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Isolation and taxonomy study of unexplored microbial resource Ktedonobacteria for discovery of novel bioactive compounds

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論文内容要旨

Isolation and taxonomy study of unexplored microbial resource *Ktedonobacteria* for discovery of novel bioactive compounds

（未開拓微生物資源クテドノバクテリアの分離と系統分類及び新規生物活性物質の探索）

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Chapter 1. General introduction

The prevalence of antibiotic resistance and decrease in discovery of novel antibiotics from the traditional producer actinomycetes in recent years necessitates the identification of potentially novel microbial resources to produce natural products [1]. Remarkably, the class *Ktedonobacteria* was established in 2006 for the gram-positive, aerobic, filamentous bacterial lineage in phylum *Chloroflexi* [2], and was characterized with their complex life cycle by forming spore-like structures on branched mycelia [2-4] and relative large genomes (5.56~13.66 Mb) [5, 6]. Similar to actinomycetes, isolates and environmental DNA clones belonging to this class were detected to be ubiquitous in various terrestrial environments [7-12]. Moreover, novel secondary metabolite compounds were discovered from this class prior to this study [13, 14]. Altogether, these above characteristics encourage us to propose the class *Ktedonobacteria* as a promising next-generation microbial resource for discovery of novel antibiotics [15, 16].

However, 1) only two orders, three families, four genera, and seven species have been formally proposed prior to this study [2-4, 17-19], which are extremely unenough; 2) phylogenetic position, genome features, and biosynthetic potential of this class remain unclear; 3) no studies yet had been focused on discovering novel bioactive compounds from the class *Ktedonobacteria*.

Accordingly, in this study, I attempted to isolate novel *Ktedonobacteria* strains and comprehensively study the taxonomic properties of the class in Chapter 2. In Chapter 3, I performed whole genome sequencing for 18 *Ktedonobacteria* strains, and studied the genome features and biosynthetic potential of this class. In Chapter 4, I screened the antimicrobial activity of the class *Ktedonobacteria* and attempted to isolate novel bioactive compounds. In Chapter 5, I attempted to clone a type II polyketide synthase (PKS) gene cluster to heterologously express an anthraquinone compound discovered in Chapter 4. Given our findings here, I concluded in Chapter 6 that, the gram-positive, aerobic, and filamentous *Ktedonobacteria* represents a versatile and promising microbial resource for pharmaceutical and biotechnological use.

Chapter 2. Isolation and taxonomic study of the class *Ktedonobacteria*

According to previous metagenomic studies, the class *Ktedonobacteria* predominate in geothermal and high-altitude oligotrophic environments although they also exist in common soil at low relative abundance [10-12]. Thus, we collected soil samples from Onikobe geothermal area, "Tengu-no-mugimeshi" (1,870 m above sea level), and Mt. Zao (1,600 m), to efficiently isolate novel *Ktedonobacteria* strains, as shown in **Fig. 1**. Onikobe geothermal area was the isolation source from which two previously proposed *Thermogemmatispora* species were recovered [4]. The "Tengu-no-mugimeshi" is soil-like orange-to-brown colored microbial mass that has been recognized in the volcanic zone of central Japan, and is also known as the "eatable soil". According to the investigation of bacterial community composition in this study, the "Tengu-no-mugimeshi" is predominated by the class *Ktedonobacteria* at 17.31~30.06% relative abundances (**Fig. 2**). Mt. Zao is a complex and active volcano nearby. The collected soil samples were directly spread on selective medium and incubated at 30 °C or 65 °C for weeks [18]. Consequently, seven novel *Ktedonobacteria* strains were successfully isolated: strains A1-2^T and A1-2^T from Onikobe, strains Uno3^T, Uno11^T, Uno16^T, Uno17 from "Tengu-no-mugimeshi", and strain W12^T from Mt. Zao. Simultaneously, eight unclassified Italian *Ktedonobacteria* isolates (strains SOSP1-1, SOSP1-9, SOSP1-30, SOSP1-52, SOSP1-85, SOSP1-142, 150039, and 150040), which were isolated together from various soil samples (soil collected from an ant house, Honduras; black locust wood soil, Italy; pine wood soil, Spain; soil collected from a solfatara volcano, Italy; and soil under a bush, France) with *K. racemifer* SOSP1-21^T [2], were obtained from Dr. Cavaletti, our co-researcher. Collectively, these *Ktedonobacteria* isolates were subjected to a comprehensive phylogenetic, morphological, physiological, and chemotaxonomic analysis to study the taxonomic common features and diversities of the class.

As given in **Fig. 3**, members in families *Ktedonobacteraceae*, *Thermosporotrichaceae*, and *Thermogemmatisporaceae* of the class *Ktedonobacteria* are all filamentous and form exospores (1.0~2.0 μm in size) on branched mycelia by budding. As for the *Dictyobacteraceae* family, however,

Dictyobacter aurantiacus S27^T and *Dictyobacter* sp. SOSP1-9 formed putative sporangiospores (8.5~10.0 μm) where other strains formed unclear structures (1.0~2.0 μm in size). Sporangia enclose sporangiospores inside and are commonly seen in plants and some fungal phyla. In this study, however, the formation of sporangia by *D. aurantiacus* S27^T was accidentally observed (**Fig. 4**), thus represented the third report of prokaryotic sporangia formation following some genera of actinomycetes (such as *Actinoplanes*) and myxobacteria [20, 21]. Unlike that of *Actinoplanes* [21], sporangiospores of *D. aurantiacus* S27^T are non-motile. Besides the morphological similarities, the class *Ktedonobacteria* also share some important phenotypic traits with the members of actinomycetes: Gram-stain positive, aerobic, and heterotrophic metabolism on various carbohydrates substrates including cellulose (**Table 1**). However, *Ktedonobacteria* differentiate with actinomycetes in lower genomic G+C content (50~60 mol%), type of major menaquinone (MK-9(H_2) or MK-9), and the cell wall composition (**Table 1**). Moreover, members of the class *Ktedonobacteria* contain an unusual amino acid of β-alanine on the cell wall peptidoglycan, and unusual cellular fatty acids of $C_{16:1}$-2OH and 12,17-Dimethyl $C_{18:0}$. However, our reconstruction of the phylogenetic position basing on 27 core genes extracted from genomes separated the class *Ktedonobacteria* from actinomycetes, and clearly determined its affiliation to the phylum *Chloroflexi* at 100% bootstraps supporting rate (**Fig. 5**). Nonetheless, the class *Ktedonobacteria* still represent a unique bacterial lineage in the phylum *Chloroflexi* given the dissimilarities in morphological, physiological, and chemotaxonomic data between the two.

Comprehensively considering the taxonomic differences compared with known type strains, the seven novel isolates recovered from Onikobe geothermal area, "Tengu-no-mugimeshi", and Mt. Zao were formally proposed with the designated names: *Thermogemmatispora aurantia* sp. nov. (strain A1-2^T), *Thermogemmatispora argillosa* sp. nov. (A3-2^T) within family *Thermogemmatisporaceae*, *Dictyobacter kobayashii* sp. nov. (Uno11^T), *Dictyobacter alpinus* sp. nov. (Uno16^T), *Dictyobacter vulcani* sp. nov. (W12^T), and *Tengunoibacter tsumagoiensis* gen. nov., sp. nov. (Uno3^T) within the

Dictyobacteraceae fam. nov. As for the eight Italian isolates, *Dictyobacter* sp. strain SOSP1-9 represented novel species in the genus *Dictyobacter*, where strains SOSP1-30, SOSP1-52, and SOSP1-85 are classified novel species in the genus *Ktedonobacter*. *Dictyobacteraceae* bacterium SOSP1-142 and *Ktedonobacteraceae* bacterium SOSP1-1 represented two new genera within the families *Dictyobacteraceae* and *Ktedonobacteraceae*, respectively. *Ktedonobacterales* bacterium 150039 and *Ktedonobacterales* bacterium 150040, however, formed independent clades on the phylogenetic tree (**Fig. 5**). Basing on the huge differences on physiological and chemotaxonomic properties, these two strains were therefore proposed as two new families in the order *Ktedonobacterales*. These Italian strains will be formally proposed after name designating in cooperation with Dr. Cavaletti.

Chapter 3. Whole genome sequencing, general genome features, and biosynthetic and cellulolytic potential of the class *Ktedonobacteria*

Non-contiguous genomic DNA of the 18 taxonomic studied *Ktedonobacteria* strains were extracted and sequenced on Illumina or PacBio platforms. As given in **Table 2** and **Fig. 6**, complete genome of *Tg. argilla* A3-2^T contains a circular chromosome (5.54 Mb) whereas *Ts. hazakensis* COM3 contains a linear chromosome (7.67 Mb). *T. tsumagoiensis* Uno3^T possesses a circular putative chromosome (5.30 Mb) and a circular putative mega-plasmid (2.40 Mb). *D. aurantiacus* S27^T and *D. alpinus* Uno16^T each comprises a linear putative chromosome (6.13 Mb and 5.58 Mb, respectively) and a linear putative mega-plasmid (2.75 Mb and 3.14 Mb, respectively). Additionally, *D. alpinus* Uno16^T also possesses two circular plasmids (199 Kb and 43 Kb, respectively) (**Fig. 6**). Initially, putative mega-plasmids of these strains were thought to be part of the chromosomes because they were quite large in size compared with normal bacterial plasmids. However, these sequences were determined to be incomplete due to the absence of most bacterial house-keeping genes, translation genes, DNA replication and repair genes, and genes involved in TCA cycle and oxidative phosphorylation [22], which are essential genes for growth thus suggesting that they may be "mega-plasmid" [23, 24]. Moreover, genome sizes of the thermophilic

strains in the families *Thermosporotrichaceae* (7.28 to 7.67 Mb) and *Thermogemmatisporaceae* (5.54 to 5.61 Mb) were the relatively small in the class *Ktedonobacteria*, but were still quite large among thermophilic bacteria given that growth temperature and genome size in bacteria are negatively correlated and thermophilic bacteria tend to have a small genome [25]. By contrast, the other mesophilic families *Dictyobacteraceae* and *Ktedonobacteraceae* two novel families harbored genomes ranging from 7.21 to 13.66 Mb, which were comparable to that of the *Streptomyces* strains [26].

To evaluate the biosynthetic potential of the class *Ktedonobacteria*, I used antiSMASH v5.0 [27] to predict putative biosynthetic gene clusters (BGCs) for secondary metabolite in the 23 available *Ktedonobacteria* genomes listed in **Table 2**. As shown in **Fig. 7**, a large number of 5~22 putative BGCs per genome encoding for secondary metabolites were predicted in these *Ktedonobacteria* genomes, which far exceeded the number of BGCs annotated in other *Chloroflexi* species (0~4 BGCs), and were comparable to well-known antibiotic-producing actinomycetes (6~29 BGCs identified in this study). Moreover, these identified BGCs exhibited very limited similarity with known clusters, indicating they may produce novel natural products. Also, I observed that BGCs encoding for peptide compounds including non-ribosomal peptide synthase (NRPS), NRPS/T1PKS hybrid, and ribosomally synthesized and post-translationally modified peptide (RiPP) family predominate in the *Ktedonobacteria* genomes, which may assist them in fighting against their competitors and predators in their niches [28, 29]. Considering that class *Ktedonobacteria* constitutes a relatively new bacterial taxa, to date only very limited knowledge is available regarding their secondary metabolites. Thus, the domain-specific phylogenetic analysis of the *Ktedonobacteria*-originated PKS keto-synthesis (KS) and NRPS condensation (C) domains may provide a better understanding of their functional and evolutionary classification [30]. As shown in **Fig. 8A**, the most abundant functional type among the *Ktedonobacteria* PKS-KS domains was assigned to hybrid KS and modular KS, whereas LCL, DCL, and epimerization types were the most abundant in the NRPS-

C domains (**Fig. 8B**). Moreover, the majority of *Ktedonobacteria*-derived KS and C domains formed independent clusters from those derived from other phyla.

Members within the class *Ktedonobacteria* exhibit a broad range of utilization of carbohydrates or degradation abilities in our physiological assays in Chapter 2, indicating that they may represent a potential cellulolytic bacterial group. However, comprehensive characterization of CAZymes in the genomes of *Ktedonobacteria* are still rare in the literature. Accordingly, I performed genome-wide analysis to profile the composition and distribution of CAZymes in the 23 available *Ktedonobacteria* genomes. As shown in **Fig. 9**, a large number of 153~320 genes per genome encoding for putative CAZymes were predicted, which far exceeded the number of CAZymes annotated in other *Chloroflexi* species (12~165 CAZymes per genome), and were comparable to well-known cellulolytic actinomycetes (100~244 CAZymes). The most abundant CAZyme class in the genomes of *Ktedonobacteria* were GHs and GTs, with 63-139 GHs and 53-108 GTs per genome, and were assigned to 85 GH families, 18 GT families, 11 CE families, 8 AA families, 5 PL families, and 18 CBM families, as given in **Fig. 10**. Remarkably, GH3 and GH5 families, which predominate in the *Ktedonobacteria* genomes, are characterized as plant polysaccharide-degrading enzymes, and have played important roles in cellulose and hemicellulose degradation [31].

Chapter 4. Antimicrobial activity of the class *Ktedonobacteria* and discovery of novel bioactive compounds

The above studies indicate the class *Ktedonobacteria* may produce numerous novel natural products. Herein, culture broth of six representative *Ktedonobacteria* species were extracted with acetone and subjected to *in vitro* antimicrobial screening. As a result, mesophilic strains of *D. aurantiacus* S27^T, *D. alpinus* Uno16^T, *T. tsumagoiensis* Uno3^T, exhibited broad antibacterial spectra against both gram-positive and gram-negative bacterial strains. The thermophilic strains *Ts. hazakensis* COM3 and *Tg. argilla* A3-2^T also strongly inhibited the gram-positive bacterial strains. Given that gram-negative bacterial strains are becoming increasingly antibiotic resistant owing to their protective outer membranes and

constitutively active efflux pumps [32, 33], these mesophilic *Ktedonobacteria* strains may contribute to the development of novel antibiotics targeting gram-negative pathogens. Herein, we decided to fractionate the crude extract and isolate novel bioactive compounds from strains *Ts. hazakensis* COM3 and *D. alpinus* Uno16^T.

Ts. hazakensis COM3 was cultured in a liquid medium (0.2% Peptone, 0.1% Yeast extract, 0.1% $MgSO_4$, 0.1% NaCl) added with 2% Diaion® HP-20 resin at 50 °C for a total volume of 72 L. The culture broth was extracted with acetone and fractionated with Sephadex® LH-20 gel filtration chromatography eluted with 20% and 80% MeOH (**Fig. 11A**). The 80% MeOH fraction 1 showed broad antimicrobial activity against gram-positive bacterial strains (**Fig. 11B**), thus was purified with various chromatography and the chemical structure was revealed using various NMR (^{1}H-, ^{13}C-, and 2D-). Unfortunately, the target compound was determined to be 2,4,6-triphenyl-1-hexene ($C_{24}H_{24}$, MW 312.4), a known metabolite of the fungus *Phellinus pini* [34]. Moreover, the isolated compound showed no antimicrobial activity after purification, which was in accordance with previous research [35]. However, given that the 80% MeOH fraction 1 showed strong anti-*M. bovis* activity in **Fig. 11B**, the real antimicrobial compound remained to be discovered in the future studies for discovery of anti-*Mycobacterium tuberculosis* drugs.

Following a similar compound discovery scheme including ODS column fractionation-HPLC purification-NMR/MS structure determination (**Fig. 11A**), a novel anthraquinone compound, designated COM1, was isolated from 80% MeOH fraction 2. Anthraquinones are a large class of aromatic secondary metabolites produced by many plants, fungi, and some inserts, and their biological activities are usually determined by functional group decorations [36]. In the putative chemical structure of COM1 ($C_{15}H_8Cl_2O_5$, NW: 336.97), some hydrogen atoms on the benzene ring are replaced by one methyl group, three hydroxyl groups, and two additional Chloro groups (positions not determined), thus forming a rare structure in natural products [37]. Moreover, in addition to the strong antimicrobial activity against MSSA and Mu50, COM1 also inhibited the growth of Hela cells

(cervical cancer cell) (**Fig. 11B**), thus may has promising applications in the discovery of anti-cancer agent [38]. In addition, COM1 is proposed to be the biosynthetic product of a type II PKS gene cluster according to its chemical structure [39].

Chapter 5. Cloning of a type II PKS gene cluster from *Ts. hazakensis* COM3

The above studies indicated the class *Ktedonobacteria* as a promising microbial resource for discovery of novel bioactive compound. However, the efficient transfer of novel biosynthetic gene cluster to molecules are extremely important. Moreover, only one type II PKS gene cluster was identified in the genome of *Ts. hazakensis* COM3 (**Fig. 7**), thus was proposed to be responsible for the biosynthesis of COM1. Herein, I attempted to apply a transformation-associated recombination (TAR) approach (**Fig. 12A**) [40] and used a commercial *Saccharomyces cerevisiae*/*E. coli* shuttle-actinobacterial chromosome integrative vector pCAP01 [41] to directly clone and heterologously express the type II PKS gene cluster. As shown in **Fig. 12B~E**, the type II PKS gene cluster was directly cloned successfully from the genomic DNA of *Ts. hazakensis* COM3 via a constructed capture vector in yeast and was verified with PCR screening. However, unknown DNA recombinations or fragmentations occurred when the COM1-pCAP01 construct was transformed into *E. coli* Top10 and *E. coli* Stbl4 strains (**Fig. 12E**), thus hampered the following conjugal DNA transfer into the *Streptomyces* host for further heterologous expression.

Chapter 6. Discussion and Conclusion

In this study, in Chapter 2, we 1) successfully isolated seven novel *Ktedonobacteria* strains from "Tengu-no-mugimeshi", Mt. Zao, and Onikobe geothermal area; and formally proposed one novel family, one novel genus, seven novel species. Moreover, two novel families, two novel genera, and four novel species are prepared to propose in future, thus significantly expanded this class and determined it as an unique bacterial lineage in the phylum *Chloroflexi*; 2) successfully isolated one of the predominate bacteria (*Ktedonobacteria*) from "Tengu-no-mugimeshi", thus may contribute to the regeneration and preservation

of the eatable soil; 3) reported the first discovery of sporangiospores formation by *D. aurantiacus* S27^{T}, thus may have important values on the studies of bacterial evolution and cell differentiation. In Chapter 3, we 1) performed whole genome sequencing for 18 *Ktedonobacteria* strains and observed huge genome size, mixture of both circular and linear genomes, and putative "mega-plasmid" in the genomes of *Ktedonobacteria*; thus enabled the further characterization of *Ktedonobacteria* genomes; 2) identified large numbers of novel secondary metabolite BGCs and CAZymes in 23 available *Ktedonobacteria* genomes, thus determined the secondary metabolites biosynthetic and biomass cellulolytic potential of the class via in silico analysis. In Chapter 4, we 1) screened the *in vitro* antimicrobial activity of six representative *Ktedonobacteria* strains, thus revealed a broad-spectrum antibacterial activity of the class; 2) successfully isolated the novel anthraquinone compound COM1 from *Ts. hazakensis* COM3, thus represented the first bioactive compounds discovered from the class. Collectively, I propose here the Gram-positive, aerobic, and filamentous *Ktedonobacteria* not only represents a promising next-generation microbial resource for pharmaceutical and biotechnological uses, but also provides important research materials and values on the studies of bacterial evolution and cell differentiation.

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Figures and Tables

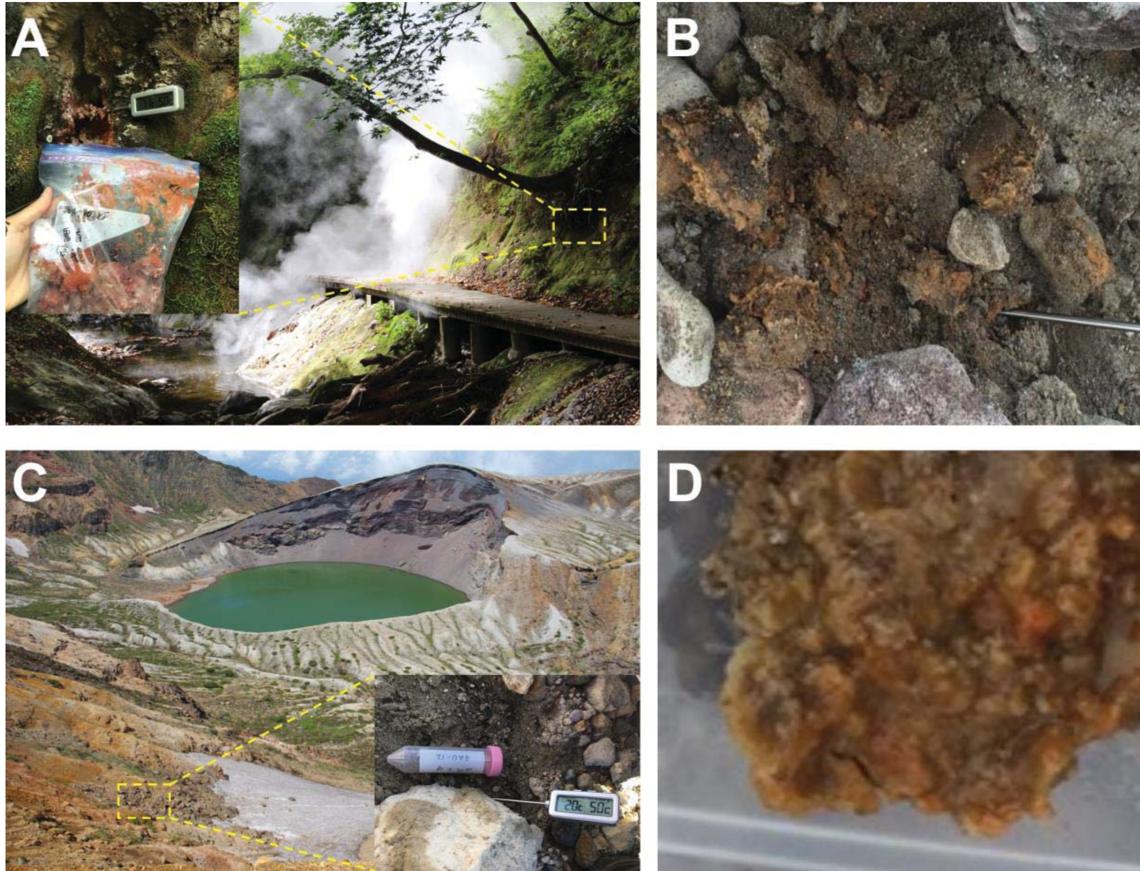


Figure 1. Collection of soil samples from Japan for strain isolation. (A), Onikobe geothermal area, Miyagi Prefecture; **(B),** "Tengu-no-mugimeshi", mountainous region of Gunma and Nagano Prefectures; **(C),** Mt. Zao, Yamagata and Miyagi Prefectures; **(D),** the photograph of "Tengu-no-mugimeshi".

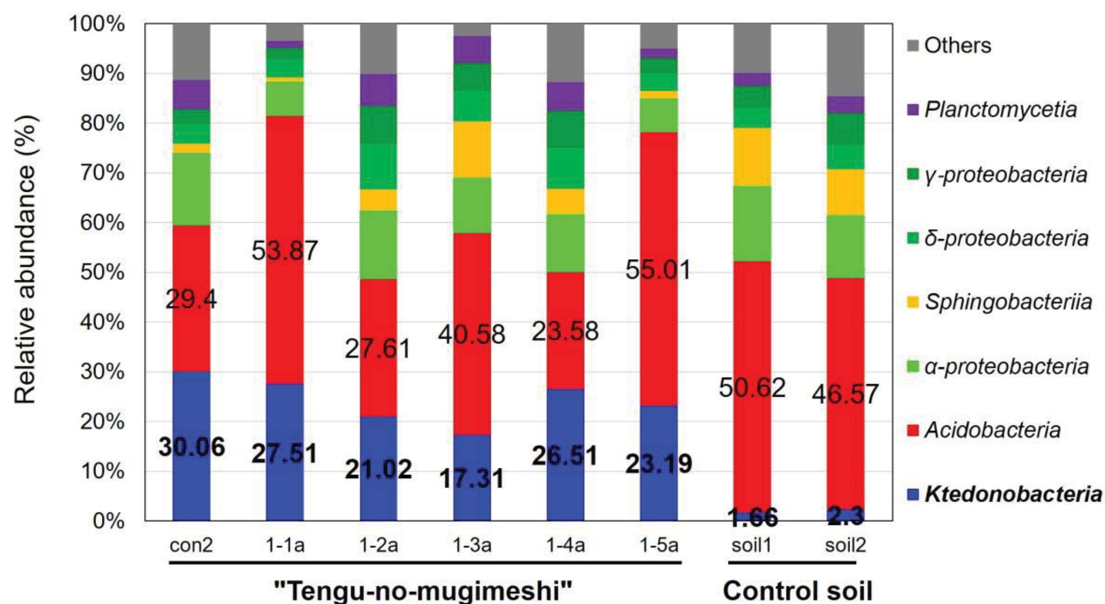


Figure 2. Bacterial community composition of the "Tengu-no-mugimeshi" at class level. Environmental DNA were extracted from the soil-like microbial mass and were used as templates to PCR amplify the V3-V4 regions of bacterial 16S rRNA gene. The amplicons were sequenced commercially on Illumina MiSeq platform and the generated reads were assigned to operational taxonomic units (OTUs) using Usearch software at 97% identity. Taxonomic information of each OTUs were annotated using RDP classifier [42]. Relative abundance of the predominate classes (>5%) in each sample are shown as numbers in the figure.

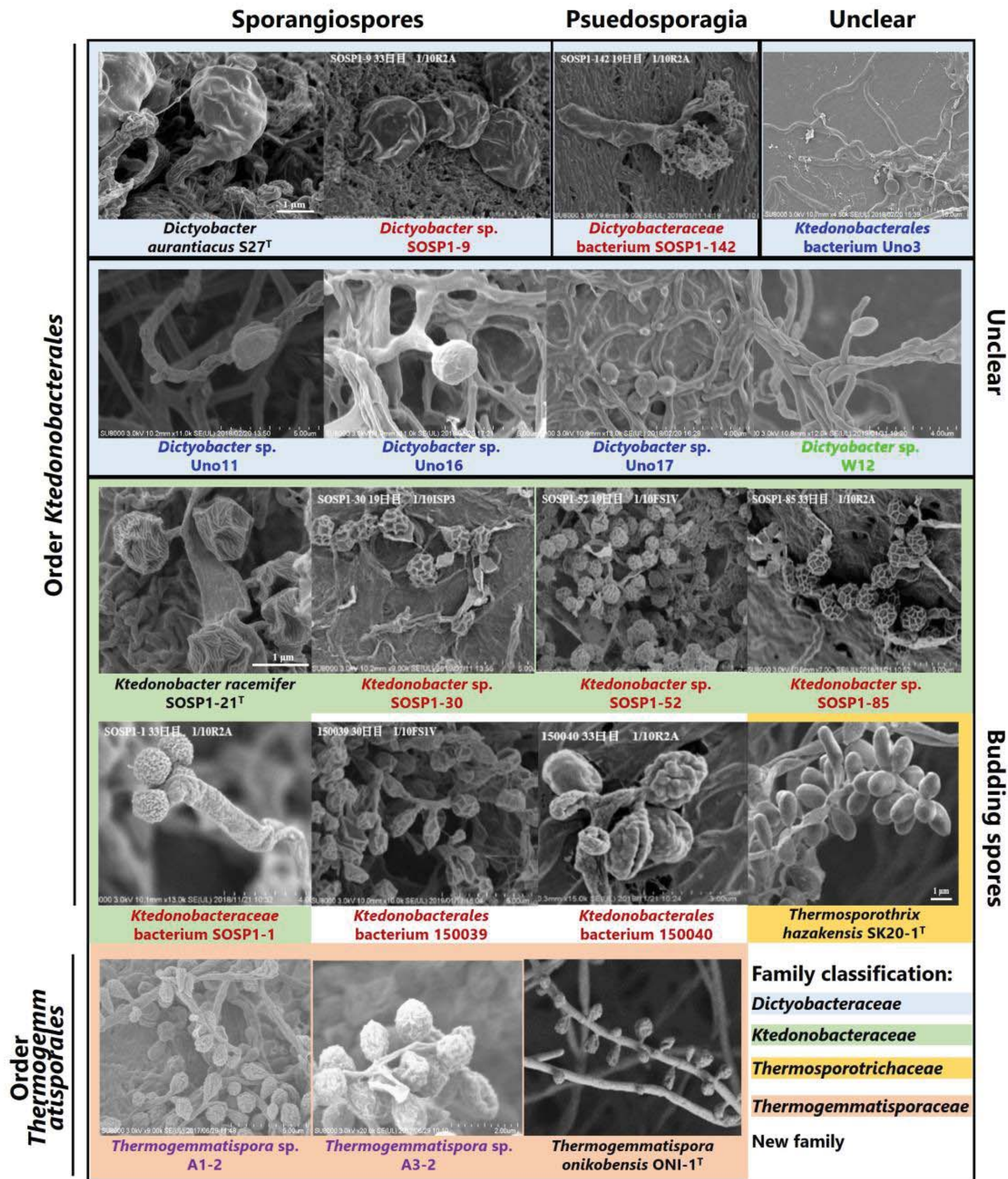


Figure 3. Scanning electron morphology of the class *Ktedonobacteria*. Isolates originated from Onikobe, "Tengu-no-mugimeshi", Mt. Zao, and Dr. Cavaletti in Italy are emphasized in bold purple, bold blue, bold green, and bold red, respectively. Families *Dictyobacteraceae*, *Ktedonobacteraceae*, *Thermosporotrichaceae*, and *Thermogemmatisporaceae* are shaded in blue, green, yellow, and red, respectively. Order classification of these strains are given as bars on the left. Types of spore formation are given at the top or on the right of the figure.

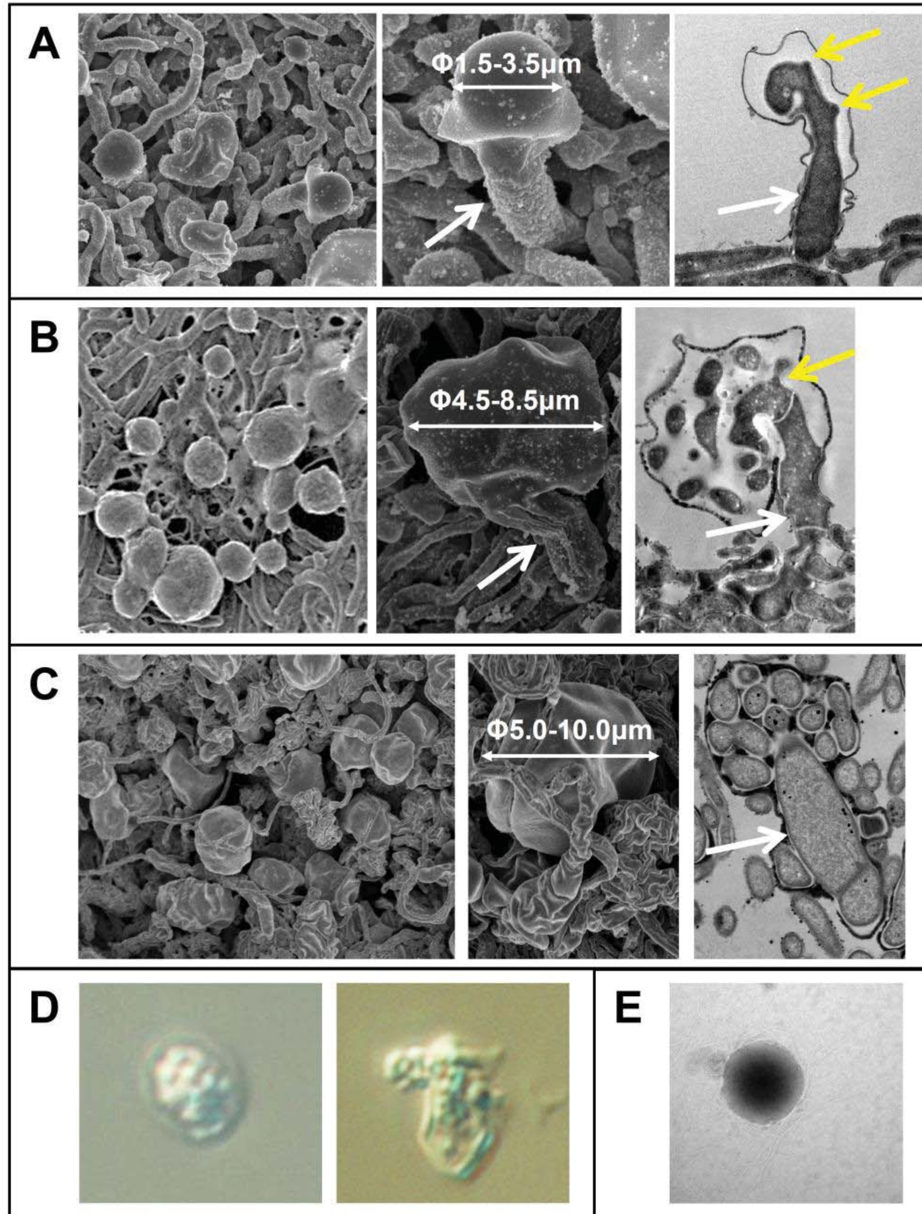


Figure 4. SEM, TEM, and phase-contrast microscopy observation of sporangia formation by *D. aurantiacus* S27^T. (A), early stage sporangia at 7 days; **(B)**, middle stage sporangia at 14 days; **(C)**, mature stage sporangia at 21 days; **(D)**, sporangium dehiscence and sporangiospores releasing; **(E)**, negatively stained TEM micrograph of sporangiospore. Stalk cells and budding spores are indicated by white and yellow arrows, respectively. Diameter of the sporangia at different stages are marked in the figures directly. *D. aurantiacus* S27^T as cultured on 10-fold diluted R2A gellan gum plates at 30 °C anaerobically (H_2:CO_2=8:2).

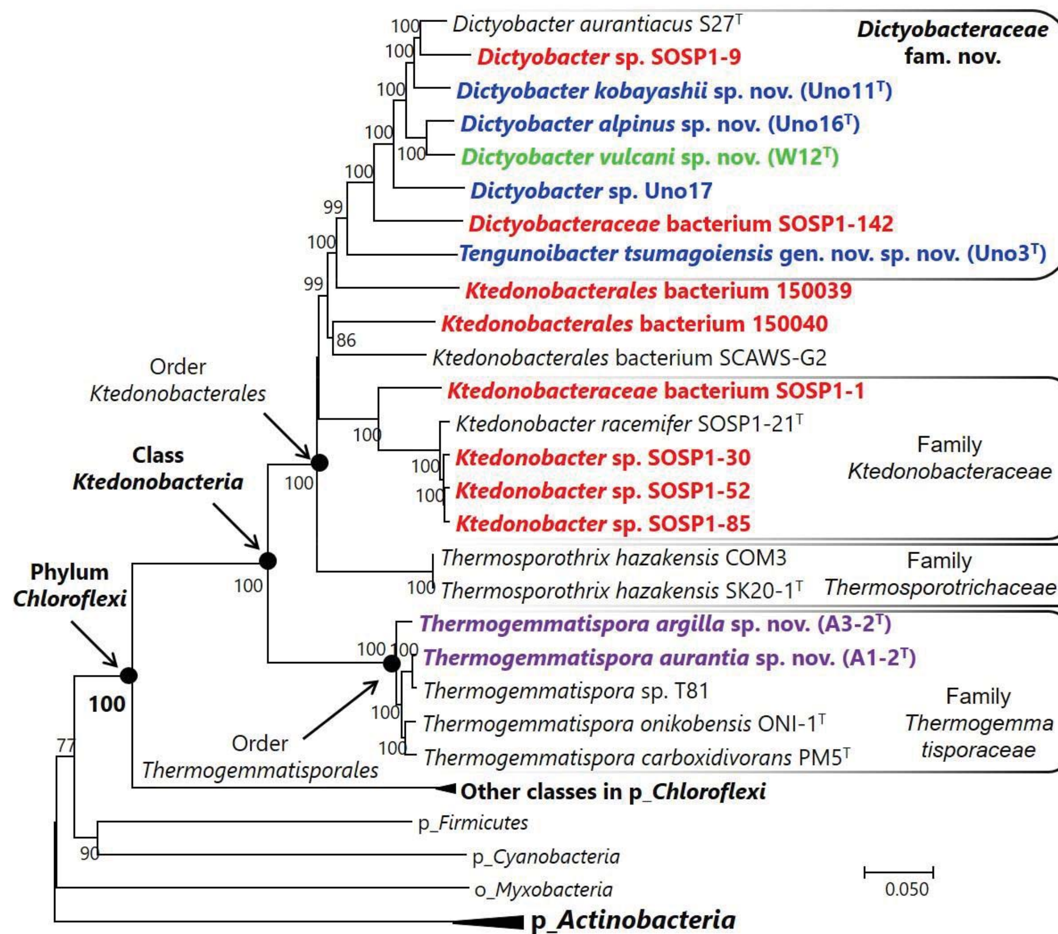


Figure 5. Phylogenetic analysis of the fifteen novel *Ktedonobacteria* isolates and reconstruction of the phylogenetic position of the class *Ktedonobacteria*. The phylogenomic tree was reconstructed on the basis of 13 core genes extracted from those genomes using the USEARCH algorithm (50% sequence identity cut-off by default). Neighbor-joining (NJ) method in MEGA v. 7.0 software was used to build the tree. Bootstrap support rates based on 1000 replicates are shown as numbers at nodes; only values larger than 70% are shown. Scale bar, 5% amino acids sequence dissimilarity. The fifteen novel *Ktedonobacteria* isolates originated from Onikobe, "Tengu-no-mugimeshi", Mt. Zao, and Dr. Cavaletti in Italy are emphasized in bold purple, bold blue, bold green, and bole red, respectively.

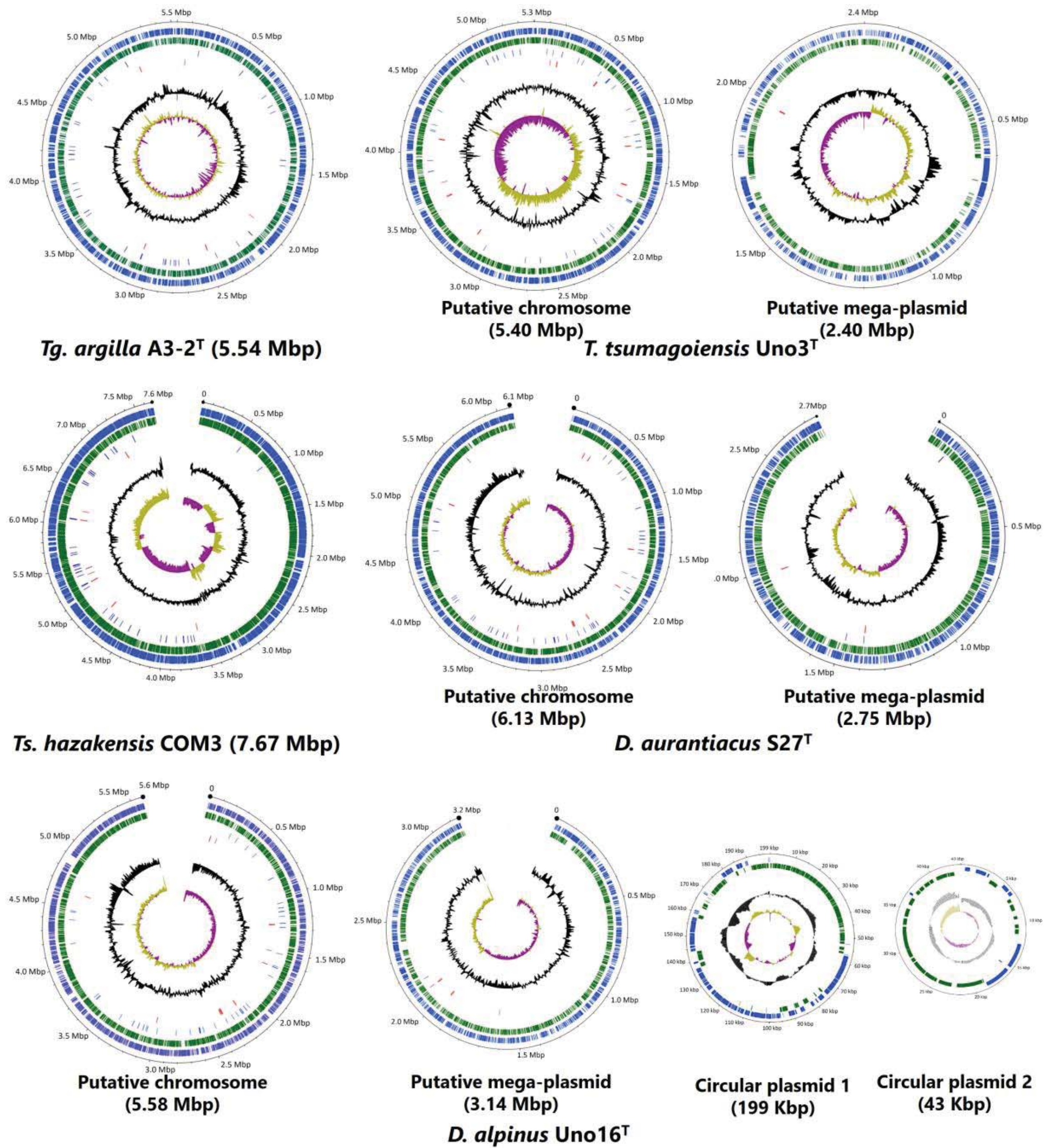


Figure 6. Complete genome plot of strains of *Tg. argilla* A3-2^{T}, *Ts. hazakensis* COM3, *T. tsumagoiensis* Uno3^{T}, *D. aurantiacus* S27^{T}, and *D. alpinus* Uno16^{T}. From outer layer to inner later: Circular 1 & 2, ORFs on the forward strand (blue) and reverse strand (green) respectively. Circular 3 & 4, tRNA genes (blue) and rRNA genes (red), respectively. Circle 5, GC content. Circular 6, GC skew (Orange above average, purple below average).

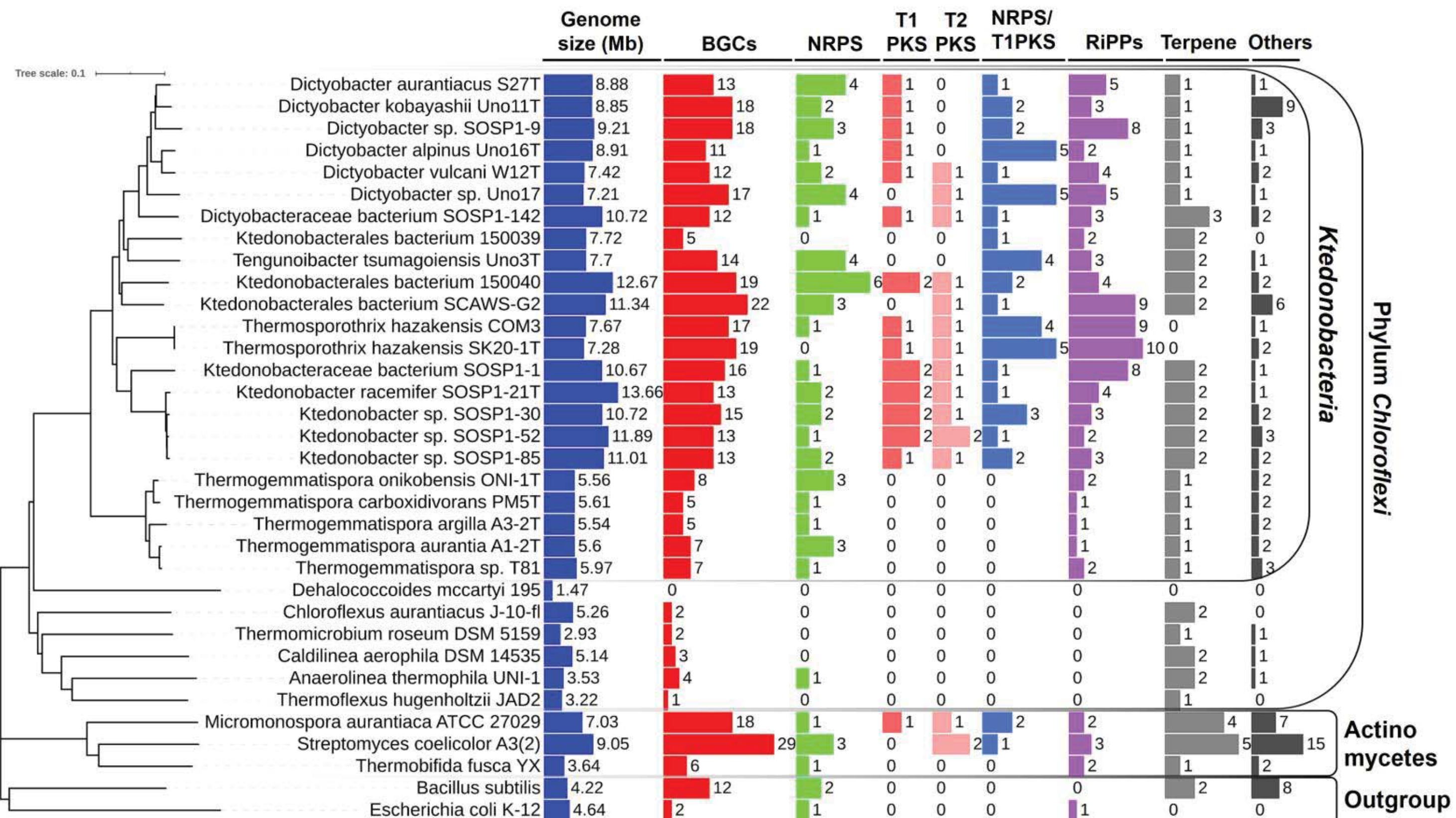


Figure 7. Composition and distribution of putative biosynthetic gene clusters (BGCs) for secondary metabolites in 23 available *Ktedonobacteria* genomes. The putative BGCs were identified by antiSMASH v5.0 at default mode and visualized using the iTOL v4 tool [43]. NRPS, non-ribosomal peptide synthase; PKS, polyketide synthase; RiPP, ribosomally synthesized and post-translationally modified peptide family (lanthipeptide, lasso peptide, thiopeptide, bacteriocin, and linear azol(in)e-containing peptide (LAP)).

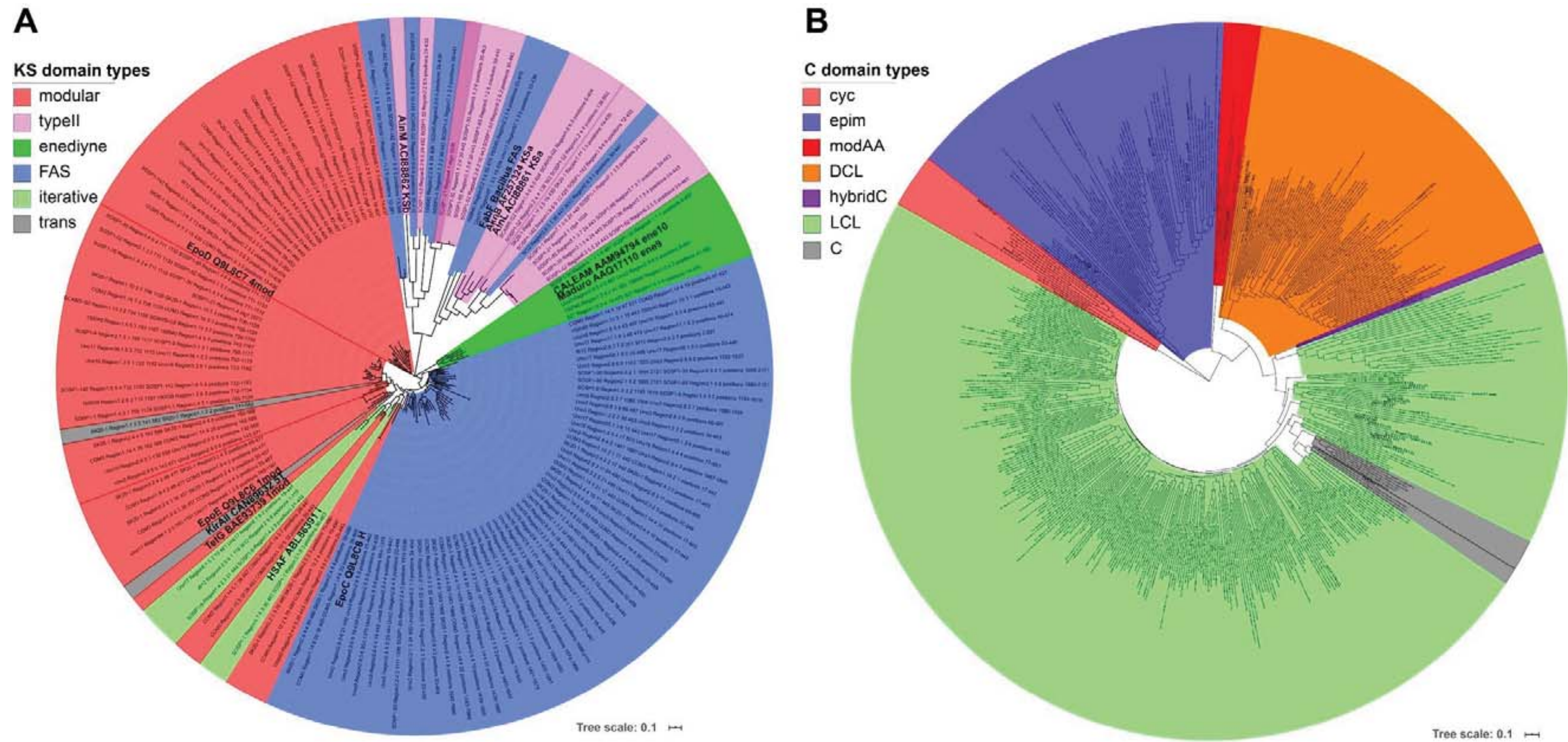


Figure 8. Functional and evolutionary analysis of *Ktedonobacteria* PKS-KS (A) and NRPS-C (B) domains. Amino acid sequences of the two domains were extracted and functionally classified with natural product domain seeker (NaPDoS) [30]. The phylogenetic tree was built by the same method with Fig. 5 and visualized using the iTOL v4 tool [43].

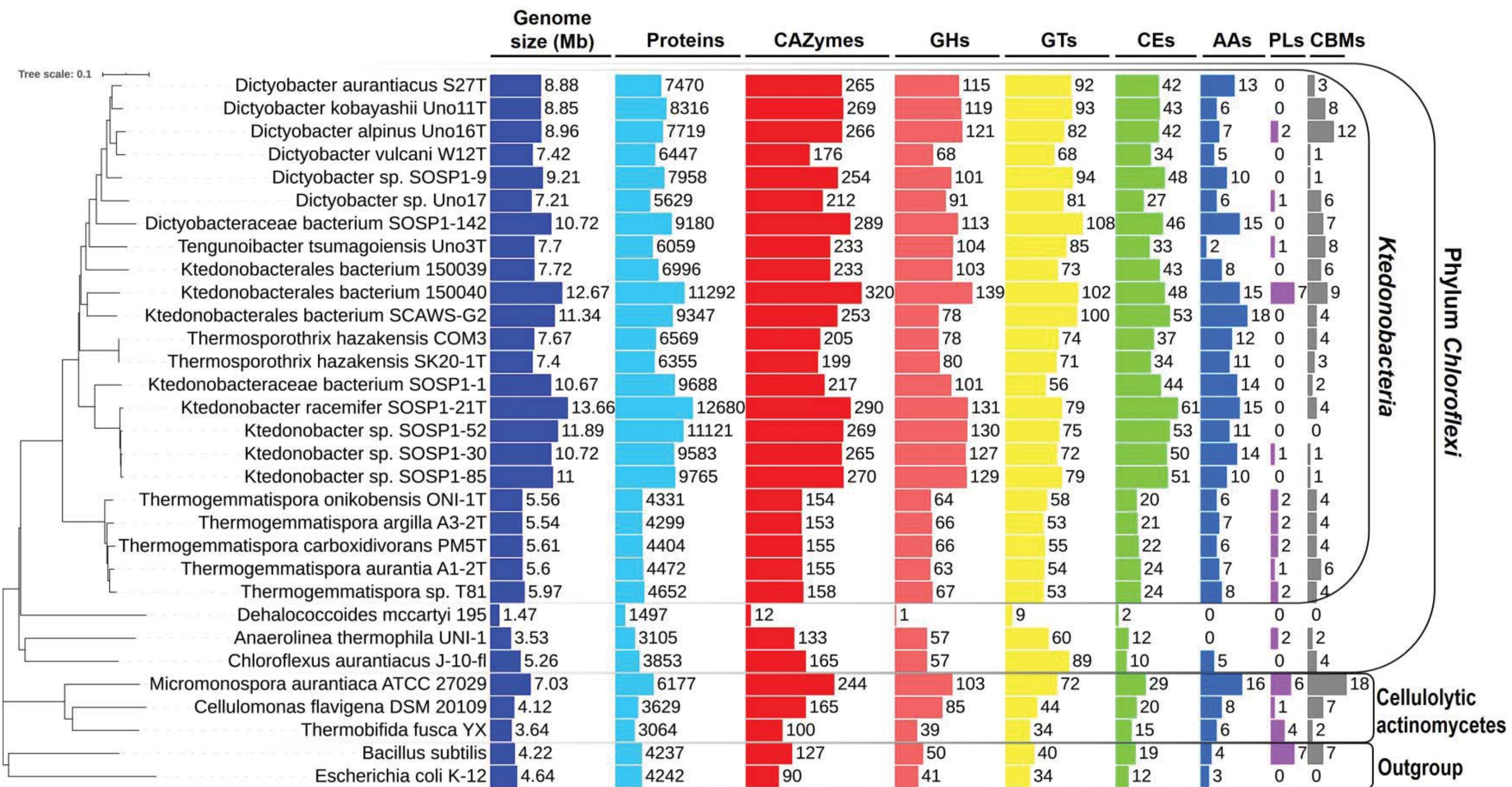


Figure 9. Composition and distribution of CAZymes in 23 available *Ktedonobacteria* genomes. The putative CAZymes were annotated with the dbCAN2 web server [44], and visualized. GH, glycoside hydrolases; GTs, glycosyltransferases; CEs, carbohydrate esterases; AAs, auxiliary activities; PLs, polysaccharide lyases; CBMs, carbohydrate-binding modules.

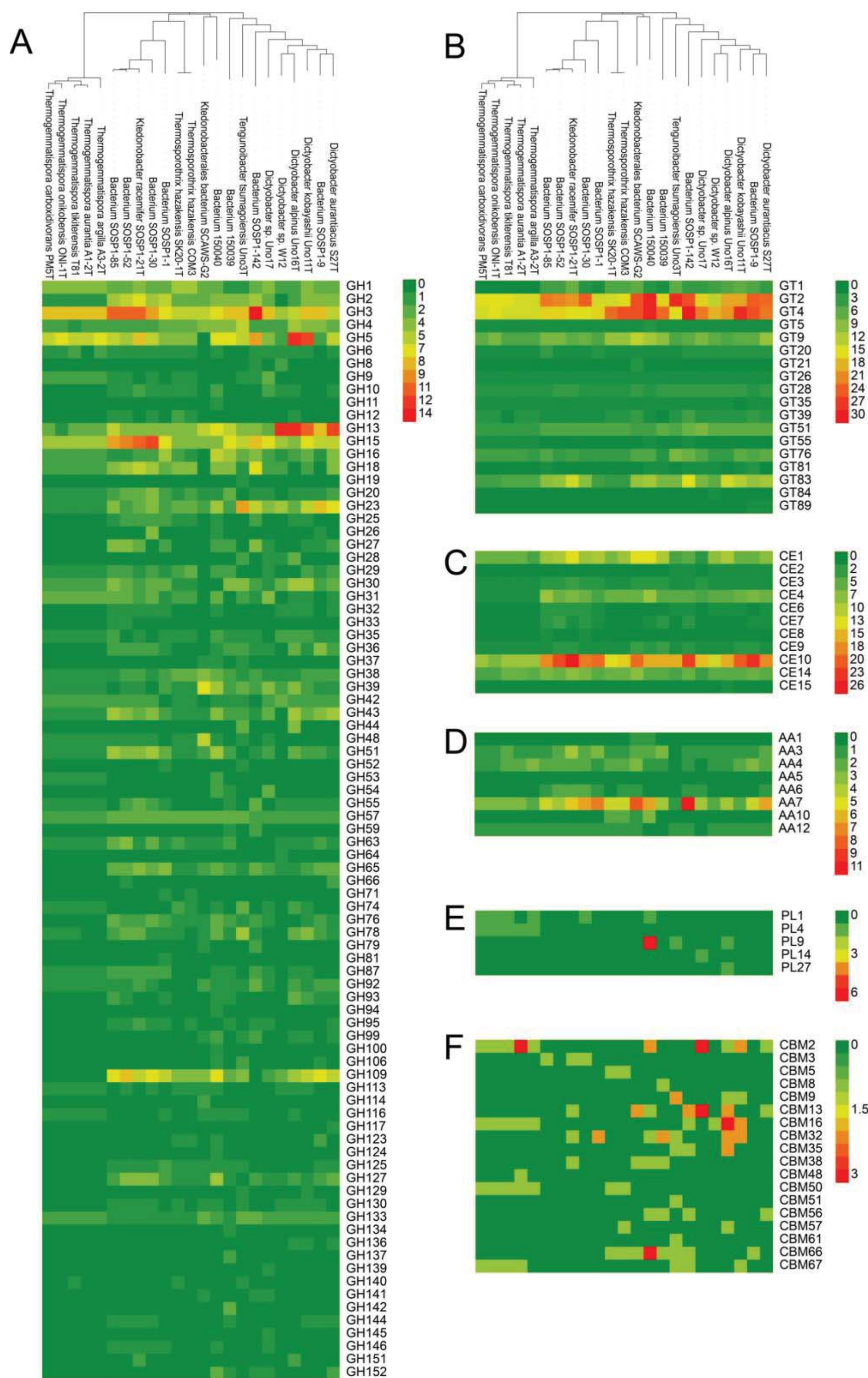
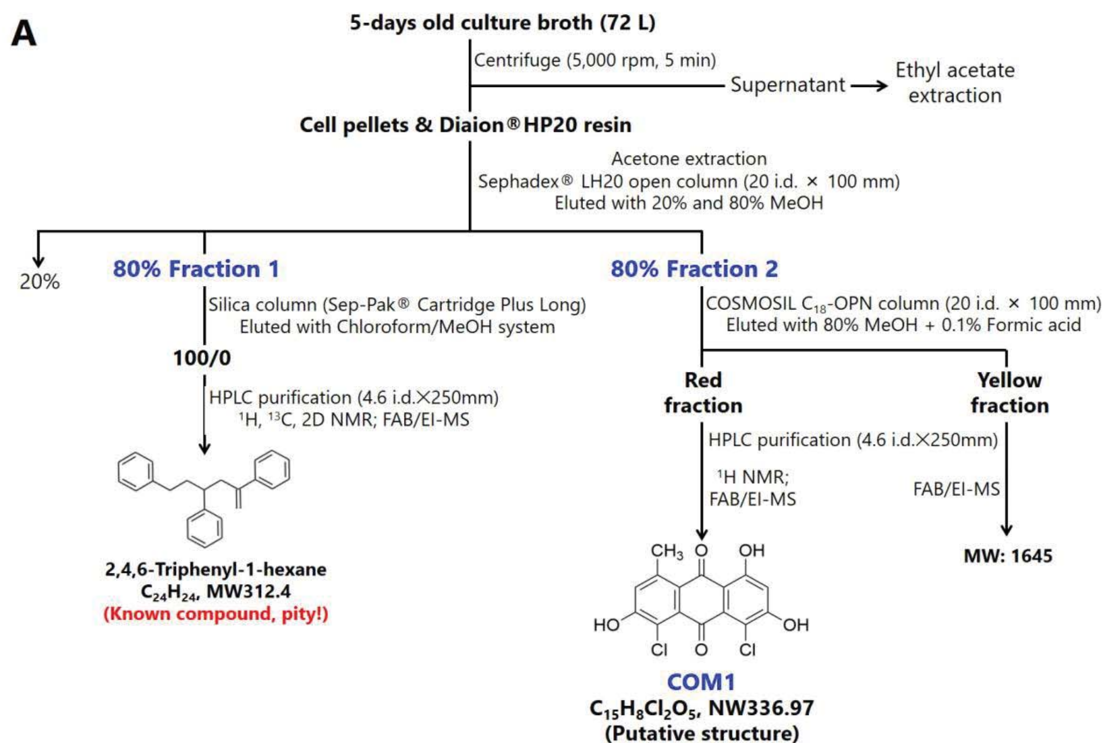


Figure 10. Composition and distribution of CAZyme families in 23 *Ktedonobacteria* genomes. (A) GH families, **(B)** GT families, **(C)** CE families, **(D)** AA families, **(E)** PL families, and **(F)** CBM families.



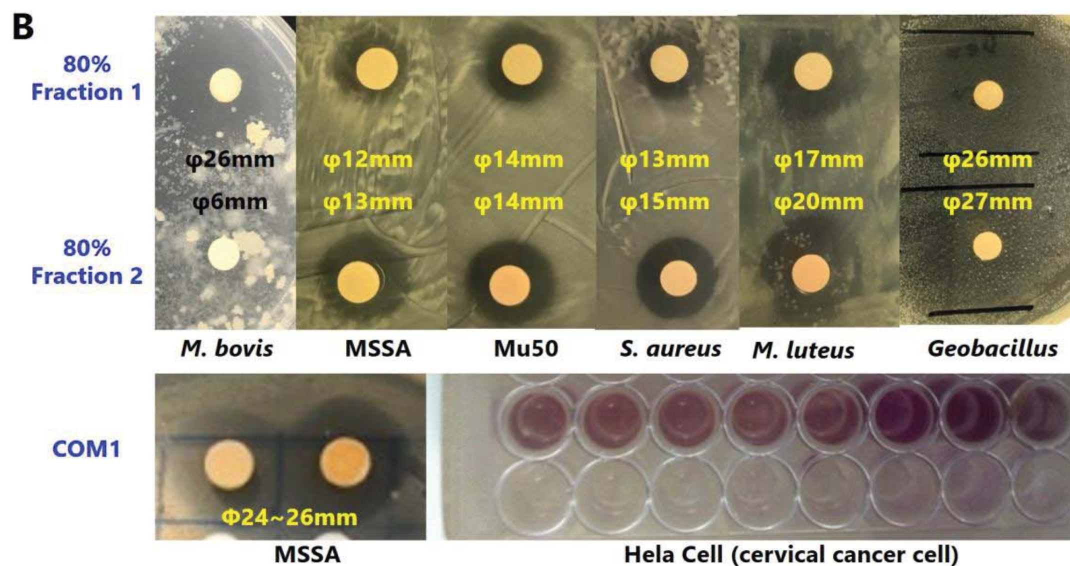


Figure 11. Discovery of novel bioactive compound from *Ts. hazakensis* COM3. (A) Purification of 2,4,6-Triphenyl-1-hexene and novel anthraquinone compound COM1. **(B)** Bioactivity screening against *Mycobacterium bovis*, *Staphylococcus aureus* NBRC 13276, *S. aureus* NTCT8325 (MSSA), *S. aureus* Mu50 (VRSA), *Micrococcus luteus* NBRC 13867, *Geobacillus stearothermophilus* NBRC 13737, and Hela Cell.

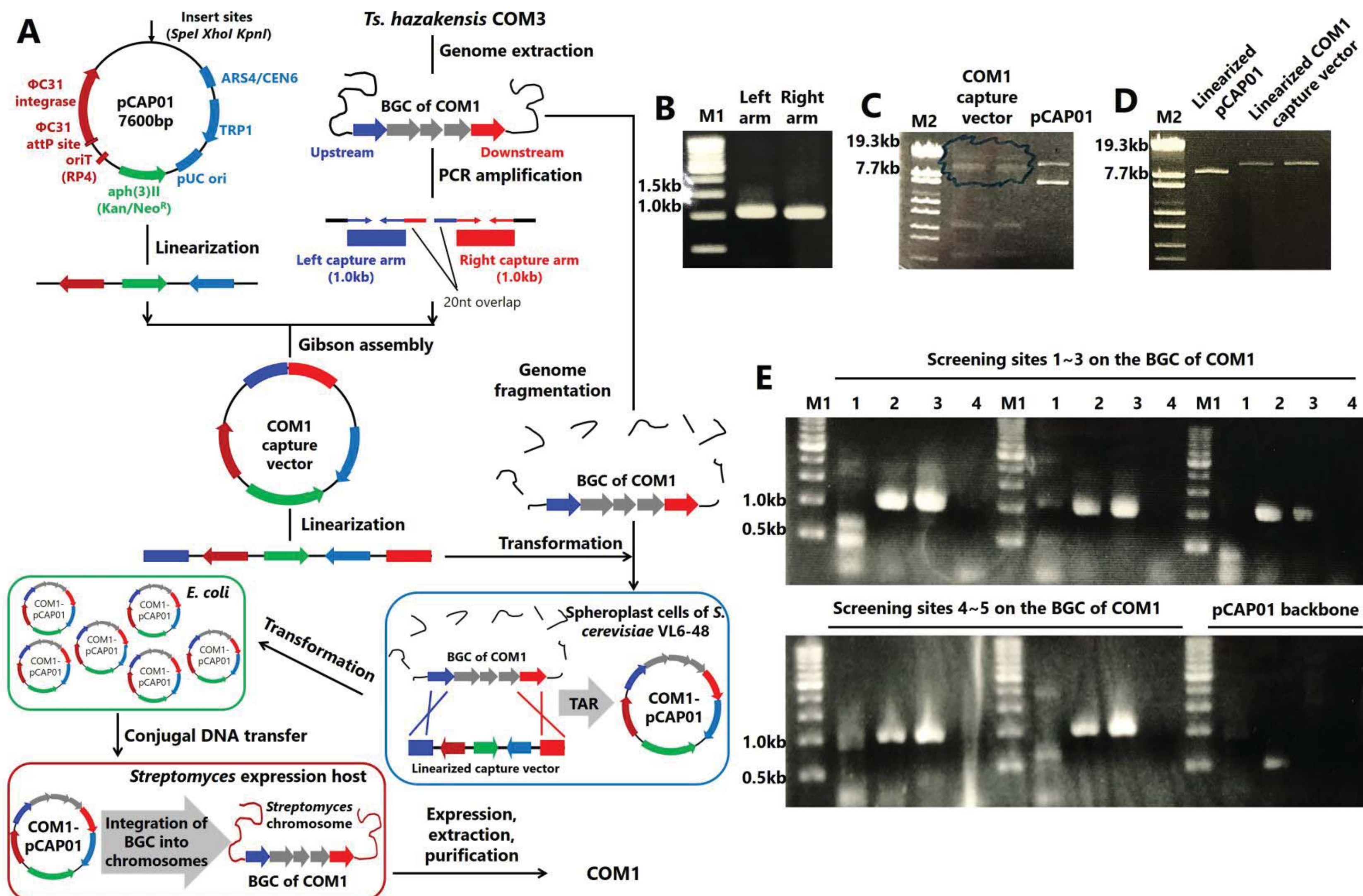

A
Insert sites
(SpeI XhoI KpnI)
ΦC31 integrase
ΦC31 attP site
oriT (RP4)
pCAP01 7600bp
ARS4/CEN6
TRP1
pUC ori
aph(3)II (Kan/Neo^R)
Linearization
Ts. hazakensis COM3
Genome extraction
BGC of COM1
Upstream
Downstream
PCR amplification
Left capture arm (1.0kb)
Right capture arm (1.0kb)
20nt overlap
Gibson assembly
COM1 capture vector
Linearization
Genome fragmentation
BGC of COM1
Transformation
E. coli
COM1-pCAP01
Transformation
Spheroplast cells of S. cerevisiae VL6-48
BGC of COM1
TAR
Linearized capture vector
COM1-pCAP01
Conjugal DNA transfer
Streptomyces expression host
COM1-pCAP01
Integration of BGC into chromosomes
Streptomyces chromosome
BGC of COM1
Expression, extraction, purification
COM1
B
M1
Left arm
Right arm
1.5kb
1.0kb
C
M2
COM1 capture vector
pCAP01
19.3kb
7.7kb
D
M2
Linearized pCAP01
Linearized COM1 capture vector
19.3kb
7.7kb
E
Screening sites 1~3 on the BGC of COM1
M1 1 2 3 4 M1 1 2 3 4 M1 1 2 3 4
1.0kb
0.5kb
Screening sites 4~5 on the BGC of COM1
pCAP01 backbone
1.0kb
0.5kb

Figure 13. Cloning of the type II PKS gene cluster from *Ts. hazakensis* COM3. (A) Scheme of the TAR-based cloning and expression approach [40, 41]. The pCAP01 vector was bought from Addgene. **(B)** PCR amplification of the left and right capture arms (1.0 kb) using genomic DNA of *Ts. hazakensis* COM3 as a template. M1, OneSTEP Ladder 500 (0.5-5 kb) DNA marker. **(C)** Gibson assembly of the left and right capture arms with pCAP01 vector to construct the specific COM1 gene cluster capture vector. M2, λ-EcoT14 I digest DNA marker. **(D)** Linearization of the specific COM1 capture vector with BamHI-HF® DNA restriction enzyme for yeast co-transformation. **(E)** Verification of the generated COM1-pCAP01 construct. Five sites (1.0 kb) of the gene cluster and one site (0.5 kb) of the pCAP01 backbone were PCR amplified. Lane 1, COM1-pCAP01 construct extracted from *E. coli* Stbl4 transformants; Lane 2, COM1-pCAP01 construct extracted from culture broth of the positive yeast colonies; Lane 3, genomic DNA of *Ts. hazakensis* COM3; Lane 4, negative control.

Table 1. Physiological and chemotaxonomic characteristics of the class *Ktedonobacteria*.

Phylum	*Chloroflexi*							*Actinobacteria*		
Class	***Ktedonobacteria***				*Chloroflexi*	*Anaerolineae*	*Caldilineae*	*Actinomycetes*		*Actinobacteridae*
Order	*Ktedonobacterales*			*Thermogemmatisporales*	*Chloroflexales*	*Anaerolineales*	*Caldilineales*	*Actinomycetales*		*Micromonosporales*
Family	*Dictyobacteraceae*	*Ktedonobacteraceae*	*Thermosporotrichaceae*	*Thermogemmatisporaceae*	*Chloroflexaceae*	*Anaerolineaceae*	*Caldilineaceae*	*Actinomycetaceae*	*Micromonosporaceae*	*Micromonosporaceae*
Spore formation	**Sporangiospore**	**Budding**			-			Segmentation		Sporangiospores
Oxygen demand	Aerobic				Aerobic	Anaerobic		Aerobic		
Gram stain	**Positive**				Negative			Positive		
G+C (mol%)	50			60	54~60	54~59	59	54~>70	>70	
Optimal temp. (°C)	25~30		50	**55~65**	20~25, 55	55		25-35		
Optimal pH	6.0-7.0				7.0		7.5-8.0	6.5-8.0		7
Nutrition metabolism	Heterotrophic				Mixotrophic	Heterotrophic		Heterotrophic		
Cellulose hydrolysis	Variable		**Cellulolytic**		-	Cellulolytic	-	Cellulolytic		
Major menaquinone	**MK-9(H2) or MK-9**	**MK-9(H2)**			MK-10	-	MK-10	MK-9 (H6)	MK-9(H8)	MK-9(H4 or H6)
Cell wall amino acids	Variable	Ser			Glu, Ala, Orn	ND	ND	LL-DAP, Gly	meso-DAP, Gly	
	Glu, Gly, Ala, **β-Ala**, Orn									
Cell wall sugars	Complex mix	Xyl	Man	Man, Ara, Xyl	ND	ND	ND	-	Ara, Xyl	
Major cellular fatty acid	$C_{16:0}$, **$C_{16:1}$-2OH**, iso-$C_{17:0}$, iso-$C_{17:0}$			iso-$C_{17:0}$, iso-$C_{19:0}$, **12,17-dimethyl $C_{18:0}$**	$C_{18:0}$, $C_{16:0}$, $C_{18:1}$	$C_{16:0}$, $C_{15:0}$, $C_{14:0}$, $C_{18:0}$	$C_{18:0}$, $C_{16:0}$, $C_{17:0}$	Complex mix		

Abbreviations: Ala, alanine; β-Ala, β-alanine; Glu, glutamic acid; Gly, glycine; Ser, serine; Orn, ornithine; DAP, 2,6-diaminopimelic acid. Man, mannose; Ara, arabinose; Xyl, xylose. 12,17-dimethyl $C_{18:0}$, 12,17-Dimethyloctadecanoic acid.

Table 2. Sequencing information and general features of the class *Ktedonobacteria*.

Order	Family	Strain	Coverage	Contigs/ Complete	Genome size (Mb)	G+C (mol%)	CDSs	rRNA	tRNA	Reference
Ktedonobacterales	*Dictyobacteraceae*	*Dictyobacter aurantiacus* S27^T	423x 127x	**2 linear complete**	8.88	54.0	7470	27	67	This study
		Dictyobacter sp. SOSP1-9	84x	63 contigs	9.21	51.1	7958	25	59	This study
		Dictyobacter sp. Uno17	150.1x	256 contigs	7.21	49.7	5629	11	67	This study
		Dictyobacter kobayashii Uno11^T	47x	2 contigs	8.85	50.3	8316	27	64	This study
		Dictyobacter vulcani W12^T	53x	7 contigs	7.42	49.7	6447	28	64	This study
		Dictyobacter alpinus Uno16^T	204x 128x	**4 linear complete**	8.91	49.7	7628	28	64	This study
		Dictyobacteraceae bacterium SOSP1-142	78x	5 contigs	10.72	50.7	9180	26	67	This study
		Tengunoibacter tsumagoiensis Uno3^T	860x 83x	**2 circular complete**	7.70	49.4	6059	28	64	This study
	Ktedonobacteraceae	*Ktedonobacter racemifer* SOSP1-21^T	24.6x 10.1x	10 contigs	13.66	53.8	12680	23	63	[5]
		Ktedonobacteraceae bacterium SOSP1-1	81x	15 contigs	10.67	51.6	9688	19	65	This study
		Ktedonobacter sp. SOSP1-30	75x	9 contigs	10.72	53.7	9583	22	67	This study
		Ktedonobacter sp. SOSP1-52	50x	9 contigs	11.89	53.7	11121	22	62	This study
		Ktedonobacter sp. SOSP1-85	89x	3 contigs	11.01	53.9	9765	23	66	This study
	Nov.	*Ktedonobacterales* bacterium 150039	115x	4 contigs	7.72	54.4	6996	8	50	This study
	Nov.	*Ktedonobacterales* bacterium 150040	74x	4 contigs	12.67	52.0	11292	14	71	This study
	Unknow	*Ktedonobacterales* bacterium SCAWS-G2	205x	1 contig	11.34	51.8	9626	12	53	No data
	Thermosporotrichaceae	*Thermosporothrix hazakensis* SK20-1^T	24x	3 contigs	7.28	53.1	6355	15	62	This study
		Thermosporothrix hazakensis COM3	663x 148x	**1 linear complete**	7.67	53.2	6569	15	63	This study
Thermogemmatisporales	*Thermosporotrichaceae*	*Thermogemmatispora onikobensis* ONI-1^T	140x	112 contigs	5.56	61.1	4331	2	47	[6]
		Thermogemmatispora carboxidivorans PM5^T	ND	1 contig	5.61	60.9	4404	8	49	No data
		Thermogemmatispora aurantia A1-2^T	152.4x	18 contigs	5.60	60.9	4472	2	48	This study
		Thermogemmatispora argilla A3-2^T	287x 161x	**1 circular complete**	5.54	60.4	4299	8	49	This study
		Thermogemmatispora sp. T81	25.9x	61 contigs	5.97	59.9	4652	3	46	No data

論文審査の結果の要旨及び担当者

氏　　　　名	鄭　宇
審　査　委　員	主査：教授 阿部　敬悦 副査：教授 米山　裕　　准教授 新谷　尚弘　　准教授 矢部　修平
学位論文題目	Isolation and taxonomy study of unexplored microbial resource *Ktedonobacteria* for discovery of novel bioactive compounds （未開拓微生物資源クテドノバクテリアの分離と系統分類及び新規生物活性物質の探索）

論　文　審　査　の　結　果　の　要　旨

近年、多剤耐性菌による感染症が拡大する中、創薬資源「放線菌」からの新規抗生物質の創出は急減しており、探索源の開拓が急務である。2006 年に創設された新奇系統「クテドノバクテリア（綱）」は、共通して放射状の気菌糸に胞子を形成する放線菌様の形態を持ち、種々の生物活性を示すことから、放線菌の次の世代の探索源として期待できる。この分類群は未培養菌群からなる巨大な系統であるが、培養株が少なく遺伝資源として未開拓であるため多様な培養菌種を得ることが課題であった。

候補者の研究は、クテドノバクテリア（綱）の新しい系統に属する細菌の分離と分類及びゲノム解析、新規二次代謝物の探索に取り組んだものである。本研究において候補者はいくつかの新しい知見を得たので以下に報告する。

（1）各種自然界からクテドノバクテリアを探索したところ、宮城県の鬼首温泉地熱地帯の土壌から 2 株、蔵王山御釜湖付近の土壌から 1 株、群馬県や長野県の山岳地帯に棲息し「食べられる土」として知られる微生物塊「天狗の麦飯」から 16 株の分離培養に成功した。それらの培養生理学的性質、化学分類学的性質及び分子系統を解明し、1 新科（*Dictyobacteraceae* fam. nov.）、1 新属・1 新種（*Tengunoibacter tsumagoiensis* gen. nov., sp. nov.）、5 新種（*Thermogemmatispora aurantia* sp. nov., *T. argillosa* sp. nov., *Dictyobacter kobayashii* sp. nov., *Dictyobacter alpinus* sp. nov., *Dictyobacter vulcani* sp. nov.）を提唱した。さらに *Dictyobacter aurantiacus* が胞子嚢を形成することを見出した。胞子嚢を形成する細菌系統の存在が明らかとなったのは、放線菌、粘液細菌に次いで 3 例目である。

（2）クテドノバクテリア 18 菌種の全ゲノムを解読して解析したところ、ゲノムサイズが 5.54~13.66 Mb と原核生物としては大きく、巨大なプラスミド（2.4-3.1Mb）を保有する菌種が存在した。二次代謝物生合成遺伝子クラスターは 5-22 個と放線菌に匹敵するほど多く、分子系統解析の結果、それらのほとんどは新規であることが強く示唆された。さらに CAZymes 分類のうち，セルラーゼやヘミセルラーゼ を含む糖質加水分解酵素に属する遺伝子が 63-139 個存在した。

（3）クテドノバクテリア綱に属する好熱菌 *Thermosporothrix hazakensis* COM3 株から抗メチシリン耐性黄色ブドウ球菌に対する抗菌活性を指標に新規二次代謝物を探索したところ、強い抗菌作用を示す化合物を見出した。それを精製し、HR-MS 及び各種 NMR で構造を解析したところ、新規と推定されるアントラキノン化合物であることが判明した。

以上、候補者の研究は、分類学的知見の乏しかったクテドノバクテリア綱の系統を飛躍的に拡充して分類学的性質を解明し、その二次代謝物生合成ポテンシャルの高さを証明するに至った。これらは、候補者によって拡充されたクテドノバクテリア綱に属する基準株が、進化、細胞分化及びゲノム構造の重要な基礎研究の材料となるだけでなく、本分類群が、第二の放線菌とも称し得る、人類にとって有益な遺伝資源と成り得ること示した成果であり、審査委員一同は本論文が博士（農学）の学位論文として価値あるものと認めた。