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Cloning and characterization of an rRNA methyltransferase from *Sorangium cellulosum*

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ABSTRACT

A locus (*kmr*) responsible for aminoglycosides-resistance of *Sorangium cellulosum* was cloned and characterized in *Myxococcus xanthus*. The gene *kmr* encodes a putative rRNA methyltransferase. Expression of the complete ORF endowed the *Myxococcus* transformants with the resistance to aminoglycosidic antibiotics of kanamycin, apramycin, gentamycin, neomycin, and tobramycin at an extraordinary high-level (MIC, higher than 500 μg/ml). However, the gene did not function in *Escherichia coli* cells. In *Sorangium* genome, the gene *kmr* was followed by a putative integrase gene, and was highly homologous in different *Sorangium* strains. The *Sorangium* rRNA methyltransferase sequence was in low similarity to the reported 16S rRNA methyltransferases, and their resistance spectrums were also different. The results indicate that the rRNA methyltransferase (Kmr) in *Sorangium* strains is a new member of the rRNA methyltransferases family.

Antibiotics resistance is an efficient means of organisms to the repression of antibiotics produced by other rivals in environments [1], and is rather easily spread among different microorganisms. This resistance is also a balance mechanism of microorganisms to release the feedback repression of their own overproduced metabolites [2], which is important for those microbes with strong production ability of antibiotics [3]. *Sorangium* is a special myxobacterial genus for their degradation ability of cellulosic materials. *Sorangium* strains possess not only the excellent ability to produce various bioactive secondary metabolites [4], but also the multiple resistances to many different kinds of antibiotics, some of which are intrinsic [5]. For example, studies of Reichenbach [5] and our unpublished data indicated that, whether or not kanamycin was added to the isolation plates, all *Sorangium* strains isolated from different soil samples were able to grow on the media plates containing kanamycin at a high concentration, normally 1000 μg/ml or higher. The resistance characteristic has been routinely used as an efficient isolation technique to limit growth of the other microbes.

The resistance mechanisms to antibiotics include three ways: limiting entrance of antibiotics into cells, modifying antibiotics, or modifying the targets of antibiotics. Because kanamycin entered *Sorangium* cells without modifications, the resistance was suggested to be the results of modification of ribosomes [6]. However, owing to the lack of efficient genetic methods, the resistance mechanism has not yet been elucidated. Due to the high G+C content in *Sorangium* genome sequence (71.4% for *Sorangium cellulosum* So ce 56 [7]), it is difficult to study its genetics in heterogeneous hosts such as *Escherichia coli*. Recently, we discovered an autonomously replicating plasmid pMF1 from a *Myxococcus* strain and developed a gene cloning system in *Myxococcus xanthus* [8], which provided a valuable tool to investigate the resistance mechanism in *Sorangium*. We cloned and characterized a gene locus responsible for the resistance of *Sorangium* in kanamycin sensitive *Myxococcus xanthus* cells using a vector derived from pMF1 and determined that the expression of an rRNA methyltransferase from *Sorangium* made the *Myxococcus* transformants tolerant of kanamycin and also many other aminoglycosidic antibiotics. Interestingly, the gene did not function in *E. coli* cells. The rRNA methyltransferase was highly homologous among *Sorangium* strains, but had a long phylogenetic distance from the known rRNA methyltransferases.

Materials and methods

Strains, plasmids, and culture conditions. The *S. cellulosum* strains used in this study were isolated from different soil samples collected in China [9]. The other strains and plasmids are listed in Table 1. The *Myxococcus* strains were routinely cultivated in CTT medium [10], and the *Sorangium* strains were cultured in M26 medium [11] or on VY/2 medium plate [5] at 30 °C. The *E. coli* cells were grown in LB medium at 37 °C. Solid medium was prepared by the addition of 1.5% agar. If required, 100 μg/ml ampicillin or 100 μg/ml kanamycin was supplemented for selection. Other antibiotics were described in detail in the text. All antibiotics used in this study were purchased from BBI (Canada).

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Table 1
Bacterial strains and plasmids

Strain or plasmid	Genotype or description[a]	Source
Strains		
M. xanthus DZ1	Nonmotile, nonfruiting, dispersed growing	D.R. Zusman, University of California, Berkeley
S. cellulosum So0157-2	Wide type	[12]
E. coli DH5α	*supE*44 Δ*lac*U169 (φ80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	Life Technologies Inc.
Plasmids		
pGEM-T Easy Vector	Amp^r, cloning vector	Promega
pZJY41	Amp^r, Km^r, a stable shuttle vector in *E. coli-M. xanthus*	[8]
pZJY42	Amp^r, pZJY41 cut off *aphII* by *Hin*dIII	This study
pZJY42_31	Amp^r, Km^r, pZJY42 with an insert of a 3.4-kb chromosomal fragment of So0157-2	This study
pZJY42_46	Amp^r, Km^r, pZJY42 with an insert of a 3.2-kb chromosomal fragment of So0157-2	This study
pZJY42_51	Amp^r, Km^r, pZJY42 with an insert of a 3.9-kb chromosomal fragment of So0157-2	This study
pZJY42_53	Amp^r, Km^r, pZJY42 with an insert of a 3.0-kb chromosomal fragment of So0157-2	This study
pZJY42_64	Amp^r, Km^r, pZJY42 with an insert of a 3.8-kb chromosomal fragment of So0157-2	This study
pZJY42_13	Amp^r, Km^r, pZJY42 with an insert of a 768-bp PCR product from So0157-2 chromosome DNA	This study
pZJY42_26	Amp^r, Km^r, pZJY42 with an insert of a 672-bp PCR product from So0157-2 chromosome DNA	This study
pZJY42_37	Amp^r, pZJY42 with an insert of a 492-bp PCR product from So0157-2 chromosome DNA	This study

[a] Amp^r, ampicillin resistance; Km^r, kanamycin resistance.

Chromosomal DNA extraction from S. cellulosum strains. The *S. cellulosum* strains were cultivated in M26 medium at 30 °C, shaking at 200 rpm for 5–6 days. 0.5 g cell pellet was harvested by centrifugation, and suspended in 9.5 ml TE buffer containing 10 mM Tris–HCl (pH 8.0) and 1 mM EDTA (pH 8.0). 0.5 ml 10% sodium dodecyl sulfate and 50 μl RNase (25 mg/ml) were added, and the mixture was incubated at 37 °C for 1 h. Then 40 μl Proteinase K (25 mg/ml) was added and the mixture was incubated at 55 °C for another 1 h. After the addition of 1.8 ml 5 M NaCl and 1.5 ml CTAB/NaCl solution, the mixture was incubated at 65 °C for 20 min. Then the mixture was extracted using an equal volume of Tris-saturated phenol–chloroform–isoamyl alcohol (25:24:1) twice. The final supernatant was mixed with 0.1 volume of 3 M sodium acetate and an equal volume of isopropyl alcohol to precipitate the DNA. After centrifugation, the DNA pellet was washed with 70% ethanol, air-dried and dissolved in TE buffer.

Cloning and sequencing of the kanamycin resistance gene (kmr). To clone the *Sorangium* kanamycin resistance gene, a plasmid pZJY42 was constructed from pZJY41 [8]. After digestion with HindIII, a 5.4-kb DNA segment of pZJY41 was purified using Agarose Gel DNA Extraction Kit (Roche) and self-ligated by T4 DNA Ligase (TaKaRa). Then the solution was transformed into *E. coli* DH5α to form the cloning vector pZJY42 (Fig. 1).

The *S. cellulosum* So0157-2 [12] genome was extracted using the method described above and incompletely digested with SacI. Then the digestion mixture was incubated in a ligation solution containing SacI and alkaline phosphatase pretreated plasmid pZJY42. The ligation mixture was precipitated using ethanol, dissolved in sterile deionized water, and electroporated into *M. xanthus* DZ1, following the protocol described by Kashefi and Hartzell [13]. The resistant clones from CTT plates containing 100 μg/ml kanamycin were selected and then purified. The presence of plasmids was determined using the method described previously [8]. The plasmids with different size from the *Myxococcus* transformants were further transferred into *E. coli* DH5α and the *E. coli* transformants were selected from LB plates containing 100 μg/ml ampicillin. Clones containing different insert segments were sent for sequencing (Biosune, China).

Fragments of the *kmr* gene were amplified from the chromosomal DNA of 13 other *Sorangium* strains by PCR using the primer set of ckmu1 (5′-CGCGGCGGCC GGCCGAAC-3′) and ckmd (5′-GAGGTGCTGCCCCCAGCTC-3′) and *Ex Taq* DNA Polymerase (TaKaRa). The 375-bp PCR products were separately inserted into the pGEM-T easy vector (Promega), and the ligation mixture was transformed into *E. coli* DH5α, respectively. The transformants were selected from LB plates containing 100 μg/ml ampicillin and the inserts were sequenced.

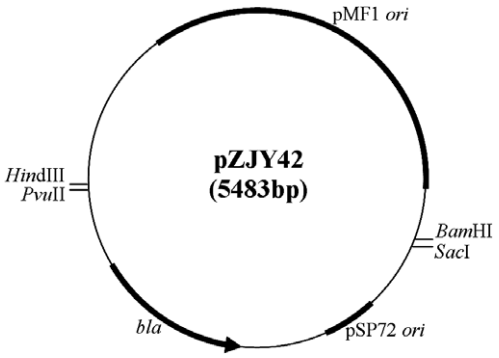

Fig. 1. Schematic map of pZJY42. Antibiotic selection marker *bla* is indicated by the bold arc. The cloning sites used in this study are shown.

Location of the kanamycin resistance gene (kmr). To locate the kanamycin resistance gene, three DNA fragments of *S. cellulosum* So0157-2 genome were amplified from the plasmid pZJY42_53 by PCR using three primer sets and *Pfu* DNA polymerase (Fermentas). A 768-bp DNA fragment containing the complete orf2 and its upstream 96-bp sequence was obtained using the primers kmu1 (5′-GAGCTCGCGGCGGCGAG-3′) and kmd1 (5′-CTAGCCTGGTGCAGCGCTGGC-3′). The other two fragments (672-bp and 492-bp in size) were amplified from the sites of two putative start codons in orf2 by using the primers of kmu2 (5′-ATGATCGTGCAGCTCGGCAAGG-3′) and kmd1, and kmu3 (5′-ATGCGCGAGGTGTCGTGGC-3′) and kmd1. After purification, the three DNA fragments were inserted into PvuII-digested plasmid pZJY42, respectively. The ligation solutions were transformed into *E. coli* DH5α separately, resulting in recombinant plasmids pZJY42_13, pZJY42_26, and pZJY42_37. Each plasmid was sequenced for validation and transferred into *M. xanthus* DZ1. After 7 days of incubation, transformants were selected from CTT plates containing 100 μg/ml kanamycin and purified twice. The plasmids were extracted for confirmation.

Resistance spectrum of the M. xanthus transformants. The *M. xanthus* transformants containing different plasmids were determined of the MICs on different aminoglycosidic antibiotics. *S. cellulosum* So0157-2 and *M. xanthus* DZ1 were used as controls. The *M. xanthus* transformants were grown in CTT media containing 100 μg/ml kanamycin for 48 h. After centrifugation, the cells were washed once with CTT media, and suspended to the final concentration of 1×10^9 cells/ml. A 3-μl aliquot of cells of each strain was inoculated on CTT plates containing different antibiotics. After 5 days of incubation at 30 °C, the MICs of the antibiotics were determined. *S. cellulosum* strain So0157-2 was grown on VY/2 plates containing different antibiotics.

Bioinformatics analysis. The sequenced segments were assembled using ContigExpress software (InforMax Inc). The ORFs in the inserts were predicted using FramePlot 3.0 [14]. The amino acid sequences of the rRNA methyltransferase and the referenced sequences, extracted from the GenBank database, were aligned using ClustalX (2.0) programs [15]. Phylogenetic reconstruction of the sequences was carried out by using distance/neighbor joining (NJ) programs with the Poisson correction distance model in MEGA v.3.1 software [16].

Accession numbers in GenBank. The assembled sequence of clones that resulted in kanamycin resistance in *M. xanthus* transformants was submitted to GenBank with the Accession No. EU429565. The partial coding sequences of the *kmr* genes from 13 *S. cellulosum* genomes were submitted to GenBank with the Accession Nos. EU429552–EU429564. The complete sequence of plasmid pZJY41 was submitted to GenBank with the Accession No. EU328349.

Results and discussion

Cloning of the Sorangium kanamycin resistance gene (kmr)

To explore the resistance mechanism of *Sorangium* cells to kanamycin, the *aphII* gene was cut off from the shuttle vector pZJY41, forming the plasmid pZJY42 (Fig. 1). When the plasmid pZJY42 was transferred into *M. xanthus* DZ1, no transformant was grown on CTT plates containing 100 μg/ml kanamycin. The chromosomal DNA of *S. cellulosum* So0157-2 was digested with different restriction enzymes that cut the plasmid pZJY42 only once,

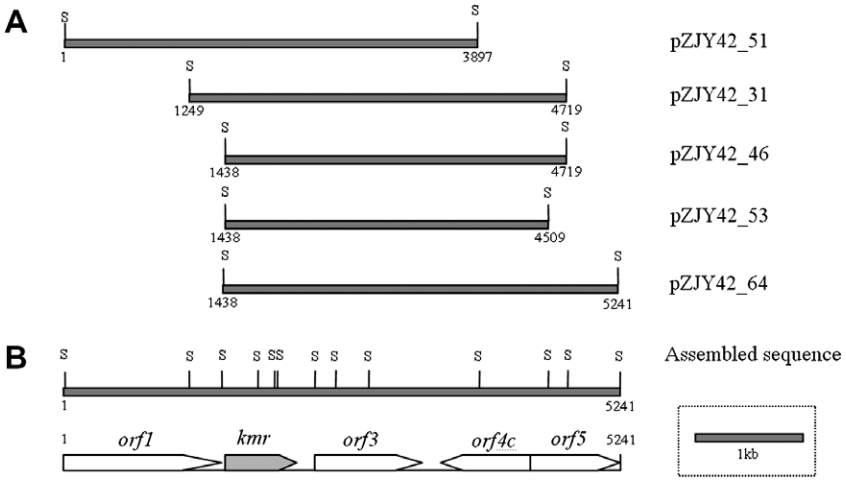

Fig. 2. Schematic maps of different insert sequences (A) and their assembled sequence and the predicted ORFs (B) of *S. cellulosum* So0157-2 genome in plasmid pZJY42. S means the SacI recognition site. The positions of the insert sequence in each plasmid are shown by sequence numbers. The predicted ORFs are indicated by open boxes except the *kmr*, which is shown as a filled box. The direction of transcription is indicated by arrowhead.

and the digestion with PvuII, SacI or BamHI produced segments of appropriate sizes for cloning. The chromosomal DNA was thus incompletely digested with SacI, and the digested DNA segments were inserted into pZJY42. After the ligation mixture had been transformed into *M. xanthus* DZ1, the cells were grown on CTT plates containing 100 μg/ml kanamycin. Five transformants were obtained, and the plasmids extracted from the resistant transformants were determined to be in different size.

The insert sequences from the five transformants were sequenced. After assembling the insert segments we got a sequence of 5,241 bp with a G+C content of 72.7%, and all of the inserts contained a 2.4-kb DNA fragment (Fig. 2), which should include the gene(s) responsible for the kanamycin resistance and may be the only reason for the kanamycin resistance in strain So0157-2. There were five predicted ORFs in the assembled sequence; four on the sense strand and one (orf4c) on the complementary strand (Fig. 2B). The mutual 2.4-kb DNA fragment contained two ORFs, orf2 and orf3. Orf2 is a putative rRNA methyltransferase, which is homologous to the rRNA methyltransferase of *S. cellulosum* So ce 56 with 92% identity, but to the 16S rRNA methylase or methyltransferase of *E. coli*, *Saccharothrix*, and *Streptomyces* with only 29% to 35% identity. Orf3 is homologous to the integrase of *Solibacter* with 48% identity, and the transposase of *Azoarcus* and *Marinobacter* with 46% identity. Methylation of 16S rRNA is thus suggested to be responsible for the kanamycin resistance in *Sorangium* strains.

Resistance to aminoglycosides of the Sorangium Kmr in M. xanthus

Methylation of 16S rRNA is able to confer an extraordinary high-level of resistance to many clinically important aminoglycosides [17]. Based on the resistance to different aminoglycosidic antibiotics, the methylation enzymes are divided into two groups. One is like the KamC of *Saccharopolyspora hirsute*, which methylates residue A1408 (numbered according to *E. coli*) of 16S rRNA, allowing growth on media containing kanamycin and apramycin, but not with gentamycin [18]. The other is like the Sgm of *Micromonospora zionensis*, which methylates residue G1405, permitting growth in the presence of kanamycin and gentamycin, but not with apramycin [19]. Both are susceptible to the 4,5-substituted deoxystreptamine aminoglycosides such as neomycin [19,20]. We inoculated the *Myxococcus* transformants containing pZJY42_31, pZJY42_46, pZJY42_51, pZJY42_53, or pZJY42_64 on CTT plates containing various aminoglycosidic antibiotics for MIC analysis,

Table 2
MICs (μg/ml) of parental strains and transformants

Strains[a]	MIC (μg/ml)[b]				
	KAN	APR	GEN	NEO	TOB
S. cellulosum So0157-2	>1000	>500	500	500	500
M. xanthus DZ1	10	10	200	10	<5
M. xanthus DZ1 (pZJY42_31)	>1000	>1000	>1000	>1000	>500
M. xanthus DZ1 (pZJY42_46)	>1000	>1000	>1000	>1000	>500
M. xanthus DZ1 (pZJY42_51)	>1000	>1000	>1000	>1000	>500
M. xanthus DZ1 (pZJY42_53)	>1000	>1000	>1000	>1000	>500
M. xanthus DZ1 (pZJY42_64)	>1000	>1000	>1000	>1000	>500
M. xanthus DZ1 (pZJY42_13)	>1000	>1000	>1000	>1000	500
M. xanthus DZ1 (pZJY42_26)	>1000	>1000	>1000	>1000	>500

[a] *S. cellulosum* So0157-2 was grown on VY/2 plates containing various antibiotics for 6 days; *M. xanthus* DZ1 and the *M. xanthus* DZ1 transformants with different plasmid were grown on CTT plates containing various antibiotics for 5 days.
[b] *Abbreviations:* KAN, kanamycin sulfate; APR, apramycin sulfate; GEN, gentamycin sulfate; NEO, neomycin sulfate; TOB, tobramycin.

Table 3
Transformation and the frequency of *M. xanthus* DZ1 with the plasmid pZJY42 containing different segments of *S. cellulosum* So0157-2 genome (positions shown by sequence numbers)

Plasmid	Segments cloned in plasmid pZJY42	Transformation efficiency in strain DZ1 (CFU/μg DNA)
pZJY42_53	1438 *kmr* *orf3* *orf4c* 4509	6×10^5
pZJY42_13	1438 *kmr* 2205	1.1×10^6
pZJY42_26	1534 *kmr* 2205	1.0×10^6
pZJY42_37	1714 *kmr* 2205	0

The predicted genes are indicated by open boxes except the *kmr* (putative rRNA methyltransferase gene), which is shown as a filled box. The direction of transcription is indicated by arrowhead.

see Table 2. These plasmids conferred resistance of *M. xanthus* transformants to kanamycin, apramycin, gentamycin, neomycin and tobramycin at an extraordinary high-level (MIC, >500 μg/ml). Interestingly, when the plasmids isolated from the *M. xanthus* transformants were separately transferred into *E. coli* cells, all *E. coli* transformants were able to grow on LB plates containing 100 μg/ml ampicillin, but not on LB plates containing 100 μg/ml kanamycin. The phenomenon is probably due to the unrecognizable promoter or the codon bias of the gene in *E. coli*. There are nine codons for proline (CCC) in orf2, which is the rare codon in *E. coli*, online analyzed with the Rare Codon Calculator (RACC) (http://nihserver.mbi.ucla.edu/RACC/).

Location of the Sorangium kanamycin resistance gene (kmr)

The 16S rRNA methylase or methyltransferase confers the resistance to aminoglycosidic antibiotics, not only in those nosocomial pathogens including *Pseudomonas*, *Serratia*, and *Klebsiella* [17], but also in those aminoglycoside-producing organisms, such as *Streptomyces* and *Saccharopolyspora* [18]. Usually, the gene is neighbored with some mobile genetic elements such as the integron [21] or transposon [22] to help it spread. The cloned sequences of *S. cellulosum* So0157-2 genome contained a putative rRNA methyltransferase gene (orf2) and an integrase gene (orf3), suggesting that the resistance gene was also able to spread horizontally. However, the Orf2 contains two in-frame methionine residues (the start codon for the orf). It is difficult to predict the actual start codon because the bigger putative peptide (223 aa) was homologous to the 16S rRNA methylase of *E. coli* and the CmnU of *Saccharothrix mutabilis* subsp. *capreolus* (with 29% and 35% identity), while the small one (163 aa) was rather homologous to the 16S rRNA methyltransferase of *Streptomyces* (with 32% identity). In the cloned mutual sequence, there was only a 96-bp upstream segment in front of the first methionine residue, in which no obvious promoter structure existed. To locate the *kmr* gene, three fragments from the beginning of the 96-bp sequence, and from the two methionine residues spanning to the stop codon of orf2 gene were amplified (768-, 672-, and 492-bp sizes, respectively). The three

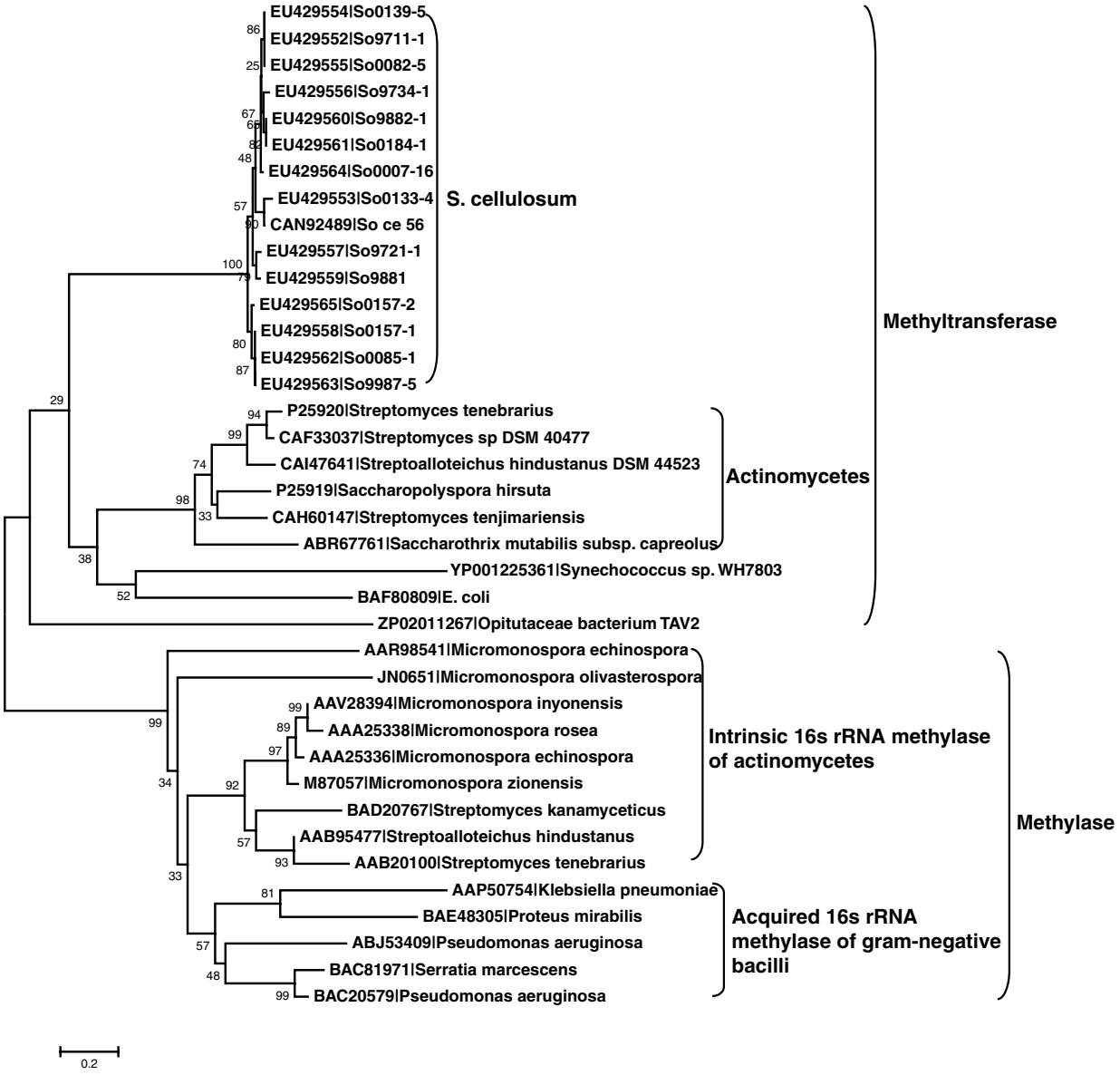

Fig. 3. Phylogeny of rRNA methylases and methyltransferases from *Sorangium* strains and the references. The reconstruction was computed by the distance method (NJ, Poisson correction distance model) with interior branch length supports from 1000 replicates.

fragments were separately inserted into pZJY42, forming recombinant plasmids pZJY42_13, pZJY42_26, and pZJY42_37. These three plasmids were separately transformed into *M. xanthus* DZ1 cells, then the cells were cultivated on the CTT plates containing 100 μg/ml kanamycin. Transformants of pZJY42_13 and pZJY42_26 were able to grow, but no transformants of pZJY42_37 appeared (Table 3). Therefore, the bigger orf2 (designated *kmr*) was the genetic element that endowed *M. xanthus* transformants with the resistance to kanamycin. In this gene locus, the sequence in front of the small orf2 probably functioned as the promoter of the rRNA methyltransferase gene, whose product was started from the second methionine residue.

Coherence of the Sorangium kanamycin resistance gene (kmr)

Doi and Arakawa [20] analyzed some enzyme sequences that are involved in the methylation of 16S rRNA, and found that the methylases were divided into two independent groups, intrinsic enzymes of actinomycetes and acquired enzymes of gram-negative bacilli. The conserved regions (about 375 bp in size) of the 16S rRNA methyltransferase gene were amplified from 13 different *Sorangium* strains. The amino acid sequences of the PCR products were aligned and phylogenetically analyzed in the tree of 16S rRNA methylases and methyltransferases (Fig. 3). The sequences from the *Sorangium* strains were all located in the 16S rRNA methyltransferase group, forming a highly coherent subgroup of their own. It is presumed that the rRNA methyltransferase gene (*kmr*) sequences in *S. cellulosum* may evolve independently.

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