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Articles

Development of PCR-Based Screening Methods for Macrolide Type Polyketides in *Actinomycetes*

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About two thirds of the naturally occurring antibiotics have been discovered from actinomycetes. Therefore, the probability of discovering further new antibiotics from actinomycetes is declining as many known metabolites are isolated repeatedly. However, various efforts have been made in order to enhance the probability of discovering novel compounds. In the present study, we have developed new screening strategies based on the antibiotic biosynthetic pathway, and the genetic information, utilizing polymerase chain reaction. We have selected macrolide type polyketides. In order to divide the ansamycin group antibotic of macrolide type polyketides, we have selected 3-amino-5-hydroxybenzoic acid (AHBA) moiety which contains a biosynthetically unique structural element in this group as a target molecules. Oligonucleotide primers were designed to amplify DNA fragments of macrolide type polyketide synthase and AHBA synthase genes from fourteen actinomycetes species. This method was successfully applied to all three of the known macrolide type polyketide producing actinomycetes tested. In addition, it also identified the presence of potential macrolide type polyketide producing genes from seven actinomycetes that were known to produce none of macrolide type polyketides, and AHBA biosynthetic genes in one actinomycetes. This technique is potentially useful for the screening of new antibiotics and cloning of their biosynthetic genes.

Key words : Actinomycetes, *3-amino-5-hydroxybenzoic acid, macrolide type polyketide.*

Most antibiotics of prokaryote origin are produced by organisms belonging to the actinomycetes family. Isolation and selection of actinomycetes have been dependent on taxonomists who are highly experienced for cultural property and morphology of actinomycetes. Over the last decade, they have shown increasing interest in rare actinomycetes as a potential source for bioactive secondary metabolites. It is likely that actinomycetes will continue to play a major role in providing novel bioactive substance in the future. However, the possibility of finding novel metabolites is declining as the known metabolites are increasing. Therefore, various efforts have been made in order to enhance the possibility of discovering novel compounds such as targeted screening and the improvement of novel fermentation conditions as well as selection of rare actinomycetes.[1]

Polyketide-derived metabolites are amongst the most numerous and diverse, and include many clinically important members of antibiotics. Assembly of the skeleton of such compounds by oligomerization of small precursor fatty acid thioesters to form polyketide backbone, is synthesized by PKS's. These are formed either by large multi-functional enzymes (modular type enzymes) or multi-enzyme complexes, so called type I PKS's and Type II PKS's, respectively.[2] Biosynthesis of macrolide type polyketide has been well studied in the case of erythromycin, and is known to be catalyzed by multifunctional enzymes produced by macrolide type genes.[3] In addition, the domain order in each module is as follows; KS, AT, DH, ER, KR, and ACP. However, DH, ER, and KR domains are not present sometimes in the order.

We have developed novel screening strategies understanding antibiotic biosynthetic pathway, applying genetic information, and utilizing polymerase chain reaction. The PCR method is a very valuable and powerful tool for the identification of actinomycetes genes, since the G+C contents is as high as 70%. Due to the biased codon usage of actinomycetes, oligonucleotide primers colud be designed rather unambiguously even from short consensus sequences determined by amino acid sequence comparison.[4,5] In this study we have investigated on macrolide type polyketides such as macrolide, ansamycin, polyether, and polyene, and some antibiotics containing AHBA moieties such as ansamycin and mitomycin as target molecules (Fig. 1). We developed a PCR method to amplify gene fragments coding macrolide type polyketide synthase and AHBA synthase which are involved in the formation of macrolide type polyketides and AHBA moiety, respectively.

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Abbreviations: ACP, acyl carrier protein; AHBA, 3-amino-5-hydroxybenzoic acid; AT, acyltransferase; DH, dehydratase; ER, enoyl reductase; KR, ketoreductase; KS, ketosynthase; PKS, polyketide synthase.

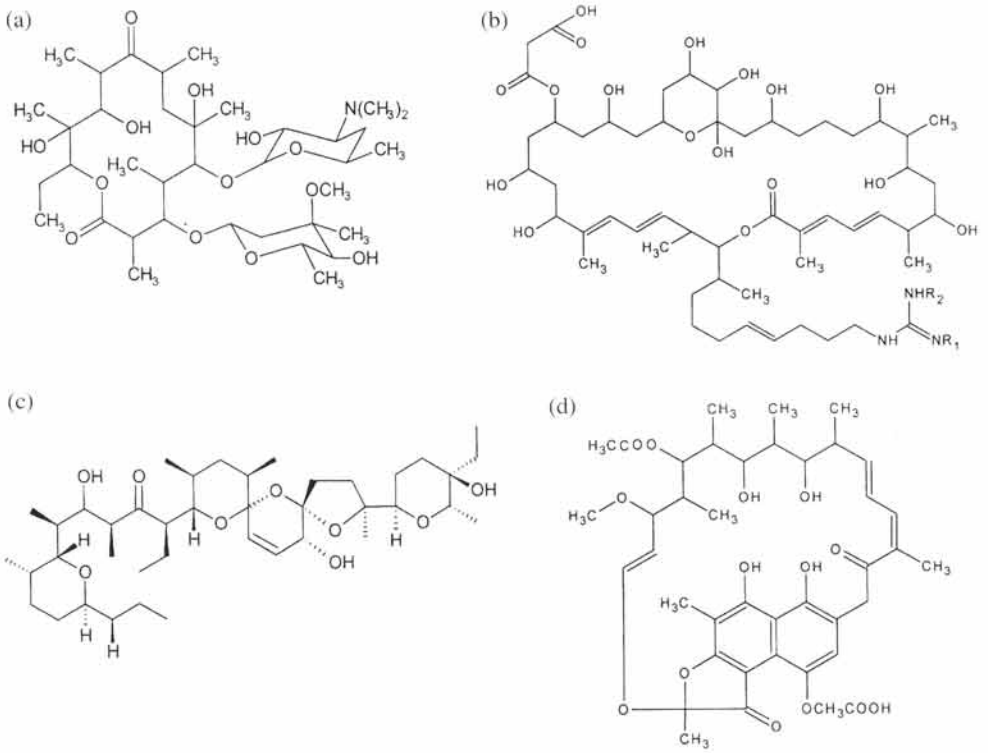

Fig. 1. Structure of representative macrolide type polyketides. (a) macrolide antibiotic, erythromycin (b) polyene antibiotic, azalomycin F (c) polyether antibiotic, salinomycin (d) ansamycin group, and rifamycin.

Materials and Methods

Bacterial strains, plasmids, and growth conditions. The strains used in this study are listed in Table 1. *Micromonospora inyoensis, M. olivasterospora, M. purpurea, Streptoalloteichus hindustanus, Streptomyces albus, S. bluensis, S. cinnamonensis, S. griseus, S. kanamyceticus,* and *S. rimosus* were obtained from the American Type Culture Collection (Rockville, MD). *S. flavopersicus* and *S. tenebrarius* were obtained the Korean Collection for Type Cultures (Taejon, Korea). *S. fladiae* was obtained from the ARS Culture Collection (Peoria, III., U.S.A). *Escherichia coli* DH5αF' was used as host strains for the recombinant plasmids. Plasmids pGEM-T easy (Promega Biotech, Madison, WI) or pT7Blue (Novagen, Madison, WI) were used as vectors for subcloning of the PCR products. All *E. coli* transformants were grown on LB agar plate with 50 μg/ml ampicillin. *Streptomyces bluensis* was grown at 28°C on YPC plates (proteose peptone 1.5%, yeast extract 0.5%, KH_2PO_4 0.4%, sucrose 0.25%, glucose 0.2%, L-cysteine 0.05%, Na_2SO_3 0.02%, pH 7.2) or NZY liquid media (yeast extract 0.5%, N-Z amine A 1.0%, $NaCl_2$ 0.5%, $MgCl_2$ $6H_2O$ 0.2%, pH 7.2). Other actinomycetes were grown on R2YE plates or TSB liquid media.[6]

General methods. Genomic DNAs were prepared from actinomycetes according to Hopwood *et al.*[6] General procedures for manipulating DNA were carried out according to Sambrook *et al.*[7] DNA was isolated from agarose gels with the Qiagen kit (Chartworth, CA). Purified plasmid was prepared using a Wizard plasmid kit (Promega Biotech, Madison, WI) according to the manufacturer's instructions.

PCR and sequecing of PCR products. The PCR reaction was performed in 50 μl reaction mixture containing 100 pmol of each primer, 300 ng of genomic DNA, 5 μl of 10× PCR buffer, 0.2 mmol of dNTP, and 2.5 units of Taq polymerase (Takara, Japan). After overlaying with mineral oil, the reaction mixtures were preheated at 94°C for 3 min, thereafter, 30 amplification cycles were carried out. Each cycle consisted of 20 sec at 98°C and 1 min at 67°C. Finally, an additional 10 min of extension reaction was performed at 72°C for complete extension. Amplification was performed in a thermal cycler (model 480, Perkin-Elmer Cetus, CT). A PCR product of the expected size was recovered by 1.5% agarose gel electrophoresis and ligated into pT7Blue (Novagen, Madison, WI) or pGEM-T easy vector (Promega Biotech, Madison, WI).

Results and Discussion

Isolation of biosynthetic genes for macrolide type polyketides. The PCR primers for the screening of the macrolide type polyketide were prepared from the comparison of the sequence of known macrolide type polyketide synthases from *Saccharopolyspora erythraea*-erythromycin producer,[3] *Streptomyces hygroscopicus*-

Table 1. Actinomycetes strains which were used for PCR amplification of modular type polyketide synthase and AHBA synthase genes.

Strain	Known antibiotic produced by the strain	Classification of the antibiotics	Macrolide type PKS primers	AHBA primers	Source
Micromonospora inyoensis ATCC27600	sisomicin	aminoglycoside	●		[a]ATCC
Micromonospora olivasterospora ATCC21819	fortimicin	aminoglycoside			ATCC
Micromonospora purpurea ATCC15835	gentamicin	aminoglycoside			ATCC
Micromonospora sagamiensis ATCC21826	sagamicin	aminoglycoside	●	●	ATCC
Streptoalloteichus hindustanus ATCC31219	nebramycin	aminoglycoside			ATCC
Streptomyces albus ATCC21838	salinomycin	polyether	●		ATCC
Streptomyces bluensis ATCC27420	bluensomycin	aminoglycoside	●		ATCC
Streptomyces cinnamonensis ATCC15413	monensin	polyether	●		ATCC
Streptomyces flavopersicus KCTC9221	spectinomycin	aminoglycoside	●		[b]KCTC
Streptomyces fradiae NRRL2702	tylosin	macrolide	●		[c]NRRL
Streptomyces griseus ATCC10137	streptomycin	aminoglycoside	●		ATCC
Streptomyces kanamyceticus ATCC12853	kanamycin	aminoglycoside	●		ATCC
Streptomyces rimosus ATCC14827	paromomycin	aminoglycoside	●		ATCC
Streptomyces tenebrarius KCTC9047	nebramycin	aminoglycoside			KCTC

● : positive signals with respect to each primers.
[a]ATCC, American Type Culture Collection, Rockville, Md., U.S.A.
[b]KCTC, Korean Collection for Type Cultures, Taejon, Korea.
[c]NRRL, ARS Culture Collection, Northern Regional Research Center, Peoria, Ill., U.S.A.

rapamycin producer,[8] *Streptomyces caelestis*-niddamycin producer,[9] and *Amycolatopsis mediterranei*-rifamycin producer.[10] The first conserved region is located at DLGFDSL motif site of ACP domain (ASRDLGFDS: 5-GCSTCSCGSGACCTSGGCTTCGACTC-3) and the second one is at GPTACSSS active site of KS domain. Both of these are perfectly conserved in all known KS domains of modular PKSs (VD/ETACSSS: 5-SGASGASGAGCASGCSGTSTCSAC-3).

In order to identify the sensitivity of these primers, the primers were used to amplify DNA fragments from *Streptomyces albus* ATCC21838, *Streptomyces cinnamonensis* ATCC15413, and *Streptomyces fradiae* NRRL2702 which produce macrolide type polyketide compounds, such as salinomycin, monensin, and tylosin, respectively. It did detect the presence of macrolide type polyketide synthase gene. In the case of *S. fradiae* NRRL2702, the sequence of PCR product is identical to that of a part in tylosin biosynthetic genes (GenBank accession number U78289). Furthermore, this PCR-based screening was able to detect the presence of genes to produce macrolide type polyketide in seven known actinomycetes. Interestingly, we found the presence of this type of genes in the organism which have not been known as macrolide type polyketide producers previously. The detection of novel macrolide type PKS-like genes in seven out of tweleve none macrolide type polyketide producing actinomycetes showed that this type of molecular genetic screening to find out novel macrolide type polyketide producers is potentially applicable as a rapid and simple, preliminary screening method.

The size of the amplified fragments was about 700 bp in each cases. Amplified fragments obtained from the strains listed in Table 1 were subcloned and sequenced. A database search with the deduced amino acid sequences of the PCR products was performed. The deduced amino acid sequences of the isolated fragments revealed remarkable similarities to each other and to the macrolide type polyketide synthase from actinomycetes involved in the biosynthesis of different macrolide type polyketide antibiotics. A multiple alignment of derived amino acid sequences of the cloned gene fragments for macrolide type PKS is given in Fig. 2.

The nucleotide sequences of macrolide type polyketide synthase genes have been deposited in the GenBank database under accession numbers, AF144045 (*S. albus*), AF144046 (*S. bluensis*), AF144047 (*S. cinnamonensis*), AF144048 (*S. flavopersicus*), AF144049 (*S. griseus*), AF144050 (*S. kanamyceticus*), AF144051 (*S. rimosus* subsp. *paromomycinus*), AF144052 (*M. sagamiensis*), and AF144053 (*M. inyoensis*), respectively.

Isolation of the biosynthetic genes for AHBA. The clinically important ansamycin antibiotics, such as rifamycin, conatins a unique structural element called a mC_7N unit. The mC_7N unit is derived from AHBA which serves as the starter unit for the assembly of a linear polyketide, resulting macrolide type PKS. To narrow down for the producers of ansamycin antibiotics among the producers of various macrolide type polyketides we have developed ansamycin-specific primers on the basis of AHBA biosynthesis.

AHBA which is derived from erythrose 4-phosphate and phosphenolpyruvate is used in the biosynthesis of a wide variety of antibiotics. AHBA is generated by the aminoshikimate pathway which parallels the first three steps of the

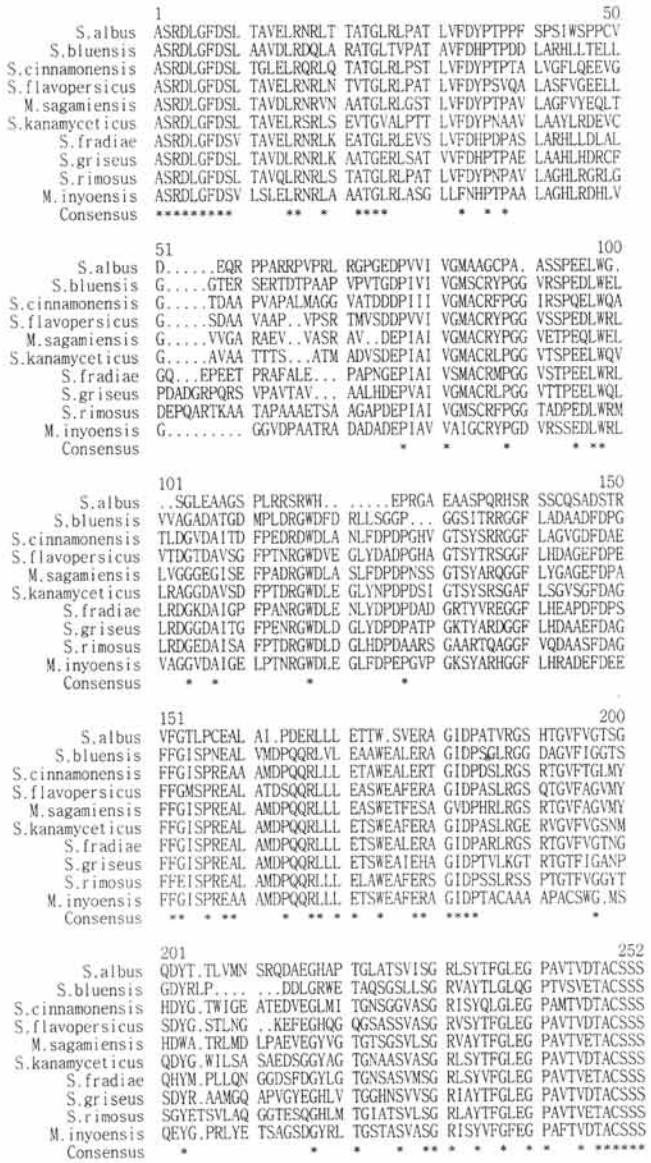

Fig. 2. Multiple alignment of the PCR products of the modular type polyketide synthases. The multiple sequence alignment was performed by CLUSTAL program, after which the resulting comparisons were refined manually.[11] Residues that match highly with the consensus sequences are indicated as astrisks. Gaps inroduced to maximize the fit are shown as dashed lines.

shikimate pathway but is modified by the introduction of nitrogen in the first step to give 3,4-dideoxy-4-amino-D-arabino-heptulosonic acid 7-phosphate (aminoDAHP). Cyclization and dehydration leads to the 5-deoxy-3-dehydroshikimic acid (aminoDHS) which is then followd by aromatization by the enzyme, AHBA synthase (Fig. 3A).

The AHBA synthase genes for ansamycin group antibiotics (Fig. 3B), such as rifamycin,[10] ansatrienin,[12] and ansamitocin (GenBank accession number U33059) show amino acid sequence homology with each other. Multiple alignment among the amino acid sequences of these biosynthetic genes was used to design degenerative PCR primers considering the codon preference. The DNA primers were designed as follows: the sequence of AHBA-1 primer was 5'-ACSGAGGTSATCGTSCCSGCSTTCACSTTC-3' (TEVIVPAFTF); and that of AHBA-2 primer was 5'- SGCS-CCGTGSGCGTGSGCSGCGTCCTG-3' (QDAAHAHGA).

These pimers were used to amplify DNA fragments from fourteen different actinomycetes species producing a variety of different antibiotics. As a result of PCR experiments, we have identified the presence of a AHBA synthase-like gene in *Micromonospora sagamiensis* ATCC21826 which were not known to produce ansamycin. The size of the amplified fragment was 264 bp. The deduced amino acid sequences of the PCR products were compared with that of the AHBA synthase genes from the rifamycin-, rubradirin-, and ansatrienin-producing strains, all of which showed high homology (Fig. 4). AHBA moiety was a starter unit for the linear polyketide of ansamycin group antibiotics or the component of mitomycin C-related as well as unclassified antibiotics. Therefore, chromosomal DNA of *Micromonospora sagamiensis* ATCC21826 can be amplified by both AHBA primers and macrolide type polyketide primers is a very interesting finding. This result suggested that *M. sagamiensis* ATCC21826 strain have a possibility to produce ansamycin group antibiotic containing AHBA moiety. The nucleotide sequences of AHBA synthase genes have been deposited in the GenBank database under accession numbers, AF144043 (*M. sagamiensis*).

Conclusions

Approximately 66% out of 11,900 antibiotics discovered up to 1994 were produced from actinomycetes.[13] Therefore, the possibility of finding further novel metabolites is unlikely. Accordingly, various new strategies should be designed to further isolate the novel strains to find new bioactive metabolites. Over the last decade, taxonomists have shown interests incresingly in rare actinomycetes as a potential source of new bioactive metabolites. They developed methods to isolate wide varieties of rare actinomycetes and were successfully found several numbers of novel bioactive metabolites.

As one of screening strategies, we have developed PCR-based screening methods for macrolide type polyketides and antibiotics containing AHBA moiety as a target molecules. This PCR-based screening methods could be applied to isolate novel metabolites from rare actinomycetes. Furthermore, the advantage of the PCR-based screening technique can exclude the problems associated with traditional screening methods, such as antibiotic production condition, development of specific and highly sensitive bioassay strain, variation in the amount of antibiotics, genetic change in the testing, and the presence of polyketide inhibitors such as cerulenin that present difficulties in screeing programs. A similar screening method for other antibiotic pathways such as for the aminoglycoside,[14,15] β-lactam antibiotics,[16] and peptide antibiotics[17] is also a

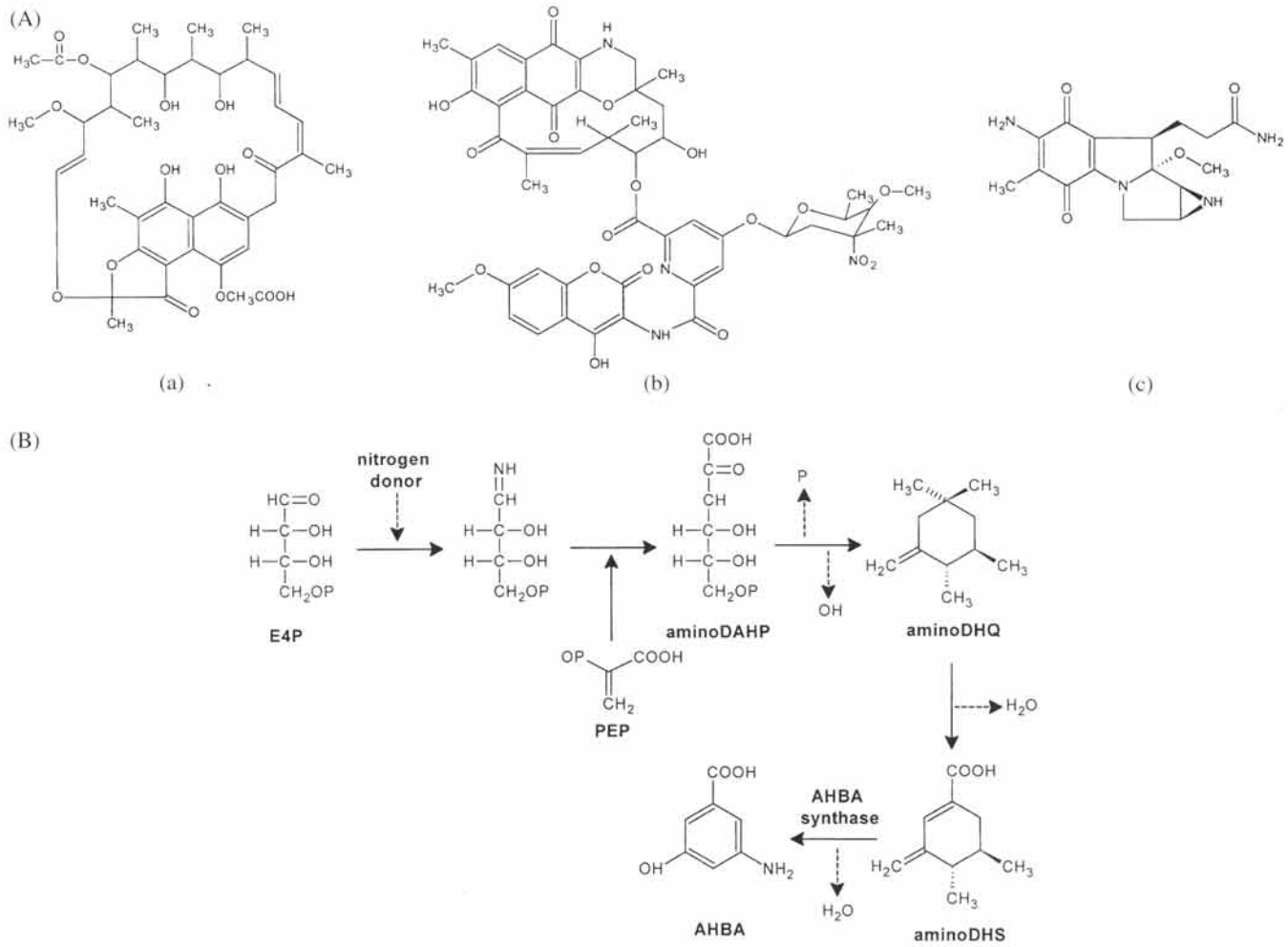

Fig. 3. Proposed pathway of AHBA moiety (A)[18] and several antibiotics containing AHBA moiety (B). a, rifamycin; b, rubradirin; and c, mitomycin C.

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                1                                                  50
M. sagamiensis  TEVIVPAFTF ISSSLAAQRL GAVAVPVDVD LDTYCIQPEA VAAAITDRTR
   S. collinus  TEVIVPAFTF ISSSQAAQRL GAVVVPVDVD PETYCIDPAE AAKAITPRTR
  A. pretiosum  TEVIVPAFTF ISSSQAVQRL GAVAVPVDVD PDTYCLDVAA AEDAVTSRTS
     Consensus  ********** **** * *** *** ******   **         * * **

                51                                      88
M. sagamiensis  VIMSVHMAGQ MSDMDALDKI AADAGVSILQ DAAHAHGA
   S. collinus  AIMPVHMAGQ LADMDALEKV AADSGVPLIQ DAAHAQGA
  A. pretiosum  AIMPVHMAGQ FADMDRLDKL SASTGVPVVQ DAAHAHGA
     Consensus  ** ******   *** *       **   * ***** **
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Fig. 4. Comparison of the deduced amino acid sequences of PCR products derived from AHBA synthase primers and other AHBA synthases. Amino acid sequences identical to each other are shown by astrisks. The amino acid sequences were taken from the following sources: Rifamycin, *Amycolatopsis mediterranei* (AF040570); ansatrienin, *Streptomyces collinus* (Z54208); ansamitocin, *Actinosynnema pretiosum auranticum* (U33059). The sequence of *M. sagamiensis* AHBA synthase was determined in this study.

possibility to obtain the available information on the biosynthetic genes.

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References

1. Okami, Y. and Hotta, K. (1988) Search and discovery of new antibiotics. In *Actinomycetes in Biotechnology*, Goodfellow, M., Williams, S. T. and Mordarski, M. (eds.) pp. 33-67, Academy Press, London.
2. Hutchinson, C. R. and Fujii, I. (1995) Polyketide synthase gene manipulation; A structure-function approach in engineering novel antibiotics. *Annu. Rev. Microbiol.* **49**, 201-238.
3. Donadio, S. and Katz, L. (1992) Organization of the enzymatic domains in the multifunctional polyketide synthase involved in erythromycin formation in *Saccharopolyspora erythraea*. *Gene* **111**, 51-60.
4. Bibb, M. J., Findlay, P. R. and Johnson, M. W. (1984) The relationship between base composition and codon usage in bacterial genes and its use for the simple and reliable identification of protein-coding sequences. *Gene*

30, 157-166.

5. Hyun, C.-G., Kim, J. M., Hong, S. K. and Suh, J.-W. (1998) An efficient approach for cloning P450 hydroxylase genes from *Actinomycetes*. *J. Microbiol. Biotechnol.* **8**, 295-299.
6. Hopwood, D. A., Bibb, M. J., Chater, K. F., Kieser, T., Bruton, C. J., Kieser, H. M., Lydiate, D. J., Smith, C. P. and Schrempf, H. (1985) In *Genetic Manipulation of* Streptomyces: *A Laboratory Manual*, John Innes Foundation, Norwich.
7. Sambrook J., Fritsch, E. F. and Maniatis, T. (1989) In *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York.
8. Molnar, I., Aparicio, J. F., Haydock, S. F., Khaw, L. E., Schwecke, T., Konig, A., Staunton, J. and Leadlay, P. F. (1996) Organisation of the biosynthetic gene cluster for rapamycin in *Streptomyces hygroscopicus*: Analysis of genes flanking the polyketide synthase. *Gene* **169**, 1-7.
9. Kakavas, S. J., Katz, L. and Stassi, D. (1997) Identification and characterization of the niddamycin polyketide synthase genes from *Streptomyces caelestis*. *J. Bacteriol.* **179**, 7515-7522.
10. Tang, L., Yoon, Y. J., Choi, C. J. and Hutchinson, C. R. (1998) Characterization of the enzymatic domains in the modular polyketide synthase involved in rifamycin B biosynthesis by *Amycolatopsis mediterranei*. *Gene* **216**, 255-265.
11. Higgins, D. G. and Sharp, P. M. (1988) Clustal: A package for performing multiple sequence alignments on a microcomputer. *Gene* **73**, 237-244.
12. Wilson, D. J., Patton, S., Florova, G., Hale, V. and Reynolds, K. A. (1998) The shikimic acid pathway and polyketide biosynthesis. *J. Ind. Microbiol. Biotechnol.* **20**, 299-303.
13. Strohl, W. R. (1997) Industrial antibiotics: Today and the future. In *Biotechnology of Antibiotics*, Strohl, W. R. (ed.) pp. 1-47, Marcel Dekker, New York.
14. Ahlert, J., Distler, J., Mansouri, K. and Piepersberg, W. (1997) Identification of *stsC*, the gene encoding the L-glutamine:scyllo-inososе aminotransferase from streptomycin-producing *Streptomycetes*. *Arch. Microbiol.* **168**, 102-113.
15. Decker, H., Gaisser, S., Pelzer, S., Schneider, P., Westrich, L., Wohlleben, W. and Bechthold, A. (1996) A general approach for cloning and characterizing dNDP-glucose dehydratase genes from *Actinomycetes*. *FEMS Microbiol. Lett.* **141**, 195-201.
16. Krallis, M. and Kirby, R. (1998) Development of a PCR/Southern dot blot based detection system for the presence of genes involved in beta-lactam biosynthesis. *Actinomycetologica* **12**, 29-36.
17. Stachelhaus, T. and Marahiel, M. A. (1995) Modular structure of encoding multifunctional peptide synthetases required for non-ribosomal peptide synthesis. *FEME Microbiol. Lett.* **125**, 3-14.
18. Kim, C.-G., Yu, T. W., Fryhle, C. B., Handa, S. and Floss, H. G. (1998) 3-Amino-5-hydroxy-benzoic acid synthase, the terminal enzyme in the formation of the precursor of mC_7N units in rifamycin and related antibiotics. J. Biol. Chem. **273**, 6030-6040.