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ABSTRACT

β-Lactamases are the main cause of β-lactam resistance in many pathogenic bacteria. To elucidate the relationships of β-lactamases in soil-dwelling Gram-positive β-lactam-producing Actinobacteria and those in other bacteria, the molecular phylogenetics among β-lactamases in 19 Actinobacteria, whose genome sequences were completely clarified, were analyzed. The precise phylogenetic and amino acid alignment analyses revealed that 10 class A, 5 class B and 1 class D probable β-lactamases were present. A class D sequence detected in *Thermomonospora curvata* belonged to OXA-5-type β-lactamase, an enzyme detected in Gram-negative bacteria. Another interesting finding is that as many as 5　class B　β-lactamases were detected in this study and these class B β-lactamases were limited to subclass B3[8] like Alaskan soil[3]. β-Lactamase candidates picked up through the primary and secondary screenings were distributed randomly in Actinobacteria irrespective of their taxonomic position. In some species over 5 putative β-lactamases were detected, but in some others no β-lactamase was detectable. Thirty five putative class C β-lactamase candidates were identified by the preliminary phylogenetic analysis. Although these presumed β-lactamases were found to be probableβ-lactamases or acyltransferases, it should be taken care that a few nucleotide substitutions can convert them into active β-lactamases in the next generation.　It is intriguing in this connection that about 11kb transposon-like sequences including β-lactamases were encountered in *Nocardia farcinica* and *Saccharopolyspora erythraea*. This is the first report describing a transposon-like sequence including β-lactamase gene in Actinobacteria. Although this paper does not represent a comprehensive survey of β-lactamases in Actinobacteria, it will provide distinct characteristics of β-lactamases in Actinobacteria.

1. Introduction

β-Lactamases are the enzymes which hydrolyze the β-lactam antibiotics to produce antibacterially inactive compounds. Because of this, β-lactamases are responsible for β-lactam resistance in many pathogenic bacteria[57]. These enzymes, however, are also produced by nonpathogenic bacteria such as *Streptomyces*[45, 48], *Bacillus*[2] and the cyanobacteria[36, 73]. In addition, recent whole genome analyses expand the β-lactamase world to β-lactamase superfamily proteins[55], β-lactamase domain-containing proteins[15] and RNA-metabolising metallo-β-lactamases[18,74].

Consequently, β-lactamase -producing organisms were found to include a thermobacterium[67] (GenBank accession number: ADD27888), a green sulfur bacterium (GenBank accession number: ACF43212), a green non-sulfur bacterium (GenBank accession number: ABX07048), a methylotrophic bacterium[37] (GenBank accession number: ACT50532), a fungus[78] (GenBank accession number: CAA93219.1) and even a plant[38] (GenBank accession number: AAC49866) and mammals[22, 61] (GenBank accession numbers: EDL84248 and NP_116246). Furthermore, an alkalophilic and halotolerant Gram-positive bacterium *Oceanobacillus iheyensis* isolated from the bottom of the Pacific Ocean at a depth of 1050m[71] and *Pseudomonas fluorescens* isolated from a remote Antarctic coastal area also produced β-lactamases[43]. Moreover, screening of metagenomic libraries from soil bacterial communities for clones that confer antibiotic resistance gave new types of β-lactamases such as oligomeric[33] and bifunctional β-lactamases[3, 19]. A bifunctional β-lactamase was also isolated from a pathogen *Mycobacterium tuberculosis* H37Rv, an actinomycete[64]. Owing to its diverse microbial community, the soil is a large environmental reservoir of antibiotic resistance[16] and is called the antibiotic resistome[17, 58]. The human microflora is also the antibiotic resistance reservoir[62].

Streptomyces species are filamentous, soil-dwelling, Gram-positive bacteria and are characterized by their ability to undergo complex cellular differentiation like filamentous fungi[12]. In addition, *Streptomyces* species produce a wide variety of secondary metabolites including β-lactam antibiotics, the substrate of β-lactamases[39]. *Streptomyces* species are prokaryotic microorganisms and hence they must protect themselves from the attack of β-lactam antibiotics. The β-lactam antibiotic biosynthetic gene cluster contains a β-lactamase coding sequence in *Streptomyces clavuligerus*, a β-lactam producing *Streptomyces*[39, 54]. This is also the case with *Lysobacter lactamgenus*, another prokaryotic microorganism producing a β-lactam antibiotic[34]. Therefore, it is possible that β-lactamases in these bacteria are involved in self-resistance against β-lactam antibiotics. However, β-lactamases are produced abundantly in *Streptomyces* irrespective of their resistance against β-lactam antibiotics[45, 48, 50]. The structural change of their targets, penicillin-binding proteins is supposed to be the major mechanism of resistance against β-lactam antibiotics in *Streptomyces*[49, 52].

β-Lactamases are grouped into four classes on the basis of their amino acid sequences[4, 8, 31, 56]. This classification reflects more or less the substrate specificity: class A (penicillin-hydrolyzing), class C (cephalosporin-hydrolyzing) and class D (oxacillin-hydrolyzing) β-lactamases are active site serine enzymes and class B β-lactamases require one or two zinc ions for their activity and are called as metallo-β-lactamases[9, 23]. A previous phylogenetic study indicated that class A β-lactamases could be divided into three subgroups[47]. β-Lactamases are not essential bacterial proteins in themselves, but are speculated to have evolved from the essential penicillin-binding proteins in some β-lactam-producing bacteria such as *Streptomyces* or related bacteria, because these bacteria have to have some self-protective strategies against β-lactam antibiotics[46, 73]. Penicillin-binding proteins are involved in peptidoglycan biosynthesis in prokaryotic microorganisms. Therefore, it is interesting to know the relationships of β-lactam antibiotics, β-lactamases and penicillin-binding proteins in *Streptomyces*, β-lactam-producing microorganisms. In addition, the relationship of β-lactamases in *Streptomyces* and those in Actinobacteria should be clarified. In this paper, the phylogenetic relationships were analyzed among β-lactamases in *Streptomyces* and some of closely related Actinobacteria, whose genome sequences were wholly clarified.

2. Materials and methods

2. 1. Selection of candidate β-lactamases

Four *Streptomyces* and closely related 15 Actinobacteria listed in Table 1 were used for mining of β-lactamase genes in Genome Information Broker[24]. β-Lactamase genes were selected at three steps. From the database, putative β-lactamase genes in these bacteria were screened with keywords “lactamase” and “penicillin-binding protein” at the

Table 1. The list of Actinobacteria used in this paper.

Actinosynnema mirum DSM 43827
Frankia alni ACN14a
Frankia sp. CcI3
Mycobacterium bovis BCG str. Tokyo 172
Mycobacterium leprae TN
Mycobacterium tuberculosis H37Rv
Nocardia farcinica IFM 10152
Nocardioides sp. JS614
Propionibacterium acnes KPA171202
Rhodococcus erythropolis PR4
Saccharomonospora viridis DSM 43017
Saccharopolyspora erythraea NRRL 2338
Streptomyces avermitilis MA-4680
Streptomyces coelicolor A3(2)
Streptomyces griseus subsp. *griseus* NBRC 13350
Streptomyces scabiei 87.22
Streptosporangium roseum DSM 43021
Thermobispora bispora DSM 43833
Thermomonospora curvata DSM 43183

first step. For each species, the screened sequences were analyzed at the second step by aligning and constructing phylogenetic trees by using ClustalW2[69] as implemented by MEGA4[66] together with the representative sequences of β-lactamases (Table 1 of Supplement) with the default parameters. On the basis of the phylogenetic trees and the alignments thus obtained, candidate sequences were tentatively chosen and classified into class A, class B, class C and class D β-lactamases. Thereafter, the statistical significance of the classification was verified by pair-wise aligning a selected candidate and the representative β-lactamases in either class with the default parameters (Pearson). When the E-value was over 1.0, the candidate sequence was excluded from further analysis.

2. 2. Phylogenetic analyses

For the final selection or at the third step, phylogenetic analyses were used. For phylogenetic analyses, the amino acid sequences were aligned using ClustalX 2.0[68] and MUSCLE version 3.8[20] with default parameters. Ambiguous regions were automatically trimmed according to Gblocks[11, 65] and concatenated alignments were constructed. Phylogenetic analyses were performed by the neighbor-joining method with the bootstrap tree option using the MEGA4 program and allowing for 1000 bootstrap iterations[66], the maximum likelihood method of the PhyML program package with 100 bootstrap iterations[28] and Baysian analyses in MrBayes 3.1[59]. As an outgroup, a class C β-lactamase (an *Escherichia coli* K-12 chromosomal enzyme, U14003) was used for class A and D trees and a class A β-lactamase (a *Streptomyces cacaoi* enzyme, D90201) was used for class C tree. A glyoxylase II (GLO2_ECOLI), a member of metallo-β-lactamase superfamily, was used as an outgroup for class B tree. For maximum likelihood (PhyML) and Bayesian analyses, the best fit amino acid substitution models for each gene were selected based on Akaike Information Criteria (AIC) using ProtTest[1].

3. Results

3. 1. General feature

As a result of secondary screening, 59 sequences in total were tentatively selected as candidates for further analysis among 529 putative β-lactamase and penicillin-binding protein sequences in 19 species (Table 2). They were 18 class A, 5 class B, 35 class C and 1 class D β-lactamase candidates. For example, 19 putative β-lactamase and 24 putative penicillin-binding proteins were picked up at the first selection in *Streptomyces coelicolor* A3(2). However only 2 (Sco0088 and Sco3774) were screened as class A and 2 (Sco0830 and Sco3912) were as class C β-lactamase candidates by pair-wise alignment. There were some characteristic features in the 59 selected candidate sequences.

First, β-lactamase candidates were not distributed uniformly at least in the strains analyzed in this study: while *Saccharomonospora viridis*, *Streptomyces avermitilis*, *Streptomyces scabiei* and *Streptosporangium roseum* possessed as many as 5, 12, 5 and 5 candidate genes, respectively, *Mycobacterium leprae*, *Nocardioides* sp. JS614 and *Rhodococcus erythropolis* had no candidate gene. In general, *Streptomyces* species produced β-lactamase candidates at higher rates. However, the numbers of candidates were not necessarily related to the taxonomic classification. For

example, although *Streptosporangium* and *Nocardioides* were closely related taxonomically[63], the numbers of β-lactamase candidates in *Streptosporangium* were 5 but those in *Nocardioides* were zero. Even in the same genus, *Mycobacterium tuberculosis* produced 4 but *M. leprae* produced none. β-Lactamases detected by their enzymatic activity also distributed independently of actinobacterial taxonomy[50]. These results indicate strongly that β-lactamases evolve and are produced independent of actinobacterial taxonomy.

Second, most of the β-lactamases detected so far by their enzymatic activity in Gram-positive bacteria belong to class A and a few belong to class B. However, this was not the case with whole genomic level. In this study, twice as many as class C β-lactamase candidates than class A were detected. The similar results were obtained with *Bacillus subtilis* and *Bacillus cereus* (unpublished results).

Third, the nucleotide sequence and hence the amino acid sequence of *Mycobacterium tuberculosis* Rv2068c were completely identical with that of *Mycobacterium bovis* JTY2081, indicating that a horizontal gene transfer occurred between these two species very recently. Thus, *Mycobacterium bovis* JTY2081 β-lactamase was precluded from further analyses.

Fourth, it is intriguing that *Thermomonospora curvata*, a high G+C Gram-positive soil thermophilic bacterium, produced two class B and one class D β-lactamase candidates but no class A and class C enzyme. Usually, Gram-positive bacteria produce class A and class B β-lactamases, but not class C and class D. However, this was not due to the fact that *T.curvata* is thermophile because another thermophilic Actinomycete, *Thermobispora bispora* DSM43833 produced two class C but no class D β-lactamase candidate. Hereafter, 59 putative β-lactamases in these groups are analyzed in more detail at the third step.

Table 2. The tentative classification of putative ß-lactamase genes.

Bacteria	Prefix	Class A	Class B	Class C	Class D
Actinosynnema mirum DSM 43827	Amir	2178*, 3962	none**	3080, 4262	none
Frankia alni ACN14a	Fraal	none	none	6499	none
Frankia sp. CcI3	Francci3	none	none	0661	none
Mycobacterium bovis BCG str. Tokyo 172	JTY	2081	none	17443	none
Mycobacterium leprae TN	ML	none	none	none	none
Mycobacterium tuberculosis H37Rv	Rv	2068	1913	0907, 1923	none
Nocardia farcinica IFM 10152	NFA	0820, 23080	none	none	none
Nocardioides sp. JS614	Noca	none	none	none	none
Propionibacterium acnes KPA171202	Ppa	none	0914	none	none
Rhodococcus erythropolis PR4	Rer	none	none	none	none
Saccharomonospora viridis DSM 43017	Svir	none	none	15330, 16190, 23940, 33850, 35970	none
Saccharopolyspora erythraea NRRL 2338	Sace	0046, 1374, 4283	none	4808	none
Streptomyces avermitilis MA-4680	SAV	4452	none	1261, 1304, 1783, 2663, 3611, 4283, 4455, 5004, 5764, 5791, 5918	none
Streptomyces coelicolor A3(2)	Sco	0088, 3774	none	0830, 3912	none
Streptomyces griseus subsp. *griseus* NBRC 13350	SGR	0457	4992	3563, 3667	none
Streptomyces scabiei 87.22	SCAB	38731	none	26931, 46171, 52151, 55861	none
Streptosporangium roseum DSM 43021	Sros	1443, 5574, 5706, 5713	none	2550	none
Thermobispora bispora DSM 43833	Tbis	none	none	0343, 1664	none
Thermomonospora curvata DSM 43183	Tcur	none	2765, 4662	none	2040

* The numbers in the table are the names of the genes in each bacterium. The prefixes for A. mirum, F. alni, Frankia sp. , M. bovis, M. leprae, M. tuberculosis, N. farcinica, Nocardioides sp., P. acnes, R. erythropolis, S. viridis, S. erythraea, S. avermitilis, S. coelicolor, S. griseus, S. scabiei, S. roseum and T. curvata are Amir, Fraal, Francci3, JTY, ML, Rv, NFA, Noca, Ppa, Rer, Svir, Sace, SAV, Sco, SGR, SCAB, Sros and Tcur, respectively.

** Not detectable.

3. 2. Class A + class D group

A phylogenetic tree was constructed with 18 selected sequences (17 class A excluding *M. bovis* JTY2081 and 1 class D) together with 6 class A (*Streptomyces cacaoi* D90201, *Streptomyces cellulosae* D12653, *Streptomyces*

albus M28303, *Streptomyces clavurigerus* Z54190, *Staphylococcus aureus* M15526 and *Mycobacterium fortuitus* L25634) and one class D enzymes (*Pseudomonas aeruginosa* Z22590) and the result is shown in Fig. 1. These sequences were divided roughly into three groups (A1, A2 and A3 in Fig. 1). Tcur2040, Sace0046 and NFA0820 made one group together with Z22590 (OXA-10). The amino acid alignment of these sequences together with Z22590 (OXA-10) and M25261 (OXA-2) showed that although Sace0046 and NFA0820 carried some of the signature amino acid residues of Class D β-lactamases[75] ($S^{67}TFK^{70}$ and $K^{205}TG^{207}$ in Table 3 and Fig. 1 of supplement), they had no other conserved residues such as $Y^{142}G$, W^{154}, $W^{221}XXG$ corresponding to box IV, box A and box VI of Joris *et al*.[32], respectively. Furthermore, neither of them carried conserved residues of class A β-lactamases such as $S^{130}DN$, $W^{165}EP$ and D^{179} and many extra amino acid sequences were inserted into the sequences. Molecular distance analysis by using FASTA Sequence Comparison at the University of Virginia (Pearson) confirmed that neither NFA0820 nor Sace0046 belonged to any of the β-lactamase classes. Moreover, Blast analysis indicates that these two sequences were closely related to 3LO7_A (penicillin binding protein A of *Mycobacterium tuberculosis*[21]) in Molecular Modeling Database (MMDB[77]) of the National Center for Biotechnology Information not only in their amino acid sequence but also in their three dimensional levels. Thus, Sace0046 and NFA0820 were probable acyltransferases. On the other hand, Tcur2040 carried two other signature sequences (S^{115} XV^{117} and $Y^{142}GX$) of class D β-lactamases. In addition, statistical significance scores from shuffles (Pearson) of an alignment by aligning Tcur2040 and class D β-lactamases of OXA-10 (2WGW_B, 1E4D_C or 2X01_A) were less than 4.9e-42, substantiating that Tcur2040 is closely related with 2WGW_B, 1E4D_C or 2X01_A not only in their amino acid sequence but also in their three dimensional levels. These sequences were retrieved in MMDB[77] with Tcur2040 as a query. It is concluded, therefore, that only Tcur2040 belonged to the class D β-lactamase group among three candidates. At the present time, most of the class D β-lactamases are detected in Gram-negative bacteria and only a few have been reported in Gram-positive bacteria[6]. Thus, it is intriguing to know to which group Tcur2040 enzyme belongs, Gram-positive or Gram-negative class D β-lactamases. Phylogenetic analysis (Fig. 2) clearly showed that Tcur2040 was more closely related to Gram-negative class D β-lactamases, especially to OXA-5 and OXA-27 type β-lactamases. Molecular distance analysis by MEGA4 and FASTA (Pearson) again confirmed this suggestion.

Table 3. The list of active site residues discussed in this paper.

Class A: $S^{70}XXK^{70}$, $S^{130}DN^{132}$, $R^{164}W/YE^{166}$, D^{179}, $K^{234}T/SG^{236}$
Class B: $H^{116}XHXD^{120}$, G^{123}, H^{196}, H^{263}
Class C: $S^{64}XXK^{67}$, $Y^{150}XN^{152}$, $K^{315}TG^{317}S/A^{318}$
Class D: $S^{67}TFK^{70}$, $S^{115}XV$, $Y^{142}G$, W^{154}, $K^{205}TG$, $W^{221}XXG$

Sco0088, Sros5713, Sco3774 and Sros1443 formed one group (A2 in Fig. 1) and Sros5574 was an isolated gene. The amino acid alignment showed that these sequences possessed signature amino acid residues of class A β-lactamases ($S^{70}XXK^{73}$, $S^{130}DN^{132}$ and $K^{234}T/SG^{236}$ in Fig. 2 of supplement). However, all the molecular distances analyzed by MEGA4 and FASTA Sequence Comparison at the University of Virginia (Pearson) were above 0.8 and 0.001 between a class A β-lactamase such as D90201 and the one of the above sequences (Sco0088, Sros5713, Sco3774, Sros1443 or Sros5574), respectively. In addition, except for Sros5574 they had E^{166}, another conserved residue, and a conserved basic residue (arginine in this case) in the omega loop region of residue 164 to 179 of class A β-lactamases[41]. Thus they may be probable β-lactamases. In fact, Blast analysis by using MMDB indicated that Sco0088, Sros5713, Sco3774 and Sros1443 were more related to BlaB protein (2WUQ_B), a regulatory protein from *Streptomyces cacaoi*[72] in their three dimensional level, and Sros5574 was only related to β-lactamase-like proteins from Actinobacteria such as *Catenulispora acidiphila* (YP_03117446.1), *Mycobacterium smegmatis* (YP_890415.1) and *Leifsonia xyli* (YP_061292.1).

The third of class A + class D group consisted of 11 members including *Mycobacterium bovis* JTY2081 (A3 in Fig. 1). The phylogenetic (Fig. 3) and molecular distance analyses indicate that all of them and the representative sequences were closely related each other and belonged to typical class A β-lactamases. The amino acid alignment shown in Fig. 3 of supplement indicates that these sequences conserved most of the amino acid residues ($S^{70}XXK^{73}$, $S^{130}DN^{132}$, $R^{164}W/YE^{166}$, D^{179}, $K^{234}T/SG^{236}$) involved in catalytic reactions with the exception of Amir2178 and a basic residue in the omega loop region of residue 164 to 179 of class A β-lactamases[41]. In Amir2178, lysine at 73, serine at 130, asparagine at 132 and threonine at 235 were replaced by serine, aspartic acid, serine, and proline, respectively. Therefore, Amir2178 may be a probable acyltransferase, even though its amino acid sequence and three-dimensional structure closely resembles 1YLT_A, Ctx-M β-lactamase[13]. More careful scrutiny of the phylogenetic and molecular distance analyses indicated that Sros5706 was closely related to Gram-negative carbapenem-hydrolyzing β-lactamases such as *Serratia marcescens* SME-1 (U60295) and *Klebsiella pneumoniae* KPC-2 (AAK70220) β-lactamases, and cephalosporin-hydrolyzing β-lactamases such as *Serratia marcescens* GES-16 (ADJ94120) and *Bacteroides fragilis* CepA (AAA21538) than to Gram-positive enzymes (Fig. 3). Other 8 sequences formed a cluster with those from *Streptomyces* such as *S. cellulosae* (D12653), *S. albus* (M28303), *S. clavuligerus* (Z54190) and *S. cacaoi* (D90201). It is concluded therefore that the class A β-lactamases in Actinobacteria consisted of a mixture of Gram-negative carbapenem-hydrolyzing and Gram-positive type enzymes.

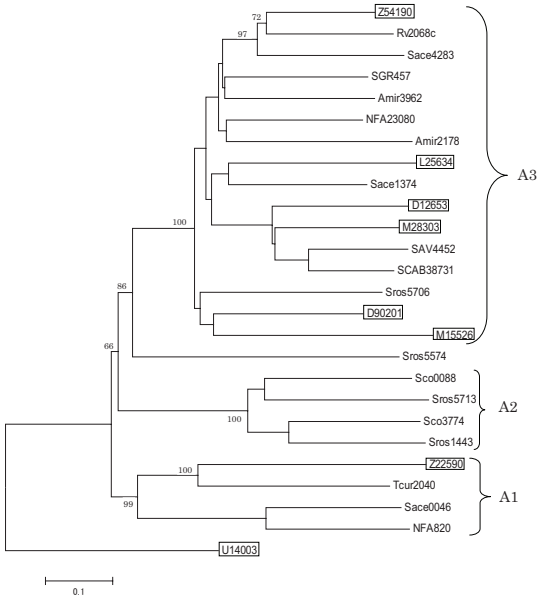


Fig. 1. Phylogenetic tree based on amino acid sequences of 18 putative and 6 representative class A and 1 class D (Z22590) β-lactamases (square boxed) constructed by MEGA4[66] and the neighbor-joining program with the default parameters. Branch lengths were drawn to scale and were proportional to the numbers of amino acid changes. Bootstrap values (%) calculated from 1,000 replicates were shown at the nodes. A class C β-lactamase (U14003) was used as an outgroup. The representative β-lactamases are as shown in Table 1 of supplement.

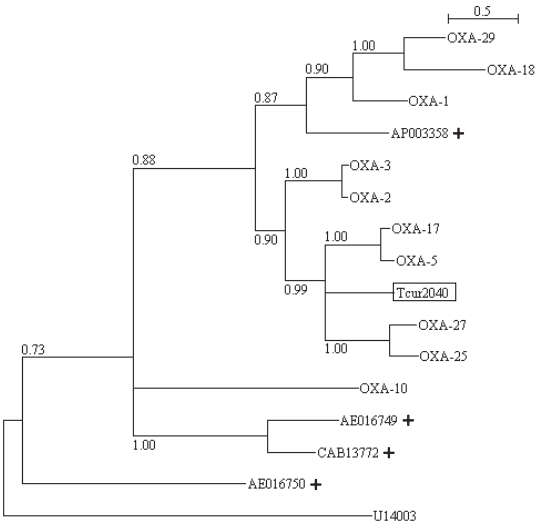


Fig. 2. Bayesian evolutionary tree of aligned amino acid sequences of Tcur2040 (square boxed) and 14 representative class D β-lactamases. Bayesian posterior probabilities were shown at the nodes. The representative β-lactamases from Gram-negative bacteria (accession numbers are indicated in parentheses) are OXA-1 (AJ238349), OXA-2 (M25261), OXA-3 (L07945), OXA-5 (X58272), OXA-10 (U37105), OXA-17 (AF060206), OXA-18 (U85514), OXA-25 (AF201826), OXA-27 (AF201828) and OXA-29 (AJ400619), and those from Gram-positive bacteria (bacterial names were indicated in the parentheses) were AP003358 (*Staphylococcus aureus*), AE016749 (*Staphylococcus epidermis*), CAB13772 (*Bacillus subtilis*) and AE016750 (*Staphylococcus epidermis*). β-Lactamases from Gram-positive bacteria were marked with "+". A class C β-lactamase (U14003) was used as an outgroup.

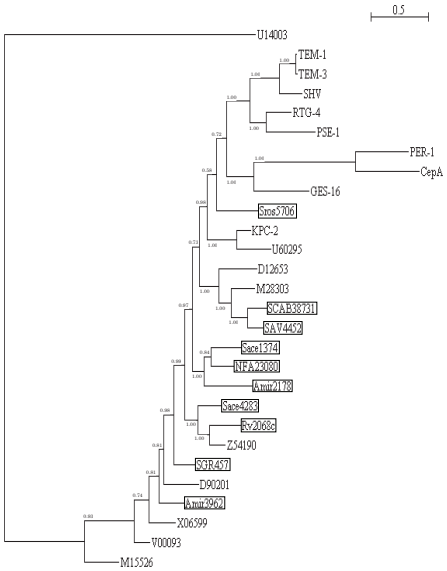


Fig. 3. Bayesian evolutionary tree of aligned amino acid sequences of 10 putative (square boxed) and 18 representative class A β-lactamases. Bayesian posterior probabilities were shown at the nodes. The representative β-lactamases from Gram-negative bacteria (accession numbers were indicated in parentheses) were TEM-1 (EU491958), TEM-3 (CAA45828), SHV (M59181), RTG-4 (ACJ61335), PSE-1 (ACL31204), PER-1 (AAW62294), CepA (AAA21538), GES-16 (ADJ94120), SME-1 (U60295) and KPC-2 (AAK70220) and those from Gram-positive bacteria (bacterial names are indicated in parentheses) are D12653 (*Streptomyces cellulosae*), M28303 (*Streptomyces albus*), Z54190 (*Streptomyces clavuligerus*), D90201 (*Streptomyces cacaoi*), X06599 (*Bacillus cereus*), V00093 (*Bacillus licheniformis*) and M15526 (*Staphylococcus aureus*). A class C β-lactamase (U14003) was used as an outgroup.

3. 3. Class B metallo-β-lactamase group

Class B β-lactamases were composed of five members: Rv1913, Ppa0914, SGR4992, Tcur2765 and Tcur4662 (Table 2). Amino acid alignment of these sequences together with three representative sequences disclosed that these sequences contained the essential sequence of $H^{116}XHXD^{120}$, G^{123} and two other histidine residues (His^{196} and His^{263}) proposed to be involved in the coordination of zinc atom[26, 76] (Fig. 4 of supplement). Molecular distance analysis confirmed that these proteins belonged to Class B metallo-β-lactamases. A phylogenetic tree constructed by using MrBayes method showed that SGR4992, Rv1913, Tcur4662 and Tcur2765 formed a distinctive cluster different from that of *Aeromonas* CAA40386, *Pseudomonas* AJ492820, *Serratia* P52699, *Bacillus* M15350, *Bacillus* AAA22276, *Bacteroides* AAA22904 and *Bacteroides* M63556 (Fig. 4). However, Ppa0914 was an isolated gene. A phylogenetic tree constructed by using PhyML program gave an essentially similar topology. BLAST search with SGR4992 and Rv1913 as query sequences revealed that these were more nearly related to metallo-β-lactamases from Actinobacteria such as *Streptomyces*, *Mycobacterium*, *Nocardioides, Amycolatopsis*, *Stackebrandtia*, *Propionibacterium, Streptosporangium* and *Saccharopolyspora*, but not to those from *Bacillus*, *Bacteroides*, *Pseudomonas*, *Serratia* and *Aeromonas*. Tcur4662 and Tcur2765 were highly related to metallo-β-lactamases from Actinobacteria such as *Frankia*, *Catenulispora*, *Streptosporangium*, *Amycolatopsis* and *Nocardiopsis* as well as polyketide cyclases from *Streptomyces*[7] and *Sulfolobus* (NP_343860). On the other hand, Ppa0914 was closely related to metallo-β-lactamases from *Mycobacterium*, *Streptomyces*, *Saccharomonospora* and *Saccharopolyspora*.

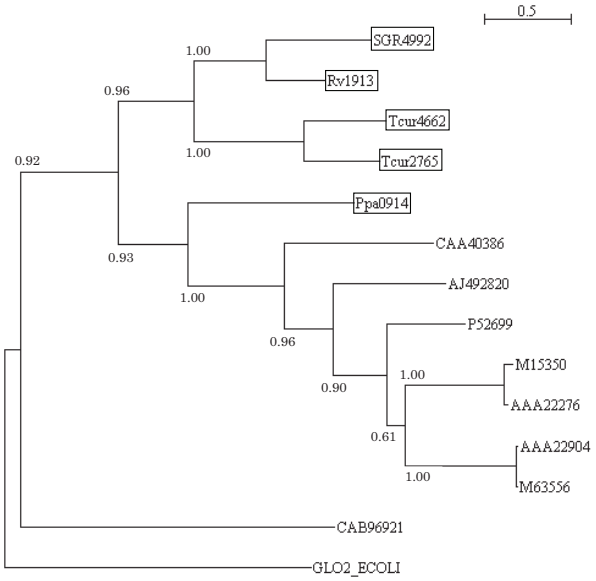


Fig. 4. Bayesian evolutionary tree of aligned amino acid sequences of 5 putative (square boxed) and 8 representative class B β-lactamases. Bayesian posterior probabilities were shown at the nodes. A glyoxylase II (GLO2_ECOLI) was used as an outgroup. The representative β-lactamases are as shown in Table 1 of supplement.

3. 4. Class C group

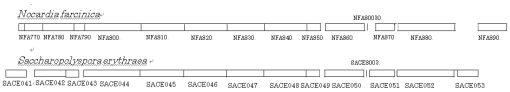
Comparative amino acid alignment and phylogenetic analyses of 35 sequences tentatively classified to Class C β-lactamases (Table 2) and 8 representative enzymes (*Escherichia coli* U14003, *Citrobacter freundii* AAM93471, *Enterobacter cloacae* P05364, *Morganella morganii* AAC68582, *Serratia marcescens* X52964, *Lysobacter lactamgenus* CAA39987, *Mycobacterium smegmatis* AY442183 and *Laribacter hongkongensis* AAT46346) indicated that these sequences were grouped roughly into 3 clusters (C1, C2 and C3 in Fig. 5 of supplement). The first cluster included fifteen sequences: Sco3912, SAV4283, SCAB46171, SGR3667, Svir33850, Fraal6499, SAV2663, SAV4455, SAV5004, Svir23940, Tbis0343, SCAV26931, Amir4262, JTY17443 and SCAB55861 (C1 in Fig. 5 of supplement). The amino acid alignment in Fig. 6 of supplement shows that these sequences carried active site Ser^{64}, Lys^{67} and Tyr^{150} (References[30, 40]). However, other residues involved in the catalytic reaction like Lys^{315}, Thr^{316}, Gly^{317} and Ser/Ala^{318} were missing, suggesting that these are probable β-lactamases. However, a close inspection of the amino acid sequence alignment in Fig. 6 of supplement revealed that in these sequences there was a conserved H/KxG motif about 25 residues before KTG motif of AAM93471, which was detected in acyltransferases of the SxxK superfamily of diverse functions like endopeptidase of *Bacillus cereus* and D-aminopeptidase of *Ochrobactrum anthropi*[27]. So these may be probable D-aminopeptidases but not β-lactamases. The second cluster contained 5 sequences: SAV5764, SCAB52151, Sros2550, Sco0830 and Sace4808 (C2 in Fig. 5 of supplement). In these sequences the active site amino acid residues Lys^{315}, Thr^{316}, Gly^{317} and Ser/Ala^{318} and the conserved H/KxG motif were also missing. The third cluster consisted of 10 members: SAV1304, SAV3611, Svir16190, Rv1923, Rv0907, SAV5791, Fracci3_0661, Svir15330, SGR3563, SAV1783, Tbis1664, Svir35970, SAV1261 and SAV5918 (C3 in Fig. 5 of supplement). These sequences again contained active site Ser^{64}, Lys^{67}, Tyr^{150} and Asn^{152}, but the active site residues Lys^{315}, Thr^{316} and Gly^{317} and the conserved H/KxG motif were missing. In addition to these residues, Lys^{67} was also absent in Amir3080. The important defect of these sequences tentatively classified as Class C was that all of them lacked commonly the conserved amino acids of Lys^{315}, Thr^{316} and Gly^{317}, which are involved in the catalytic reaction[30, 40].

Instead of this, C1 group in Fig. 5 of supplement had a conserved H/KxG motif detected in D-aminopeptidases. In accord with this fact, 8 representative class C β-lactamases formed a distinct cluster from that of the sequences from Actinobacteria (Fig.5 of supplement), although these representative class C enzymes were selected from various sub-groups[9, 30]. Therefore, these sequences tentatively classified as class C β-lactamases are considered to be probable or reserved β-lactamases or acyltransferases for the next generation.

3. 5. Genomic organization

A comparative analysis of *Nocardia farcinica* and *Saccharopolyspora erythraea* disclosed that the genomic organizations of the β-lactamase genes (NFA0820/Sace0046) and the nearby regions were arranged in very similar orders even though these bacteria were not so closely related taxonomically[63]: two protein kinases, NFA0820 or Sace0046, a cell division protein, a protein phosphatase, two hypothetical proteins and tRNA (Fig. 5). In addition, amino acid identities and similarities of these corresponding proteins from *Nocardia farcinica*[29] and *Saccharopolyspora erythraea*[51] were over 50 and 75%, respectively. However, the genomic organization upstream of a reductase (NFA0780) and a transposase (Sace0042) and a hypothetical protein (NFA0790 or Sace0043) and that downstream of a transcriptional regulator (NFA0870) and a phosphatase (Sace0051) differed completely, indicating that about 11kb DNA fragment was inserted in this region by transposition or other mechanisms. A reductase (NFA0780) and a transposase (Sace0042), and a transcriptional regulator (NFA0870) and a phosphatase (Sace0051) were located about 4,000bp upstream and about 7,000bp downstream of the β-lactamase (NFA0820/Sace0046) gene in each bacterium, respectively (Fig. 5). It is interesting that two transposases were present in *Saccharopolyspora* but not in *Nocardia*, indicating that these transposases may function in gene transfer between these Actinobacteria.

(A)



(B)

Function	Nocarida farcinica		Saccharopolyspora erythreae		Homology
	Gene name	Gene size (bp)	Gene name	Gene size (bp)	
pabA/transposase	NFA770	645	SACE041	807	over
reductase/transposase	NFA780	1222	SACE042	1380	over
hypothetical protein	NFA790	471	SACE043	357	over
protein kinase	NFA800	1908	SACE044	1962	2.00E-85
protein kinase	NFA810	1524	SACE045	1310	4.50E-40
β-lactamase	NFA820	1467	SACE046	1452	4.30E-66
cell division protein	NFA830	1479	SACE047	1437	1.20E-68
protein phosphatase	NFA840	1530	SACE048	1419	5.80E-54
hypothetical protein	NFA850	468	SACE049	465	1.90E-38
hypothetical protein	NFA860	1431	SACE050	1314	2.80E-19
tRNA-Leu	NFA80030	86	SACE8003	83	1.60E-04
regulator/phosphatase	NFA870	633	SACE051	1002	over
hypothetical protein	NFA880	2163	SACE052	1542	over

Fig. 5. Genomic organizations of the β-lactamase genes and nearby regions. (A) Gene arrangements of 14 genes nearby β-lactamase genes in *Nocardia farcinica* and *Saccharopolyspora erythreae*, and (B) function, gene name, gene size and homology between the corresponding genes. Homology was calculated according to the method of Pearson. "over" means that the homology was not detected between the two genes with the default parameters.

Depending on the binding to blue-dextran, the β-lactamases of *Streptomyces* can be divided into two groups[35, 48]. *S. cacaoi* (blue-dextran nonbinding) group and *S. cellulosae*/*S. fradiae* (blue-dextran-binding) group. This reflects somehow the genomic organization of the β-lactamase gene and the adjacent region. The genomic arrangement of *Actinosynnema mirum* Amir3962 was very similar to that of *Streptomyces cacaoi* β-lactamase[72]: Amir3960/BlaB, Amir3961/BlaA and Amir 3962/β-lactamase. In addition, the percentage identity and similarity at their amino acid sequence level were 62.8 and 82.6%, 57.9 and 81.4%, and 47.1 and 68.1%, respectively. BlaA and BlaB are transcriptional regulator or activator proteins of *S. cacaoi* β-lactamase. This may be also a case of the gene transfer between these species. No other β-lactamase detected in this study belonged to this class. However, near a β-lactamase in *N. farcinica* (NFA23080) and that of *S. avermitilis* (Sace1374), there was a transcriptional regulator whose sequence had some similarity with BlaA. Moreover, a MerR family transcriptional regulator and a putative β-lactamase was present near a *S. avermitilis* β-lactamase (SAV4452). In *S. roseum*, three

β-lactamase-like sequences (Sros5713, Sros5714 and Sros5715) existed in tandem together with an upstream transcriptional regulator (Sros5712). Sros5713 was shown to be very similar to BlaB in the three dimensional level, as described before.

4. Discussion

In the Genome Information Broker[24], 529 sequences are deposited as putative β-lactamases and penicillin-binding proteins in 4 *Streptomyces* and 15 closely related Actinobacteria. On the basis of primary screening using the phylogenetic trees and the amino acid alignments, 59 sequences were selected as β-lactamase candidates for further analysis. However, through more dense and cautious examination the numbers of β-lactamases were restricted to 10 class A, 5 class B and one class D enzymes. No candidate remained as class C β-lactamase. The fact that most of the β-lactamases detected in this study belonged to class A is in accord with the result of enzymatic study[50]. However, in contrast to the result on the basis of their enzymatic activity, the preliminary phylogenetic analysis and/or the secondary screening noticed twice as many as class C than class A β-lactamase candidates in whole genomic level (Table 2). This result may be accounted for by the fact that most of the presumed class C β-lactamase candidates are considered to be enzymatically inactive probable β-lactamases or acyltransferases. However, because only a few nucleotide substitution can convert them into active β-lactamases, strategies must be prepared for protecting us against these next generation β-lactamases. In addition, considering that β-lactam-interacting proteins including β-lactamases are very flexible to adapt to carry out their catalytic reactions[27, 43, 62, 70], it is possible that some of these class C β-lactamase candidates catalyze the hydrolysis of β-lactams.

It is interesting that the amino acid sequence and the three-dimensional structure of Amir2178 in class A was very closely related to 1YLT_A, Ctx-M (TEM-3-type) β-lactamase, although active site lysine73, serine130, asparagine132 and threonine235 were replaced by serine, aspartic acid, serine and proline, respectively. So Amir2178 maybe a probable β-lactamase or acyltransferase. Nine other class A sequences formed a cluster with β-lactamases from Gram-positive bacteria except Sros5706. Sros5706 falled into the cluster of carbapenem-hydrolyzing β-lactamases from Gram-negative bacteria in the phylogenetic tree (Fig. 3). Similar topology was obtained by PhyML program. Blast analysis indicates that another sequence SAV4452 in class A was narrowly related to 2OV5_A (KPC-2 carbapenemase).

It should be pointed out here that a Gram-negative OXA-5 and OXA-27 type class D sequence of Tcur2040 was found in a thermophilic soil bacterium, *Thermomonospora curvata* (Fig.2). *T. curvata* produced two class B and one class D β-lactamases but no class A and class C enzymes. This is very peculiar in Gram-positive bacteria, because most of β-lactamases produced in Gram-positive bacteria belong to class A and a few class B β-lactamases. Another intriguing thing is that as many as 5 class B sequences were detected in this study and, in addition, all of them belonged to one (B3) of the three subgroups[8] of known metallo-β-lactamases (Fig. 4). A similar situation, that is, the presence of a relatively large number of subgroup B3 metallo-β-lactamases, was reported in remote Alaskan soil bacteria[3].

Other interesting features emerged in this study are: First, the nucleotide sequence and hence the amino acid sequence of *Mycobacterium tuberculosis* Rv2068c[10] are completely identical with that of *Mycobacterium bovis* JTY2081[61]. Comparison of these two bacteria disclosed that the nucleotide sequences of at least 50kb segment including the β-lactamase and the adjacent vitamin B12 biosynthetic gene cluster were almost completely identical with each other, implying that a horizontal gene transfer occurred very recently. Probably, these two bacteria belong to the same species, because the nucleotide sequences of 16S rRNAs were completely identical with each other. In this connection, it is intriguing that comparison of the nucleotide and the amino acid sequences of putative *Bacillus cer-*

*eus*β-lactamases BCA2625 and BCA3308 revealed that 97% of the amino acid sequences were identical with each other, suggesting that the gene duplication occurred very recently in this bacterium (unpublished data). These two genes were separated by about 650kb. Thus, it is supposed that a horizontal gene transfer and a gene duplication are not rare events in natural environment, especially in the soil.

Blast analysis in the National Center for Biotechnology Information indicated that NFA0820 and Sace0046 in A1 of Fig. 1 were narrowly related to penicillin-binding protein A of *M. tuberculosis*, and Sros5706, SAV4452 and Amir2178 in A3 of Fig. 1 were to extended type β-lactamases of Gram-negative bacteria like NMC-A, KPC-2, and CTX-M. It is concluded therefore that Actinobacteria, members of soil bacteria, have a potential to produce a various kind of β-lactamases including canonical class A, extended class A, class B, class C and class D β-lactamases and form a reservoir of these β-lactamases in the next generation.

No β-lactam biosynthesis and/or related gene was found out near the β-lactamase sequences analyzed so far, although thioesterases (Sace4285 and Sace4286) and a LD-carboxypeptidase (SGR456) involved in non-ribosomal peptide biosynthesis and peptidoglycan recycling existed immediately downstream of a β-lactamase (Sace7283) and upstream of a β-lactamase (SGR457), respectively. Therefore, the present analysis could not clarify the relationship between the biosynthesis of β-lactam antibiotics and that of β-lactamases in Actinobacteria.

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Supplement information

Table 1 of Supplement. The list of ß-lactamases used for the classification

Accession number	Origin	Class
AF297554	*Klebsiella pneumoniae*	A
AF395881	*Klebsiella pneumoniae*	A
D12653	*Streptomyces cellulosae*	A
D90201	*Streptomyces cacaoi*	A
L25634	*Mycobacterium fortuitus*	A
M15526	*Staphylococcus aureus*	A
M28303	*Streptomyces albus*	A
U60295	*Serratia marcescens*	A
V00093	*Bacillus licheniformis*	A
X06599	*Bacillus cereus*	A
Z54190	*Streptomyces clavuligerus*	A
AAA22276	*Bacillus cereus*	B1
AAA22904	*Bacteroides fragilis*	B1
AJ492820	*Pseudomonas aeruginosa*	B2
CAA40386	*Aeromonas hydrophila*	B2
CAB96921	*Fluoribacter gormanii*	B3
M15350	*Bacillus sp.*	B1
M63556	*Bacteroides fragilis*	B1
P52699	*Serratia marcescens*	B1
AAC68582	*Morganella morganii*	C
AAM93471	*Citrobacter freundii*	C
AAT46346	*Laribacter hongkongensis*	C
AY442183	*Mycobacterium smegmatis*	C
CAA39987	*Lysobacter lactamgenus*	C
P05364	*Enterobacter cloacae*	C
U14003	*Escherichia coli*	C
X52964	*Serratia marcescens*	C
Z22590	*Pseudomonas aeruginosa*	D
AP003358	*Staphylococcus aureus*	D
AE016749	*Staphylococcus epidermidis*	D
AE016750	*Staphylococcus epidermidis*	D
CAB13772	*Bacillus subtilis*	D

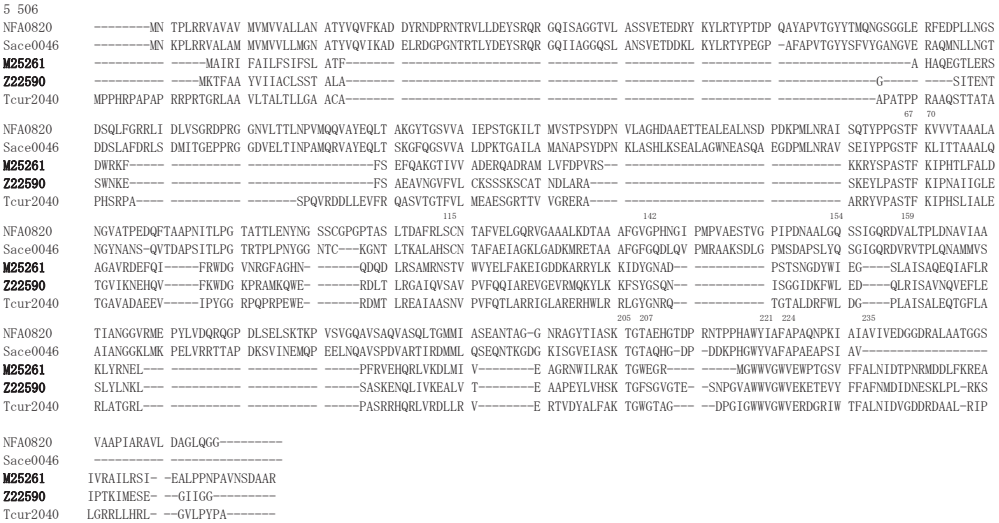

Fig. 1 of Supplement. Amino acid alignment of 3 putative and 2 representative (marked with bold letters) class D β-lactamases. The alignment was generated using the MUSCLE version 3.8[20]. The amino acid numbering was in accord with Ambler *et al.*[5].

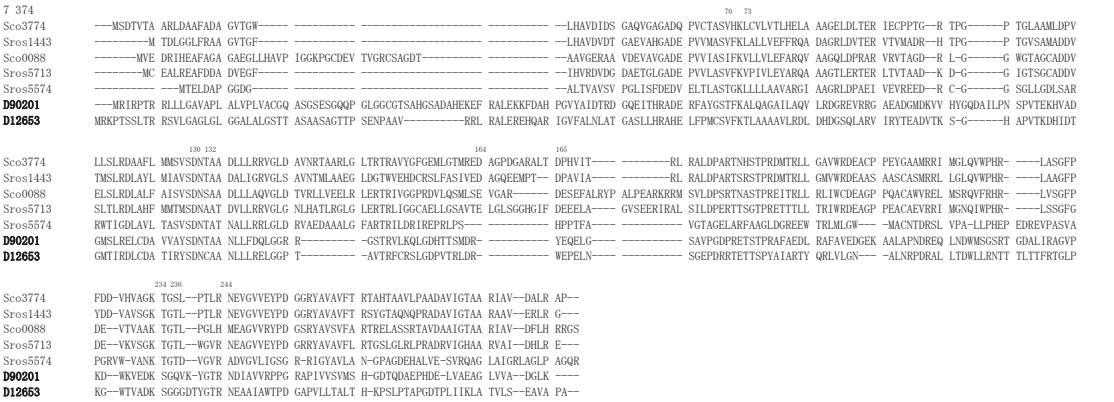

Fig. 2 of Supplement. Amino acid alignment of 5 putative and 2 representative (marked with bold letters) class A β-lactamases. The alignment was generated using the MUSCLE version 3.8[20]. The amino acid numbering was in accord with Ambler *et al.* [5].

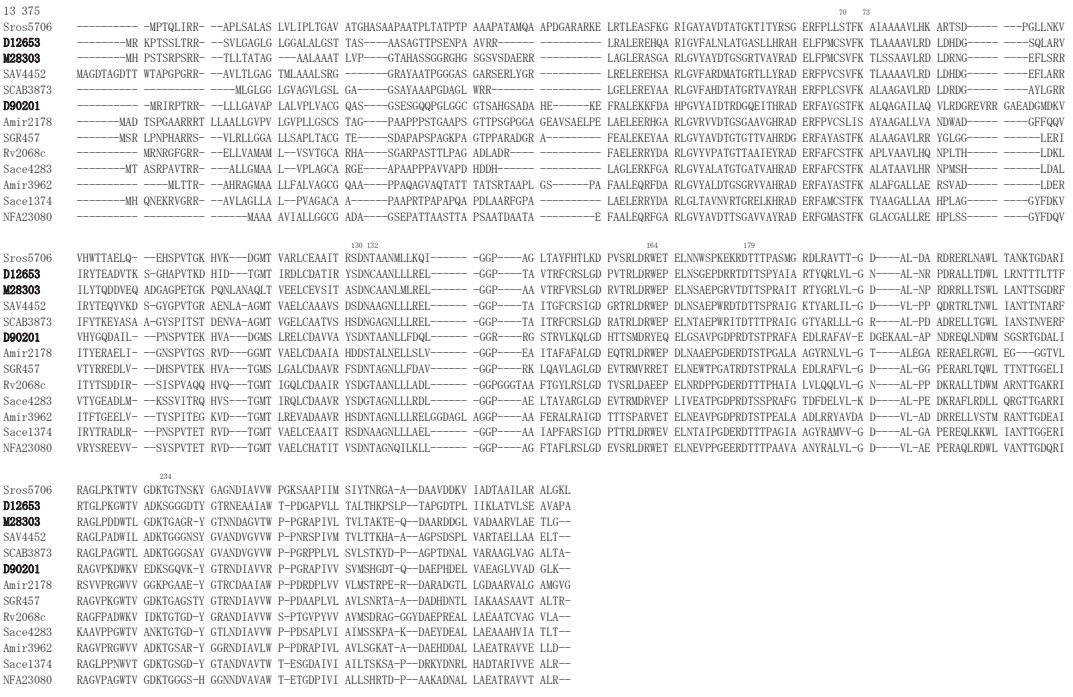

Fig. 3 of Supplement. Amino acid alignment of 10 putative and 3 representative (marked with bold letters) class A β-lactamases. The alignment was generated using the MUSCLE version 3.8[20]. The amino acid numbering was in accord with Ambler *et al.*[5].

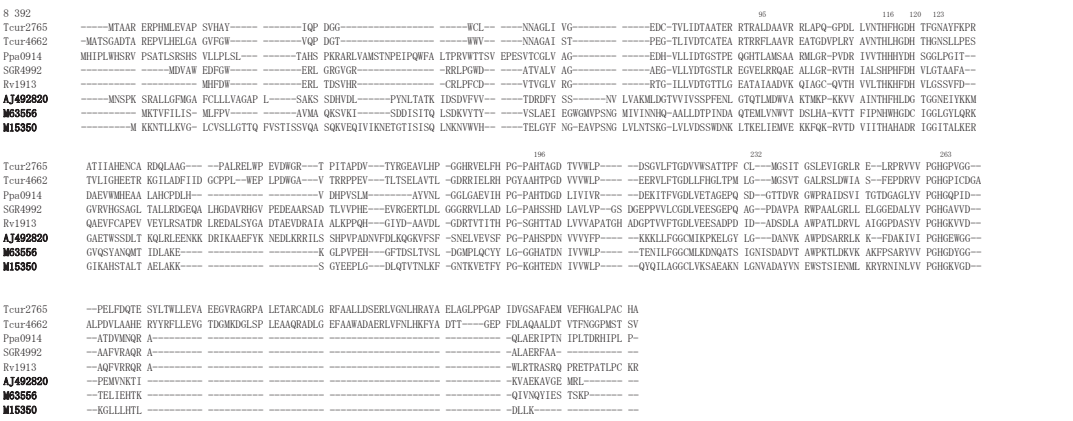

Fig. 4 of Supplement. Amino acid alignment of 5 putative and 3 representative (marked with bold letters) class B β-lactamases. The alignment is generated using the MUSCLE version 3.8[20] The amino acid numbering is in accord with Galleni *et al.*, [25].

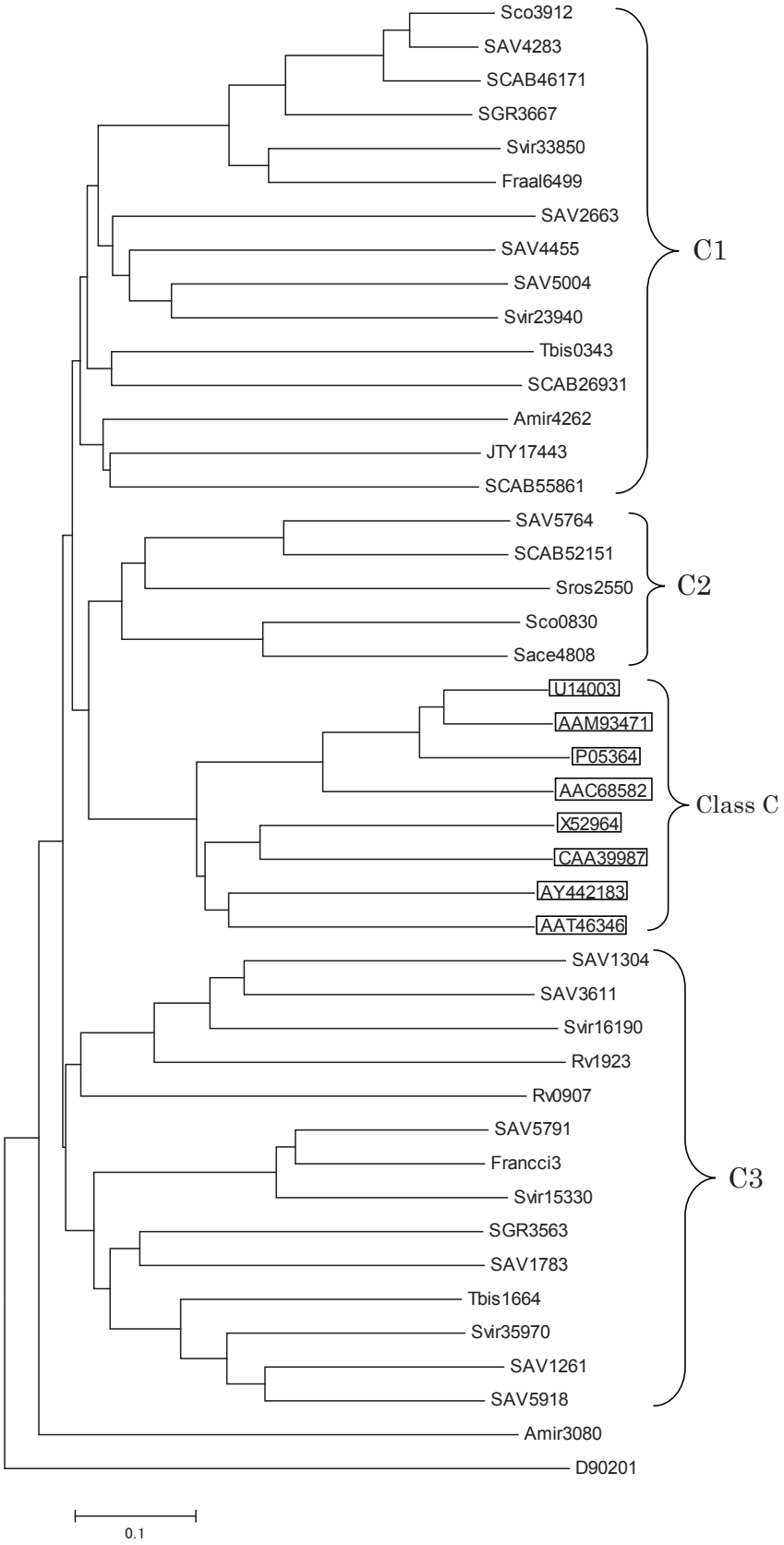

Fig. 5 of Supplement. Phylogenetic tree based on amino acid sequences of 35 putative and 8 representative class C β-lactamases (square boxed). The tree was constructed by MEGA version 4[66] and the neighbor-joining program with the default values. A class A β-lactamase (D90201) was used as an outgroup.

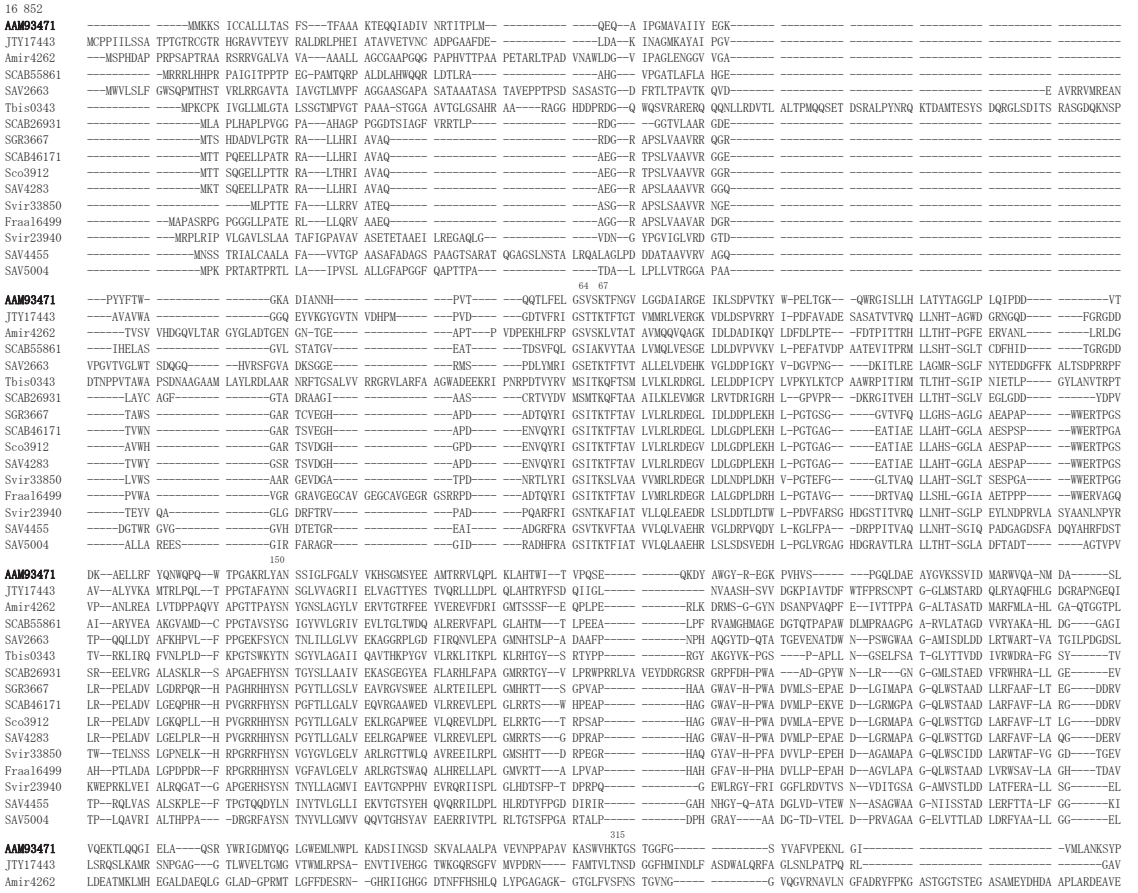

Fig. 6 of Supplement. Amino acid alignment of 15 putative and one representative (marked with bold letters) class C β-lactamases. The alignment was generated using the MUSCLE version 3.8[20]. The amino acid numbering was in accord with Lobkovsky *et al*, [40].