Genome Sequence of *Kitasatospora setae* NBRC 14216<sup>T</sup>: An Evolutionary Snapshot of the Family Streptomyctaceae

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Abstract

*Kitasatospora setae* NBRC 14216<sup>T</sup> (=KM-6054<sup>T</sup>) is known to produce setamycin (bafilomycin B1) possessing antitrichomonal activity. The genus *Kitasatospora* is morphologically similar to the genus *Streptomyces*, although they are distinguishable from each other on the basis of cell wall composition and the 16S rDNA sequence. We have determined the complete genome sequence of *K. setae* NBRC 14216<sup>T</sup> as the first *Streptomycetaceae* genome other than *Streptomyces*. The genome is a single linear chromosome of 8 783 278 bp with terminal inverted repeats of 127 148 bp, predicted to encode 7569 protein-coding genes, 9 rRNA operons, 1 tmRNA and 74 tRNA genes. Although these features resemble those of *Streptomyces*, genome-wide comparison of orthologous genes between *K. setae* and *Streptomyces* revealed smaller extent of synteny. Multilocus phylogenetic analysis based on amino acid sequences unequivocally placed *K. setae* outside the *Streptomyces* genus. Although many of the genes related to morphological differentiation identified in *Streptomyces* were highly conserved in *K. setae*, there were some differences such as the apparent absence of the AmfS (SapB) class of surfactant protein and differences in the copy number and variation of paralogous components involved in cell wall synthesis.

Key words: *Kitasatospora setae*; complete genome; *Streptomyces*
1. Introduction

Among many filamentous bacteria belonging to the phylum Actinobacteria, Streptomyces species have been extensively studied because of the ability to produce various bioactive secondary metabolites and the complex process of morphological differentiation. The life cycle of Streptomyces is initiated by the germination of spores that develop into vegetative mycelia. In response to environmental signals, aerial mycelia emerge from the colony surface and differentiate into chains of spores. Many pioneering works about the regulation of secondary metabolism and differentiation of Streptomyces species were done using model organisms such as S. coelicolor A3(2) and S. griseus IFO 13350.1,2 The complete genome sequences of three Streptomyces species, S. coelicolor A3(2),3 S. avermitilis MA-4680T4 and S. griseus IFO 13350,5 have been reported and further accelerated the studies. Kitasatospora setae is a soil-habiting mycelial bacterium belonging to the same family Streptomyctaceae. All 23 validly published species belonging to the genus Kitasatospora exhibit a similar life style and morphology with Streptomyces species. Kitasatospora may also be comparable with Streptomyces in its capacity to produce bioactive secondary metabolites. The type strain of K. setae, NBRC 14216T, is known to produce setamycin (bafilomycin B1) and bafilomycin A1, specific inhibitors of vacuolar ATPase and commonly used as biochemical reagents for investigation of molecular transport in eukaryotic cells. This genus also includes several other strains reported as producers of bioactive compounds including a proteasome inhibitor and an anti-fungal agent.6,7

After the first proposal of the genus Kitasatospora (originally Kitasatosporia) by Omura et al. in 1982,8 the taxonomic position of Kitasatospora had been under a debate. It was once reclassified as a synonym of Streptomyces based on morphology and partial 16S rDNA analysis reported by Wellington et al.9 and Ochi and Hiranuma.10 Afterward, Zhang et al.11 reported that Streptomyces and Kitasatospora form distinct phyletic groups in the detailed inspection of the 16S rDNA and the 16S–23S rDNA internal spacer region and proposed the revival of the genus Kitasatospora. Such a history made Kitasatospora a suitable model for the development of methods distinguishing closely related species and genera based on the DNA sequence.12,13 Besides the molecular phylogenetic approach, there are clear phenotypic criteria to distinguish Kitasatospora from Streptomyces, of which most notable is the chemical composition of cell wall peptidoglycan.14,15 Cell wall peptidoglycan of streptomycetes contains the l, isomer of diaminopimelic acid (DAP), whereas many other spor actinomycetes contain the meso isomer. The peptidoglycan of Kitasatospora, in contrast, contains both l- and meso-DAP.16 In K. setae NBRC 14216T, aerial spores on solid culture and submerged spores in liquid culture both contain exclusively l-DAP, whereas mycelia in both cultures contain mainly meso-DAP.14,15,17 This suggests that the composition of cell wall peptidoglycan would be regulated in Kitasatospora depending on differentiation stages. Many of the genes involved in the formation of aerial mycelium (bld genes and others) and sporulation (whi genes and others) have been identified by genetic analysis of Streptomyces species,1,2 but the genetic and molecular basis for the differential incorporation of DAP isomers in Kitasatospora raises new questions. Considering the unique taxonomic position of Kitasatospora, as well as the common and distinct features of Kitasatospora and Streptomyces, Kitasatospora would be a key microorganism for further understanding of the evolution of not only actinobacteria within the family Streptomyctaceae but also other mycelial actinobacteria.

We determined the complete genome sequence of K. setae NBRC 14216T (=KM-60547T) and compared it with Streptomyces genomes. Although the overall topology and gene organization of the K. setae NBRC 14216T genome showed close resemblance to Streptomyces genomes, there are discriminative distances between them. We established a robust phylogenetic position of the genus Kitasatospora by multilocus phylogenetic analysis and confirmed the previous results based on 16S rDNA sequences. We also describe the conservation and variation of differentiation-related genes predicted from the annotation of K. setae NBRC 14216T genome, as well as the possible coding capacity of the genome for bioactive secondary metabolites.

2. Materials and methods

2.1. Genome sequencing, assembly and validation

DNA shotgun libraries with average insert sizes of 1.6 and 6.5 kb were constructed in pUC118 (TaKaRa), whereas a fosmid library with average insert size of 37 kb was constructed in pCC1FOS (EPICENTRE) as described previously.18,19 A total of 50 400 clones (34 560, 10 752 and 5088 clones from libraries with 1.6, 6.5 and 37 kb inserts, respectively) were subjected to sequencing from both ends of the inserts on either ABI 3730xl DNA Analyzer (Applied Biosystems) or Base Station DNA Fragment Analyzer BST-0100 (MJ Research, Inc.). Sequence reads were trimmed at a threshold quality value of 20 by Phred and assembled by Phrap and CONSED assembly tools.20,21 For alignment and validation of contigs, Optical Mapping (OpGen) was used. Gaps
between contigs were closed by sequencing PCR products, which bridge two neighboring contigs. Finally, each base of \textit{K. setae} NBRC 14216\textsuperscript{T} genome was ensured to be sequenced from multiple clones and from both directions with Phrap quality score \(\geq 70\) or from one direction with Phrap quality score \(\geq 40\). Chromosomal terminus was determined after attaching adenine and thymine homopolymers to the naked 3’ ends of the chromosome as described previously.\textsuperscript{5}

2.2. Data analysis and annotation

The prediction of open reading frames (ORFs) was performed using Glimmer3.\textsuperscript{22} The initial set of ORFs was manually selected from the prediction result in combination with BLASTP\textsuperscript{23} and FramePlot\textsuperscript{24} results. Each ORF was annotated manually using in-house genome annotation system OCSS (unpublished). Similarity search results against Uniprot,\textsuperscript{25} Interpro\textsuperscript{26} and HAMAP\textsuperscript{27} databases were used for functional prediction. The KEGG\textsuperscript{28} database was used for the reconstruction of metabolic pathways. If necessary, annotation was confirmed by molecular phylogenetic analysis using ClustalW, NJplot or GARLI (http://www.bio.utexas.edu/faculty/antisense/garli/Garli.html). Putative transporters and peptidases were independently evaluated using TransportDB\textsuperscript{29} and MEROPS\textsuperscript{30} databases, respectively. Non-coding genes were predicted using the Rfam,\textsuperscript{31} tRNAscan-SE\textsuperscript{32} and ARAGORN\textsuperscript{33} programs. Putative \textit{oriC} region was located using origin\textsuperscript{x} program. Putative ORFs related to mobile genetic elements were predicted and their boundaries were inferred with the assistance of GenomeMatcher\textsuperscript{34} software. For accurate assignment of orthologs from actinobacterial genomes, comparative data compiled in the MBGD\textsuperscript{36} database were used with further molecular phylogenetic evaluation, if necessary.

3. Results and discussion

3.1. General features of the \textit{K. setae} NBRC 14216\textsuperscript{T} genome

The \textit{K. setae} genome was composed of a single linear chromosome of 8 783 278 bp with 127 148 bp of terminal inverted repeats (TIRs). These characteristics in the genome topology were similar to those of \textit{Streptomyces},\textsuperscript{3–5} although the microorganism does not harbor a linear or circular plasmid. The general features of the \textit{K. setae} genome are summarized in Table 1 and Fig. 1. The chromosome was predicted to encode 74 tRNA genes, 9 copies of ribosomal RNA operon and 7569 protein-coding genes. Among the predicted protein-coding genes, 53.5\% (4049 ORFs) were assigned putative functions. The average \(\text{G} + \text{C}\) content of the chromosome was 74.2\%, which is among the highest in actinobacteria, being nearly equal to that of \textit{Kineococcus radiotolerans} (http://genome.jgi-psf.org/finished_microbes/kinra/). The putative replication origin containing 20 DnaA box-like sequences was located at the center of the chromosome. This putative origin was flanked by \textit{dnaA} and

| \textbf{Table 1. General features of \textit{K. setae} NBRC 14216\textsuperscript{T} genome and \textit{Streptomyces} genomes} |
|---|
| **Strain** | **Length (bp)** | **TIR (bp)** | **G + C Content (%)** | **CDS (no.)** | **rRNA operons (no.)** | **tRNA genes (no.)** | **Average CDS length (bp)** | **Coding density (%)** | **Reciprocal BLAST best-hit pair (no.)\textsuperscript{a}** |
| \textit{K. setae} NBRC 14216\textsuperscript{T} | 8 783 278 | 127 148 | 74.2 | 7569 | 9 | 74 | 1012 | 87.0 | — |
| \textit{S. coelicolor} A3(2) | 8 667 507 | 21 653 | 72.1 | 7825 | 6 | 63 | 991 | 88.9 | 3550 |
| \textit{S. avermitilis} MA-4680\textsuperscript{b} | 9 025 608 | 49 | 70.7 | 7582 | 6 | 68 | 1027 | 86.3 | 3498 |
| \textit{S. griseus} IFO 13350 | 8 545 929 | 132 910 | 72.2 | 7138 | 6 | 66 | 1055 | 88.1 | 3513 |
| \textit{S. scabies} 87.22 | 10 148 695 | 18 488 | 71.5 | 8746 | 6 | 75 | 1005 | 86.2 | 3534 |

\textsuperscript{a}Numbers of best-hit pair between \textit{K. setae} NBRC 14216\textsuperscript{T} and each \textit{Streptomyces} are calculated using BLASTP program with a threshold \textit{E}-value of \(1 \times 10^{-20}\).

\textsuperscript{b}On the basis of the latest annotation data of \textit{S. avermitilis} MA-4680\textsuperscript{T} maintained by H. Ikeda (http://avermitilis.ls.kitasato-u.ac.jp).
*dnaN* as in the cases of almost all bacteria, including *Streptomyces* species.37

The finished sequence consisted of a big contig representing the major part of the chromosome connected to the same small contig at both termini. As neither sequence variation nor assembly inconsistency was found in the terminal contig, we concluded that the chromosome has inverted identical sequences at both extremities. Terminal sequences of linear chromosomes and plasmids of *Streptomyces* and *Rhodococcus* can be classified into at least six groups. The terminal sequence of the *K. setae* chromosome was distinct from any of these groups; while the first 13 bp sequence exactly matched with that of major *Streptomyces* groups I and II (Supplementary Fig. S1), the subsequent region containing palindrome structures and loops, which are known in *Streptomyces* to be required for binding of telomere-associated protein (Tap), was not conserved.38,39 Tap and terminal protein (Tpg) encoding genes (*KSE_73020* and *KSE_73030*) were detected in the *K. setae* chromosome with lower similarity (42–45% amino acid identity) to those of *Streptomyces*. *Kitasatospora setae* thus seems to have similar mechanisms for chromosome maintenance; it possesses a linear chromosome with TIRs, replicates bidirectionally from a centrally located oriC region and maintains terminal sequences by Tap and Tpg. However, the binding specificity of Tap might be different even from those recognizing group I and II replicons of *Streptomyces*.

No critical difference was found between *K. setae* and *Streptomyces* species in the predicted primary metabolism such as carbohydrate metabolism, amino acid metabolism, nucleic acid metabolism and respiration. The numbers of other ubiquitous components, such as membrane transporters, peptidases, transcriptional regulators and sigma factors, were also almost equivalent to those of *Streptomyces*.

### 3.2. Taxonomic reevaluation and comparative analysis of *K. setae* NBRC 14216^T^  

From morphological similarity and rDNA relatedness to *Streptomyces* species, genus *Kitasatospora* was once regarded as a synonym of *Streptomyces*.9 Although phylogenetic analysis based on the 16S rDNA sequence usually separates *Streptomyces* and *Kitasatospora* species into distinct sister groups, the results sometimes depend on the choice of the outgroup11 or the region used for the alignment, depicting the difficulties in determining correct taxonomic relationships only from the nucleotide sequences of rDNA. In order to obtain a more robust measure of the taxonomic position of *Kitasatospora* by taking advantage of its genomic information, we performed a multilocus phylogenetic analysis using 31 conserved amino acid sequences. Ciccarelli et al.40 reported the construction of a tree of life across all three domains using 191 species including 14 actinobacteria. We adopted the same method by utilizing 58 actinobacterial genomes with the *Escherichia coli* genome as an outgroup (Supplementary Tables S1 and S2). Amino acid sequences were aligned for each of the 31 conserved protein genes, and then all 31 alignments were concatenated and ambiguous portions were deleted before performing phylogenetic reconstruction. Figure 2 shows a phylogenetic tree obtained by the neighbor-joining method (a phylogenetic tree obtained by the maximum-likelihood method is shown in Supplementary Fig. S2). Relationships between major taxonomic groups were broadly consistent with the previous results obtained by 16S rDNA sequences. Within the phylogenetic tree, 82% of all predicted branches were supported by bootstrap proportions greater than 90% (i.e. 900 of 1000). Four *Streptomyces* species were grouped in the same branch, with *S. griseus* IFO 13350 branching out at the deepest position. *Kitasatospora setae* NBRC 14216^T^ was placed within the same clan as

Figure 1. Schematic representation of the *K. setae* NBRC 14216^T^ chromosome. (i) G + C content. (ii) GC skew. (iii) ORFs encoded in forward (upper) and reverse (lower) strand. Each ORF is colored on the basis of the predicted function. (iv) RNA encoding genes. rRNA operons are colored in red and tRNAs are colored in blue. (v) Putative insertion sequences. (vi) Secondary metabolism gene clusters. (vii) Red bars indicate ORFs conserved commonly in all four *Streptomyces* genomes.
Streptomyces creating the outermost branch. All these results were supported by bootstrap proportions of 100%, reinforcing the idea that the genera Kitasatospora and Streptomyces were generated from a common progenitor and have diverged into distinct sister groups. The closest to this group was Catenulispora acidiphila DSM 44928, a mycelial actinobacterium with a circular chromosome.

For further analysis of the relationship between K. setae and Streptomyces, we compared all annotated proteins of K. setae and four Streptomyces species. More than half of ORFs predicted in K. setae had orthologs (reciprocal best-hit pairs) in each of four Streptomyces species using a BLASTP threshold of $E < 10^{-20}$ (Table 1). About 34% of K. setae ORFs had orthologs in all four Streptomyces genomes. The average amino acid identity between orthologous pairs from K. setae and Streptomyces species was around 60%. Despite such high similarities observed between orthologs, genome-wide comparison using

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**Figure 2.** Phylogenetic tree based on amino acid sequences of 31 protein-coding genes analyzed by the neighbor-joining method. Branches with less than 90% bootstrap support are represented in dashed lines. Lists of organisms and genes used for the analysis are shown in Supplementary Tables S1 and S2, respectively. Names of the organisms mentioned in the text are shown in bold type.
ortholog plots demonstrated smaller extent of synteny between *K. setae* and *Streptomyces* genome (Fig. 3A–C). Many short synteny blocks were observed along either of the diagonal lines, suggesting that frequent inversions around the replication origin have had occurred. This is in contrast to the higher extent of colinearities with only 2–4 inversions observed in comparative analysis among *Streptomyces* genomes.3–5

Conserved core region of the *K. setae* genome predicted by threading major synteny blocks was \( \sim 5 \) Mb in length ranging from KSE_13770 to KSE_57600, which is \( \sim 1 \) Mb smaller than the core regions deduced from comparison among *Streptomyces* genomes.5 Long-range synteny between *K. setae* and *C. acidiphila* genomes was much less obvious compared with that between *K. setae* and *Streptomyces* genomes (Fig. 3D), although the number of orthologous gene pairs between *K. setae* and *C. acidiphila* (3185) was only 10% smaller than that between *K. setae* and *Streptomyces* (3498–3550, Table 1).

### 3.3. Conservation and variation of genes related to developmental regulation

*Streptomyces* is well characterized as a model organism by its complex life cycle; it grows as a thread-like mycelium and forms aerial mycelium in the air under nutrient-limited conditions.1,2,42,43 A series of genes whose mutations cause defects in aerial growth (‘bld’ genes) were characterized in *S. coelicolor* A3(2). A complex interaction cascade among bld gene products results in the secretion of surfactant proteins, which assist *Streptomyces* in extending aerial mycelium upward and in differentiating into spores. A number of genes in ‘whi’ loci were also characterized mainly in *S. coelicolor* A3(2) as causing sporulation deficiency. Most of these classical differentiation genes were found to have orthologs in *K. setae*. The number of conserved bld and whi genes and their similarity (amino acid identity) to *S. coelicolor* A3(2) counterparts were higher than those in other mycelial actinobacteria, such as *C. acidiphila* DSM 44928,

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**Figure 3.** Synteny between the genomes of *K. setae* NBRC 14216\(^T\) and *S. coelicolor* A3(2) (A), *K. setae* NBRC 14216\(^T\) and *S. avermitilis* MA-4680\(^T\) (B), *K. setae* NBRC 14216\(^T\) and *S. griseus* IFO 13350 (C) and *K. setae* NBRC 14216\(^T\) and *C. acidiphila* DSM 44928 (D). Reciprocal BLAST best-hit pairs with a threshold value of \( E < 10^{-20} \) were plotted. The direction of each chromosome was adjusted so that the dnaA gene faces the same direction. Green bar in each panel represents the conserved core region on the *K. setae* chromosome.
Saccharopolyspora erythraea NRRL 23338 and Salinispora arenicola CNS 205 (Table 2), consistent with the closer morphological similarity of K. setae to Streptomyces. Notably, bld gene pairs orthologous between K. setae and Streptomyces species represented 80–97% amino acid identities, which are much higher than the average (60%) of all orthologous pairs and comparable with those between Streptomyces species. Higher than the average conservation of bld was also observed between C. acidiphila and Streptomyces (58–95% amino acid identities). The bldA tRNA gene was also highly conserved in K. setae, with its cognate codon UUA being the rarest in K. setae; used only in 69 predicted ORFs, most of which were either of unknown function or with predicted regulatory functions. In four completely sequenced Streptomyces genomes, only three genes were found to have a conserved UUA codon at the same position in each ortholog.45 One of these, the bldH gene, shared a UUA codon at an equivalent position also in K. setae. The high conservation of these regulatory components may further imply that each component in the regulatory cascade undergoes a higher than the usual number of interactions with other bld gene products or with other cellular components.

On the contrary, we could not identify most of the amf genes in K. setae (Table 2). The amf genes are highly conserved among Streptomyces species and are necessary for the synthesis of Amfs [SapB in S. coelicolor A3(2)] surfactant protein, which is known in Streptomyces to be secreted before the initiation of aerial growth. The presence of amfAB homologs in C. acidiphila and S. erythraea mycelial actinobacteria more distantly related to Streptomyces, may suggest that the pre-existing Amfs system was eliminated in the Kitasatospora lineage, although other possibilities such as the horizontal acquisition of these genes in each lineage cannot be excluded. In addition to the lack of the amf gene cluster, differences from Streptomyces were also suggested in the variation of paralogous components (Supplementary Table S3) such as SsgA-like proteins involved in peptidoglycan synthesis in sporogenic cell division, WhiB family transcriptional regulators and chaplins, another class of surfactant protein shown in S. coelicolor to play a part in aerial mycelium formation.46,47

A number of whi genes control the process of sporulation septation and spore maturation in Streptomyces. Of the classical whi genes, whiA, whiB and whiD are commonly found in actinobacteria, and their exact functions in simpler (non-sporulating) actinobacteria need to be elucidated.1 All other whi genes, which had been considered specific to Streptomyces species,1,42 were also conserved in K. setae with remarkably high amino acid identities (69–90%). Despite such close similarities in genetic background, preliminary experimental results suggest that K. setae produces seemingly less mature spores compared with Streptomyces; (i) although K. setae genome encodes full set of whiE gene cluster (encoding biosynthetic enzymes for polyketide spore pigment), no expression of whiE genes nor the production of spore pigment was observed in any culture conditions for sporulation, (ii) both aerial and submerged spores produced by K. setae are much more sensitive to freeze-thaw cycles than those of Streptomyces (S. Kitani and H. Ikeda, unpublished observations). Further studies are needed to elucidate mechanisms underlying such differences.

The initiation of secondary metabolite synthesis is known to be linked with morphological differentiation in S. griseus via the gamma-butyrolactone autoregulator cascade. In this regard, it would be noteworthy that K. setae possesses three homologs of the autoregulator receptor: KsbA (KSE_58650), KsbB (KSE_01050t and KSE_75690t; identical genes encoded in the TIR) and KsbC (KSE_44580). However, KsbA in K. setae was experimentally confirmed to be involved only in secondary metabolism,18 but not in morphological differentiation, whereas the involvement of other two remains to be clarified. In addition, the AfsA family protein, which contains two A-factor biosynthesis repeat motifs, has been reported to be a crucial enzyme to synthesize the gamma-butyrolactone autoregulator in Streptomyces.59 Interestingly, three AfsA family proteins were encoded on the genome of K. setae: KsbS2 (KSE_01060t and KSE_75680t; identical and present in the TIR, similar to the case of KsbB), KsbS3 (KSE_22970) and KsbS4 (KSE_44600). These findings suggested that there might be a more complicated signaling network for secondary metabolism and/or morphological differentiation in K. setae.

3.4. Genes involved in peptidoglycan biosynthesis

One of the most important features of the genus Kitasatospora from the chemotaxonomic viewpoint is that the cell wall peptidoglycan contains both ll-DAP and meso-DAP.14,15 DAP analysis of Kitasatospora strains showed that spores contain only ll-DAP, whereas mycelia contain mostly meso-DAP.16,17 This observation suggests that Kitasatospora incorporates different DAP isomers into the cell wall depending on differentiation stage. We can speculate two steps which might be responsible for the differential incorporation of DAP isomers. (i) DAP biosynthesis and isomerization: ll-DAP is isomerized to meso-DAP by dapF gene product in the course of lysine biosynthesis.50 (ii) Incorporation of
Table 2. Conservation of aerial mycelium and spore formation-related genes in *K. setae* NBRC 14216<sup>T</sup> and other mycelial actinobacteria

| Aerial mycelium | Kitasatospora setae NBRC 14216<sup>T</sup> | Streptomyces coelicolor A3(2) | Streptomyces avermitilis MA-4680<sup>T</sup> | Streptomyces griseus IFO 13350 | Streptomyces scabies 87.22 | Catenulispora acidiphila DSM 44928 | Saccharopolyspora erythraea NRRL 23338 | Salinispora arenicola CNS 205 |
|----------------|---------------------------------|-------------------------------|---------------------------------|-------------------|-----------------|-----------------|-----------------|-----------------|
| **bldA**       | KSE_10069                       | SCO124                        | SAV_157                         | SGR_TRNA42        | SCAB_150        | Caci_R0007      | SACE_8016       | Sare_R0014      |
| **bldB**       | (KSE_16220)<sup>§</sup> (64)   | SCO5723                       | SAV_2529 (SAV_1241)             | SGR_1796          | SCAB_25271 (SCAB_84941) | ND            | SACE_6156       | Sare_3856       |
| **bldC**       | KSE_41620 (97)                  | SCO4091                       | SAV_4130                        | SGR_3882          | SCAB_47901      | Caci_0301       | SACE_6926       | Sare_0170       |
| **bldD**       | KSE_13950 (92)                  | SCO1489                       | SAV_6861                        | SGR_6045          | SCAB_75171      | Caci_2399       | SACE_2077       | Sare_1844       |
| **bldG**       | (=rsbV)                         | KSE_35840 (96)                | SCO3549                         | SGR_3307          | SCAB_40861      | Caci_8450       | SACE_4194       | Sare_4413       |
| **bldH**       | (=adpA)                         | KSE_26930 (80)                | SCO2792                         | SGR_4742          | SCAB_57831      | Caci_5972       | SACE_4523       | ND              |
| **bldKA–bldKE**| KSE_48250–KSE_48290 (41–78)    | SCO5112–SCO5116               | SAV_3152–SAV_3156; SAV_3172–SAV_3176 | SGR_2414–SGR_2418 | SCAB_31501–SCAB_3154 | Caci_7147–Caci_7151 | ND | ND |
| **bldM**       | (=whiK)                         | KSE_31060 (97)                | SCO4768                         | SAV_4998          | SGR_2759        | SCAB_36231      | Caci_1011       | Sare_4229       |
| **bldN**       | (=adsA)                         | KSE_33690 (92)                | SCO3323                         | SAV_4735          | SGR_4151        | SCAB_39121      | Caci_8330       | Sare_0420       |
| **amfC**       | KSE_43740 (54)                  | SCO4184                       | SAV_4026                        | SGR_3974          | SCAB_49711      | Caci_8239       | SACE_7115       | ND              |
| **amfR**       | (=ramR)                         | ND                            | SCO6685                         | SAV_7499          | SGR_2393        | SCAB_8642<sup>c</sup> | ND | ND |
| **amfS**       | (=ramS)                         | ND                            | SCO6682                         | SAV_7502          | SGR_2396        | SCAB_8621       | Caci_4240       | SACE_4231       |
| **amfB**       | (=ramA)                         | ND                            | SCO6683                         | SAV_7501          | SGR_2395        | SCAB_8631       | Caci_4242       | SACE_4232       |
| **amfA**       | (=ramB)                         | ND                            | SCO6684                         | SAV_7500          | SGR_2394        | SCAB_8641       | Caci_4243       | SACE_4233       |
| **amfT**       | (=ramC)                         | KSE_63000 (44)                | SCO6681                         | SAV_7503          | SGR_2397        | SCAB_8611       | Caci_4239       | SACE_4230       |
| **Sporulation**|                                |                               |                                 |                   |                 |                 |                 |                 |
| **whiA**       | KSE_55390 (89)                  | SCO1950                       | SAV_6294                         | SGR_5572          | SCAB_69681      | Caci_5609       | SACE_2141       | Sare_3326       |
| **whiB**       | KSE_29410 (83)                  | SCO3034                       | SAV_5042                         | SGR_4503          | SCAB_55081      | Caci_7609       | SACE_6464       | ND              |
| **whiD**       | (=wblB)                         | KSE_31070 (90)                | SCO4767                         | SAV_4997          | SGR_2760        | SCAB_36241      | Caci_1009       | SACE_5583       |
| **whiE**       | KSE_72410–KSE_72480 (46–67)     | SCO5314–SCO5321               | SAV_2837–SAV_2844               | ND                | SCAB_43281–SCAB_43341 | Caci_1083–Caci_1090 | ND | Sare_2682–Sare_2690 |
| **whiG**       | KSE_52540 (79)                  | SCO5621                       | SAV_2630                         | SGR_1866          | SCAB_26051      | Caci_1526       | SACE_6040       | ND              |
DAP into peptidoglycan: the addition of DAP to UDP-N-acetylmuramyl-L-alanyl-D-glutamate precursor is catalyzed by an enzyme encoded by murE. As in the case of Streptomyces species whose peptidoglycan is composed of LL-DAP, K. setae had only one murE gene (KSE_21280), which is located in the center of a long conserved dcw (division cell wall) cluster. Amino acid residues known to be responsible for substrate recognition in the enzymes from meso-DAP containing bacteria are also conserved in the enzymes from LL-DAP containing bacteria including Streptomyces. The MurE protein purified from E. coli can incorporate LL-DAP in addition to meso-DAP, the natural substrate in this bacterium. Thus, the MurE protein may not be responsible for the differentiation-dependent alterations in peptidoglycan synthesis, unless the substrate specificity is regulated by unknown factor(s). On the other hand, K. setae had three dapF genes (KSE_32600, KSE_32630 and KSE_53750) whose products share highly conserved active site residues. Phylogenetic analysis based on amino acid sequences showed that two of the DapF proteins (encoded by KSE_32600 and KSE_53750) were closely related to those from LL-DAP containing actinobacteria such as Streptomyces species and Nocardioides sp. BAA-499/JS614. In contrast, the third DapF protein (KSE_32630) was positioned apart from the actinobacterial groups, but in the vicinity of proteins from meso-DAP containing Listeria, Lactobacillus and Clostridium. In the dapF mutant strain of E. coli, a n increase in LL-DAP containing peptidoglycan was observed, suggesting that the relative size of LL- and meso-DAP pools would affect the composition of peptidoglycan. Differential regulation of the multiple dapF genes, or their protein products, in the course of morphological differentiation might play an important role in the change in peptidoglycan composition.

### Table: Orthologs of K. setae NBRC 14216T ORF and S. coelicolor A3(2)

| Gene | K. setae ORF | S. coelicolor A3(2) ORF | S. avermitilis MA-4680T ORF | E. coli ORF | 
|------|--------------|-------------------------|-----------------------------|-------------|
| whiH | KSE_54050 (74) | SCO5819 | SAV_2445 | SGR_1702 | SCAB_24461 | Caci_1760 | ND | ND |
| whil | KSE_55090 (77) | SCO6029 | SAV_2230 | SGR_1475 | SCAB_20881 | Caci_6781 | SACE_1833 | ND |
| sigF | KSE_37310 (69) | SCO4035 | SAV_4185 | SGR_3551 | SCAB_47291 | Caci_7708 | ND | ND |
| sigN | KSE_37320 (80) | SCO4034 | SAV_4186 | SGR_3552 | SCAB_47281 | ND | ND | ND |
| crpA | KSE_39340 (50) | SCO3854 | SAV_4331 | SGR_3718 | SCAB_45631 | Caci_0043 | SACE_0037 | Sare_0058 |

*The amino acid identity between K. setae NBRC 14216^T ORF and S. coelicolor A3(2) ortholog was shown in parentheses. The range of amino acid identity was shown where multiple genes were assigned.

*The bldB gene paralog found in S. coelicolor A3(2) and S. avermitilis MA-4680^T are also included. KSE_16220 corresponds to an ortholog of the bldB paralog.

This ORF tentatively named SCAB_8642 (corresponding to position 975 194–975 802 on the complementary strand) has not yet been incorporated in the submitter annotation.
Figure 4. Amino acid phylogenetic trees of DapF (A) and HMW-PBPs (B). Branches with less than 70% bootstrap support are represented in dotted line. The distribution of peptidoglycan amino acid component is also presented in (A). Streptomyces coelicolor A3(2) PBPs and their orthologs were grouped and expression patterns were assigned according to Noens et al.55 in (B); those known to be expressed in aerial mycelium and vegetative mycelium are shown by AM and VM, respectively. Amino acid sequences were derived from the HAMAP database (MF_00197).
gene (SCO5039) specifically expressed in vegetative mycelium in S. coelicolor seemed not conserved in K. setae (Fig. 4B). Such differences in stage-specific PBPs could also be reflected in differentiation specific incorporation of peptidoglycan units in K. setae. In this context, it is also interesting to note that SsgA-like family proteins (SALPs), likely involved in the regulation of peptidoglycan synthesis in sporogenic cell division, are much diversified in K. setae compared with Streptomyces. Kitasatospora setae encodes at least 12 SALPs, about twice as many as those found in Streptomyces species (Supplementary Table S3).

3.5. Gene clusters for secondary metabolite biosyntheses

Bacteria belonging to the genus Kitasatospora have been explored as potential new sources of various bioactive metabolites. Kitasatospora setae NBRC 14216\(^7\) is known to produce setamycin (bafilomycin B1), which bears antitrichomonal activity.\(^5^7\) A total of 24 genes or gene clusters in the K. setae genome were predicted to be involved in the biosynthesis of secondary metabolites (Table 3). Of the 24 clusters, more than 60% (16 clusters) were located in subtelomeric regions; 11 were in the right subtelomeric region, whereas 5 were in the left subtelomeric region. The number of predicted gene clusters for secondary metabolism was slightly lower than that predicted in Streptomyces species (36 in S. griseus IFO 13350, 37 in S. avermitilis MA-4680\(^7\), 30 in S. coelicolor A3(2) and at least 20 in S. scabies 87.22), but apparently higher than that in other prokaryotes, underscoring the importance of the genus Kitasatospora as the source of bioactive compounds.

Of the 24 clusters, 5 were estimated for terpene biosynthesis, 12 for polyketides or non-ribosomal peptides, 2 for siderophores and 5 for lantibiotics and others. Five ORFs containing the terpene synthase domain (IPR005630) were classified by the phylogenetic analysis described by Komatsu et al.,\(^5^9\) indicating that KSE_46080 was in the group of germacradienol/geosmin synthase\(^5^0,^6^1\) and KSE_70210 in the group of 2-methylisoborneol synthase. Consistently, geosmin and 2-methylisoborneol were identified from the culture of K. setae. Of the 12 clusters for polyketides or non-ribosomal peptides, setamycin (bafilomycin B1) cluster was estimated and experimentally proved to be KSE_73410–KSE_73580 (H. Ikeda, unpublished results). An 84 kb region containing the cluster KSE_70570–KSE_70620 showed striking resemblance in features of gene organization and in deduced amino acid sequences of each ORF to the kirromycin biosynthetic gene cluster, the first characterized combined cis–trans-PKS cluster in Streptomyces collinus Tu 365.\(^6^2\)

Regarding the two siderophore clusters, the one (KSE_12660–KSE_12700) showed good similarity in gene arrangement and deduced amino sequence of each ORF to a cluster in S. avermitilis (SAV_7320–SAV_7323, lacking a homolog corresponding to KSE_12680), both of which were similar to vibriofermin cluster identified in Vibrio parahaemolyticus.\(^6^3\) Another one (KSE_53800–KSE_53830) was similar in gene arrangement to the rhizobactin cluster in Sinorhizobium meliloti,\(^6^4\) but differed from any of the known siderophore biosynthesis clusters of Streptomyces. Interestingly, K. setae lacks a gene cluster...
for nocardamin (desferrioxamine) biosynthesis that was commonly found in genome-sequenced Streptomyces species.58,65

3.6. Conclusion

Streptomyces are thought to have emerged ~440 million years ago. At present, Streptomyces species are some of the most highly differentiated microorganisms with a complex life cycle. The cell wall peptidoglycan composed of \(\kappa\)-DAP is another feature to be considered to distinguish Streptomyces from other sporoactinomycetes. Here, we analyzed the first genome sequence of a Streptomycetaceae bacterium other than Streptomyces species. Phylogenetic analysis based on amino acid sequences, together with a genome-wide comparison of the predicted genes such as those related to morphological differentiation and cell wall biosynthesis, suggests that the genera Streptomyces and Kitasatospora were diverged directly from their last common ancestor. The chromosomal linearity and the presence of the TIR sequence would also be the features inherited from the common ancestor. Many differentiation-related genes highly conserved in K. setae and Streptomyces species might have been acquired sequentially in the evolution, as previously suggested from the comparison of Streptomyces with other groups of mycelial actinobacteria, Thermobifida fusca and Frankia species.1,42 If additional genomic sequences determined more recently such as C. acidiphila, S. erythraea, Salinispora species and K. setae were taken into consideration, however, the evolutionary pathway of differentiation-related genes seemed not necessarily be straightforward, and gene losses and horizontal acquisitions, in particular lineage, might also have to be considered (Table 2). Phylogenetic data presented in Fig. 2 and Supplementary Fig. S2 also suggest that mycelial actinobacteria are not monophyletic; some organisms such as S. erythraea and T. fusca belong to sub-branches that also contain bacteria without most of the developmental genes. Extraordinary high similarity observed in \(\text{bld}\) regulatory genes, which are dispersed in the genome, might also reflect recent horizontal transfer. Although the highly regulated differentiation system of Streptomyces has attracted much attention, reasons for the unique abundance of \(\kappa\)-DAP in peptidoglycan may have been overlooked. We provided the possible background of the relative abundance of \(\text{meso}\)- and \(\kappa\)-DAP in K. setae. Further analysis and comparison of two genera, including genome sequencing of another Kitasatospora species, will provide deeper understanding of the evolution of actinobacteria.

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