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NOTE

Molecular Detection of α-Glucosidase Inhibitor-producing Actinomycetes

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In this study, we demonstrate the use of a PCR-based method for the detection of the specific genes involved in natural-product biosynthesis. This method was applied, using specifically designed PCR primers, to the amplification of a gene segment encoding for *sedo*-heptulose 7-phosphate cyclase, which appears to be involved in the biosynthetic pathways of C_7N aminoacyclitol or its keto analogue-containing metabolites, in a variety of actinomycetes species. The sequences of DNA fragments (about 540 bp) obtained from three out of 39 actinomycete strains exhibited a high degree of homology with the *sedo*-heptulose 7-phosphate cyclase gene, which has been implicated in acarbose biosynthesis. The selective cultivation conditions of this experiment induced the expression of these loci, indicating that the range of C_7N aminoacyclitol or its keto analogue-group natural products might be far greater than was previously imagined. Considering that a total of approximately 20 C_7N aminoacyclitol metabolites, or its keto analogue-containing metabolites, have been described to date, it appears likely that some of the unknown loci described herein might constitute new classes of C_7N aminoacyclitol, or of its keto analogue-containing metabolites. As these metabolites, some of which contain valienamine, are among the most potent antidiabetic agents thus far discovered, the molecular detection of specific metabolite-producing actinomycetes may prove a crucial step in current attempts to expand the scope and diversity of natural-product discovery.

Key words: *sedo*-heptulose 7-phosphate cyclase, C_7N aminoacyclitol, α-glucosidase inhibitor, molecular method

Microbial natural products constitute one of the most important sources of lead compounds in the pharmaceutical industry. Despite a trend towards the use of alternative sources, including synthetic combinatorial libraries, computer-based molecular modeling and, most recently, combinatorial biology or the manipulation of biosynthetic gene clusters, the pharmaceutical pipeline remains crowded with traditional, microbial-derived natural products, and their derivatives (Jansen *et al.*, 2003).

Members of the order *Actinomycetales*, like the streptomycetes, belong to the most prominently known group of microbes, due to their demonstrated importance in the medical field. About two-thirds of the naturally-derived antibiotics in current use, and most of the important bioactive metabolites, are synthesized by these Gram-positive soil bacteria (Kim *et al.*, 1998; Yoo *et al.*, 2002; Weber *et al.*, 2003). As the result of the complete sequencing of two genomes, *Streptomyces coelicolor* and *S. avermitilis*, a striking discovery was made--the identification of a staggering number of gene clusters wholly devoted to the synthesis of secondary metabolites (Bentley *et al.*, 2002; Ikeda *et al.*, 2003). Bentley *et al.* (2002) counted 23 such clusters, while attempting to locate genes typical for secondary metabolism. This number probably represents a fairly low estimate, as it excludes clusters that are carried on large plasmids, such as SCP1, and also does not include a host of smaller clusters, which may consist of as-yet-uncharacterized genes involved with secondary metabolism. Therefore, it seems relatively safe to surmise that the average actinomycete harbors the genetic potential to produce at least 10-20 secondary metabolites (Donadio *et al.*, 2002). This explains, at least in part, the overall success with which the streptomycetes and related genera have been identified as viable sources of bioactive metabolites.

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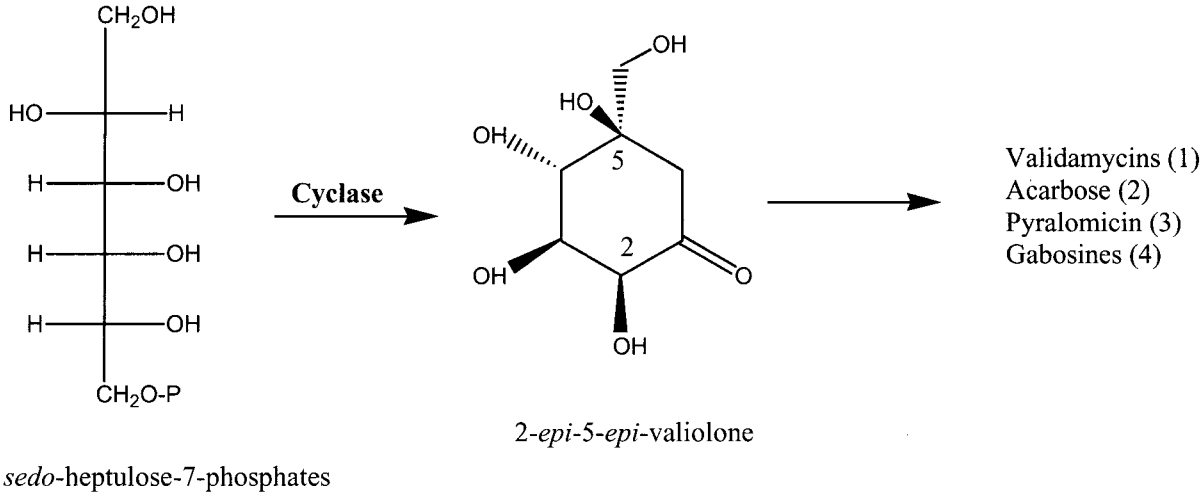

Fig. 1. Formation of 2-*epi*-5-*epi*-valiolone, the precursor of the cyclitol moieties in validamycin, acarbose, pyralomicin, and the gabosines.

The C_7N aminocyclitol family is a relatively newly-discovered class of natural products, and is gaining increasing recognition, due to the significant biomedical and agricultural applications with which it has been associated. The common chemical structures of this family include an unsaturated aminocyclitol moiety, valienamine, which has been implicated in the α-glucosidase inhibitory effects observed in conjunction with many of the members of this family, although the epoxy, hydroxy, and dihydro forms of valienamine have been detected in several other compounds (Mahmud *et al.*, 2001; Chen *et al.*, 2003; Mahmud, 2003). Recently, several studies have reported the isolation of a number of unusual C_7N aminocyclitol-containing natural products which exhibit antibiotic, anti-inflammatory, and anti-tumor effects, as well as antiviral and α-glucosidase inhibitory properties (Namiki *et al.*, 1982; Yokose *et al.*, 1983; Vertesy *et al.*, 1984). Studies regarding the biosynthesis of the C_7N aminocyclitols and their keto-analogues, e.g. acarbose (Mahmud *et al.*, 2001), validamycin (Dong *et al.*, 2001), pyralomicin (Naganawa *et al.*, 2002), and the gabosines (Regina *et al.*, 2000), have established several closely-related novel metabolic pathways involving 2-*epi*-5-*epi* valiolone (ketocyclitol intermediate), all of which appear to be derived from a common open-chain C_7 sugar phosphate, which is itself involved in the pentose phosphate pathway (Fig. 1). The isolation of a variety of aminoacyclitol keto-analogues, including the glyoxalase I inhibitor, KD16U1, and the gabosines detected in a host of *Streptomyces* species, indicate that this type of compound may be widely distributed among soil bacteria (Fig. 2).

In the present study, we developed a set of oligonucleotide primers for the amplification of DNA fragments of the genes for *sedo*-heptulose 7-phosphate cyclase from actinomycetes. These primers proved efficient with regard to the amplification and isolation of several types of C_7N aminocyclitols or their keto-analogue biosynthetic genes, which encode for 2-*epi*-5-*epi* valiolone intermediates. This approach exploits the fact that the genes involved in secondary metabolite biosynthesis are typically found clustered together within the bacterial genome. As a first target, we selected the C_7N aminocyclitol or its keto analogue-containing metabolites. This selection was predicated on the assumption that one of these groups, either valienamine or one of its analogues, could be used in the treatment of type II diabetes. Most C_7N aminocyclitols, like its keto analogue moieties such as acarbose, validamycin, pyralomicin, and gabosine, harbor a common pentose phosphate pathway at the early stage. *sedo*-Heptulose 7-phosphate was converted into 2-*epi*-5-*epi*-valiolone via the activity of *sedo*-heptulose 7-phosphate cyclase at the first step of the biosynthesis of C_7N aminocyclitol, or that of its keto analogues (Fig. 1).

Recently, Piepersberg and coworkers (Stratmann *et al.*, 1999) demonstrated that the synthesis of the intermediate 2-*epi*-5-*epi*-valiolone is catalyzed by cyclase AcbC, which is itself encoded in the acarbose biosynthetic (*acb*) gene cluster found in *Actinoplanes* sp. SE50/110. Therefore, the PCR primers constructed for the screening of the C_7N aminocyclitol or its keto analogue-containing metabolites were designed on the basis of the comparison of the sequence of known *sedo*-heptulose 7-phosphate cyclase (*acbC*) from that of *Actinoplanes* sp. SE50/100, and that of several 3-hydroquinate synthetases which exhibit a high degree of similarity with the AcbC protein. Due to the biased codon usage manifested by actinomycetes, primers can be designed unambiguously, even from the short consensus sequences obtained as the result of amino acid sequence comparisons. Therefore, PCR primers for the amplification of the *sedo*-heptulose 7-phosphate cyclase gene were prepared from amino acid consensus sequences within the known *sedo*-heptulose 7-phosphate cyclase, as well as several 3-hydroquinate synthetases.

The sequence of each of the primers used in the exper-

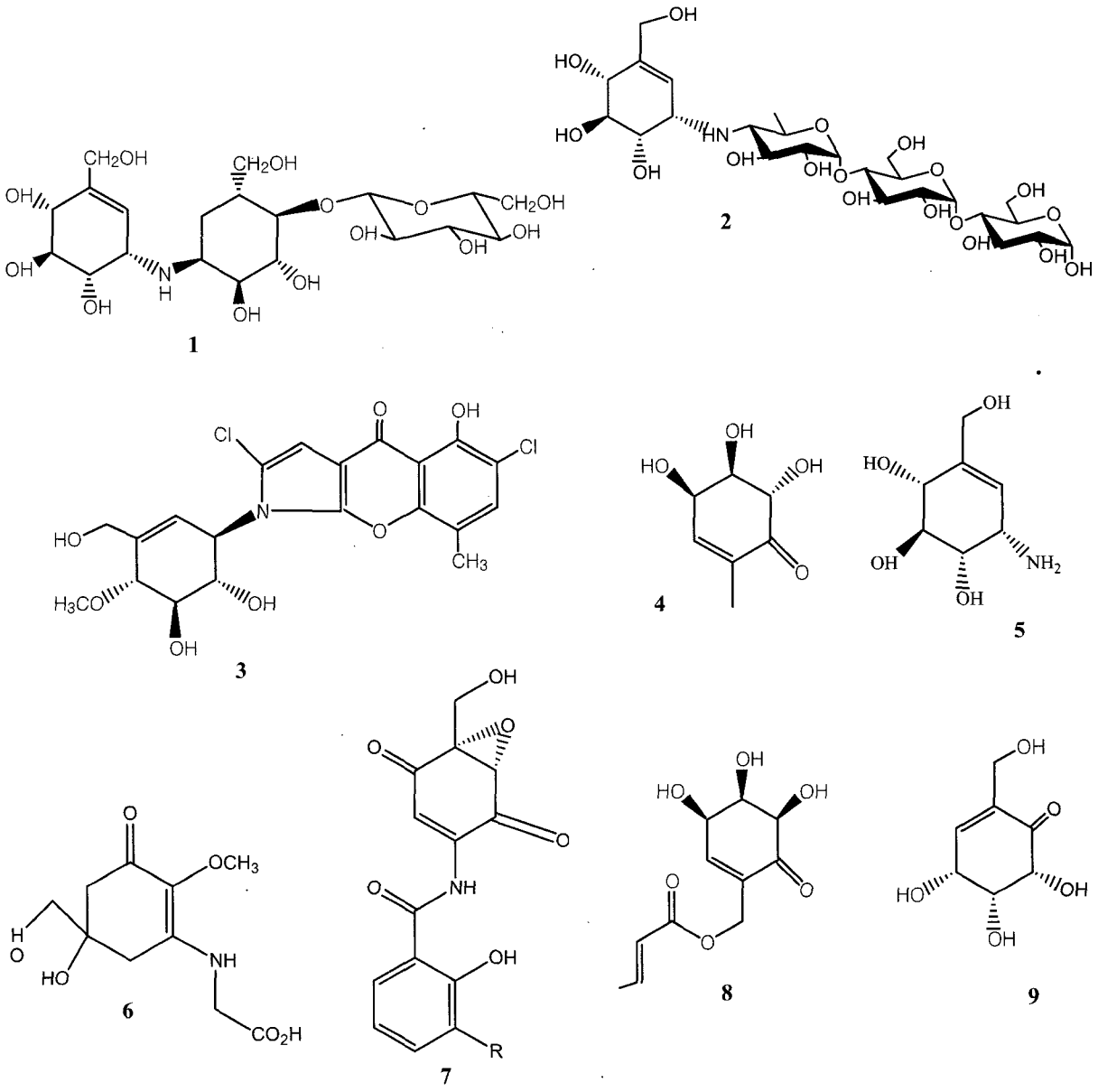

Fig. 2. Chemical structures of C_7N aminocyclitol and its keto analogues : (1) Validamycin A, (2) acarbose, (3) pyralomicin, (4) gabosine A, (5) valienamine, (6) mycosporine-Gly, (7) epoxyquinomicin, (8) COTC (glyoxalase I inhibitor), and (9) KD16-U1.

iments was as follows: the sequence of the VOG-F primer was 5'-GGSGGSGGSGTSCTSATGGACGTSGCSGG-3' (GGGVLMDVAG); and the sequence of the VOG-R primer was 5'-GCCATGTCSACGCASACSGCSGCCTC-SCCGAG-3' (HGEAVCVDMA). These primers were employed in the amplification of DNA fragments from a total of 39 actinomycetes species. PCR was conducted as described previously, with slight modifications (Hyun *et al.*, 2000). PCR mixtures used for the amplification of the 40 total DNAs contained 200 μM deoxynucleoside triphosphates, 2.5 mM $MgCl_2$, 1× PCR buffer, 100 pmol of each primer, and 2.5 U of Ex Taq polymerase (Takara, Japan). The amplification reactions were conducted using a thermal cycler (model 480, Perkin-Elmer, USA), with a 5-minute initial denaturation at 94°C, 30 cycles of 20 sec of denaturation at 98°C, and 1 min of primer annealing at 67°C, and a final extension period of 10 min at 72°C. The resultant PCR products (about 540 bp) were then purified on agarose gel (Qiagen, USA), subcloned into pGEM-T (Promega, USA) or pST-Blue (Novagen, USA), and subsequently transformed into *E. coli* TOP10. The plasmid DNA which encoded the subclones was then extracted with a Wizard plasmid DNA purification kit (Promega, USA).

In order to assess the sensitivity of these primers, the primers were used to amplify DNA fragments from *Act-*

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NAIST13/40      LVGSLYRRGTPFVRVPTTLIGLVDAGVGAKTGVNFNGHKNRLGTYFPAELTLLDRAFLAT
KACC20281       LVANLYRRGTPVLRVPTTLIGLVDAGVGVKTGVNFNGHKNRLGTYFETSLTLLDRSFLST
ValK            LAASLYRRATPYVRIPTTLIGMIDAGIGAKTGVNFREHKNRLGTYHPSSLTLIDPGFLAT
KACC20296       LAASLYRRGIPFIRVPTTLIGLVDAAVGVKTAINHHSHKNRLGTYFPAATTLLDQSFLRT
AcbC            LVASLYRARHAVLRVPTTLVGLIDAVS-REDRVNFNGHKEPAGYVRPADLTLLDRRFLAT
                *...***   . :*:****:*::**    :  :*.. **:  *    :  **:*  ** *

NAIST13/40      LDRRHIGNGLAEILKIALIKDARLFDLLEQYGPVLLDEKFQGVSERGDLVAEEVLRRAIH
KACC20281       VERRHISNGLAEILKMALIKDVRLFDLLEEHGSRLLDIRFQGDTAAEQRVAAEVVQRAVH
ValK            LDARHLRNGLAEILKVALVKDAELFDLLEGHGASLVEQRMQPGEGGTGGAALTVLRRAVQ
KACC20296       LPTRHVANGLAEIVKIALVRDRVLFDLLADRAADVVELVKHGGVGG------EIIERAVG
AcbC            LDRRHLSNGLAEMLKIALIKDAELFQLLERHGRVLIEERFQGVPEPVTGPPSGPCARHP-
                :  **: *****::*:**::*  **:**   .  :::   :                 *

NAIST13/40      GMLEELQPNLWEAKLERTVDYGHTFSPTVEMRALPELL
KACC20281       GMLEELQPNLWGTELERVVDYGHTFSPTIEMHALPALL
ValK            GMLEELQPNLWEHQLRRLVDFGHSFSPSVEMAALPELL
KACC20296       GMLAELAPNLWEQELRRLVDFGHTFSPGFELNADHPLL
AcbC            WHAGGTRPNLWESRLERSVDYGHTFSPTIEMRALPALL
                ****  .*.* **:**:*** .*: *   **
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Fig. 3. Comparison of the deduced amino acid sequences of the PCR products derived from the *sedo*-heptulose 7-phosphate cyclases. Multiple sequence alignment was conducted using the CLUSTAL program, after which the resultant comparisons were manually optimized. Residues which matched sufficiently with the consensus sequences are indicated as asterisks. Gaps introduced in order to maximize the fit are shown as dashes. The amino acid sequence of AcbC was taken from *Actinoplanes* sp. SE50/110 (Q9ZAE9). The DNA and protein sequences of the PCR products determined in this study are deposited in the GenBank database under the accession numbers, DQ001144, DQ001145, and DQ001146.

inoplanes sp. SE50/100. They were successful in amplifying the known *sedo*-heptulose 7-phosphate cyclase gene. Furthermore, our PCR-based screening proved capable of detecting the presence of the genes involved in the biosynthesis of C_7N aminocyclitol or its keto analogue-containing metabolites, in a series of organisms which were not previously known to be producers of C_7N aminocyclitol nor of its keto analogue-containing metabolites. The size of the amplified fragments was about 540 bp in each case. The amplified fragments were then subjected to subcloning and sequencing. The sources used in this step are listed in Table 1. The resultant sequences were then compared with the non-redundant sequences retrieved from the GenBank database, using a BLAST search (Altschul *et al.*, 1990). The protein sequences were aligned using either the Genetic Computer Group software package, or the CLUSTAL program (Higgins and Sharp, 1988).

The deduced amino acid sequences of the amplified DNA fragments revealed a remarkable degree of similarity with one another, and with the *sedo*-heptulose 7-phosphate cyclase involved in acarbose biosynthesis in *Actinoplanes* sp. SE50/110. A multiple alignment of the derived amino acid sequences of the cloned gene fragments for *sedo*-heptulose 7-phosphate cyclase is shown in Fig. 3. Ultimately, the fact that novel *sedo*-heptulose 7-phosphate cyclase genes were detected in three out of 39 actinomycetes species reveals that this type of molecular genetic screening may represent a simple and rapid scheme for the preliminary identification of producers of novel C_7N aminocyclitol, or of its keto analogue-containing metabolites.

In order to evaluate the α-glucosidase inhibitory activity of the test strains, we validated the production of either C_7N aminocyclitol or its keto analogue-containing metabolites, as well as the activity of α-glucosidase inhibitor, by culturing strains which harbored *sedo*-heptulose 7-phosphate cyclase gene cassettes, in a variety of fermentation media.

These organisms were initially grown in 20 ml of R2YE seed medium for 60 h at 28°C, and were then diluted by a factor of 30-fold in 25 ml of production medium. The production medium (P medium) for *Streptomyces abikoensis* KACC 20281 and Actinomycete NAIST13/40 consisted of 10 g of beef extract, 10 g of polypeptone, 15 g of glucose, 10 g of glycerol, and 2 g of $CaCO_3$ per liter. The production medium (K media) for *Saccharothrix espanaensis* KACC 20296 consisted of 25 g of soluble starch, 15 g of soybean meal, 2 g of yeast extract, and 4 g of $CaCO_3$ per liter (pH 6.2 - 7.0). The production cultures were incubated for 7 days at 28°C, under constant agitation. A small aliquot (1.5 ml) of culture broth was sampled, after which the supernatants were prepared via centrifugation.

The α-glucosidase inhibitory activity was then evaluated, using a slightly modified version of the ρ-nitrophenyl-α-D-glucoside (PNPG) method (Dahlqvist, 1970). This revealed that three of the strains which harbored *sedo*-heptulose 7-phosphate cyclase genes also exhibited α-glucosidase inhibitory activity (Table 2).

Interestingly, *Saccharothrix espanaensis* KACC 20296 inhibited the activity of α-glucosidase when grown in specialized media (P media) selected for its ability to support the production of either C_7N aminocyclitol or its keto analogue-group-containing natural products. Out of a total of 39 analyzed actinomycete strains, three (8%) were ultimately determined to contain biosynthetic loci which encoded for *sedo*-heptulose 7-phosphate cyclase, indicating that these strains might potentially produce C_7N aminocyclitol or its keto analogue-group-containing natural products, and also inhibit α-glucosidase. To date, none of the three strains had previously been determined to produce C_7N aminocyclitol or its keto analogues-group-containing natural products, nor were any of the three previously demonstrated to inhibit α-glucosidase. However, our results indicate that the gene loci encoding for C_7N aminocyclitol or its keto analogue-group-containing natural products, which inhibit α-glucosidase, occur at a remarkably high frequency in the actinomycetes.

This finding is interesting, in that we determined that the molecular detection of specific metabolite-producing actinomycetes can be used to facilitate attempts to locate different sources of aminocyclitol or its keto analogue-containing metabolites. A recent study regarding high-throughput genome scanning has also revealed that a set of 12 functional enediyne PKS genes were dispersed among the genomes of a widely diverse group of organisms, none of which had previously been implicated as enediyne producers (Zazopoulos *et al.*, 2003).

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