RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2022.087

ScienceAsia 48 (2022): **635–641**

Characterization, genome annotation, and antibacterial properties of *Actinopolyspora saharensis* BKK2

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Received 19 Nov 2021, Accepted 3 Apr 2022
Available online 6 Jul 2022

ABSTRACT: Strain BKK2 was highly similar to *Actinopolyspora saharensis* (*A. saharensis*) DSM 45459^T based on phenotypic characteristics, chemotaxonomic properties, and genotypic features. It had NaCl ranges of 10–30% (w/v), light-yellow diffusion pigment, Type IV cell wall, Type A whole-cell sugar pattern, Type PIII phospholipid pattern, and no mycolic acid. Moreover, the genotypic features of the strain BKK2 were 98.56% average nucleotide identity-Blast (ANIb), 99.03% average nucleotide identity-MUMmer (ANIm), and 90.90% digital DNA-DNA hybridization (dDDH). It was, therefore, identified as *A. saharensis*. Gene clusters of biosynthetic pathways of this strain are predicted to synthesize desferrioxamine E by complete biosynthesis genes (IucA/IucC and DesABC). *A. saharensis* BKK2 displayed antibacterial activity against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 at 1.5–2.0 folds weaker than the reference drugs. The results suggested that *A. saharensis* BKK2 produced at least one bioactive compound likely to be therapeutically useful in the future.

KEYWORDS: halophilic actinomycete, *Actinopolyspora*, desferrioxamine E, antibacterial

INTRODUCTION

Actinobacteria, i.e., *Micromonospora oryzae*, *Saccharopolyspora erythraea*, *Streptomyces parvulus*, *St. lasiicapitis*, *St. tubercidicus*, and *St. armeniacus*, produce numerous bioactive metabolites including antifungals, antibacterials, stress relief/protectives, and antitumors [1, 2]. The genus *Actinopolyspora* was first described by Gochnauer et al [3] comprising halophilic actinomycetes of the class Actinobacteria, order Actinomycetales, and family Actinopolysporaceae. Currently, twelve species of *Actinopolyspora* have been described with validly published names: *A. halophila* ATCC 27976^T, *A. mortivallis* JCM 7550^T [4], *A. xinjiangensis* KCTC 19656^T [5], *A. alba* DSM 45004^T, *A. erythraea* KCTC 19372^T [6], *A. algeriensis* DSM 45476^T [7], *A. saharensis* DSM 45459^T [8], *A. mzabensis* DSM 45460^T [9], *A. lacussalsi* KCTC 19657^T [10], *A. righensis* DSM 45501^T [11], *A. biskrensis* DSM 46684^T [12], and *A. salinaria* BCC 51286^T [13]. Halophilic actinomycetes have advantages over non-halophiles by growing optimally in the presence of high salt concentration (15% w/v) and producing unique bioactive compounds [14]. They are also considered a useful resource for the discovery of novel bioactive metabolites [15].

However, there have been not many studies on bioactive compounds production by *Actinopolyspora*, such as erythromycin and desferrioxamine [3–13], while whole-genome sequencing and gene prediction analysis warrant an effective and non-time-consuming methodology for exploration of secondary metabolism gene clusters [15, 16]. In 2014, genome sequencing of *A. erythraea* YIM90600 revealed a new erythromycin biosynthetic gene cluster encoding two novel erythromycins as erythronolide H and erythronolide I [14]. Desferrioxamine, an iron-chelating bacterial siderophore, was recently approved by the FDA for thalassemia treatment [17]. Genome analysis and annotation are simple and productive methods for the discovery of novel/known bioactive compounds in *Actinopolyspora* species with medical benefits as biosubstances.

Therefore, here, a halophilic actinomycete (strain BKK2) was isolated from salted eggs purchased at Bangkapi Market in Bangkok Province, Thailand and characterized based on its phenotypic and chemotaxonomic properties. The genome sequence of strain BKK2 was analyzed to annotate the secondary metabolite genes and determine antibacterial activity.

MATERIALS AND METHODS

Phenotypic and chemotaxonomic characteristics of strain BKK2

A halophilic actinomycete strain BKK2 was isolated from salted eggs collected from Bangkapi Market in Bangkok Province, Thailand. The spread-plate technique was performed on complete medium (CM) containing (g/l) 150 g, NaCl; 7.5 g, casamino acid; 3 g,

tri-sodium citrate; 10 g, yeast extract; 2 g, KCl; 10 g, $MgSO_4 \cdot H_2O$; 50 mg, $FeCl_2 \cdot 4H_2O$; and 20 g, agar at pH 7.2 [7, 8, 12]. Spore morphology was determined using a scanning electron microscope (JSM-5410LV, Japan). Cultural characteristics were investigated on a medium consisting of 15% (w/v) NaCl, CM agar, nutrient agar (NA) and the international *Streptomyces* project (ISP 2 and ISP 4) [18]. Colors of aerial and substrate mycelia and any soluble pigments were observed as described previously [19]. Acid production from carbohydrates, utilization of carbohydrates, lysozyme sensitivity, and production of nitrate reductase were assessed following Gordon et al [18]. All media used for characterization testing were supplemented with 15% (w/v) NaCl (except for the NaCl concentration test), with incubation at 30 °C for 2 weeks.

Polar lipids and menaquinones were determined as described previously in chemotaxonomic studies [20, 21]. The isomeric form of diaminopimelic acid and the presence (or absence) of glycine in the cell wall were also analyzed [22], while compositions of whole-cell sugars and fatty acid proïňĄles were examined [23].

16S rRNA gene sequencing and phylogenetic tree analysis

The 16S rRNA gene was amplified according to the standard procedure as initial denaturation (95 °C for 0.5 min), 30 cycles (95 °C for 1 min, 45 °C for 1 min, and 71 °C for 2 min), and final extension (68 °C for 5 min). The purified PCR products were sequenced on a DNA sequencer (Macrogen, Korea) using universal primers: 16F358 (5′-CTCCTACGGGAGGCAGCAGT-3′); 16F536 (5′-CAGCAGCCGCGGTAATAC-3′); 16F926 (5′-AACTCAAAGGAATTGACGG-3′); 16F1112 (5′-AGTCC CGCAACGAGCGCAAC-3′); 16F1241 (5′-GCTACACAC GTGCTACAATG-3′); 16R339 (5′-ACTGCTGCCTCCCG TAGGAG-3′); 16R519 (5′-GTATTACCGCGGCTGCTG-3′); and 16R1093 (5′-GTTGCGCTCGTTGCGGGACT-3′) [24]. The 16S rRNA sequence was deposited in the GenBank data library and assigned accession number LC493221. The aligned 16S rRNA gene sequence of strain BKK2 against sequences of *Actinopolyspora* type strains was constructed using a phylogenetic tree based on neighbor-joining algorithms using MEGA 7.0 [25].

Genome sequencing and function annotation

Whole-genome sequencing of strain BKK2 was performed at the Japan Collection of Microorganisms using an Illumina Miseq platform (Illumina, Inc., San Diego, CA, USA). A *de novo* assembly of the reads into contigs using the St. Petersburg genome assembler (SPAdes) version 3.12 was accomplished [26]. The draft genome was deposited in GenBank with accession number JAANTE000000000. The genome was annotated following the method of Seemann [27] using Prokka software 1.12 in line with the NCBI prokaryotic genome annotation pipeline (PGAP). An average nucleotide identity (ANI) was calculated amongst the genomes of strain BKK2 and *Actinopolyspora* type strains based on ANI-Blast (ANIb) and ANI-MUMmer (ANIm) algorithms within the JSpeciesWS web service [28]. Digital DNA-DNA hybridization (dDDH) was analyzed using the genome-to-genome distance calculator (GGDC 2.1) of the BLAST$^+$ method [29]. Secondary metabolism gene clusters were evaluated using antiSMASH (version 6.0.1) to allow prediction of the biosynthetic gene clusters of secondary metabolites [30].

Antibacterial activity assay

Antimicrobial activity was determined by the disc diffusion method as described previously [31]. The test bacteria, *S. aureus* ATCC 25923 (Gram-positive bacteria) and *E. coli* ATCC 25922 (Gram-negative bacteria), were cultured in nutrient broth for 24 h and adjusted to a final concentration of 1.5×10^8 CFU/ml. Sterile filter paper discs were introduced to the surface of the inoculated nutrient agar with the bacterial strains. The test extract was impregnated into 6 mm discs and incubated overnight at 37 °C. The positive control antibiotics were gentamicin (10 mg per disc), chloramphenicol (30 μg per disc), and ampicillin (10 mg per disc). The outer diameter of the inhibitory zone was measured in millimeters (mm) using a vernier caliper. Tests were carried out in triplicate, and inhibition zone averages were reported.

RESULTS AND DISCUSSION

Phenotypic and chemotaxonomic characteristics of strain BKK2

The strain BKK2 grew well on various media: CM agar, NA, and ISP2); supplemented with 15% (w/v) NaCl, and an optimal growth was observed on the CM agar. After cultivation on CM agar for 2 weeks at 37 °C, development of fragmented yellow substrate mycelia and long chain spores on white aerial mycelia were observed. The spores were rod-shaped and non-motile (Fig. 1). The isolate utilized various sugars for growth in a wide range of NaCl concentrations. The physiological and biochemical properties of strain BKK2 compared with two type strains, *A. saharensis* DSM 45459^T and *A. biskensis* DSM 46684^T, were summarized in Table 1. Chemotaxonomic characteristics consisted of meso-diaminopimelic acid (but not glycine, type IV cell wall), arabinose including galactose in whole-cell hydrolysates (pattern type A), and phosphatidylcholine (type PIII phospholipid pattern) (Fig. 2). Three fatty acids and two menaquinones were obtained (Table 1). The chemotaxonomic features of strain BKK2 were similar to typical members of the genus *Actinopolyspora* as described previously [3–13]. Both phenotypic and chemotaxonomic characteristics

Table 1 Phenotypic and chemotaxonomic characteristics of strain BKK2, *A. saharensis* DSM 45459^T and *A. biskensis* DSM 46684^T. Strains: 1, strain BKK2; 2, *A. saharensis* DSM 45459^T (chemotaxonomic dada from Meklat et al [8]); 3, *A. biskensis* DSM 46684^T (chemotaxonomic dada from Saker et al [12]).

Characteristic	1	2	3
Diffusible pigment	Light yellow	Light yellow	Darkbrown
pH range	5–8	5–8	6–8
NaCl range (%)	10–30	10–30	10–30
Hydrolysis of			
Skim milk	−	−	+
Starch	+	+	+
Tween 80	+	+	+
Acid from:			
Cellobiose	−	−	−
Galactose	−	−	−
D-Melizitose	−	−	−
Salicin	−	−	−
Utilization from:			
Cellobiose	+	+	+
Glycerol	+	+	+
D-Melizitose	−	−	−
Salicin	+	+	−
Fatty acid proliles	anteiso-$C_{17:0}$, iso-$C_{15:0}$,	anteiso$C_{17:0}$, iso-$C_{15:0}$	anteiso-$C_{17:0}$, iso-$C_{15:0}$
Polar lipids	PG, DPG, GL, PC, PL	PG, DPG, GL, PC	PG, DPG, GL, PC
Menaquinones	MK-10(H_4), MK-9(H_4)	MK-10(H_4), MK-9(H_4)	MK-10(H_4), MK-9(H_4)
Diaminopemelic acid (DAP)	*meso*-DAP (no glycine)	*meso*-DAP (no glycine)	*meso*-DAP (no glycine)
Whole-cell hydrolysates	arabinose, galactose, glucose	arabinose, galactose, ribose	arabinose, galactose, ribose

+, positive; −, negative; GL, glycolipid; PC, phosphatidylcholine; PG, phosphatidylglycerol; DPG, diphosphotidylglycerol; PL, phospholipid.

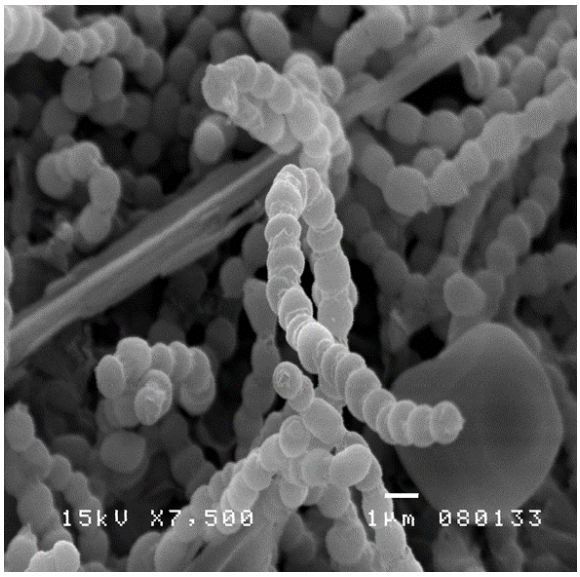

Fig. 1 Scanning electron micrograph of strain BKK2 grown on CM agar containing 15% (w/v) NaCl for 14 days. Bar, 1 μm.

suggested that strain BKK2 belonged to the genus *Actinopolyspora*.

16S rRNA gene and genome sequencing

The neighbor-joining phylogenetic tree based on 16S rRNA gene sequences of strain BKK2 (LC493221) and the representative bacterial species proved that it was closely related to members of the genus *Actinopolyspora* (Fig. 3). The 16S rRNA gene sequence similarity between strain BKK2 and *A. saharensis* DSM 45459^T at 99.63% confirmed their common evolutionary origin. A more precise evolutionary descent of different species has previously been reported by comparing whole-genome sequencing instead of a single gene, providing highly detailed information about how organisms either relate to or differ from one another on a genetic level [28, 29].

Genome statistics of *Actinopolyspora* species showed that they were 4 231 650–5 353 291 bp in size, with 67.5–69.5% G-C content, 3938–4967 predicted protein-coding gene sequences (CDSs), 0–12 tRNA gene sequences, 55–67 tRNA gene sequences, and 4–16 CRISPRS gene sequences (Table 2). Genome size (4 838 056 bp), G+C content (69.4%), CDSs (4348 sequences), tRNA gene (64 sequences), and CRISPRS (4 sequences) of strain BKK2 concurred with the range of the genus *Actinopolyspora*. As summarized in Table 3, average nucleotide identity-Blast (ANIb) and average nucleotide identity-MUMmer (ANIm) of the *Actinopolyspora* genome closest species were

Table 2 Genome statistics of strain BKK2 and related type strains.

Geonome of	Feature							
	Accession no.	Genome size (bp)	G+C content (%)	No. of Contigs	Protein coding gene	rRNA gene	tRNA gene	CRISPRS
1	JAANTE000000000	4,838,056	69.4	21	4,348	0	62	4
2[a]	FNKO00000000	4 682 228	69.5	2	4211	16	59	8
3[a]	AQUI00000000	5 353 291	68.0	2	4967	12	57	7
4[a]	AQZN00000000	4 231 650	68.8	18	3938	9	57	16
5[a]	CP022752	5 243 930	68.8	1	4747	10	67	12
6[a]	FNJR00000000	5 038 150	68.5	34	4620	1	57	9
7[a]	FOMZ00000000	5 240 643	67.7	42	4686	0	58	11
8[a]	FPAT00000000	4 924 010	67.5	23	4435	0	60	8
9[a]	FNFM00000000	5 004 253	67.7	25	4455	0	55	4

Strains: 1, BKK2; 2, *A. saharensis* DSM 45459^T; 3, *A. halophila* ATCC 27976^T; 4, *A. mortivallis* JCM 7550^T; 5, *A. erythraea* KCTC 19372^T; 6, *A. xinjiangensis* KCTC 19656^T; 7, *A. alba* DSM 45004^T; 8, *A. righensis* DSM 45501^T; 9, *A. mzabensis* DSM 45460^T; [a] Data obtained from GenBank.

Table 3 ANIb, ANIm, and dDDH values among draft genomes of strain BKK2 and their related type strains.

Genome	Reference genome	ANIb %	ANIm %	% dDDH (formula 2)[a]	Prob. DDH ⩾70%	Model C. I.
1	2	98.59	99.03	90.90	96.76	[88.6–92.7%]
1	3	90.89	92.00	43.40	84.39	[40.9–46.0%]
1	4	80.40	85.67	24.00	0.19	[21.7–26.5%]
1	5	79.81	85.54	23.60	0.01	[21.4–26.1%]
1	6	78.67	85.37	23.20	0.01	[20.9–25.7%]
1	7	78.44	85.47	23.20	0.01	[20.9–25.7%]
1	8	78.46	85.37	23.20	0.01	[20.9–25.6%]
1	9	78.37	85.41	23.30	0.01	[21.0–25.8%]

Strains: 1, BKK2; 2, *A. saharensis* DSM 45459^T; 3, *A. halophila* ATCC 27976^T; 4, *A. mortivallis* JCM 7550^T; 5, *A. erythraea* KCTC 19372^T; 6, *A. xinjiangensis* KCTC 19656^T; 7, *A. alba* DSM 45004^T; 8, *A. righensis* DSM 45501^T; 9, *A. mzabensis* DSM 45460^T. All data were obtained from the present study. [a] Recommended formula (identities/HSP length) liberated of genome length and thus prosperous against the use of incomplete draft genomes.

78.37–98.59% and 85.37–99.03%, respectively. The digital DNA-DNA hybridization (dDDH) values were highest at 90.9% (C. I. model 88.6–92.7%) for *A. saharensis* DSM 45459^T. Comparing genomes, ANI and dDDH values correlated well for the same species, with values of ⩾95 % (ANI) and ⩾70% (dDDH), respectively [29, 32]. dDDH (90.90%) and ANI (98.59–99.03%) values between strain BKK2 and *A. saharensis* DSM 45459^T were higher than the species cut off. The strain BKK2 was, therefore, identified as *A. saharensis* DSM 45459^T and named as *A. saharensis* BKK2.

Gene function annotation and secondary metabolism gene clusters

Secondary metabolism gene cluster prediction was analyzed using antiSMASH. An iron chelator cluster (desferrioxamine E), a terpene cluster, a T1PKS cluster, and an ectoine cluster were discovered through gene function annotation. Four siderophore-related genes of *A. saharensis* BKK2 showed 100% affiliation with four key enzymes, IucA/IucC, DesA, DesB and DesC, in the biosynthesis of desferrioxamine E from *Streptomyces tubercidicus* (Fig. 4) that exhibited marked inhibitory effects on the growth of Gram-positive and Gram-negative bacteria [33].

IucA/IucC is the biosynthetic gene of the siderophore encoding the enzyme-catalyzed formation of an amide bond [34]. Enzymes DesA (lysine decarboxylase), DesB (monooxygenase), and DesC (acryl transferase) have been reported as catalysts of desferrioxamine E from *St. coelicolor* [33]. Gene function annotation suggested that *A. saharensis* BKK2 produced desferrioxamine E, which functioned as an antibacterial, anti-inflammatory, and anti-cancer agent [17, 34].

Fig. 4 reveals that BKK2 consisted of three ectoine biosynthetic genes with 75% similarity to ectoine from *St. anulatus*. As for the function of protecting cells from high osmolarity, ectoine enhanced the ability of *Halomonas elongata* DSM2581^T as well as *A. saharensis* BKK2 to thrive in high salinity environments [35, 36]. Strain BKK2 showed 46% similarity with the hopene gene cluster of *St. coelicolor* A3(2). The natural compound ectoine plays an important role in stabilizing the bacterial membrane structure [33]. Hence, one gene cluster carried two enzymes (halogenase and polyketide synthases) and showed 22% similarity with

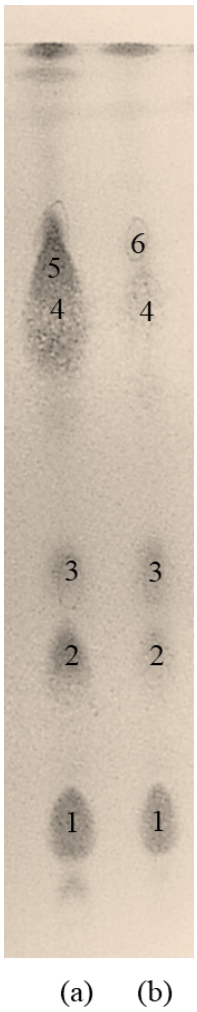

Fig. 2 Thin-layer chromatogram of total polar lipids of *A. mortivallis* JCM 7550^{T} (a) and strain BKK2 (b) after spraying the plates with specific reagents (dittmer, anisaldehyde, and dragendroff). Polar lipids: 1 and 5, glycolipids; 2, phosphatidylcholine; 3, phosphatidylglycer-rol; 4, diphosphotidylglycerol; 6, phospholipid.

the antibacterial compound armeniaspirol from *St. armeniacus* [37].

Antibacterial activity assay

Antibacterial activity was determined to confirm the prediction of biosynthetic pathways. The supernatant from *A. saharensis* BKK2 inhibited the growth of *S. aureus* ATCC 25923 and *E. coli* ATCC 25922, with size range of the clear zones of 10.5–11.2 ± 0.2 mm and 9.5–10.1 ± 0.3 mm, respectively (using DMSO as the negative control, 6.5 ± 0.2 mm). For the positive control, the inhibition zones were 17.7–29.5 ± 0.2 mm for *S. aureus* ATCC 25923 and 15.3–17.2 ± 0.3 mm for *E. coli* ATCC 25922. Strong antibacterial activity of

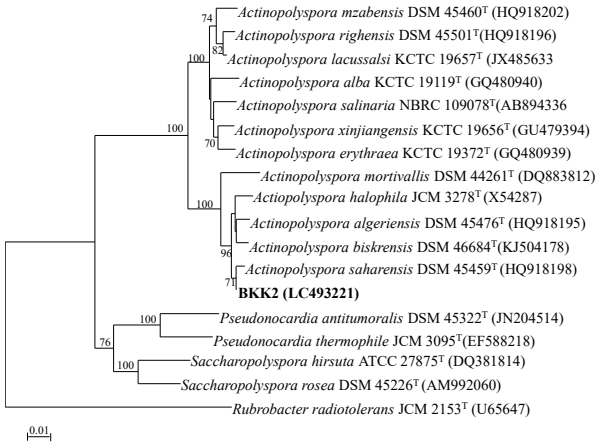

Fig. 3 Phylogenetic tree of strain BKK2 and related bacterial species based on 16S rRNA gene sequences. The branching pattern was generated by the neighbor-joining method. Bar, 10 substitutions per 1000 nucleotide positions.

bioactive compounds was suggested when the clear zone against the tested strains was higher than the reference drug by at least 1 mm [38]. Therefore, the inhibitory activity of *A. saharensis* BKK2 was weak according to its clear zone, and 1.5–2.0 folds less than the positive control. Numerous bioactive metabolites including siderophore (desferrioxamine E), ectoine, terpenes, armeniaspirol, madurastatin A1, and piericidin A1 have been reported from Actinobacteria [1, 2, 39, 40]. Siderophores as iron chelators are excreted to extract ferric iron from the environment by forming an iron-siderophore complex (ferrisiderophore) that is absorbed via specific transport mechanisms [33]. These bioactive compounds also significantly inhibited the growth of non-siderophore synthesizing bacteria caused by siderophore iron uptake systems [34]. Armeniaspirol is a potent antibiotic that is active against gram-positive pathogens by inhibiting the ATP-dependent proteases ClpXP and ClpYQ activities to dysregulate cell division [41].

Results suggested that *A. saharensis* BKK2 produced low concentrations of bioactive compounds, which were not concentrated enough to be antimicrobial by the action of iron uptake systems or cell division arrest.

CONCLUSION

The strain BKK2 isolated from salted egg was identified as *A. saharensis* based on the polyphasic taxonomy. Genome function annotation results showed that *A. saharensis* BKK2 contained four bio-cluster genes with 100% similarity to desferrioxamine E, 75% to ectoine, 46% to hopene, and 22% to armeniaspirol. The BKK2 fermentation broth exhibited weak antibacterial activity against *S. aureus* ATCC 25923 and *E. coli* ATCC 25922. Based on the results, *A. saharensis*

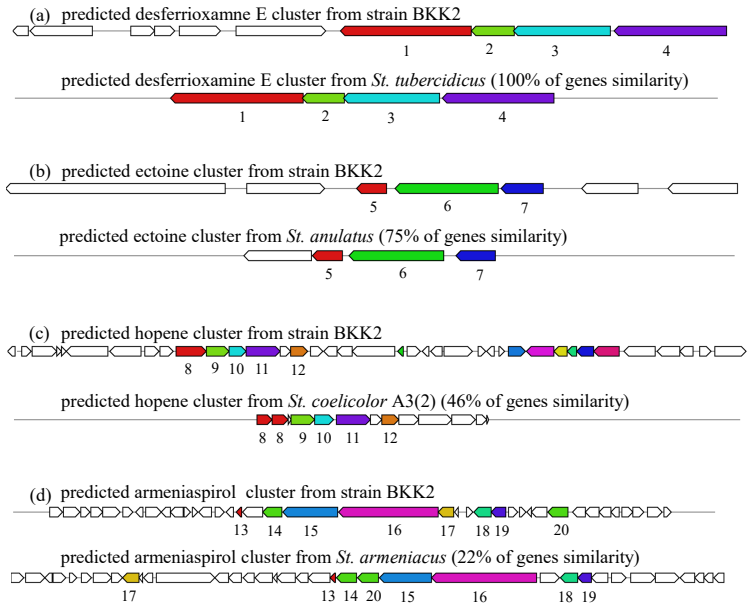

Fig. 4 Comparison of secondary metabolite gene clusters among strain BKK2 and related Species: *Streptomyces tubercidicus* (desferrioxamine E, a); *St. anulatus* (ectoine, b); *St. coelicolor* A3(2) (hopene, c); and *St. armeniacus* (armeniaspirol, d). The predicted genes: 1, IucA/IucC family siderophore biosynthesis protein; 2, acetyltransferase (DesC); 3, L-lysine 6-monooxygenase (DesB); 4, lysine decarboxylase (DesA); 5, ectoine synthase (EctC); 6, diaminobutyrate-2-oxoglutarate aminotransferase (EctB); 7, diaminobutyrate acetyltransferase (EctA); 8, squalene/phytoene synthase; 9, hydroxysqualene dihydroxylase; 10, polyprenyl synthetase; 11, squalene-hopene cyclase; 12, adenosyl-hopene transferase; 13, flavin reductase; 14, 17, 20, tryptophan halogenase; 15–16, polyketide synthase; 18, AMP-binding protein; 19, acyl-CoA dehydrogenase.

BKK2 produced a variety of bioactive compounds in low concentrations. More intensive research using response surface methodological optimization and genetic engineering approaches is required to increase the productivity of this halophilic actinomycete, which could be a valuable input into the medical industry.

Acknowledgements: We would like to thank the Institute for Research and Development, Suan Sunandha Rajabhat University for financial support and the provision of laboratory equipment and experimental space.

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