Cloning of novel ABC transporter genes from *Streptomyces* producing anti-cancer drug, actinomycin D

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Abstract

A 3.15-kb genomic DNA fragment containing a region encoding consensus ABC transporter sequences similar to that of human P-glycoprotein was isolated from *Streptoymyces* sp. NRRL11395, a producer microorganism of an anti-cancer drug actinomycin D. The sequence of this fragment revealed the presence of four ORFs, *stmd10A, stmd10B, stmd10C, and stmd10D,* which seem to foam an operon. The results of sequence analysis suggest that the putative proteins encoded by *stmd10A*, *B*, *C*, and *D* would constitute an ABC transporter complex for either exporting or importing its substrates, and that *stmd10A* and *D* would encode an ATP binding subunit and a substrate binding subunit, respectively, while *stmd10B* and *C* would encode membrane spanning subunits. By RT-PCR analysis, it was indicated that *stmd10A* was actually expressed in this bacterial strain, but the expression level was not altered even when actinomycin D was added to the medium. Homology search using BLAST suggested that the substrate of the putative ABC transporter might be a cyclic amino acid, ectoine/hydroxyectoine.

1. Introduction

ATP-binding cassette (ABC) transporters comprise a superfamily of proteins that translocates a wide range of substrates across a variety of cellular membranes [(1)]. The members of ABC superfamily transporters posses well conserved ATP binding domains in each molecules, and have been identified in mammalians, plants, yeast, and bacteria [(2-5)]. P-glycoprotein, one of the representative members of ABC transporters in human, can extrude many kinds of cytotoxic drugs from the cells before the drugs reach their intracellular targets, thus conferring resistance to many structurally dissimilar drugs, such as *Vinca* alkaloids, epipodophyllotoxins, anthracyclines and actinomycin D [(6-9)].

Actinomycin D is an anti-cancer drug that inhibits RNA polymerases by binding to DNA, and produced by some species of *Streptomyces* [(10)]. Such antibiotic-producing microorganisms must protect themselves from their own toxic products. It has been indicated that such resistance mechanisms are mediated by membrane associated proteins which secrete the antibiotics outside the cells [(11-13)].

We hypothesized that *Streptoymyces* sp. NRRL11395, one of the producer microorganisms of actinomycin D, would have genes encoding ABC transporters which could extrude actinomycin D from the cells like P-glycoprotein in mammalian cells. In this paper, we report the isolation of novel ABC transporter genes from *Streptoymyces* sp. NRRL11395.

2. Materials and Methods

2.1 Bacterial strain and growth condition.

The strain *Streptomyces* sp. NRRL11395 was obtained from Kyowa Hakko Kogyo Co., Ltd. The *Streptomyces* strain was grown in YEME medium or Yeast-Molt medium as described [(14)].

2.2 Isolation of conserved ATP binding motif by PCR

Degenerate primers were designed to amplify regions of the *Streptomyces* sp. NRRL11395 genome encoding consensus ABC transporter sequences similar to that of human P-glycoprotein. The sense primer (primer1: 5'-GCICTSGTSGGICCSTCSGG-3') corresponding to the amino acid sequence ALVGPSG, was used with the antisense primer (primer 2: 5'-CCICCIGTCTTCGTCGCWTAG -3'), the complement of the sequence encoding GGQKQRI. The PCR program was 40 cycles of melting at 94°C

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for 30 sec, annealing at 68°C for 30 sec, and extension at 72°C for 30 sec.

2.3 Genomic library Screening

Genomic DNA of *Streptomyces* sp. NRRL11395 was obtained as described [14]. A genomic library was constructed in λ-ZAP Express vector (stratagene) according to the manufacture's instruction. The library was screened with a fragment amplified by degenerate PCR described above. After three rounds of screening, a positive clone, containing a 3.15-kb insert DNA fragmented by *Bam*HI was isolated. Sequence analysis of the inserted genomic DNA revealed that this clone contained four ORFs, each of which encodes a subunit of an ABC transporter.

2.4 RT-PCR

Procedures for RNA isolation from *Streptomyces* was done as described [14]. Transcription of the novel gene was analyzed by reverse transcription (RT) PCR with a pair of the sense primer (stmd10upper: 5'-TCCTGCGGCTGCTGATGACG-3') and the antisense primer (stmd10lower: 5'-CGCTCCACC GCCTCGTCCTT-3') using Qiagen one-step RT-PCR kit (Qiagen). As a control, a fragment of 16S rRNA was amplified by RT-PCR with another pair of primers (16SrRNAsense: 5'-AGA CACGGCCCAGACTCCTACG-3', and 16SrRNAantisense: 5'-TTCGCCACCGGTGTTCCTCCTGAT -3').

3. Results and Discussion

A 348-bp DNA fragment was amplified from the genomic DNA of *Streptomyces* sp. NRRL11395 by PCR-based approach using degenerate primers designed from conserved domains of ABC transporters involved in human P-glycoprotein. Cloning and sequence analysis revealed that the amplified fragment had highly conserved amino acid sequence, characteristic of the ATP binding domains of ABC transporters. The fragment was used as a probe to screen a genomic library of this microorganism, and a positive lambda clone containing a 3.15-kb insert DNA digested by *Bam*HI was isolated. The sequence of this fragment revealed the presence of four ORFs, *stmd10A, stmd10B, stmd10C, and stmd10D* (Fig.1).

stmd10A starts at a start codon GTG, which is frequently used as an initiation codon in streptomycete [11,12,15], at nt 2142 to 2144, and terminates at TGA codon at nt 2923 to 2925, which could encode a protein of 260 amino acids. *stmd10B* starts at a start codon GTG at nt 1500 to 1502, and terminates at TGA codon at nt 2148 to 2150, encoding a protein of 216 amino acids. *stmd10C* starts at codon ATG at nt 802 to 804, and terminates at TGA codon at nt 1501 to 1503, encoding a protein of 233 amino acids. The first four nucleotides of the *stmd10B*, encoding N-terminal two amino acids, and the last four nucleotides of *stmd10C*, encoding the C-terminal amino acid and stop codon, are overlapping. This type of overlap often occurs in operons of streptomycetes, and suggests that these two genes are cotranscribed [14,15]. *stmd10D* starts at codon GTG at nt 68 to 70, and terminates at TGA codon at nt 803 to 805, encoding a protein of 245 amino acids. The first four nucleotides of *stmd10C* and the last four nucleotides of *stmd10D* are also overlapping.

From the sequence of the putative ORFs in the isolated DNA fragment, it is estimated that these four ORFs seem to foam an operon and the functions of the products from the operon would be closely related to each other. The region with which the probe for screening could hybridize locates in *stmd10A*, and the deduced amino acid sequence coded by this ORF has sequence motifs characteristic to ATP binding domain of ABC transporter, walker A motif, walker B motif, and ABC signature sequence [16,17]. Therefore, *stmd10A* is considered to encode an ATP binding subunit of an ATP transporter protein complex. Hydropathy plot of the putative proteins coded by *stmd10B* and *stmd10C* showed that both of these proteins were very hydrophobic (data not shown). BLAST analysis of the deduced amino acid

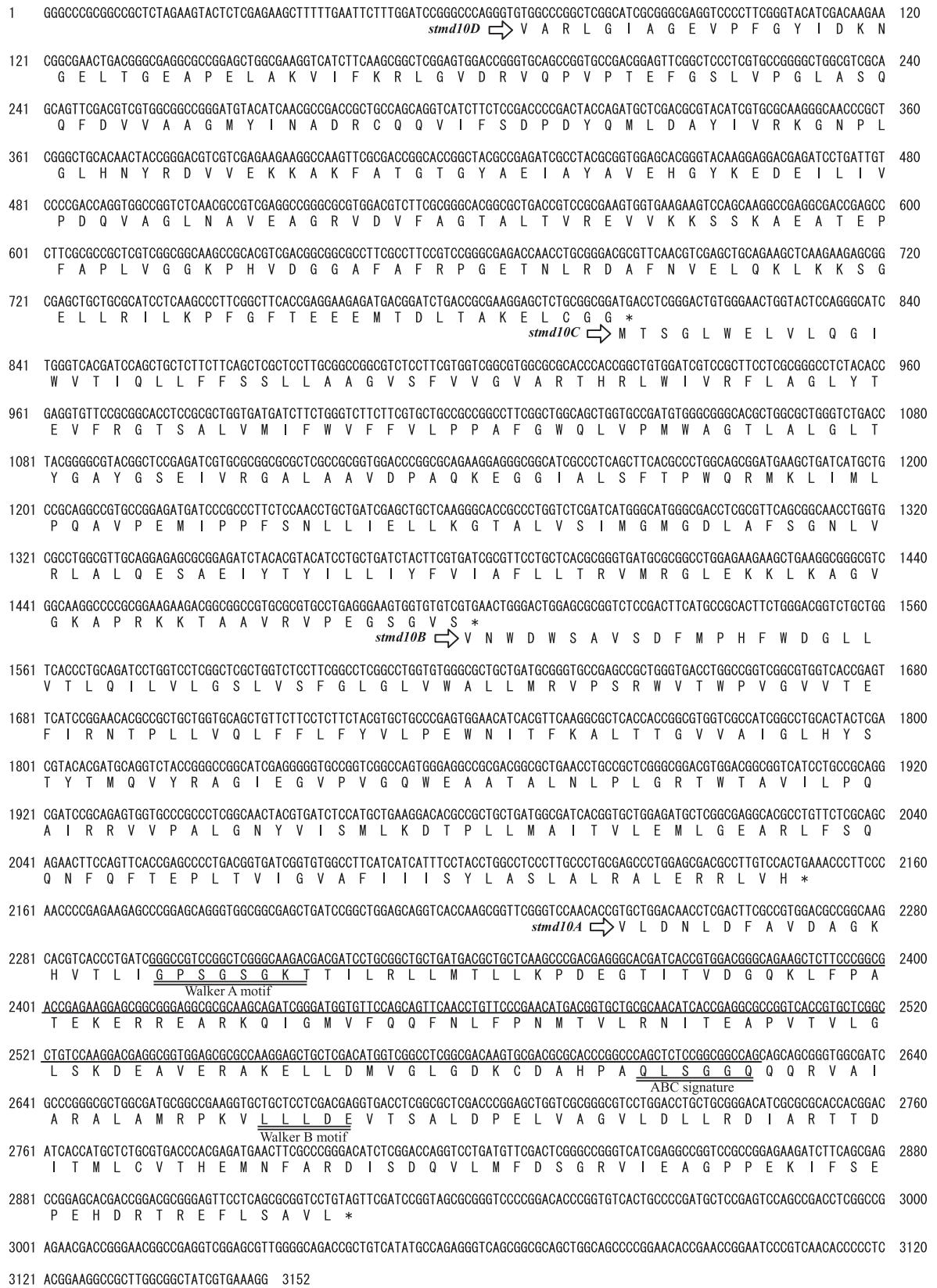

Fig.1 Nucleotide sequence of the isolated genome DNA by screening and the deduced amino acid sequences of the proteins encoded by *stmd10A, stmd10B, stmd10C, and stmd10D*. The region with which the probe for screening hybridized is indicated by underline. Double underline showed the amino acid sequence motifs characteristic to ATP binding domain of ABC transporters (walker A motif, walker B motif, and ABC signature).

sequences of them showed that these proteins have homology to the membrane spanning subunits of bacterial ABC transporters. The putative protein encoded by *stmd10D* is very hydrophilic, and the amino acid sequence of it has homology to several kinds of the substrate binding subunits of bacterial ABC transporters. These results of sequence analysis suggest that the putative proteins encoded by *stmd10A*, *B*, *C*, and *D* would constitute an ABC transporter complex for either exporting or importing its substrates, and that *stmd10A* and *D* would encode an ATP binding subunit for supply of energy to the transporter and a substrate binding subunit for transport, respectively, while *stmd10B* and *C* would encode membrane spanning subunits to foam a path for transporting substrates (Fig.2).

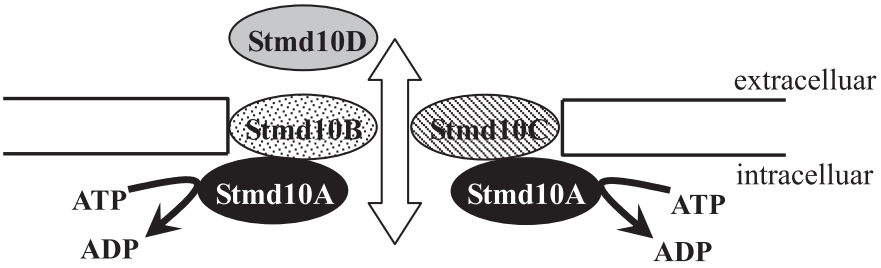

Fig. 2 Schematic drawing of the predicted structure of putative ABC transporter constituted from the proteins encoded by *stmd10A, B, C, D.* The proteins Stmd10B and Stmd10C would be integrated in the membrane, to each of which Stmd10A, an ATP hydryzing subunit, would be associated. Stmd10D might be in extracelluar space, and bind to the substrate, which would be imported or exported by the transporter.

In order to examine whether the novel genes would be actually expressed, RT-PCR analysis was done for RNA prepared from this bacteria (Fig.3). RT-PCR with a primer pair corresponding to a region of *stmd10A* amplified a specific fragment of estimated size, suggesting that stmd10A was actually expressed, and the operon containing *stmd10A*, *B*, *C* and *D* would be transcribed in this bacteria. It had been indicated that the expression of some bacterial ABC transporters increases when the substrates of them were added to the growth medium [18,19]. Thus, we investigated whether actionmycin D, which is produced by this bacteria strain, could change the expression level of *stmd10A* (Fig.3). However, the expression level of *stmd10A* was not changed even when actinomycin D was added to the medium.

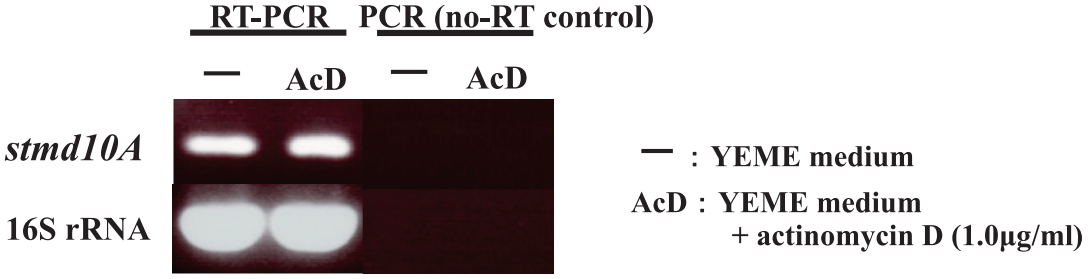

Fig.3 RT-PCR analysis of the expression of *stmd10A*. RNA was extracted at the late exponential phase (OD_{450} = 0.7-0.9). 0.1μg of RNA from each sample was used for the template of the reaction using Qiagen one-step RT-PCR kit (Qiagen). For the control of RNA quantities, 16S rRNA from the each template was also amplified.

From our data, it was not supported that actinomycin D might be transported by this putative ABC transporter encoded by *stmd10A*, *B*, *C*, and *D*. It remains to be elucidated what would be the substrates of this ABC transporter and whether this transporter would export or import the substrates. It was revealed by homology search using BLAST that the protein coded by *stmd10D* has similarity to the solute binding protein of cyclic amino acids ectoine/hydroxyectoine ABC transporter of several bacterial strains, such as *Pseudomonas aeruginosa*. Elucidation of affinity between the polypeptide encoded by *stmd10D* and ectoine/hydroxyectoine might provide a clue to identification of the substrates of the novel ABC transporter of *Streptomyces* NRRL11395.

References

(1) Higgins, C. F. (1992) ABC transporters: from microorganisms to man. *Annu Rev Cell Biol* 8**,** pp67-113

(2) Klein, I., Sarkadi, B., and Varadi, A. (1999) An inventory of the human ABC proteins. *Biochim Biophys Acta* 1461(2)**,** pp237-62

(3) Verrier, P. J., Bird, D., Burla, B., Dassa, E., Forestier, C., Geisler, M., Klein, M., Kolukisaoglu, U., Lee, Y., Martinoia, E., Murphy, A., Rea, P. A., Samuels, L., Schulz, B., Spalding, E. J., Yazaki, K., and Theodoulou, F. L. (2008) Plant ABC proteins--a unified nomenclature and updated inventory. *Trends Plant Sci* 13(4)**,** pp151-9

(4) Bauer, B. E., Wolfger, H., and Kuchler, K. (1999) Inventory and function of yeast ABC proteins: about sex, stress, pleiotropic drug and heavy metal resistance. *Biochim Biophys Acta* 1461(2)**,** pp217-36

(5) Davidson, A. L., and Chen, J. (2004) ATP-binding cassette transporters in bacteria. *Annu Rev Biochem* 73**,** pp241-68

(6) Chen, C. J., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M., and Roninson, I. B. (1986) Internal duplication and homology with bacterial transport proteins in the mdr1 (P-glycoprotein) gene from multidrug-resistant human cells. *Cell* 47(3)**,** pp381-9

(7) Endicott, J. A., and Ling, V. (1989) The biochemistry of P-glycoprotein-mediated multidrug resistance. *Annu. Rev. Biochem.* 58**,** pp137-171

(8) Gottesman, M. M., and Pastan, I. (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. *Annu Rev Biochem* 62**,** pp385-427

(9) Ueda, K., Clark, D. P., Chen, C. J., Roninson, I. B., Gottesman, M. M., and Pastan, I. (1987) The human multidrug resistance (mdr1) gene. cDNA cloning and transcription initiation. *J Biol Chem* 262(2)**,** pp505-8

(10) Aktipis, S., and Panayotatos, N. (1981) A kinetic study on the mechanism of inhibition of RNA synthesis catalyzed by DNA-dependent RNA polymerase. Differences in inhibition by ethidium bromide, 3,8-diamino-6-ethylphenanthridinium bromide and actinomycin d. *Biochim Biophys Acta* 655(3)**,** pp278-90

(11) Guilfoile, P. G., and Hutchinson, C. R. (1991) A bacterial analog of the mdr gene of mammalian tumor cells is present in *Streptomyces peucetius*, the producer of daunorubicin and doxorubicin. *Proc Natl Acad Sci U S A* 88(19)**,** pp8553-7

(12) Van Bambeke, F., Balzi, E., and Tulkens, P. M. (2000) Antibiotic efflux pumps. *Biochem Pharmacol* 60(4)**,** pp457-70

(13) Andrade, A. C., Van Nistelrooy, J. G., Peery, R. B., Skatrud, P. L., and De Waard, M. A. (2000) The role of ABC transporters from *Aspergillus nidulans* in protection against cytotoxic agents and in antibiotic production. *Mol Gen Genet* 263(6)**,** pp966-77

(14) Kieser, T., Bibb, M. J., Buttner, M. J., Chater, K. F., and Hopwood, D. A. (2000) *Practical Stereptomyces genetics.* The John Innes Foundation, Norwich

(15) Ishizuka, H., Horinouchi, S., Kieser, H. M., Hopwood, D. A., and Beppu, T. (1992) A putative two-component regulatory system involved in secondary metabolism in *Streptomyces* spp. *J Bacteriol* 174(23)**,** pp7585-94

(16) Walker, J. E., Saraste, M., Runswick, M. J., and Gay, N. J. (1982) Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *Embo J* 1(8)**,** pp945-51

(17) Bianchet, M. A., Ko, Y. H., Amzel, L. M., and Pedersen, P. L. (1997) Modeling of nucleotide binding domains of ABC transporter proteins based on a F1-ATPase/recA topology: structural model of the nucleotide binding domains of the cystic fibrosis transmembrane conductance regulator (CFTR). *J Bioenerg Biomembr* 29(5)**,** pp503-24

(18) Schlosser, A. (2000) MsiK-dependent trehalose uptake in *Streptomyces reticuli*. *FEMS Microbiol Lett* 184(2)**,** pp187-92

(19) Huang, X., Yan, A., Zhang, X., and Xu, Y. (2006) Identification and characterization of a putative ABC transporter PltHIJKN required for pyoluteorin production in *Pseudomonas* sp. M18. *Gene* 376(1)**,** pp68-78

和文抄録

抗ガン剤アクチノマイシンDを生産する放線菌からの新規ABCタンパク質遺伝子の単離

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抗ガン剤アクチノマイシンDを産生する放線菌(*Streptomyces* sp. NRRL11395)から、この抗ガン剤の排出能力をもつP-糖タンパク質と類似したABCタンパク質の遺伝子を単離しようと試みた。この菌株のゲノムライブラリーをスクリーニングした結果、プローブと特異的にハイブリダイズする約3kbのゲノムDNA断片を得た。得られたDNA断片の塩基配列を決定した結果、この断片には、４つのORFが含まれると予測された。相同性解析の結果、これらのORFの一つにはABCタンパク質特有のATP結合サブユニットをもつタンパク質がコードされていることが示唆された。また、2つのORFは、膜結合サブユニットを、残り一つは、輸送基質結合サブユニットをコードしていることが示唆された。RT-PCRによる解析の結果、単離した遺伝子群が転写されていることが確認された。これら４つのORFにコードされるタンパク質群は、全体で１つのタンパク質複合体を形成し、何らかの物質の輸送に機能していると予想された。

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