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Biosynthesis of 2-Deoxystreptamine-containing Antibiotics in *Streptoalloteichus hindustanus* JCM 3268: Characterization of 2-Deoxy-*scyllo*-inosose Synthase

Toshifumi Hirayama, Hideyuki Tamegai†, Fumitaka Kudo, Kazumasa Kojima, Katsumi Kakinuma, Tadashi Eguchi

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Abstract A part of the new biosynthetic gene cluster for 2-deoxystreptamine-containing antibiotics was identified from *Streptoalloteichus hindustanus*. The *alloH* gene in the gene cluster was deduced to encode 2-deoxy-*scyllo*-inosose synthase and the expressed protein AlloH was confirmed to have this enzyme activity. Furthermore, biochemical properties of AlloH were studied.

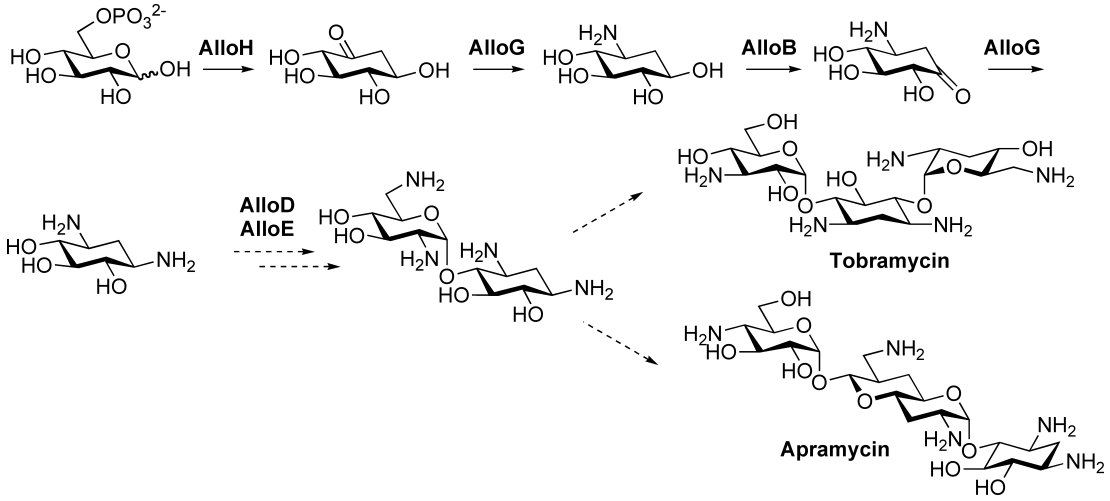
Keywords 2-deoxystreptamine biosynthetic genes, *Streptoalloteichus hindustanus*, 2-deoxy-*scyllo*-inosose synthase, kinetics

Aminoglycosides are an important group of antibiotics in clinical use for a long time. Even today, new activities of aminoglycosides are being discovered; for example, the activity of arginine complexes against HIV [1, 2]. Aminoglycosides are structurally oligosaccharides containing one of a small number of specific aminocyclitols and various amino sugars, which are connected together through the glycosidic bonds. Indeed, the biosynthetic enzymes catalyze the syntheses of each unit and the transfer reactions, which connect them. Therefore, structurally different aminoglycosides are expected to be constructed by combination of such biosynthetic enzymes through the gene organization in microorganisms. 2-Deoxy-*scyllo*-inosose (DOI) synthase catalyzes the first crucial carbocyclization in the most abundant aminocyclitol 2-deoxystreptamine (DOS). Our identification of this gene in the butirosin producer *Bacillus circulans* [3], is an important key to the identification of many DOS-containing antibiotic gene clusters. So far, in addition to the butirosin biosynthetic gene cluster [4, 5], the biosynthetic genes for tobramycin [6], kanamycin [7, 8], neomycin [9, 10], ribostamycin [11], hygromycin B (only sequence data available; AJ628642), istamycin (AJ845083), lividomycin (AJ748832), paromomycin (AJ628955), and apramycin (AJ629123) from *Streptomyces*, and gentamicin [12, 13] from *Micromonospora* have been identified. Recombination of various these genes is expected to lead novel aminoglycosides by combinatorial biosynthesis.

We here report the identification of the biosynthetic gene cluster of DOS-containing antibiotics in *Streptoalloteichus hindustanus* [14], which, as well as *Streptomyces tenebrarius*, is known as an apramycin/tobramycin producer (Scheme 1). *Streptoalloteichus hindustanus* presents a new genus as a source of aminocyclitol biosynthetic genes beyond *Streptomyces*, *Micoromonospora*, and *Bacillus*, and thus it was anticipated to provide novel features of the biosynthetic enzymes.

Among the DOS-containing antibiotic biosynthetic gene clusters, the gene for L-glutamine:DOI aminotransferase is completely conserved and the enzyme catalyzes both transaminations in the DOS-formation [15]. This type of

T. Eguchi (Corresponding author)**, T. Hirayama:** Department of Chemistry and Materials Science, Tokyo Institute of Technology, 2-12-1 O-okayama, Meguro-ku, Tokyo 152-8551, Japan,
E-mail: eguchi@cms.titech.ac.jp
H. Tamegai, F. Kudo, K. Kojima, K. Kakinuma: Department of Chemistry, Tokyo Institute of Technology, 2-12-1 O-okayama, Meguro-ku, Tokyo 152-8551, Japan
† Present address: Department of Chemistry, College of Humanities and Sciences, Nihon University, 3-25-40 Sakurajosui, Setagaya-ku, Tokyo 156-8550, Japan



Scheme 1 Biosynthesis of 2-deoxystreptamine-containing antibiotics in *Streptoalloteichus hindustanus*.

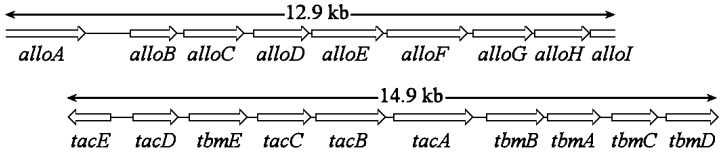


Fig. 1 Comparison of the DOS-containing antibiotic biosynthetic genes in *Streptoalloteichus hindustanus* (upper) and in *Streptomyces tenebrarius* (bottom).

The *allo* genes in *Streptoalloteichus hindustanus*, except for *alloA*, showed significant homology to the tobramycin biosynthetic genes in *Streptomyces tenebrarius* and the arrangement of these homologous genes is also the same. See also Table 1.

Table 1 Gene cluster from *Streptoalloteichus hindustanus*

Genes	Size (bp)	Products of homologous genes	Origins	Homology
alloA	1687	putative dehydrogenase	*Bradyrhizobium japonicum*	36%
alloB	1020	putative dehydrogenase (*tacD*)	*Streptomyces tenebrarius*	79%
alloC	1299	putative transport protein (*tbmE*)	*Streptomyces tenebrarius*	62%
alloD	1188	putative aminotransferase (*tacC*)	*Streptomyces tenebrarius*	81%
alloE	1527	putative dehydrogenase (*tacB*)	*Streptomyces tenebrarius*	78%
alloF	1713	putative carbamoyltransferase (*tacA*)	*Streptomyces tenebrarius*	87%
alloG	1275	DOI aminotransferase (*tbmB*)	*Streptomyces tenebrarius*	87%
alloH	1158	DOI synthase (*tbmA*)	*Streptomyces tenebrarius*	81%
alloI	>558	putative dehydrogenase (*tbmC*)	*Streptomyces tenebrarius*	69%

aminotransferase gene is also seen in the streptomycin [16] and fortimicin (AJ628421) biosynthetic gene clusters and was proposed to be a common gene among any aminocyclitol biosynthetic systems [16]. In fact, using the genetic information of this enzyme, many DOS-containing antibiotic biosynthetic gene clusters have been identified.

Similarly, by PCR screening against a cosmid library (using pOJ446 as a vector) of *Sau*3AI digested genomic DNA of *Streptoalloteichus hindustanus* using the degenerate primers to amplify the aminotransferase gene [17], a cosmid callo16-16 was identified (Fig. 1 and Table 1). As a result of DNA sequence analysis, seven complete and two incomplete open reading frames (ORFs) were found in the gene cluster (deposited as AB103327) and, with the exception of *alloA*, showed significant homology to the tobramycin biosynthetic genes from *Streptomyces*

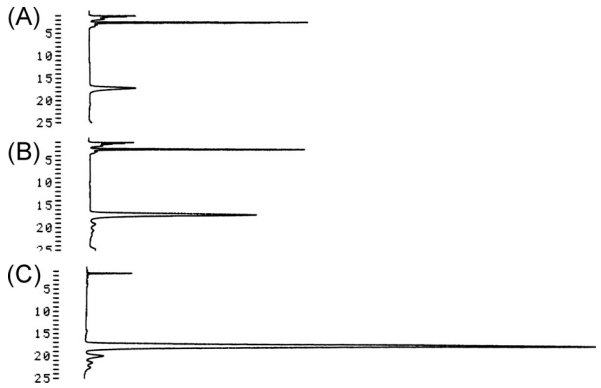


Fig. 2 HPLC of the derivatives from enzyme reaction using AlloH (A), (A)+authentic DOI oxime derivatives (B), authentic DOI oxime derivatives (C).

tenebrarius (Table 1). The arrangement of these homologous genes is also the same. Thus, the present identified gene cluster appears to be responsible to the tobramycin biosynthesis in *Streptoalloteichus hindustanus*. The *alloA* gene encodes a putative PQQ-dependent alcohol dehydrogenase that has not been seen in other aminoglycoside biosynthetic gene clusters, suggesting that possibly the *alloA* gene may be outside the biosynthetic cluster.

To confirm involvement of the *allo* genes in the antibiotic biosynthesis, the function of *alloH* gene encoding DOI synthase was investigated. Although several DOI synthases were heterologously expressed and found to be active, their biochemical properties have not been reported except for the single case of BtrC from *B. circulans* [3]. Since DOI is a versatile material for fine chemicals such as catechol [18] and carbaglucose [19], a highly efficient enzyme is desirable. Therefore, the enzymatic properties of AlloH were determined in the present study.

The *alloH* gene was thus expressed in *Escherichia coli* in a standard manner and the expressed AlloH was purified to homogeneity by DEAE Sepharose chromatography and gel filtration. Following the previously reported conditions for the BtrC reaction [3], AlloH was assayed with 5 mM of glucose-6-phosphate and 5 mM of NAD^+ at 46°C for 30 minutes. As shown in Fig. 2, AlloH was clearly confirmed as DOI synthase, indicating that the *allo* gene cluster is responsible for the biosynthesis of DOS-containing aminoglycoside antibiotics in *Streptoalloteichus hindustanus*.

Using the pure enzyme, the kinetic constants of AlloH were determined in a standard manner to be 0.075 s^{-1} and 0.69 mM for k_{cat} and K_m (for G-6-P), respectively (Table 2). Compared with those of the BtrC, 1.0 s^{-1} (k_{cat}) and 0.21 mM (K_m), AlloH was less efficient than BtrC. The k_{cat} value was significantly lower, indicating that the catalytic amino acid residues may not be well organized in the active site of AlloH. The probable catalytically important amino acids presumed from the crystal structure of 3-dehydroquinate synthase [20], which catalyzes a similar reaction in the shikimate pathway, are highly conserved in AlloH and BtrC [3, 21]. No metal was required during purification of AlloH, whereas BtrC was inactive without Co^{2-} ion. In addition, an association with NAD^+ was observed in the reaction at low concentrations (data not shown). Although these properties may affect on the catalytic efficiency, the reason for the lower activity of AlloH is unclear at the moment. Amino acid sequences of all known DOI synthases from actinomycete are very similar but somewhat different to that of BtrC from *Bacillus*. This may suggest that all of the actinomycete DOI synthases are of lower activity. Detailed studies of DOI synthases including BtrC on the molecular level may open a way to engineer the enzymes to be more efficient.

Table 2 Kinetic constants of AlloH and BtrC

	K_m (mM)	k_{cat} (sec^{-1})	k_{cat}/K_m (mM^{-1} sec^{-1})
AlloH	0.69	0.075	0.11
BtrC	0.21	1.0	4.8

The K_m values for G-6-P are shown.

In the present paper, a new aminoglycoside biosynthetic gene cluster from *Streptoalloteichus hindustanus* was identified showing that the *allo* genes are quite similar to the other known aminoglycosides biosynthetic genes from *Streptomyces*, *Micromonospora*, and *Bacillus*. This suggests that the biosynthetic genes were horizontally transferred during evolution and organized in each microorganism, and raises the likelihood that combinatorial fashioned arrangement of the genes beyond species could produce structurally diverse natural/unnatural aminoglycoside antibiotics. Indeed, the unknown functional genes in the biosynthetic gene clusters should be characterized for creation of made-to-order antibiotics.

While this manuscript was in preparation, a part of the apramycin biosynthetic gene cluster derived from *Streptoalloteichus hindustanus* DSM44523 (accession number AJ875019) was opened to public on the web. This cluster is different from the present identified cluster. Therefore, *Streptoalloteichus hindustanus* seems to have two sets of DOS-containing antibiotic biosynthetic gene cluster for tobramycin and apramycin.

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