresponding recovery of 3′,4′-dideoxy IST production (Fig. 3C).

Besides, for inactivation of the *forP* gene, an in-frame deletion of a conserved 501 bp sequence was carried out using pKC1139. Primers were designed using the sequence of the FOR biosynthetic gene cluster (GenBank Accession No. AJ628421) from *M. olivasterospora* DSM43868 as a template, and their sequences are shown in Table S1. In accordance with the above-mentioned process employed for the *istP* disruption plasmid, a *forP* disruption plasmid pFP501 was also constructed. To create a ΔforP mutant strain, the corresponding plasmid pFP501 was introduced into the wild-type strain of *M. olivasterospora* by conjugation on MS agar plates supplemented with 10 mM $MgCl_2$ and 10% sucrose. After selection on the plates overlaid with apramycin (25 μg/ml) and nalidixic acid (25 μg/ml), exconjugants were first selected for an apramycin-resistant phenotype as the single crossover event and then for an apramycin-sensitive phenotype (the double crossover event) via a series of liquid cultures without an antibiotic. Screening of the resulting progeny for an apramycin-sensitive phenotype gave rise to the ΔforP mutant strain. The genomic DNA of the ΔforP mutant was isolated, and the PCR product (2,427 bp) amplified with the primer pair (forP-L1 and forP-R2, see Table S1) was sequenced and compared with that from the wild-type strain (2,928 bp) (Fig. S2). The mutant and its wild-type strains of *M. olivasterospora* were cultivated in the NZ-amine liquid medium at 28°C for 8 days, and then their FOR congener profiles were analyzed by LC-ESI-MS/MS. The wild-type strain produced diverse kinds of FOR congeners, in line with one report [15]; a number of 3′,4′-dideoxy FORs including FOR-AP, FOR-B/KH/KR, FOR-A, and dactimicin (DCM) were found to be the main components (Fig. 4A). In contrast, in case of the ΔforP mutant, the synthesis of the above 3′,4′-dideoxy FORs was definitely abrogated, with accumulation of FOR-KL1 and FOR-KK1 (Fig. 4B). Nevertheless, as a slight difference from the typical IST metabolite profiles of a ΔistP mutant (Fig. 3), a new and discrete FOR peak at a retention time of 11.4 min was detected in the ΔforP mutant stain of *M. olivasterospora* (Fig. 4B). High resolution-MS analysis was performed on a Waters XEVO G2-XS Q-TOF mass spectrometer (Waters Corp., USA), whereas NMR spectra were acquired using a Varian INOVA 500 MHz spectrometer (Palo Alto, USA) operated at 500 MHz for ^{1}H and 125 MHz for ^{13}C nuclei. According to its fragmentation pattern and MS/MS spectra (Fig. S3), this FOR with the protonated molecular ion $[M+H]^+$ at *m/z* 381.3 was proposed to be 3-*O*-methyl-FOR-KK1, and finally, we confirmed its new chemical structure by subsequent high-resolution MS spectra and NMR assignments (Fig. S4).

This FOR has not been reported as a FOR congener and instead is present only in the ΔforP mutant. Therefore, 3-*O*-

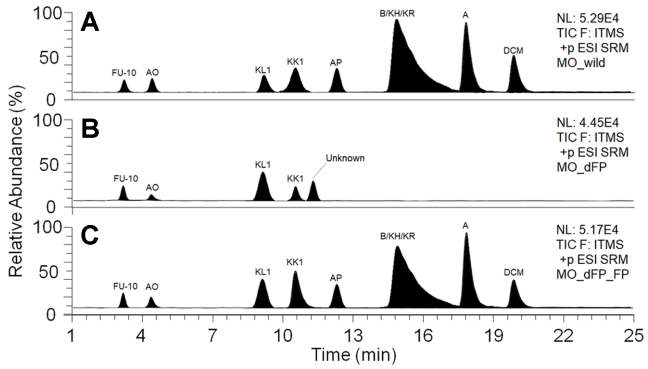

Fig. 4. LC-ESI-MS/MS analysis of the fortimicin congener profiles produced by (**A**) the wild-type strain, (**B**) the ΔforP mutant strain, and (**C**) the complemented (ΔforP::*forP*) strain of *Micromonospora olivasterospora* DSM43868.

Tracing was performed using MS/MS operated in the SRM mode by choosing mass pairs specific to the fortimicin congeners to detect the transition of the protonated parent ion to typical product ion; FOR-FU-10 (*m/z* 341.3 > 145.1), FOR-AO (*m/z* 354.3 > 145.1), FOR-KL1 (*m/z* 353.3 > 144.1), FOR-KK1 (*m/z* 367.3 > 158.1), FOR-AP (*m/z* 335.3 > 126.1), FOR-B (or FOR-KH or FOR-KR) (*m/z* 349.3 > 126.1), FOR-A (*m/z* 406.3 > 126.1), DCM (*m/z* 433.3 > 126.1) and unknown (*m/z* 381.3 > 158.1).

methyl-FOR-KK1 appears to be a shunt metabolite derived from the disruption of FOR biosynthetic genes. As a product of some part of the FOR biosynthetic gene cluster (GenBank Accession No. AJ628421), gene product ForO was *in silico* predicted to act as 3-*O*-methyltransferase on a 3′,4′-dideoxy FOR-AP. Although there is still no biochemical evidence for the function of ForO, detection of the above-mentioned shunt product in a ΔforP mutant implies substrate flexibility of this tailoring enzyme ForO, thus supporting the feasibility of mutasynthesis as one of the biological tools for the synthesis of some novel AGs. Next, to verify the biochemical role of the *forP* gene, a complementation plasmid was prepared by cloning *forP* into pNPBE2 (a derivative of the pSET152 plasmid, into which a thiostrepton resistance gene, *tsr*, was inserted along with apramycin resistance gene *aac(3)-IV* under the control of the P*erm*E* promoter). The PCR products prepared for the cloning of *forP* were inserted into pNPBE2 between the BamHI and XbaI sites to obtain pFORP (Tables S1 and S2). This plasmid was introduced into the ΔforP mutant strain of *M. olivasterospora* by conjugation, and then the complemented exconjugants (ΔforP::*forP*) were verified based on thiostrepton resistance and confirmed by PCR (data not shown). LC-ESI-MS/MS analysis of the culture extracts of this genetically complemented strain of *M. olivasterospora* revealed total restoration of 3′,4′-dideoxy FORs (Fig. 4C). Nonetheless, the above-mentioned shunt product 3-*O*-methyl-FOR-KK1 detected in the culture extracts of ΔforP mutant disappeared from those of the complemented strain of *M. olivasterospora*.

At last, to investigate the cross-compatibility of each phosphotransferase-encoding gene, we carried out bilateral complementation of the heterologous ΔistP or ΔforP mutant with a phosphotransferase-encoding gene analog *forP* or *istP*, respectively. To this end, the *forP* (or *istP*) gene located in the corresponding pFORP (or pISTP) plasmid was cut out with restriction enzymes BamHI and XbaI and then ligated into the empty pNPBE1 (or pNPBE2) vector cut at the same restriction sites, thereby yielding pFORP1 and pISTP2. These constructed plasmids were reciprocally introduced into the ΔistP mutant strain of *S. tenjimariensis* and ΔforP mutant strain of *M. olivasterospora* by conjugation, and then the exconjugants (ΔistP::*forP* and ΔforP::*istP*) were verified via antibiotic resistance and confirmed by PCR (data not shown). Subsequent LC-ESI-MS/MS analyses of the culture extracts derived from the above exconjugants clearly indicated that the bilateral complementation of both phosphotransferase-encoding genes in two different strains, namely *M. olivasterospora* and *S. tenjimariensis*, leads to restoration of their natural FOR or IST metabolite profiles, equivalent to their wild-type strains (Figs. S5 and S6) [13, 15].

Hence, in spite of the structural distinction at the C2 position between FOR-KK1 (as a natural substrate of ForP) and IST-KL1 (also as a natural substrate of IstP), these findings pointed to a similar function of the two genes (*istP* and *forP*) in the 1,4-diaminocyclitol biosynthetic pathways, as well as their cross-compatibility for the biosynthesis of 3′,4′-dideoxy FORs and ISTs. Indeed, according to the in vivo characterization of *istP* and *forP* genes using both gene disruption and subsequent complementation, along with the mutasynthetic approach, the results were as follows: i) the function of a putative *istP* gene, homologous to other (known) *forP* and *genP*, is proven to be a phosphotransferase, ii) the ΔforP mutant strain of *M. olivasterospora* is ready for the synthesis of a shunt FOR metabolite, namely 3-*O*-methyl-FOR-KK1, iii) genes *istP* and *forP* can be interchangeable as a launcher of the biosynthesis of 1,4-diaminocyclitol antibiotics, owing to acceptability of both substrates IST-KL1 and FOR-KK1 for the gene products of *istP* and *forP*. Future studies on enzymatic kinetics and biochemical characterization of IstP will not only provide a better understanding of the biosynthetic routes of 3′,4′-dideoxy AGs, including GENs as well as FORs and ISTs, but also

give some clues to the enigmatic C3′,4′-dideoxygenation biosynthetic pathway.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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