



Identification of a novel bacteriocin-like protein and structural gene from *Rhodococcus erythropolis* JCM 2895, using suppression-subtractive hybridization

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Abstract

A novel bacteriocin-like protein and its structural gene (*rap*) were identified from *Rhodococcus erythropolis* JCM 2895. The *rapA* and *B* genes are located on a 5.4-kb circular plasmid, and were obtained using a modified suppression-subtractive hybridization method. The *rapA* and *B* genes were heterologously expressed in *Rhodococcus* sp. or *Escherichia coli*, and then characterized. The results indicated that RapA is a small, water-soluble, heat-stable antimicrobial protein, and that RapB is an immunity protein against RapA, estimated to be located on the cell membrane. RapA showed antimicrobial activity particularly against *R. erythropolis*, and the activity persisted even after SDS-PAGE analysis. For the heterologous expressed RapA protein, N-terminal amino acid sequence was also confirmed. This is the first report of a bacteriocin-like substance obtained from the genus *Rhodococcus*.

Introduction

Species of the genus *Rhodococcus* have recently been described as prolific producers of secondary metabolites, including antibiotics [1–3]. The antibiotic aurachin RE is the first antimicrobial secondary metabolite isolated from a member of *Rhodococcus*. The aurachin RE biosynthesis gene cluster has been cloned and characterized in the producing strain, *R. erythropolis* JCM 6824 [4, 5].

Antibiotic peptides produced by members of *Rhodococcus* have also been reported. The Lariatins A and B isolated from *R. jostii* K01-B0171 are ribosomally

synthesized peptides with antimycobacterial activity [2, 6]. The mature lariatins A and B are small peptides (18 and 20 amino acids respectively) that contain a unique lasso structure. Another peptide antibiotic, rhodopeptin, has also been reported in *Rhodococcus* sp. Mer-N1033. Rhodopeptin consists of cyclic tetrapeptides with hydrocarbon chains [3]. They have antifungal activity, and a number of structural analogues have been designed and chemically synthesized to improve their activity [7–9]. The biosynthesis gene cluster of lariatins has been reported [10], but, that of rhodopeptin has not been reported.

To date, numerous bacteriocins have been reported from diverse bacterial groups. Colicins produced by Gram-negative *E. coli* are typical bacteriocins and have been extensively studied. For related strains, it was reported that colicins promote strain diversity, instead of just attacking and killing their relatives [11, 12]. Bacteriocins from Gram-positive bacteria have been mostly isolated and studied in the low-GC content members of the phylum *Firmicutes* [13–15]. The well-known nisin produced by *Lactococcus lactis* shows strong activity against a large variety of Gram-positive bacteria. The nisin antimicrobial peptide is widely used as a food additive or a preservative, since it shows remarkable biological activity, stability, and safety. In contrast to *Firmicutes*, only a limited number of bacteriocins were identified from the high-GC content Gram-positive phylum, *Actinobacteria*. Bacteriocins or

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Table 1 Bacterial strains and plasmids used in this study

| Strain or plasmid | Relevant characteristic(s) | Reference or origin |
|--|---|---------------------|
| Strains | | |
| <i>Rhodococcus</i> strains | | |
| <i>R. erythropolis</i> JCM 2895 | Wild type, antimicrobial protein producer, GI ⁺ | JCM |
| <i>R. erythropolis</i> M1218 | Mutant strain of JCM 2895, antimicrobial protein sensitive, GI ⁻ | This study |
| <i>R. erythropolis</i> JCM 2893 | Wild type, antimicrobial protein producer, GI ⁺ | JCM |
| <i>R. erythropolis</i> JCM 2894 | Wild type, antimicrobial protein producer, GI ⁺ | JCM |
| <i>R. erythropolis</i> DSM 9675 | Wild type, antimicrobial protein producer, GI ⁺ | DSM |
| <i>R. erythropolis</i> DSM 43200 | Wild type, antimicrobial protein producer, GI ⁺ | DSM |
| <i>R. erythropolis</i> JCM 20154 | Wild type, antimicrobial protein producer, GI ⁺ | JCM |
| <i>R. erythropolis</i> JCM 20186 | Wild type, antimicrobial protein producer, GI ⁺ | JCM |
| <i>R. erythropolis</i> JCM 3201 ^T | Wild type, antimicrobial protein sensitive, Type strain | JCM |
| <i>E. coli</i> Rosetta (DE3) pLysS | Expression host strain for pET vectors | Novagen |
| Plasmids | | |
| pREC01 | Cryptic plasmid, isolated from JCM 2895 | This study |
| pBluescriptII KS | Cloning vector, Ap ^r | Stratagene |
| pTip-QC2 | Expression vector for <i>Rhodococcus</i> sp., <i>tip</i> promoter (thiostrepton inducible), Cm ^r , Ap ^r | 28 |
| pNit-QC2 | Expression vector for <i>Rhodococcus</i> sp., <i>nit</i> promoter (constitutive), Cm ^r , Ap ^r | 28 |
| pNit-RT2 | Expression vector for <i>Rhodococcus</i> sp., <i>nit</i> promoter (constitutive), Tet ^r , Ap ^r | 27 |
| pNit-BB4.4 | pTip-QC2 harboring 4.4-kb BglIII–BamHI fragment of pREC001 | This study |
| pNit-C01K | pNit-QC2 harboring <i>rapA</i> and <i>B</i> (ORFs 1 and 2) | This study |
| pNit-C01L | pNit-QC2 harboring <i>rapB</i> (ORF2) | This study |
| pNRT-C01L | pNit-RT2 harboring <i>rapB</i> (ORF2) | This study |
| pNit-C01M | pNit-QC2 harboring <i>rapA</i> (ORF1) | This study |
| pTip-C01K | pTip-QC2 harboring <i>rapA</i> and <i>B</i> (ORFs 1 and 2) | This study |
| pET26, pET28 | Expression vector for <i>E. coli</i> , Km ^r | Novagen |
| pET-rapA | pET26 harboring <i>rapA</i> | This study |
| pET-rapB | pET26 harboring <i>rapB</i> | This study |

GI⁺ growth inhibition activity positive (against type strain of *R. erythropolis*), GI⁻ growth inhibition activity negative, Ap^r ampicillin resistance, Cm^r chloramphenicol resistance, Tet^r tetracycline resistance, Km^r kanamycin resistance, JCM Japan Collection of Microorganisms, DSM Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

bacteriocin-like peptides from the phylum *Actinobacteria* were reported in genera such as *Streptomyces*, *Actinoplanes*, *Micrococcus*, and *Corynebacterium* [15–17]. However, no bacteriocin or bacteriocin-like substance has been isolated and studied from genus *Rhodococcus*.

In our previous study, we reported that seven *R. erythropolis* strains produce unknown antibiotic [1]. The producing strains showed growth inhibition zones particularly against the same species, *R. erythropolis*. The compounds were exported outside the cells and were active only against closely related species. These antimicrobial properties were well matched with that of bacteriocin. As mentioned above, the antibiotic compound aurachin RE has been isolated from *R. erythropolis* JCM 6824. Interestingly, the unknown substance producing *R. erythropolis* strains showed antimicrobial activity against the aurachin RE producing strains; and contrariwise, the aurachin RE producing strains showed antimicrobial activity against the unknown substance

producing strains [1, 18]. These observations indicated that entirely different antibiotics were produced by *R. erythropolis* strains. In order to identify the unknown antimicrobial compound and the biosynthesis gene, a representative strain, *R. erythropolis* JCM 2895, was selected from the seven producing strains. Then the strain was subjected to the further research. In this study, isolation of the structural gene of a novel bacteriocin-like protein using a modified suppression-subtractive hybridization (SSH) method, followed by protein characterization is reported.

Materials and Methods

Bacterial strains, plasmids, and culture conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *R. erythropolis* JCM 2895 was used as the original

antimicrobial protein-producing strain. The spontaneous antibiotic-deficient mutant strain, M1218, was used as the gene-expression host strain and was also used as the driver DNA source for SSH. The type strain of *R. erythropolis*, JCM 3201 was used as the antibiotic-negative control strain. *E. coli* Rosetta (DE3) pLysS (Novagen) was also used as the host strain. Bacterial strains were cultured in Luria-Bertani (LB) medium or W-minimal medium [19] supplemented with succinate, sucrose, and casamino acids (0.2% w/v each), at 37 °C for *E. coli* or at 28 °C for the *Rhodococcus* strains. Kanamycin (200 µg/ml), chloramphenicol (34 µg/ml), tetracycline (10 µg/ml), or ampicillin (100 µg/ml) was added to the culture media when needed. When using an inducible expression vector, *Rhodococcus* strains were cultured in LB medium until the optical density was at 600 nm (OD₆₀₀) of 2.0. The inducing molecule, thioestrepton, was then added to the medium at a final concentration of 0.5 µg/ml, followed by a 5-h incubation. In *E. coli*, IPTG (final concentration of 400 µM) was added to the medium at the OD₆₀₀ of 0.6, followed by a 3-h incubation.

Antimicrobial activity test

The growth inhibition activity of *Rhodococcus* strains was tested on LB soft-agar medium (soft-agar overlay assay) with *R. erythropolis* JCM 2895 or strain M1218 as the indicator strain [1]. An antimicrobial activity test on an SDS-PAGE gel was performed after a 3-hour wash of the gel with distilled water, followed by a top agar overlay containing the test strain.

SDS-PAGE analysis

The *rapA* overexpressed *E. coli* cells (harboring pET-*rapA*) were disrupted by sonication in the 50 mM Tris-HCl pH8.0 buffer on ice. The samples were centrifuged at 12,000× *g* for 10 min, and the supernatants were collected as the crude cell extract samples. The electrophoresis sample was prepared with Tricine sample buffer (Bio-Rad) according to the manufacturer's instruction; i.e., combine 10 µg of cell extract, 4.75 µl of Tricine sample buffer, and 0.25 µl of β-mercaptoethanol in the total volume of 10 µl, followed by heating at 95 °C for 5 min. The electrophoresis was performed using commercial precast gels of 16.5% Mini-PROTEAN Peptide Gel and prestained protein standard of Precision Plus Protein™ Dual Xtra Standards (Bio-Rad).

Suppression-subtractive hybridization

SSH was performed as reported previously with some modifications [20]. The procedure was illustrated in Supplemental Fig. S1.

(A) SSH driver DNA preparation: Genomic DNA of antibiotic-deficient mutant strain, M1218, was used as the driver. The DNA was digested by *RsaI* or *HaeIII* and was column purified using the Wizard® SV Gel and PCR Clean-Up System (Promega). The final concentration of the driver was adjusted to 300 ng/l.

(B) SSH tester DNA preparation and adapter ligation: Genomic DNA of *R. erythropolis* JCM 2895 was used as the tester. *RsaI*- or *HaeIII*-digested tester DNA was prepared as described for the driver, and the final concentration of the tester was adjusted to 50 ng/l. Four microliters of digested tester DNA was ligated to 6 µl of SSH adapter 1 (10 µM) and SSH adapter 2 (10 µM) in separate ligation reactions in a total volume of 20 µl. Structure and nucleotide sequence of the adapters were;

SSH adapter 1 (partially double stranded):

5'-GTAATACGACTCACTATAGGGCTCGAGCGGC
CGCCCGGGCAGGT-3'

3'-GGCCCGTCCA-5'

SSH adapter 2 (partially double stranded):

5'-TGTAGCGTGAAGACGACAGAAAGGGCGTGGT
GCGGAGGGCGGT-3'

3'-GCCTCCCGCCA-5'

After ligation, 2 µl of 0.2 M EDTA was added and the samples were heat inactivated at 65 °C for 10 min.

(C) Subtractive hybridization: the first and second hybridization of the tester DNA was performed as first reported [20].

(D) PCR amplification: the first PCR was performed in a total volume of 25 µl, containing 5 µl of template DNA (hybridized DNA, 4 ng/µl), 1 µl of primer P1 (10 µM), 1 µl of primer P2, and an appropriate amount of recombinant *Taq* polymerase and the supplier-provided reaction buffer (Takara, Japan). The amplification program was composed of 7 min initial denaturation at 75 °C, 50 cycles of 91 °C for 30 s, 68 °C for 30 s, 72 °C for 2.5 min, and 7 min final elongation at 68 °C. The second (nested) PCR was also performed in a total volume of 25 µl, containing 0.1 µl of the first PCR products, 1 µl of primer PN1-NotI (10 µM), and 1 µl of primer PN2-Sse8387I (10 µM). The second amplification program was composed of 1 min initial denaturation at 94 °C, 20 cycles of 94 °C for 30 s, 65 °C for 30 s, 72 °C for 1.5 min, and 3 min final elongation at 72 °C.

(E) Cloning and nucleotide sequence analysis of the subtracted DNAs: PCR products from the second amplification were column purified and double digested with NotI and Sse8387I. The digested DNA was then ligated with NotI-PstI double digested pBluescript II KS plasmid vector (Stratagene). Nucleotide sequence analysis of each insert DNA of the plasmids was carried out using the vector-specific sequencing primers, universal -120 and reverse + 1. Primers used in this study were listed in the Supplemental Table S1.

N-terminal amino acid sequence analysis of RapA

Strain M1218 containing pTip-C01K was cultured and expression of the recombinant protein was induced by thioestrepton. The culture media was centrifuged and the supernatant was filter sterilized with 0.22- μ m pore size. Then the filtered media was mixed with acetonitrile to make 20% acetonitrile solution. The sample was concentrated with Sep-Pak C18 Plus Short Cartridge (Waters) and eluted with 60% acetonitrile followed by vacuum evaporation to remove the solvent. The sample was resuspended in water and used for SDS-PAGE analysis. The protein band was transferred to PVDF membrane and subjected to N-terminal amino acid sequence determination. The sequencing analysis was carried out at the open facility institute of Hokkaido University using Procise-cLC protein sequencer (Applied Biosystems).

Accession numbers

The nucleotide sequence determined in this study has been deposited in the DDBJ, EMBL, and GenBank databases under the accession number AB893594.

Results and Discussion

Identification of the antibiotic biosynthesis gene

The *R. erythropolis* JCM 2895-derived antibiotic was supposed to be a protein or peptide in a preliminary study [1]; however, neither the crude protein cell extract nor the culture supernatant of JCM 2895 showed antimicrobial activity. We have previously succeeded in cloning the biosynthesis gene of the antibiotic, aurachin RE, by random transposon mutagenesis with *R. erythropolis* JCM 6824 [21]. In order to identify the biosynthesis gene of the antibiotic, the transposon method was also tried in JCM 2895 [22]. After repeated trials, over 60,000 kanamycin resistant mutants were screened for loss of antimicrobial activity, without any success. In the process of the screening, a spontaneous antibiotic-deficient mutant strain M1218 was obtained. The strain lacks not only antimicrobial activity but also resistance against the wild-type JCM 2895. Based on these results, we postulated that strain M1218 had lost both the antibiotic biosynthesis genes and genes for its resistance. Using the mutant strain, we used the SSH method to identify the genes. The SSH method was first reported in 1996 as an easy and effective way to identify tissue or organ-specific expressed genes based on the differences between their cDNA [20]. Recently, the technique was widely used not only for eukaryote samples but also for prokaryote samples. In particular, SSH was used for

identifying species or strain-specific genes, using two or more closely related strains, based on the differences between their genomic DNA [23–26]. In our study, SSH is easily applicable using the genomic DNA of strain M1218 as the driver and that of wild-type JCM 2895 as the tester (see Supplemental Fig. S1). The SSH procedure was performed similarly as first reported. Two major modifications were made: (a) using a more stable adapter 1, and (b) the amplified DNA was double digested with restriction enzymes and cloned into the pBluescript vector. We designed a new adapter 1 that has a 10-bp section of double stranded DNA, whereas that in original adapter was 8-bp. This modification improves not only the stability of the adapter 1 but also ligation efficiency with the tester DNAs. For the second major modification, we designed a new nested PN2 PCR primer with an Sse8387I enzyme site (designated PN2-Sse8387I). This modification improves cloning efficiency and decreases negative background (plasmid without insert) at the ligation and *E. coli* transformation stage.

Genomic DNA of the strains was digested with HaeIII or RsaI, and the two SSH were performed independently. A total of 38 plasmids (22 from HaeIII and 16 from RsaI) were obtained and the nucleotide sequences were determined. To simplify the experiment, clones with fewer than 50 bp insert were not used for further study. Finally, 20 individual clones were selected and the PCR primers to amplify each of the 20 sequences were designed. The PCR screening with these 20 primer sets was performed using genomic DNA of wild-type JCM 2895, M1218, and the type strain of *R. erythropolis*, JCM 3201 independently as the templates. The results revealed that 10 of the 20 sequences were exclusively present in the wild-type JCM 2895 but were nonexistent in the mutant strain M1218 and in the type strain. In order to confirm the specificity of the DNA fragments in relation to the antimicrobial activity, PCR screening with these 10 candidate primers was also performed against genomic DNA of six other *R. erythropolis* strains, which have been shown to have the same antimicrobial activity of JCM 2895. The PCR screening showed two sequences that were particularly conserved in all the seven antibiotic positive strains (Supplemental Fig. S2): one sequence from HaeIII, and the other from RsaI (clone no. A05 and C05, respectively). Using the forward primer of A05 and C05, a 1.7-kb fragment of DNA was PCR amplified. It indicated that the clone numbers A05 and C05 were located closely with each other in the opposite direction on the genomic DNA of JCM 2895.

In order to isolate the flanking region of the SSH clone, a DNA probe of 1.7-kb A05–C05 nucleotide was constructed. Southern hybridization analysis was performed using a probe against the EcoRI or PstI digested genomic DNA. A positive signal, 5.4 kb in size, was obtained from both

EcoRI or PstI digested DNA. The DNA fragments were subcloned and the entire nucleotide sequences were determined. Interestingly, both are composed of 5420 nt, and these sequences are completely identical. Furthermore, a set of well-known plasmid replication protein genes, *repA* and *repB* were identified in the sequence. These results indicate that the DNA is a circular plasmid with a unique EcoRI or PstI restriction site. It was also confirmed by isolating the plasmid from the wild-type strain, followed by agarose gel electrophoresis and nucleotide sequence determination. The plasmid was designated as pREC01, and its physical map is shown in Fig. 1.

To confirm whether the plasmid DNA contained the genes that are responsible for the antimicrobial activity, a 4.4-kb BglII–BamHI fragment was subcloned to the rhodococcal constitutive expression vector, pNit-QC2 [27, 28]. The resultant plasmid, pNit-BB4.4 was introduced into strain M1218 (self-cloning) by electroporation, then the transformant was tested for antimicrobial activity and for resistance against the wild-type strain. The plasmid pNit-BB4.4 confers both antibiotic and resistance properties to the host *Rhodococcus* strain. In order to narrow down the region and to identify the causative genes, varying lengths of nucleotide sequences were PCR subcloned and tested for activity as shown in the Fig. 2 and Supplemental Fig. S3. As a result, plasmid containing both orfs 1 and 2 (indicated in Fig. 1) confers both antimicrobial and resistance properties to the host strain. On the other hand, orf2 confers only resistance properties. The antimicrobial property was obtained only when the orfs 1 and 2 were co-introduced into the cells. We could not obtain any transformant that harbored orf1 alone. This may be because orf1 codes for the antimicrobial protein, and cells that do not have resistance genes for the antibiotic do not survive with just the orf1. Based on these results, we concluded that the orf1 codes the antimicrobial protein and orf2 codes the resistance

(immunity) protein, and designated these genes as *rapA* and *rapB* (rhodococcal antimicrobial protein), respectively. The estimated amino acid sequences of both the *rapA* (93 a.a.) and *rapB* (65 a.a.) showed no similarity to any known proteins or peptides in the database. In order to identify whether the other strains have these genes, PCR screening was performed using a pair of PCR primers designed to amplify *rapA* and *B* genes and using genomic DNAs of 20 strains of *R. erythropolis* as the templates. The PCR amplified fragments were obtained only from the six strains showing similar antimicrobial activity of JCM 2895. The results demonstrated that the strain JCM 2895 and the six tested *R. erythropolis* strains shared identical or highly similar *rapA* and *B* genes, and also indicated that the other 14 *R. erythropolis* strains did not have the genes. To date, only two antimicrobial-producing gene clusters, *rau* genes for aurachin RE and *lar* genes for lariatins have been reported in *Rhodococcus* [10, 21]. The *rap* genes reported in this study are a third example. A MerR type transcriptional regulator gene was also found in pREC01; however, the activity and its relation to the *rap* genes were not identified in this study. Some other ORFs were identified in pREC01. Their estimated amino acid sequences showed no homology with any of known protein in the database.

Heterologous expression of *rapA* and *rapB* in *E. coli*

rapA, *rapB*, and their derivative genes were cloned into the pET26 or pET28 vector (Novagen), and heterologously expressed in *E. coli*. As observed in *Rhodococcus*, SDS-PAGE analysis of the proteins from *E. coli* transformants could not identify any specifically expressed proteins. However, antimicrobial activity was obtained after the SDS-PAGE, by overlaying the top agar on the distilled-water-washed SDS-PAGE gels. A growth inhibition zone was observed on the recombinant *rapA* sample, in between the molecular size markers of 5 kDa and 10 kDa (Fig. 3). These observations are consistent with the estimated molecular weight of RapA proteins, 9.0 kDa or 6.3 kDa, with or without signal peptides, respectively. The results also demonstrated that RapA is a heat-stable protein and is not inactivated by SDS, because the protein sample was treated with heat for 5 min at 95 °C prior to electrophoresis. Some bacteriocin-like substances from Gram-positive bacteria have been initially proposed as heat labile, but to date they have been described as heat stable [13].

N-terminal amino acid sequence analysis of RapA protein

Wild-type strain JCM 2895 and the recombinant strain containing *rapA* and *B* produced a growth inhibition zone

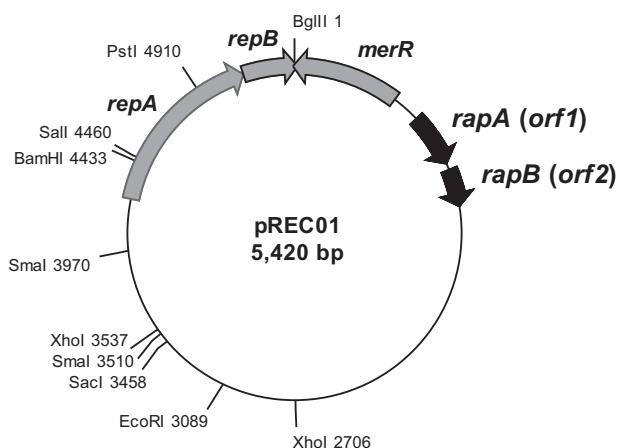


Fig. 1 Physical map of pREC01 plasmid isolated from *Rhodococcus erythropolis* JCM 2895

on the agar media against the test strain. Thus, at the least, the RapA protein must be an extracellular protein. The amino acid sequence of the protein is predicted to have signal peptides composed of 29 a.a. The mature protein is soluble according to web prediction tools, SOSUI [29], SignalP [30], TMHMM [31], and Phobius [32] (see Supplemental Fig. S4). These predictions are consistent with the hypothesis that RapA is an extracellular protein, and is in line with the structural characteristics of bacteriocin. Since it is expected to be an extracellular protein, extraction of RapA from culture supernatant and subsequent N-terminal amino acid sequence analysis was demonstrated (see materials and methods). The SDS-PAGE analysis showed an intense band at the molecular weight of 7 kDa ca., and the activity was again confirmed on the gel (data not shown). The N-terminal amino acid sequence analysis of the protein band determined the ten a.a. sequence of DSLPPSVXSS, and it well matched with the 30th to 39th amino acid residues of RapA (DSLPPSVCSS) that was deduced by the nucleotide sequence of the gene. These results indicated that the predicted 29 a.a. signal peptide of RapA was removed and the matured protein was exported to the outside of the cells.

Signal peptide and transmembrane region prediction were also tested in the case of RapB by the web tools. Using SignalP and Phobius program, signal peptide region was not indicated in RapB. On the other hand, two distinct transmembrane domains were indicated by three independent programs, Phobius, TMHMM, and SOSUI. Each of the two predicted domains constitutes the major part of N- or C-terminal half (see Supplemental Fig. S4). Based on these results, RapB protein was predicted to be a membrane protein. The mechanism of the resistance to the RapA was not indicated in this study; however, one

possible function of the RapB protein might be the protection of RapA protein penetration into the cell. Immunity proteins for bacteriocins vary on their sizes and sequences [33]. Predicted transmembrane-immunity proteins for bacteriocins were also found in some cases such as enterocin Q [34] and lactococcin G [35], though their immune mechanisms have not been clearly characterized. Further study is also needed to understand the RapB function.

Concluding remarks

A novel bacteriocin-like protein and immunity protein and their structural genes were identified from *R. erythropolis* JCM 2895. This is the first report of a bacteriocin-like substance obtained from the genus *Rhodococcus*. In this study, we successfully identified the *rapA* and *B* genes by using a modified SSH method. The modified method improves cloning efficiency and can be applied to other samples. RapA is a small, water-soluble, heat-stable, antimicrobial protein. Since it is active only against *R. erythropolis*, RapA is a strain-specific toxin. RapB is the immunity protein against RapA, which is considered to be located on the membrane. The RapB protein might inhibit the RapA–target protein reaction at the cell membrane, to avoid cell death. Further studies are needed to elucidate the molecular mechanism of the antimicrobial process. In *E. coli*, it has been reported that diversity of colicins supports diversity of *E. coli* species [11, 12]. Thus far, we have identified a number of *Rhodococcus* strains showing antimicrobial activity. However, strains producing bacteriocin or bacteriocin-like substances other than the seven strains used in this study have not been identified. Further studies are needed to understand the bacteriocin-like proteins

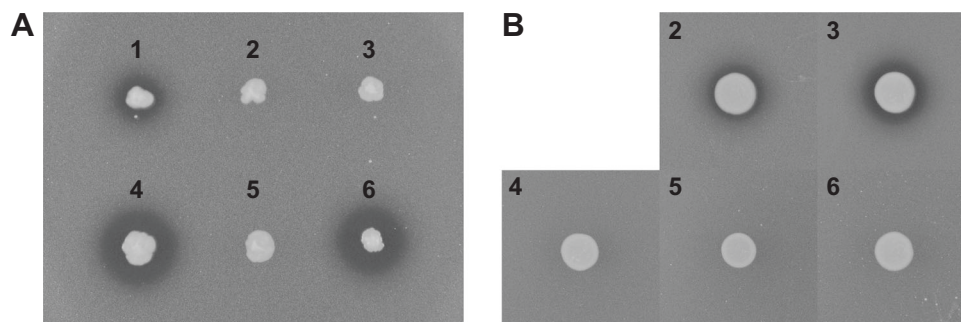


Fig. 2 Antimicrobial activity and resistance activity of *Rhodococcus* transformants containing the subcloned plasmids. **a** antimicrobial activity test. Strain M1218 was used as the susceptible test strain and overlaid on the agar plate. Spot-inoculated strains: 1, wild-type JCM 2895; 2, M1218 pNit-QC2 (vector control); 3, M1218 pNit-RT2 (vector control); 4, M1218 pNit-C01K (orf1, 2); 5, M1218 pNit-C01L (orf2); 6, M1218 pNit-C01M (orf1) with pNRT-C01L (orf2). Clones with growth inhibition zone represent the existence of the antibiotic

gene in the plasmid. **b** resistance activity test against the wild-type strain JCM 2895. The test clones, M1218 pNit-QC2 (vector control), M1218 pNit-RT2 (vector control), M1218 pNit-C01K (orf1, 2), M1218 pNit-C01L (orf2), and M1218 pNit-C01M (orf1) with pNRT-C01L (orf2), were overlaid on the plates of 2, 3, 4, 5, and 6, respectively. The wild-type strain JCM 2895 was spot-inoculated at the center. Clones (around the wild-type strain) without growth inhibition zone represent the existence of the resistant gene in the plasmid

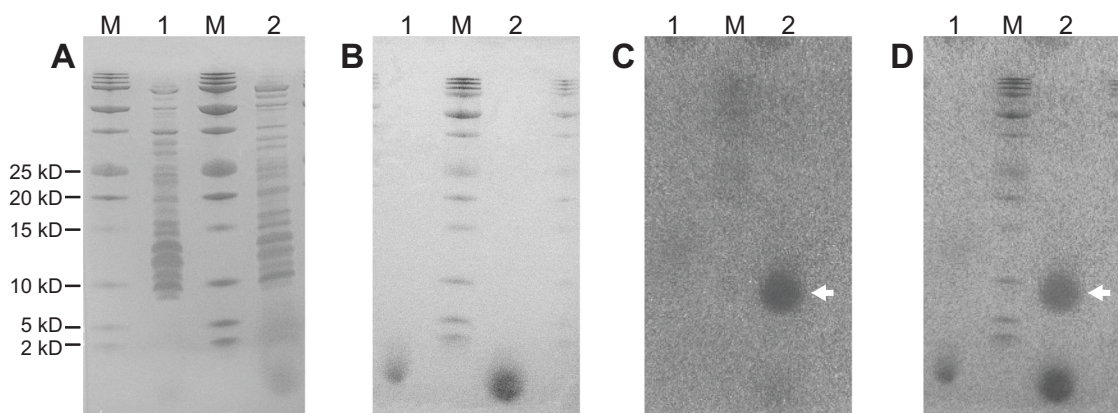


Fig. 3 SDS-PAGE analysis and subsequent antimicrobial activity test of recombinant RapA. Lanes: 1, Crude cell extract of *E. coli* pET26 (vector control); 2, Crude cell extract of *E. coli* pET-rapA; M, protein size marker (prestained). **a** SDS-PAGE analysis with coomassie brilliant blue (CBB) stain. **b** SDS-PAGE analysis without CBB stain. **c**

antimicrobial activity test of (b) gel (The susceptible test strain M1218 was dispersed in the soft agar, and was overlaid on the washed gel of panel (b). **d** merged image of panels (b) and (c). Arrows indicate the growth inhibition zones of the test strain. Bromophenol blue dye can be seen in lanes 1 and 2 of panels (b) and (d)

distributed in *Rhodococcus*, and their ecological function and significance.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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