Genetic and Functional Analyses of the DKxanthene Biosynthetic Gene Cluster from *Myxococcus stipitatus* DSM 14675^S

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DKxanthenes are a class of yellow secondary metabolites produced by myxobacterial genera *Myxococcus* and *Stigmatella*. We identified a putative 49.5 kb DKxanthene biosynthetic gene cluster from *Myxococcus stipitatus* DSM 14675 by genomic sequence and mutational analyses. The cluster consisted of 15 genes (MYSTI_06004–MYSTI_06018) encoding polyketide synthases, non-ribosomal peptide synthases, and proteins with unknown functions. Disruption of the genes by plasmid insertion resulted in defects in the production of yellow pigments. High-performance liquid chromatography and liquid chromatography-tandem mass spectrometry analyses indicated that the yellow pigments produced by *M. stipitatus* DSM 14675 might be novel DKxanthene derivatives. *M. stipitatus* did not require DKxanthenes for the formation of heat-resistant viable spores, unlike *Myxococcus xanthus*. Furthermore, DKxanthenes showed growth inhibitory activity against the fungi *Aspergillus niger*, *Candida albicans*, and *Rhizopus stolonifer*.

Keywords: Myxobacteria, *Myxococcus stipitatus*, secondary metabolite, DKxanthene, biosynthetic gene

Introduction

Myxobacteria are gram-negative soil bacteria that are well known for the production of diverse bioactive secondary metabolites [1–4]. DKxanthenes are a class of yellow pigments produced by myxobacteria belonging to the genera *Myxococcus* and *Stigmatella* (Fig. 1) [5, 6]. *Myxococcus xanthus* displays phase variations between yellow- and tan-phase cells [7]. DKxanthenes are produced by yellow-phase cells but not by tan-phase cells [8]. DKxanthenes are required for developmental sporulation in *M. xanthus*. Mutant cells of *M. xanthus* defective in DKxanthene biosynthesis produce severely reduced amounts of heat-resistant viable spores [5]. It has been suggested that tan-phase cells are the progenitors of spores and yellow-phase cells provide factors that tan-phase cells need to produce viable spores [9]. Later, DKxanthene was suggested as one of the factors needed by tan-phase cells for the maturation of spores in *M. xanthus* [5].

The majority of secondary metabolites isolated from myxobacteria are biosynthesized by polyketide synthases (PKSs) and/or non-ribosomal peptide synthases (NRPSs) [10]. DKxanthenes are also biosynthesized by PKSs and NRPSs. The structure of DKxanthenes from *M. xanthus* was elucidated and their biosynthetic gene clusters were identified in *M. xanthus* DK1622 and *Stigmatella aurantiaca* DW4/3-1 [5, 6]. The organization of the two biosynthetic gene clusters identified in *M. xanthus* DK1622 and *S. aurantiaca* DW4/3-1 was similar but not identical. Both clusters have core PKS and NRPS genes for the production of the DKxanthene backbone structure. However, *S. aurantiaca* has additional PKS genes, *dkxQR* and *dkxT*. The organization of the associated genes was also different (Fig. 2) [6]. A total of 13 DKxanthene derivatives were identified from *M. xanthus* DK1622, whereas *S. aurantiaca* DW4/3-1 was reported to produce five derivatives among them [6].

M. stipitatus is another species of the genus *Myxococcus*. *M. stipitatus* forms multicellular fruiting bodies consisting

Fig. 1. Structure of DKxanthenes [5, 6].

of a spherical mass of slime and myxospores on a long stalk on the natural substrates [1]. *M. stipitatus* is known to produce melithiazol, rhizopodin, and phenalamide [11–13]. It also produces yellow pigments that appear to be DKxanthenes similar to those of *M. xanthus*. Herein, we

report that the yellow pigments produced by *M. stipitatus* are new DKxanthene derivatives that have not been identified from *M. xanthus* and *S. aurantiaca*. We also report that DKxanthenes show growth inhibitory activity against fungi.

M. stipitatus DSM 14675

S. aurantiaca DW4/3-1 (SA type)

M. xanthus DK1622 (MX type)

Fig. 2. Biosynthetic gene clusters of DKxanthene. DKxanthene biosynthetic gene clusters from *Myxococcus stipitatus* DSM 14675, *Stigmatella aurantiaca* DW4/3-1, and *Myxococcus xanthus* DK1622 are shown. ■ Polyketide synthase (PKS); ■ Non-ribosomal peptide synthetase (NRPS); ■ PKS/NRPS hybrid. Arrows indicate the locations of the plasmid insertions, which disrupted the genes in the mutant strains.

Table 1. Microbial strains used in this study.

Strains	Relevant features	Source or references
<i>Myxococcus xanthus</i> DK1622	Wild type	[28]
<i>Myxococcus stipitatus</i> DSM 14675	Wild type	DSMZ ^a
<i>Myxococcus stipitatus</i> KYC617	DSM14675 MYSTI_06016::pHS221	This study
<i>Myxococcus stipitatus</i> KYC618	DSM14675 MYSTI_06014::pHS222	This study
<i>Myxococcus stipitatus</i> KYC633	DSM14675 MYSTI_06019::pHS243	This study
<i>Myxococcus stipitatus</i> KYC634	DSM14675 MYSTI_06018::pHS244	This study
<i>Myxococcus stipitatus</i> KYC635	DSM14675 MYSTI_06010::pHS245	This study
<i>Myxococcus stipitatus</i> KYC636	DSM14675 MYSTI_06005::pHS246	This study
<i>Myxococcus stipitatus</i> KYC637	DSM14675 MYSTI_06004::pHS247	This study
<i>Myxococcus stipitatus</i> KYC638	DSM14675 MYSTI_06002::pHS248	This study
<i>Pseudomonas aeruginosa</i> ATCC 10145	Gram-negative bacterium	KCTC ^b
<i>Staphylococcus aureus</i> ATCC 25923	Gram-positive bacterium	KCTC
<i>Aspergillus niger</i> ATCC 16404	Fungus (Ascomycota, mold)	KCTC
<i>Candida albicans</i> ATCC 18804	Fungus (Ascomycota, yeast)	KCTC
<i>Rhizopus stolonifer</i> KCCM 32398	Fungus (Zygomycota, mold)	KCCM ^c

^aDSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

^bKCTC, Korean Collection for Type Cultures.

^cKCCM, Korean Culture Center of Microorganisms.

Materials and Methods

Strains

The microbial strains used in this study are listed in Table 1. *M. stipitatus* DSM 14675 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ). *M. stipitatus* KYC617, KYC618, KYC633, KYC634, KYC635, KYC636, KYC637, and KYC638 were constructed by inserting plasmids pHS221, pHS222, pHS243, pHS244, pHS245, pHS246, pHS247, and pHS248, respectively, into the chromosome of strain DSM 14675 in this study. *Aspergillus niger* ATCC 16404, *Candida albicans* ATCC 18804, *Pseudomonas aeruginosa* ATCC 10145, and *Staphylococcus aureus* ATCC 25923 were obtained from the Korean Collection for Type Culture (KCTC). *Rhizopus stolonifer* KCCM 32398 was obtained from the Korean Culture Center of Microorganisms (KCCM).

Sequence Analysis

DKxanthene biosynthetic gene clusters were analyzed using the antiSMASH program [14, 15]. DNA and amino acids sequences were analyzed using BLAST [16] and CD-Search [17] programs.

Plasmid and Strain Construction

The plasmids used in this study are listed in the supplementary Table S1. Plasmids pHS221, pHS222, pHS243, pHS244, pHS245, pHS246, pHS247, and pHS248 were constructed by inserting an internal DNA fragment of MYSTI_06016, MYSTI_06014, MYSTI_06019, MYSTI_06018, MYSTI_06010, MYSTI_06005, MYSTI_06004, or MYSTI_06002 into the pCR2.1 plasmid (Invitrogen, USA), respectively.

Sequences of the oligonucleotide primers used to amplify the internal DNA fragments of the genes by polymerase chain reaction (PCR) are shown in the Table S2.

Plasmid insertion mutants were constructed as described previously [18]. Plasmid DNA was introduced into *M. stipitatus* DSM 14675 by electroporation using Gene Pulser II (Bio-Rad, USA). Kanamycin-resistant transformants were selected. Because pCR2.1 cannot replicate in *M. stipitatus*, only cells carrying a plasmid inserted on the chromosome by homologous recombination grew in the presence of kanamycin. The insertion of the plasmids into the designated locations was confirmed by PCR using a set of oligonucleotides as primers: one binds to the pCR2.1 vector DNA and the other binds to the chromosomal DNA near the insertion site. The primers used to confirm the insertions are listed in Table S2.

Media and Culture Conditions

CYE medium [19] was used for the vegetative growth of *M. stipitatus* and *M. xanthus*. CYS medium [20] was used for DKxanthene production. CYD agar was used for fruiting body development of *M. stipitatus*. CYD agar contained 0.25% (w/v) casitone, 0.1% (w/v) yeast extract, 0.1% (w/v) MgSO₄·7H₂O, 0.05% (w/v) CaCl₂, 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.6), 0.1% (w/v) trace element solution, and 2.5% (w/v) agar. The trace element solution contained 100 mg MnCl₂·4H₂O, 20 mg CoCl₂, 10 mg CuSO₄, 10 mg Na₂MoO₄·2H₂O, 20 mg ZnCl₂, 5 mg LiCl, 5 mg SnCl₂·2H₂O, 10 mg H₃BO₃, 20 mg KBr, 20 mg KI, and 8 g EDTA Na-Fe³⁺-salt (tri-hydrate) per liter [2]. Trypticase soy

agar (TSA) and tryptic soy broth [21] were used for the growth of fungi and other bacteria besides myxobacteria. All the strains were cultured at 30°C.

Preparation of Culture Extract

Myxobacterial cells cultured on CYS agar plates for 4 days at 30°C were collected with a scraper and extracted with methanol. The methanol was evaporated from the extract, and then a mixture of ethyl acetate and water in a 1:1 ratio (v/v) was added to the residue. The solution was centrifuged to separate the ethyl acetate and water layers. The ethyl acetate layer was recovered and dried. The dried residue was dissolved in 80% (v/v) methanol.

High-Performance Liquid Chromatography (HPLC) Analysis

HPLC was performed using an HPLC system (1260 VL Infinity Series; Agilent, USA) equipped with a Zorbax SB-C18 column (4.6 × 150 mm, 5 µm) (Agilent) and a diode array detector (1260 VL Infinity; Agilent). The mobile phase A was water and mobile phase B was acetonitrile, both of which contained 0.1% formic acid. The gradient elution at a flow rate of 0.5 ml/min was performed as follows: 0–5 min 5–40% B (linear gradient), 5–25 min 40–60% B (linear gradient), and 25–35 min 100% B (isocratic).

High-Resolution Liquid Chromatography Mass Spectrometry (LC-MS) Analysis

LC-MS was performed as previously described [22] with slight modifications. The mobile phase A was water and mobile phase B was acetonitrile, both of which contained 0.1% formic acid. The gradient elution at a flow rate of 0.4 ml/min was performed as follows: 0–1 min 5–30% B (linear gradient), 1–9 min 30–50% B (linear gradient), 9–10 min 50–100% B (linear gradient), and 10–12 min 100% B (isocratic).

Antimicrobial Assay

Paper discs of 6 mm diameter (Advantec MFS, Japan) containing 10 µl of sample solution (10 mg/ml) were dried and placed on TSA plates inoculated with test microorganisms. The plates were then incubated for 2 days.

Induction of Fruiting Body Development and Analysis of Viable Spore Formation

For inducing fruiting body development, 1×10^6 cells, which were grown on CYE broth until the absorbance of the culture broth was 1.0 at 600 nm, were placed on CYD plates as a 20 µl spot and incubated at 30°C for 5 days. Cells in the spot were harvested

Table 2. Comparison of the DKxanthene biosynthetic genes from *M. stipitatus* DSM 14675 and *S. aurantiaca* DW4/3-1.

<i>M. stipitatus</i> DSM 14675					<i>S. aurantiaca</i> DW4/3-1		
ORF No.	Product size (aa)	Predicted function	PKS/NRPS motif	Identity/Similarity (%)	Gene	ORF No.	Product size (aa)
06019	183	Hypothetical protein		-	-	-	-
06018	498	NRPS	A(N/A)	77/87	<i>dkxA</i>	4842	507
06017	380	Acyl-CoA dehydrogenase		85/91	<i>dkxB</i>	4841	379
06016	711	PKS	KS-ACP	64/73	<i>dkxE</i>	4840	739
06015	1,418	PKS	KS-KR-ACP	59/70	<i>dkxF</i>	4839	1,461
06014	2,958	NRPS/PKS	C-A(thr)-PCP-KS-AT(mal)-DH-KR-ACP	74/84	<i>dkxG</i>	4838	2,971
06013	1,834	PKS	KS-AT(mal)-DH-KR-ACP	70/81	<i>dkxH</i>	4837	1,855
06012	1,833	PKS	KS-AT(mal)-DH-KR-ACP	68/79	<i>dkxI</i>	4836	1,837
06011	1,436	NRPS	C-A(asn)-PCP-TE	69/81	<i>dkxJ</i>	4835	1,433
06010	366	Patatin-like phospholipase family protein		68/81	<i>dkxK</i>	4834	365
06009	381	Arsenical pump-driving ATPase		49/62	<i>dkxL</i>	4833	388
06008	165	Hypothetical protein		45/62	<i>dkxP</i>	4832	159
06007	931	PKS	oMT-KR-ACP	67/78	<i>dkxQR</i>	4831	941
06006	674	Radical SAM domain-containing protein		83/89	<i>dkxS</i>	4830	673
06005	1,835	PKS	KS-AT(mal)-DH-KR-ACP	72/81	<i>dkxN</i>	4829	1,866
06004	947	PKS	KS-AT(N/A)	70/80	<i>dkxT</i>	4828	949
06003	87	Hypothetical protein		75/87	-	4827	87
06002	172	Alpha-L-arabinofuranosidase		-	-	-	-

A, adenylation; aa, amino acid; ACP, acyl carrier protein; asn, asparagine; AT, acyltransferase; C, condensation; DH, dehydrase; KR, ketoreductase; KS, ketosynthase; mal, malonate; mmal, methyl malonate; N/A, not available; NRPS, non-ribosomal peptide synthetase; oMT, O-methyl transferase; ORF, open reading frame; PCP, peptidyl carrier protein; PKS, polyketide synthase; SAM, S-adenosylmethionine-dependent methyltransferase; TE, thioesterase.

Identity/Similarity: Identity and similarity to the corresponding proteins of *S. aurantiaca* DW4/3-1.

with a scrapper and suspended in distilled water. Heat-resistant spores were counted on CYE plates after inactivating vegetative cells by incubating the harvested cells to 50°C for 2 h before sonication. Fruiting bodies were observed using a stereomicroscope (SMZ1000; Nikon, Japan), and individual cells and spores were observed using a phase-contrast microscope (Eclipse E600; Nikon).

Results

Identification of a Putative DKxanthene Biosynthetic Gene Cluster of *M. stipitatus* DSM 14675

Most strains of *M. stipitatus* produce yellow pigments that are predicted to be DKxanthenes. Because the DNA sequences of the DKxanthene biosynthetic gene clusters from *M. xanthus* DK1622 and *S. aurantiaca* DW4/3-1 were available, we analyzed the genome sequence of *M. stipitatus* DSM 14675 for the presence of a similar biosynthetic gene cluster. When the genome sequence of *M. stipitatus* DSM 14675 (GenBank Accession No. CP004025) [23] was analyzed with the antibiotics & Secondary Metabolite Analysis SHeLL (antiSMASH) program [14, 15], genes in a genomic region of 7,741,562–7,791,088 bp (approximately 49.5 kb) were predicted to be a putative DKxanthene biosynthetic gene cluster. This region contained 15 genes (MYSTI_06004–MYSTI_06018). The organization of the PKS and NRPS modules encoded by the genes in the region was very similar to that of the DKxanthene biosynthetic gene cluster from *S. aurantiaca* DW4/3-1 (Fig. 2, Table 2).

Inactivation of the DKxanthene Biosynthetic Genes

To confirm whether the MYSTI_06004–MYSTI_06018 region encodes enzymes for DKxanthene production, we inactivated the genes in the region by plasmid insertion mutagenesis and tested whether the resulting mutants produce yellow pigments assumed to be DKxanthenes. We first created two mutants, KYC617 and KYC618. KYC617 was a mutant in which the MYSTI_06016 gene was disrupted by the insertion of plasmid pHS221. KYC618 was a mutant in which the MYSTI_06014 gene was disrupted by the insertion of plasmid pHS222.

The resulting two mutants were defective in the production of yellow pigments and formed tan colonies under the condition where the wild-type strain DSM 14675 produced yellow pigments and formed yellow colonies. When the culture extracts were analyzed by HPLC, approximately 11 peaks, which showed a maximum absorption at 410 nm, disappeared in the extracts of mutant strains KYC617 and KYC618 compared with the extract of the wild-type strain DSM 14675 (Fig. 3).

We also created KYC633, KYC634, KYC635, KYC636, KYC637, and KYC638 by inserting plasmids pHS243, pHS244, pHS245, pHS246, pHS247, and pHS248 into the MYSTI_06019, MYSTI_06018, MYSTI_06010, MYSTI_06005, MYSTI_06004, and MYSTI_06002 genes, respectively. All the mutants that had plasmid insertions in the MYSTI_06004–MYSTI_06018 region were defective in the production of yellow pigments (Fig. S1). However, mutants KYC633 and KYC638, which had plasmid insertions in MYSTI_06019 and MYSTI_06002, respectively, produced yellow pigments similar to the wild-type strain (Fig. S1). MYSTI_06002 and MYSTI_06019, which adjoined the MYSTI_06004–MYSTI_06018 region, were not conserved in the DKxanthene biosynthetic gene clusters from *M. xanthus* DK1622 and *S. aurantiaca* DW4/3-1. Meanwhile, MYSTI_06003 was a relatively small open reading frame with a size of 264 bp, which might encode a hypothetical protein with a size of 87 aa. Therefore, it was concluded that the MYSTI_06004–06018 region encoded enzymes for the production of yellow pigments assumed to be DKxanthenes in *M. stipitatus* DSM 14675.

Fig. 3. High-performance liquid chromatography chromatograms of the culture extracts of *M. stipitatus* strains DSM 14675, KYC617, and KYC618.

Comparison of DKxanthenes from *M. stipitatus* DSM 14675 and *M. xanthus* DK1622

M. xanthus DK1622 has been reported to produce 13 derivatives of DKxanthene, including five derivatives produced by *S. aurantiaca* DW4/3-1 [5]. Therefore, we tested whether the yellow pigments produced by the wild-type strain of *M. stipitatus* but not by the insertion mutants were the same DKxanthenes produced by *M. xanthus* DK1622. Culture extracts of *M. stipitatus* DSM 14675 and *M. xanthus* DK1622 were prepared and analyzed using an HPLC equipped with a photodiode array detector. DKxanthenes from *M. xanthus* DK1622 were analyzed as

peaks with retention times of 11–17 min. Meanwhile, yellow pigments from *M. stipitatus* DSM 14675 were analyzed as peaks with retention times of 13–17 min (Fig. S2). The absorption spectra of the 13–17 min peaks from *M. stipitatus* DSM 14675 were very similar to those of DKxanthenes from *M. xanthus* DK1622 (Fig. S2 inserts). Since derivatives of the same backbone structure generally have similar absorption spectra, this suggested that the yellow pigments from *M. stipitatus* DSM 14675 might be the derivatives of DKxanthenes.

To confirm this, the DKxanthene fraction of *M. xanthus* between 11 and 19 min and the yellow pigment fraction of

Fig. 4. High-resolution LC-MS analysis of the DKxanthenes produced by *M. stipitatus* DSM 14675 and *M. xanthus* DK1622. UV chromatograms of the DKxanthene fractions from *M. stipitatus* DSM 14675 (A) and *M. xanthus* DK1622 (B) at 410 nm. High-resolution mass spectra of the eight main peaks from *M. stipitatus* DSM 14675 (C) and *M. xanthus* DK1622 (D).

Table 3. Comparison of the DKxanthenes produced by *M. stipitatus* DSM 14675 and *M. xanthus* DK1622.

	RT (min)	<i>m/z</i> ([M+H] ⁺)	Formula ([M+H] ⁺)	Δppm	DKxanthene derivatives	Known or new
<i>M. stipitatus</i> DSM 14675	6.16	563.2880	C ₃₁ H ₃₉ O ₆ N ₄	1.935	DKxanthene-562	New
	6.44	547.2928	C ₃₁ H ₃₉ O ₅ N ₄	1.361	DKxanthene-546	New
	6.53	563.2875	C ₃₁ H ₃₉ O ₆ N ₄	0.888	DKxanthene-562 isomer	New
	6.88	547.2923	C ₃₁ H ₃₉ O ₅ N ₄	0.539	DKxanthene-546 isomer	New
	7.12	589.3031	C ₃₃ H ₄₁ O ₆ N ₄	0.883	DKxanthene-588	New
	7.34	529.2820	C ₃₁ H ₃₇ O ₄ N ₄	1.170	DKxanthene-528	New
	7.44	573.3083	C ₃₃ H ₄₁ O ₅ N ₄	1.142	DKxanthene-572	New
	7.71	529.2822	C ₃₁ H ₃₇ O ₄ N ₄	1.303	DKxanthene-528 isomer	New
<i>M. xanthus</i> DK1622	4.46	535.2556	C ₂₉ H ₃₅ O ₆ N ₄	0.524	DKxanthene-534	Known
	4.94	517.2455	C ₂₉ H ₃₃ O ₅ N ₄	0.744	DKxanthene-516	New
	5.33	561.2710	C ₃₁ H ₃₇ O ₆ N ₄	-0.516	DKxanthene-560	Known
	5.82	575.2868	C ₃₂ H ₃₉ O ₆ N ₄	-0.243	DKxanthene-574	Known
	5.94	543.2602	C ₃₁ H ₃₅ O ₅ N ₄	-0.966	DKxanthene-542	New
	6.32	575.2875	C ₃₂ H ₃₉ O ₆ N ₄	1.269	DKxanthene-574 isomer	New
	6.53	557.2758	C ₃₂ H ₃₇ O ₅ N ₄	-1.050	DKxanthene-556	Known
	6.81	589.3028	C ₃₃ H ₄₁ O ₆ N ₄	0.136	DKxanthene-588 isomer	New

The structural formula was estimated from the measured high-resolution mass spectrometry results using Xcalibur ver. 2.2 software (Thermo Scientific, USA). The molecular formula was selected within ±3 ppm mass accuracy.

M. stipitatus between 13 and 20 min were analyzed with LC-MS/MS. Four derivatives of DKxanthene reported previously (DKxanthene-534, -556, -560, and -574) were detected in the DKxanthene fraction of *M. xanthus* (Fig. 4, Table 3). Four new derivatives that have not been reported previously (DKxanthene-516, -542, -574 isomer, and -588 isomer) were also detected in the DKxanthene fraction of *M. xanthus*. Under the same condition, the retention time and mass of DKxanthene derivatives in the yellow pigment fraction of *M. stipitatus* were totally different from those produced by *M. xanthus* (DKxanthene-528, -528 isomer, -546,

-546 isomer, -562, -562 isomer, -572, and -588) (Fig. 4, Table 3).

Effect of DKxanthenes on Fruiting Body Development and Spore Formation

Because DKxanthenes have been reported to be essential for fruiting body development and spore formation in *M. xanthus* [5], we tested whether the DKxanthenes were also essential for development in *M. stipitatus*. The wild-type cells of *M. stipitatus* DSM 1467 and mutant cells of KYC617 and KYC618, which were defective in DKxanthene production, were placed on CYD plates and incubated for

Fig. 5. Effects of the inactivation of DKxanthene production on the formation of fruiting bodies. Approximately 1 × 10⁶ cells of *M. stipitatus* DSM 14675 (A), KYC617 (B), and KYC618 (C) grown in CYE broth were placed on CYD plates as 20 μl spots and incubated at 30°C for 5 days. Cells aggregated and formed fruiting bodies consisting of a mass of slime and myxospores on the agar media. All three strains developed normal fruiting bodies. DSM 14675 formed yellow fruiting bodies, but KYC617 and KYC618 formed white fruiting bodies because they were defective in DKxanthene production. Bar, 0.2 mm.

fruiting body development. However, no difference between the wild-type strain and the mutant strains were observed in fruiting body development and spore formation except for the color of the fruiting bodies after 5 days. The fruiting bodies of the mutants were white or tan, whereas those of the wild-type strain were yellow (Fig. 5). No delay in fruiting body development was observed with the mutant strains compared with the wild-type strain. The mutant cells produced the same amounts of heat-resistant viable spores compared with that of the wild-type strain. A mutant strain of *M. xanthus* defective in DKxanthene production failed to produce heat-resistant viable spores under identical conditions (data not shown). Therefore, it was concluded that *M. stipitatus* DSM 14675 did not require DKxanthenes for fruiting body development and spore formation, unlike *M. xanthus*.

Antimicrobial Activity of DKxanthenes

When we previously studied mutants that were defective in the production of melithiazols [24], we found that *M. stipitatus* DSM 14675 was producing other antifungal substances besides melithiazols. An HPLC analysis and antifungal activity assay indicated that those substances were DKxanthenes. When a paper disc containing 100 µg of the DKxanthene fraction of *M. stipitatus* DSM 14675 between 13–20 min was placed in the center of plates inoculated with *P. aeruginosa* or *S. aureus*, no growth inhibition of bacteria was observed. However, a paper disc containing the same amount of the DKxanthene fraction inhibited the

growth of fungi *A. niger*, *C. albicans*, and *R. stolonifer* (Fig. 6). The same fractions of culture extracts from mutants defective in DKxanthene (KYC617 and KYC618) did not show any growth inhibitory activity against these fungi under the same conditions. Because the DKxanthene fraction from *M. stipitatus* DSM 14675 showed antifungal activity, we tested the antifungal activity with the DKxanthene fraction from *M. xanthus* DK1622. The DKxanthene fraction from *M. xanthus* DK1622 also showed antifungal activity, although the activity against *A. niger* and *C. albicans* was lower than that of the fraction from *M. stipitatus* DSM 14675 (Fig. 6).

Discussion

Organization of DKxanthene Biosynthetic Gene Clusters and Their Products

DKxanthene biosynthetic gene clusters have been found in the genomes of strains belonging to genus *Myxococcus* and *Stigmatella*. DKxanthene biosynthetic gene clusters could be classified into two groups based on the organization of genes: MX type and SA type. The MX-type clusters are represented by the cluster identified in the genome of *M. xanthus* DK1622 (Fig. 2). SA-type clusters are represented by the cluster identified in the genome of *S. aurantiaca* DW4/3-1 (Fig. 2) [6]. Genomic sequence analysis indicated that *M. xanthus*, *M. virescens*, *M. hansupus*, and *M. macrosporus* have MX-type clusters, whereas *M. stipitatus* and *M. fulvus* have SA-type clusters. DKxanthene biosynthetic gene clusters are not found in the genomes of *Coralloccoccus*, a

Fig. 6. Antifungal activity of DKxanthenes from *M. stipitatus* DSM 14675 and *M. xanthus* DK1622.

Paper discs containing 100 µg of DKxanthene fractions from *M. stipitatus* DSM 14675 or *M. xanthus* DK1622 were placed on TSA plates that had been spread with *Aspergillus niger* ATCC 16404, *Candida albicans* ATCC 18804, or *Rhizopus stolonifer* KCCM 32398. The plates were then incubated at 30°C for 2 days.

closely related genus to *Myxococcus*.

It has been reported that the core structure of DKxanthene is biosynthesized by PKSs and NRPSs in the order of DkxA, DkB, DkxE, DkxF, DkxG, DkxN, DkxH, DkxI, and DkJ [6]. Both MX- and SA-type clusters have genes for these PKSs and NRPSs in common. However, the SA-type clusters have additional genes, *dkxP*, *dkxQR*, *dkxS*, and *dkxT*, which were not found in the MX-type clusters (Fig. 2), whereas they lack *dkxC* and *dkxD*, which were present in the MX-type clusters (Fig. 2). The *dkxP* gene was predicted to encode a hypothetical protein, the *dkxQR* gene a PKS with oMT-KR-ACP domains, the *dkxS* gene a radical SAM domain-containing protein, and the *dkxD* gene a PKS with KS-AT domains (Table 2). Meanwhile, the *dkxC* gene was predicted to encode a type 1 phosphodiesterase and the *dkxD* gene a FAD-dependent monooxygenase [6]. Therefore, it was expected that the MX- and SA-type clusters might produce different DKxanthene derivatives. Indeed, *M. stipitatus* DSM 14675 carrying an SA-type cluster produced totally different DKxanthene derivatives from those produced by *M. xanthus* DK1622 having an MX-type cluster under identical culture conditions. None of the DKxanthene derivatives produced by *M. stipitatus* DSM 14675 were identical to those produced by *M. xanthus* DK1622 in terms of molecular mass and HPLC retention time, suggesting that DKxanthenes produced by *M. stipitatus* DSM 14675 might be novel (Fig. 4, Table 3).

S. aurantiaca DW4/3-1 carrying an SA-type cluster was reported to produce five derivatives that were identical to five DKxanthenes produced by *M. xanthus* DK1622 having an MX-type cluster [6]. It was suggested that the PKS domains encoded by additional PKS genes *dkxQR* and *dkxT* in the SA-type cluster might be inactive in *S. aurantiaca* DW4/3-1 [6].

Thirteen DKxanthene derivatives were identified previously from *M. xanthus* DK1622 (MX type) [5, 6]. We found four new DKxanthene derivatives that were produced by *M. xanthus* DK1622 in addition to those previously reported (Table 3). CYS agar was used to cultivate *M. xanthus* in this study, whereas other growth media were used in the previous study [5, 6]. Thus, it appeared that *M. xanthus* produced different DKxanthene derivatives depending on the culture conditions.

Biological Function of DKxanthenes

We found that the DKxanthene fractions from *M. stipitatus* DSM 14675 and *M. xanthus* DK1622 inhibited the growth of fungi such as *A. niger*, *C. albicans*, and *R. stolonifer*. This is the first report on the antimicrobial activity of DKxanthenes.

The growth inhibitory activity of the DKxanthene fractions from both *M. stipitatus* and *M. xanthus* against *R. stolonifer* was similar, but the activity of the DKxanthene fraction from *M. xanthus* DK1622 against *A. niger* and *C. albicans* was relatively weak compared with that of the fraction from *M. stipitatus* DSM 14675 (Fig. 6). DKxanthene derivatives from *M. stipitatus* were completely different to those from *M. xanthus*. Thus, it is possible that the antifungal activity of each derivative might be different.

M. xanthus DK1622 produced new DKxanthene derivatives (DKxanthene-516, -542, -574 isomer, and -588 isomer) in addition to the derivatives previously reported (DKxanthene-534, -556, -560, and -574) in this study (Table 3). It could be possible that the antifungal activity of DKxanthenes was not observed before this study because previously known derivatives showed no or very weak activity against fungi, whereas the newly produced derivatives showed strong inhibitory activity. Differences in assay conditions might be another possible reason why the antifungal activity of DKxanthenes had not been observed before this study. DKxanthenes from *M. xanthus* DK1622 did not show growth inhibitory activity against *A. niger* on potato dextrose agar plates, whereas those from *M. stipitatus* DSM 14675 showed obvious inhibitory activity under identical conditions (data not shown).

DKxanthenes are reported to be required for developmental sporulation in *M. xanthus*. Mutant cells of *M. xanthus* defective in DKxanthene synthesis produce severely reduced amounts of viable spores [5]. Unlike *M. xanthus*, the mutant cells of *M. stipitatus* defective in DKxanthene synthesis produced normal levels of heat-resistant viable spores compared with the wild-type cells. This suggested that DKxanthenes are not required for the formation of viable spores in *M. stipitatus*. It is possible that DKxanthenes have other roles in *M. stipitatus*, such as protecting cells from eukaryotic organisms rather than being a factor required for fruiting body formation, because it has antifungal activity.

Myxobacteria produce diverse antimicrobial substances [25, 26] that would confer advantages against other microorganisms in the soil [27]. *M. stipitatus* DSM 14675 produces melithiazols, another group of antifungal substances, in addition to DKxanthenes [24]. The production of two antifungal substances, melithiazols and DKxanthenes, seems to be independent because the mutants defective in melithiazols produced DKxanthenes, and the mutants defective in DKxanthenes produced melithiazols (data not shown). Melithiazols inhibit electron transfer in the mitochondria [11]. The growth inhibitory mechanism of DKxanthenes against fungi is unknown at this moment. It

will be interesting to elucidate the mechanism because DKxanthenes are a new group of antifungal substances.

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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