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Mediation of Rubradirin Resistance by ABC Transporters (RubT1) from *Streptomyces achromogenes* var. *rubradiris* NRRL3061

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Abstract The rubradirin biosynthetic gene cluster harbors 58 ORFs within a 105.6-kb sequence, which includes all of the genes responsible for the synthesis of rubradirin, as well as the primary genes relevant to regulatory, resistance, and transport functions. This gene cluster also harbors a resistance-mediating ABC transporter, RubT1, which is located at the most upstream position in the cluster. In the present study, RubT1 was expressed heterologously in *E. coli*, and the resistance affinity of RubT1 was determined by an antibacterial activity test, as well as by HPLC and ESI-MS analyses. Evidence clearly demonstrates that RubT1 mediates rubradirin resistance as an ABC transporter.

Key words: ABC transporter, membrane-spanning domain, resistance gene, rubradirin, *S. achromogenes*

Rubradirin is an ansamycin antibiotic that has been shown to exert a profound influence on a variety of Gram-positive bacteria, most notably multidrug-resistant *Staphylococus aureus* strains [2]. Sequence analysis of the gene cluster showed 58 open reading frames (ORFs) within a 105.6-kb sequence, which harbors all of the genes responsible for the formation and modification of four distinct moieties in rubradirin. Six ORFs within the gene cluster have been assigned to function in regulation, resistance, and transport. The *rubR1* and *rubR3* encode for translation initiation factors, *rubRg1*, *rubRg2*, and *rubRg3* transcription regulators, and one of the ORFs, *rubT1*, the ATP-binding cassette (ABC) transporter.

In several known antibiotic-producing *Streptomyces* strains, antibiotic resistance is mediated by ABC transporters, which are a large family of membrane-associated export and import systems [11, 13, 17, 22] that include four membrane-associated domains. The two hydrophilic components have been suggested to bind ATP and to couple its hydrolysis with the transport process [11]. Furthermore, those domains share a highly conserved amino acid region, which consists of ATP-binding domain residues and contains two characteristic nucleotide sequences, referred to as the Walker A and Walker B motifs.

The majority of the prokaryotic genes encoding for ABC transporters are organized into operons, which harbor ABC domains and TMDs (transmembrane domains) as separated subunits, thereby requiring a process of assembly for the formation of a biologically active transporter. In some ABC transporter-encoding genes, these distinct domains are already fused into higher structural units, as seen in the so-called "half-transporters" [TMD-NBD] or "full-transporters" $[TMD\text{-}NBD]_2$ [15]. The prokaryotic drug transporters are classified as drug-proton exchange systems [23], which employ the energy generated by ATP hydrolysis to drive drug extrusion; these transporters are members of the ABC transporter family. Most known drug transporters have been shown to mediate single drug resistance (SDR) in Gram-positive bacteria. SDR transporters tend to be specific for a single drug or for a group of structurally related drugs, such as Ard1 from *S. capreolus*, which confers resistance to the aminonucleoside antibiotic A201A [1], YnrB from *S. logisporoflavus*, which mediates SDR of the polyether-ionophore antibiotic tetronasin [17], and MsrA, which is comprised of two NBDs (nucleotide-binding domains) and mediates the active efflux of the macrolide antibiotic erythromycin from MsrA-encoding plasmid-transformed *Staphylococcus aureus* [26]. Thus, resistance-mediating ABC transporters contribute to the secretion of different molecules from cells, including sugars, amino

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acids, oligopeptides, and ions. Many of the other genes encoding ATP-binding proteins have been shown to confer resistance to the macrolides and the antitumor drugs [6, 7, 10, 16, 27, 29]. *S. antibioticus*, which produces the oleandomycin, harbors two ATP-binding proteins designated OleB and OleC [18, 20, 25, 29]. The secretion of both oleandomycin B and its inactive glycosylated derivative has been shown to be mediated by OleB, which harbors two NBDs, both being approximately 200 amino acids long and featuring no hydrophobic regions. The presence of the first NBD appears to be sufficient to confer resistance to the antibiotic [18, 19].

In this study, we expressed *rubT1*, which encodes for an ABC transporter *in vitro,* and quantified its antibiotic (rubradirin)-binding properties. The rubradirin bound by the purified protein was analyzed by HPLC, ESI-MASS, and the antibacterial activity test.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Streptomyces achromogenes var. rubradiris NRRL3061 was obtained from the ARS collection (Peoria, IL, U.S.A.). *Escherichia coli* XL1-blue MRF (Stratagene, La Jolla, CA, U.S.A.) and *E. coli* BL21 (DE3) pLysS were employed as hosts for plasmid preparation and expression [21]. *S. achromogenes* was grown in R2YE medium [12] for total DNA preparation. Multidrug-resistant (MDR) strains of *Staphylococcus aureus* (KCTC 1621, Korean Collection for Type Cultures) were used for antibacterial bioassay [14].

DNA Isolation and Plasmid Construction

Recombinant DNAs were constructed for *in vitro* expression in *E. coli*. The *rubT1* (1.2 kb), which was obtained by using the primers JL1F (5'-C GGA CCA TGG GCA TGG AGA AGA TT-3') and JL1R (5'-AAG GGA TCC CTG GCT GAG GGA-3'), was cloned into NcoI- and BamHI-digested pET32a (+), resulting in the formation of pJL1. PCR was conducted in a thermocycler (Takara, Japan) under the following conditions: 30 cycles of 30 s at 94°C, 1 min at 55°C, and 1 min at 72°C. All of the PCR products were cloned into pGEM-T Easy Vector (Promega, U.S.A.) and sequenced prior to cloning into the expression vectors in order to confirm that no mutations had been introduced during PCR amplification. The *E. coli* expression constructs were introduced into *E. coli* BL21 (DE3) pLysS via heat-pulse transformation, and the ampicillin-resistant transformants were selected.

SDS-PAGE Analysis of RubT1

For the expression of *rubT1*, *E. coli* BL21 (DE3) pJL1 was grown in 50 ml of LB medium containing ampicillin at 37°C for 8 h with shaking at 250 rpm. At an OD_{600} of *E. coli*, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM, and incubation was continued for an additional 4 h at 37°C. The cells were harvested by centrifugation at 6,000 ×*g* for 10 min and washed with 25 μl of 50 mM Tris-HCl (pH 7.4), after which the cells were slowly suspended by shaking. The contents were then centrifuged for an additional 10 min at 6,000 ×*g*. The cells were subsequently resuspended in 1 ml of 50 mM Tris-HCl (pH 7.4) and subjected to ultrasonication. The soluble proteins were separated by centrifugation, and the insoluble proteins were extracted with 10% SDS and 50 mM Tris-HCl (pH 7.4). The proteins were then loaded onto 30% acrylamide SDS-PAGE for the determination of expression levels. The expression conditions were optimized for a high yield of soluble protein; *i.e.*, 36 h of incubation at 20°C and 1 mM IPTG.

Purification of RubT1 Protein

The expressed protein was purified by gel filtration on an HPLC column (300 mm×7.8 mm, Bio-Sil SEC 250, Bio-Rad), followed by filtration on an anion-exchange column (UNOQ1 and QR1, 7 mm ID×35 mm L, Bio-Rad, CA, U.S.A.). The columns were washed with washing buffer (20 mM Tris-HCl, pH 7.5), and the cell lysates were loaded onto the column. The elution of the bound protein was conducted using a mixture of 20 mM Tris-HCl and 1.0 M NaCl solution. The purified protein was then analyzed by SDS-PAGE.

Measurement of Substrate Binding of RubT1 by FPLC

The purified RubT1 protein was mixed separately with the standard rubradirin sample with vigorous shaking at a controlled temperature (5 min at 4°C). The mixture was then subjected to a pass-through gel filtration FPLC column and eluted with 20 mM Tris-HCl (pH 7.5). Rubradirin-harboring proteins were denatured with acetone (4:1 v/v) and centrifuged for 2 min at 12,000 ×*g*. The supernatants were subjected to ESI-MASS analysis. Additionally, the denatured rubradirin-harboring supernatants (250 μl) were applied to paper disks placed on LB solid medium infused with *Staphylococcus aureus*.

Measurement of Substrate Binding of RubT1 by HPLC

S. achromogenes was grown in 50 ml of R2YE medium for 72 h [12] at 28°C, and the mycelia of the culture broth were removed by centrifugation at 6,000 ×*g* for 10 min. The supernatants were mixed with purified RubT1 under continuous stirring for 15 min at 4°C. The contents were harvested with ethyl acetate, and the volume was reduced using a rotary evaporator. The ethyl acetate extracts were subjected to HPLC analysis at 335 nm using an ODS column (150×4.6 mm, Japan, ODS-H80) with a linear

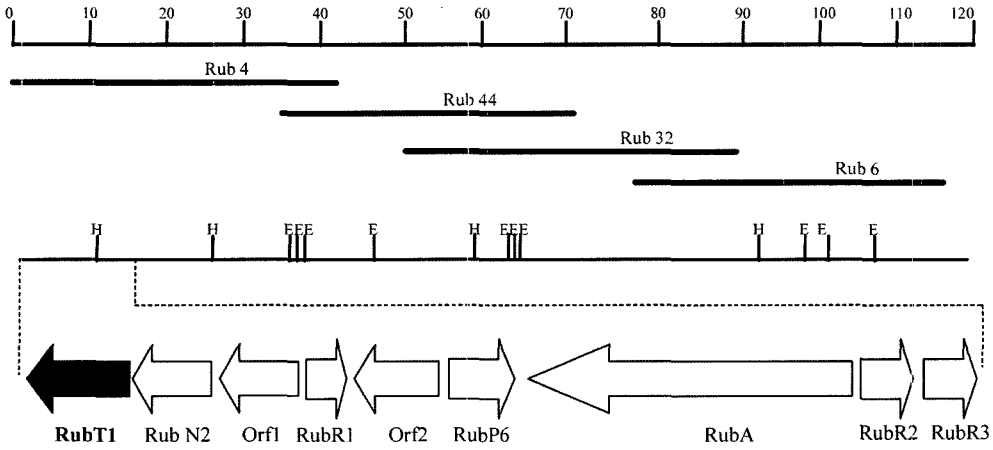

Fig. 1. Map of *rubT1* in the Rub4 cosmid of the rubradirin gene cluster.
Isolated cosmid clones are shown above the map. ORFs from cosmid Rub 4 are shown as arrows indicating the size and direction of transcription. ORF designation: RubT1, ABC transporter; RubN2, TDP-glucose 4,6-dehydratase; Orf1, Reductase; RubR1, Translation initiation factor; Orf2, Glucose-6-phosphate dehydrogenase; RubP6, cytochrome P450; RubA, polyketide synthase; RubR2, 30S ribosomal protein modification protein; RubR3, translation initiation factor.

gradient formed with 30% to 80% methanol in 50 mM ammonium acetate (pH 4.0) at a flow rate of 1 ml/min for 55 min. The HPLC profile was then further analyzed via ESI-Mass spectroscopy (Spectra-Physics Co.).

Nucleotide Sequence Accession Number

The nucleotide sequence of *rubT1* was deposited at the EMBL nucleotide database under the accession number AJ871581.

Results and Discussion

Sequence Analysis of *rubT1*

The *rubT1* is located at the most upstream region of the entire rubradirin biosynthetic gene cluster, and is flanked by TDP-glucose 4,6-dehydratase (*rubN2*) (Fig. 1). The gene encodes for a protein consisting of 311 amino acids, with a molecular mass of 34.5 kDa. The deduced amino acid sequence of RubT1 showed a high degree of similarity with other putative

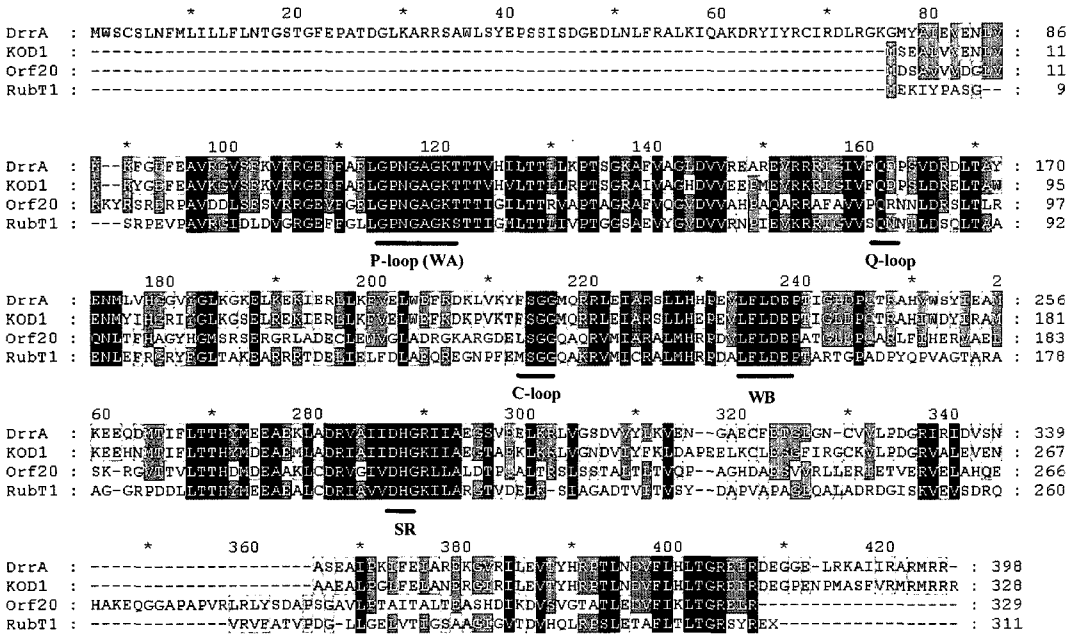

Fig. 2. Comparison of the deduced amino acid sequences of ABC transporters derived from various strains.
Archaeoglobus fulgidus (DrrA), *Thermococcus kodakarensis* (KOD1), *S. aizunensis* (Orf20), and *S. achromogenes* (RubT1). The essential conserved motifs are indicated by underlining. P-loop, Walker A; Q-loop; C-loop, signature sequence; Walker B, WB; and Switch Region, SR.

genes within the database; *e.g.*, 41% identity with the daunorubicin resistance ATP-binding protein DrrA (accession no. NP_070709); 40% identity with the ABC transporter protein of orf 20 (accession no. AAX98195); and 42% identity with the ABC-type multidrug transport system ATPase component KOD1 (accession no. BAD85333) (Fig. 2).

The protein sequence of RubT1 harbors a highly conserved signature sequence at the region spanning 133aa-139aa, MSGGQAKR, which is specific to the ABC transporter superfamily [3]. The characteristic conserved domain I of RubT1, referred to as the catalytic domain, has been shown to harbor a conserved ATP/GTP-binding site for motif A (P-loop). It is also known as the Walker A region, at 34aa-41aa, or GPNGAGKS in the sequence. The Walker B region, which harbors four aliphatic residues with aspartate followed by glutamate, was found to be conserved in the region spanning 153aa-158aa (ALFEDE) in the sequence. A Q-loop with a conserved glutamine at 81aa was also found in the sequence, as was the switch region harboring the conserved histidine at 207aa, which is believed to function as a γ-phosphate sensor. The ABC transporter genes are composed of α-helices and harbor a C-loop or signature sequence motifs. Of the ABC transporter genes, domain II, known as the signaling domain, was found to contain the conserved MSGGQAKR sequence within the RubT1 sequence at the region spanning 133aa-139aa (Fig. 2) for the interaction with the TMD [28]. The phylogenetic tree illustrates the ABC transport histories of different species (data not shown). The protein belonging to DrrA from *S. peucetius* exhibits its phylogeny with the protein from *Thermococcus kodakaraensis* KOD1, which harbors the ATPase component of an ABC-type multidrug transporter [8], whereas the CinT possessing only an ATP-binding subunit with a separated integral membrane subunit belongs to a separate group [30]. RubT1 and Orf20 are located in one group, and possess similar ABC transporters with no membrane subunits. These data support the notion that RubT1 is a resistance-mediated ABC transporter, and may function via a mechanism similar to the inherent activity of other ABC transporters.

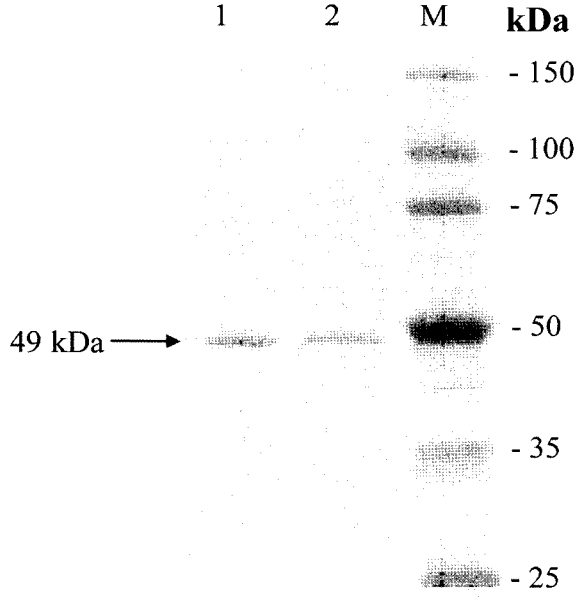

Fig. 3. SDS-PAGE analysis of the purified RubT1 protein.
1, Ion-exchange chromatography; 2, gel filtration chromatography; and M, protein marker.

Expression and Purification of RubT1

E. coli BL21 (DE3) cells were transformed with the pJL1 expression plasmid as 6×histidine and thioredoxin-tagged fusion proteins. Induction of the transformant with IPTG at 20°C for 20 h resulted in an excessive expression of soluble proteins. The soluble fractions were then purified via Ni^{2+}-affinity chromatography. The Ni^{2+} column-purified samples were further purified via ion-exchange chromatography, followed by gel filtration chromatography. Approximately 49 kDa molecular size of RubT1 was in agreement with that calculated from the amino acid sequence determined by SDS-PAGE analysis (Fig. 3).

Binding Affinity of Rubradirin to RubT1

In order to elucidate the binding affinity of RubT1, we mixed the standard rubradirin solution with the purified

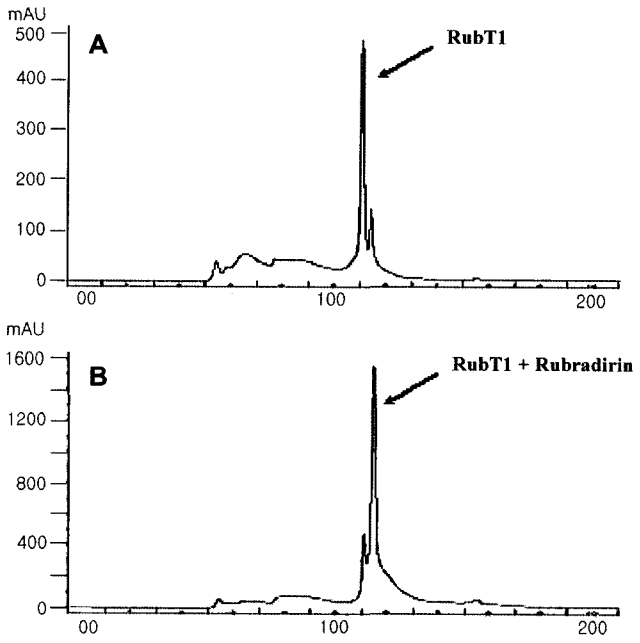

Fig. 4. Comparison of FPLC profiles, obtained before and after the binding of rubradirin with the RubT1.
A. Before binding of standard rubradirin to RubT1, peak of purified RubT1; **B.** After binding of standard rubradirin to RubT1, peak of rubradirin-bound RubT1. Increased peak height of the protein along with increased elution times shows the increase in protein size after binding with the standard rubradirin compound.

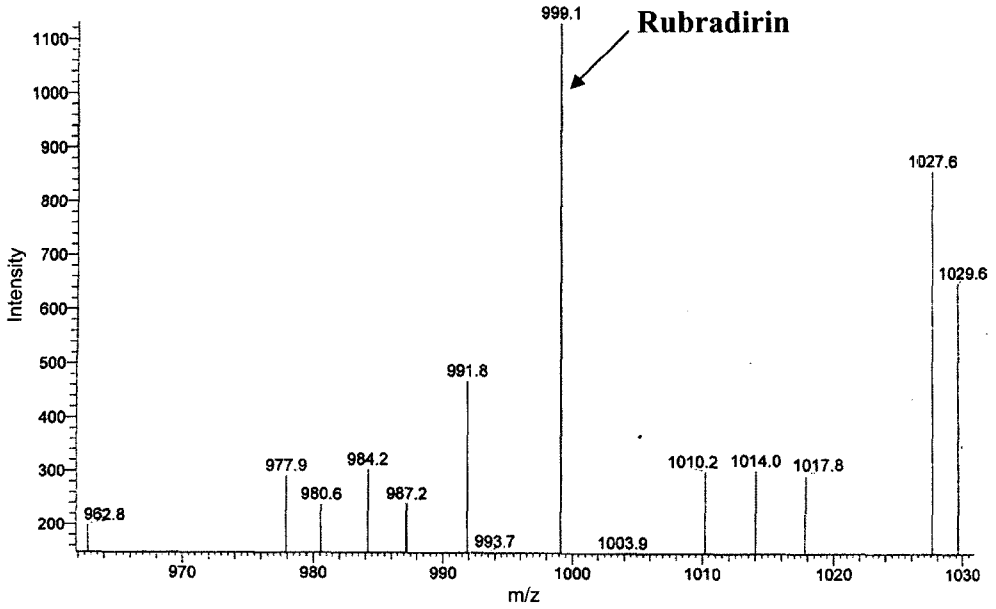

Fig. 5. ESI- MASS analysis of rubradirin as detected from the RubT1 protein-rubradirin binding complex after denaturation.
The arrow shows the molecular ion peak of rubradirin.

RubT1, and the mixture was then passed through an FPLC column, as previously described. A distinct increase in the size of the protein complex was observed in the FPLC profile, compared with that of the RubT1 protein (Fig. 4), indicating the formation of the protein complex in the presence of the binding substrate. In order to confirm that the bound substrate was rubradirin, the protein that had been eluted from the FPLC was denatured, and the supernatant was subjected to ESI-MS analysis. A mass peak of m/z^{+}=999 (Fig. 5) and 442 (positive ion modes), representing the molecular ion peaks of rubradirin and rubransarol, respectively, were consistent with the breakdown fragments of standard rubradirin. The identification of the mass peaks strongly suggested that RubT1 binds to the antibiotic, and is released after denaturation of the protein. The paper disk bioassay of the denatured solution against the Gram-positive bacterium *Staphylococcus aureus* indicated a significant inhibition zone, thus confirming its antibacterial activity (Fig. 6). HPLC analysis of the extract illustrated that the rubradirin peak was completely absent,

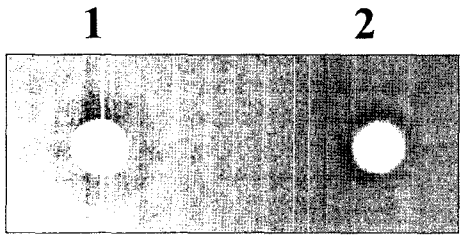

Fig. 6. Inhibition zone observed after the denaturation of the rubradirin-bound protein.
Activity of the supernatant obtained after the denaturation of the protein followed by centrifugation from 1, the standard rubradirin-protein mixture; 2, the extract of the rubradirin culture broth-protein mixture. The difference in inhibition zones may be attributed to low rubradirin concentration produced by the wild strain.

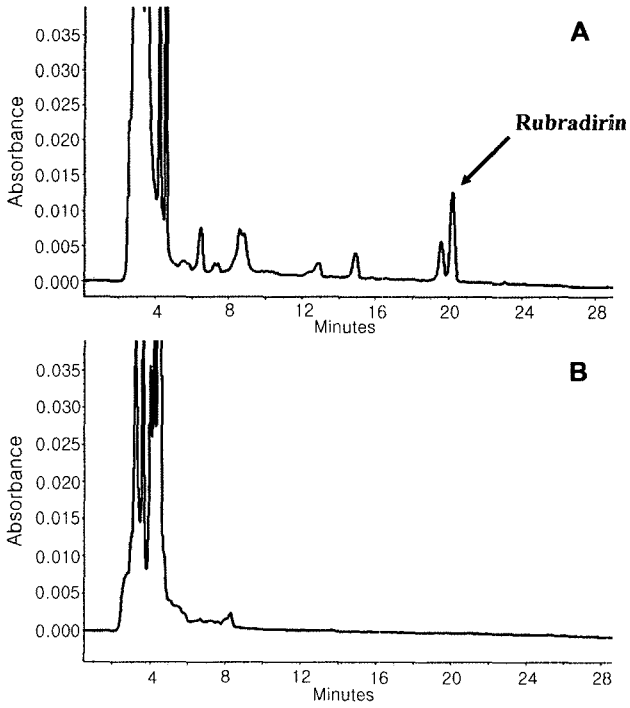

Fig. 7. HPLC analysis of the extracts from wild-type *S. achromogenes* var. *rubradiris* NRRL3061 (**A**), and after the addition of RubT1 to wild-type culture broth (**B**).

compared with the extract from the wild-type strain, indicating that the binding of the produced rubradirin was shifted by the purified RubT1 protein (Fig. 7). These data indicate that RubT1 binds rubradirin, and is employed in a bacterial system to export the rubradirin from the cell, thereby inducing resistance against rubradirin.

It has been demonstrated that one of the mechanisms inherent to the acquisition of resistance to antibiotics involves the enhanced expression of an ABC transporter, which promotes the efflux of the drug from bacterial cells [5, 9]. There are many examples to illustrate the resistance characteristics of ABC transporter-mediated MDR (multidrug resistance). The mithramycin resistance of the producer strain is mediated by the *mtrA* and *mtrB* genes. *mtrA* encodes for an ATP-binding protein of the ABC transporter superfamily [13]. It is similar to TnrB of *S. longisporoflavus*, which is involved in tetronasin resistance [17], and OleC from *S. antibioticus*, which is involved in oleandomycin resistance.

Furthermore, the extruder ABC transporter harbors a conserved intracellular domain (ICD) region, which is located between the TMD and NBD. The study of MsbA from *E. coli* shows that the ICD region (97–139 residues) harbors three α-helices, and enables the coupling of the TMD to the NBD [24]. In the gene sequence of RubT1 (311 aa), the amino acid residues (~110 aa) are believed to comprise the sequence of the ICD domain. However, this issue will remain nebulous until the crystal structure of this gene has been completed clearly. ABC exporters have evolved to fuse TMD with NBD, thereby ensuring a physically tethered catalytic domain. The presence of the peptide tether enables the dissociation of NBD from the ICD in the absence of substrate [4]. Upon substrate binding, changes in the conformation of ICD may increase its affinity for NBD, such that it significantly favors an orientation that promotes NBD dimerization, which drives both chamber closure and ATP hydrolysis. Bacteria that generate antibiotics are capable of defending themselves from the toxic effects of that antibiotic. *S. peucetius* generates daunorubicin and doxorubicin, and expresses the ABC transporter for drug resistance, DrrAB, which confers resistance to both of the cytotoxic secondary metabolites [10]. RubT1 shows 40% identity with the DrrA of *S. peucetius* and contains all necessary domains, such as an ABC transporter and ICD domains. It confirms that RubT1 has a function similar to that of DrrA. This study established the function of RubT1 as a resistance-mediating transporter gene, which may bind to rubradirin and be extruded with the help of transmembrane domains, which have yet to be characterized in detail in this strain.

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