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Antimicrobial activities and natural products of cellulolytic myxobacteria isolated from soils in Japan

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Cellulolytic myxobacteria cultured using filter paper as the only carbon source were isolated from soils in Japan. To purify isolated strains, fruiting bodies of cellulolytic myxobacteria formed on Stanier agar medium were incubated at 60℃ for 1–7 days and/or at room temperature for 3–6 months. Purified 120 strains were cultured in P- and E-liquid media for 4–6 weeks at 28℃. Approximately 75% of the strains were found to produce metabolites with antimicrobial activity, and 63% of extracts showed growth inhibitory activity against *Aspergillus niger*. Based on LC-MS analysis, we estimated that 34 known natural products of the genus *Sorangium* were produced by 100 isolates, including spirodienal, ambruticin, disorazole, and epothilone. Also, many products whose molecular weights did not match those of known products from the genus *Sorangium* were detected.

Key words: antimicrobial activity, isolation and purification methods, natural products, *Sorangium*, cellulolytic myxobacteria

Myxobacteria are Gram-negative, unicellular, aerobic, gliding bacteria with relatively large rod-shaped vegetative cells and a complex life cycle (Dworkin, 1996; Shimkets *et al*., 2006). They are isolable from soils, animal dung, and decaying plant materials (Reichenbach & Dworkin, 1992; Reichenbach, 1999). Under starvation conditions, vegetative cells aggregate into fruiting bodies and differentiate into desiccation-resistant spores (Dawid, 2000).

Myxobacteria fall into either bacteriolytic and cellulolytic nutritional subgroups. Most species of myxobacteria are bacteriolytic, lysing dead organic matter as well as dead and living microorganisms with a host of excreted enzymes. Myxobacterial genera, *Sorangium* and *Byssovorax*, are the members of the taxon which decompose cellulosic materials (Gaspari *et al*., 2005; Reichenbach *et al*., 2006). Genome sizes of myxobacteria range from 9.2 to 14.8 Mbp, and among myxobacteria, *Sorangium cellulosum* has the largest bacterial genome (Schneiker *et al*., 2007; Han *et al*., 2013). Almost half of the discovered myxobacterial secondary metabolites are produced by different strains of this species (Reichenbach, 2001; Gerth *et al*., 2003; Bode & Müller, 2006). However, there are very few reports on comprehensive analysis of antimicrobial activities and natural products in cellulolytic myxobacteria (Gerth *et al*., 2003), and on isolation of cellulolytic myxobacteria from Japan (Komaki *et al*., 2008).

We isolated cellulolytic myxobacteria from soil samples in Japan in order to obtain useful compounds from myxobacteria. In this study, antimicrobial activities of 120 isolates were determined by the paper disc assay, and natural products of the isolates were analyzed by liquid chromatography-mass spectrometry (LC-MS).

Approximately 2,000 soils in Japan except Hokkaido were collected and approximately 1 g of each was placed on filter paper (70 mm diameter, Advantec No. 1) placed on Stanier agar medium (0.1% KNO_3, 0.02% K_2HPO_4, 0.01% $MgSO_4$, 0.01% $CaCl_2$, 0.002% $FeCl_3$, 0.1% standard trace element solution (0.1 g $MnCl_2$, 0.02 g $CoCl_2$, 0.01 g $CaSO_4$, 0.01 g $NaMoO_4$, 0.02 g $ZnCl_2$, 0.005 g LiCl, 0.005 g $SnCl_2$, 0.01 g H_3BO_3, 0.02 g KBr, 0.02 g KI per liter) and 1% agar; Stanier, 1942) and incubated for 2 to 4 weeks at room tem-

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perature. The spot where the paper was decomposed was observed with an optical microscope, and it was confirmed that the fruiting bodies of genera *Sorangium* or *Byssovorax* were formed. Many cellulolytic myxobacteria strains on the filter paper produced an orange to brown color pigment (Reichenbach *et al*., 2006; Mohr *et al*., 2018), and then strains formed red-brown, sometimes yellow or black fruiting bodies. In approximately 20% of the plates, fruiting bodies of cellulolytic myxobacteria strains were observed on the filter paper, and 360 isolates were obtained from 2,000 soil samples.

To purify the isolates, the appearance of fruiting bodies on the filter papers was checked by microscope, and then transferred to fresh Stanier plates (55 mm diameter) that had filter papers (35 mm diameter) on top using a needle. After several weeks of incubation, formed fruiting bodies were picked up with a needle and transferred into sterilized 1.5 m*l* tubes. The tubes containing fruiting bodies of cellulolytic myxobacteria were placed in an oven at 60℃ for 1 to 7 days for heat treatment, and then at room temperature for 3 to 6 months for drying treatment. After these incubations, the fruiting bodies in the tubes were inoculated on filter paper that were placed on Stanier agar medium and incubated at 28℃ for 2–4 weeks. To check for purity of culture, fruiting bodies of approximately 5×5 mm^2 that formed on the filter paper were inoculated into 3 m*l* P-liquid medium (Gerth *et al*., 1984), cultured for 3 days at 30℃, and whether other bacteria were growing in the P-medium was verified using a microscope. As a simple determination method, we judged whether or not the liquid medium became clouded by growth of other bacteria over the course of 3 days' incubation, since the growth of cellulolytic myxobacteria is comparatively very slow with generation times approaching 16 h (Reichenbach, 1999). If isolate was not purified by this treatment, this treatment was repeated, and many purified strains were obtained by this method.

In contaminated samples, we attempted purification using various antibiotics, which do not kill spores of cellulolytic myxobacteria. Contaminated fruiting bodies of cellulolytic myxobacteria strains were suspended in 1 m*l* P-medium containing 7 antibiotics at 50 μg ml^{-1} each: nystatin, penicillin G, fradiomycin, gentamycin, carbenicillin, ampicillin, and kanamycin or 4 antibiotics at 50 μg ml^{-1} each: oleandomycin, spiramycin, lincomycin, and rifampicin. After incubation for 1 to 2 weeks at room temperature, fruiting bodies were inoculated on filter papers, and then the filter papers were placed on Stanier agar medium. Fruiting bodies formed on filter paper were checked for purity by the method described above. In this experiment, approximately 20% of the subjects were purified by the antibiotic treatment. Other methods for purifying cellulolytic myxobacteria fruiting bodies contaminated with bacteria were attempted, including ultrasonic treatment using a sonifier (Branson) for 2.5–5 min, washing treatment with 1.5 *l* of sterilized water using a membrane (10 μm pore size) filtration apparatus, and incubation with 1–5% sodium dodecyl sulfate, 2–3 M NaCl, 10–20% dimethyl sulfoxide or 6–18 mM H_2O_2 for 7–14 days; however, other bacteria were not removed by these treatments. The purification ratio of isolates was approximately 50% in this study, and the fruiting bodies of purified 178 strains were stocked in P-medium with 20% glycerol and stored at −80℃.

Isolated cellulolytic myxobacteria strains were precultured in 40 m*l* of P-medium for 1 week at 30℃. Following this, precultures were inoculated into 500-m*l* Erlenmeyer flasks containing 250 m*l* of P-medium or 250 m*l* of E-medium (0.4% skim milk powder, 0.4% soybean flour, 1% corn starch, 0.2% yeast extracts, 0.5% glycerol, 0.1% $CaCl_2 \cdot 2H_2O$, 0.1% $MgSO_4 \cdot 7H_2O$, 0.008% Na-Fe-EDTA, and 1.2% HEPES, pH 7.2) and 3.5 g of absorber resin XAD-16 (Sigma Aldrich), and grown at 30℃ on a rotary shaker at 100 rpm for 4 or 6 weeks, respectively. The resin and microbial cells were harvested separately and extracted with 60–100 m*l* of acetone. Extracts were concentrated using an evaporator to 5 m*l* and analyzed using antimicrobial activity analysis and LC-MS.

We first analyzed antimicrobial activities of extracts from 120 isolated strains. Antimicrobial activity was examined by paper disc assay against three bacteria, two yeasts, and two molds as indicator microorganisms. *Micrococcus luteus* NBRC 3333 and

Corynebacterium glutamicum NBRC 12168, and *Gordonia rubripertincta* NBRC 101908 were used as targets of bacteria and actinomycete, respectively. *Candida albicans* NBRC 1385 and *Wickerhamomyces anomalus* NBRC 10213 were used as representative yeast. These were prepared as overnight cultures in 20 m*l* medium consisting of 1% peptone, 0.2% yeast extract, and 0.1% $MgSO_4$ (Bacterial medium) or YM broth (Difco Laboratories), respectively. One m*l* of each culture was mixed with 20 m*l* of melted Bacterial agar medium or YM agar, and they were used in antimicrobial activity evaluations. Meanwhile, *Aspergillus niger* NBRC 33023 and *Mucor hiemalis* NBRC 9404 were grown on potato dextrose agar (Difco Laboratories). One cm^3 of each was transferred into a tube, and agar containing hyphae was crushed with a platinum loop, followed with glass beads in a vortexer, and then mixed with 20 m*l* of melted potato dextrose agar. Ten μl from the acetone extracts were spotted onto sterile filter paper discs (8 mm in diameter; Advantec) and allowed to dry. These discs were placed on plates prepared with the above described targets and incubated for 48 h at 30℃. Following this, the diameters of inhibition zones were measured.

Among 120 isolates, 91 exhibited antimicrobial activities against at least one of seven indicator microorganisms (Table 1). Gerth *et al.* have reported that approximately 90% of *S. cellulosum* isolates produced some biologically active compounds (Gerth *et al*., 2003). 63% and 35% of extracts showed inhibitory activity against *A. niger* and *M. hiemalis*, respectively (Table 1). Eight strains (No. 102, 162, 202, 250, 280, 290, 301, and 396) had broad-spectrum antimicrobial activity against bacteria, yeasts, and molds. However, most of extracts from isolated strains (70%) did not show activity against the three bacteria (*M. luteus, C. glutamicum*, and *G. rubripertincta*), indicating that secondary metabolites of cellulolytic myxobacteria have mainly antifungal activity. The extracts prepared from cultures in E-medium showed stronger antibiotic activity compared to those prepared from P-medium, likely because many isolates grew more readily in E-medium (data not shown).

We next analyzed the products of isolates by LC-MS. Fifty μl of the extracts was concentrated under a vacuum at 28℃, and then redissolved in 500 μl of acetonitrile:methanol 1:1 (v/v) for LC-MS analysis (Shimadzu LCMS-2020). A 2 μl aliquot was injected into a Kinetex 5 μm C18 100A column (2.1 mm × 100 mm, particle size 5 μm, Phenomenex) with a guard column (SecurityGuard ULTRA, Phenomenex). Mobile phase A was water and mobile phase B was acetonitrile, where both phases contained 0.1% formic acid. The gradient elution at a flow rate of 0.4 m*l* min^{-1} was performed as follows: 0–22 min 10–90% B and 22–25 min 90% B. Mass spectra were obtained in both positive and negative reflector ion mode in the range of *m/z* 200–1200. The mass spectrometer fitted with an electrospray ionization (ESI) source was used for analysis. It was operated the following parameters: probe voltage +4.5 kV (+ESI) / −3.5 kV (−ESI), nebulizer gas flow 1.5 *l* min^{-1}, drying gas flow 15.0 *l* min^{-1}, block heater 400℃, DL temperature 250℃.

Of the natural products isolated from the genus *Sorangium*, 241 are registered in Dictionary of Natural Products 29.1. Gerth *et al.* have reported that disorazole, icumazole, spirangien, tartrolon, soraphen, ambruticin, chivosazol, and epothilone were commonly found in 1,700 *S. cellulosum* isolates from Germany (Gerth *et al*., 2003). From *m/z* values obtained from LC-MS analysis, metabolites from 100 cellulolytic myxobacteria strains yielded 34 known products (Tables 1 and 2), and spirodienal, ambruticin, disorazole, epothilone, pellasoren, microsclerodermin M, and spirangien were major known products of cellulolytic myxobacteria isolates from Japan (Table 2). These are natural polyketides, with potent antifungal activity and cytotoxicity against human tumor cells. Approximately 80% of spirodienal-, ambruticin-, disorazole-, and spirangien-producing strains had antimicrobial activities against *A. niger* (Table 1). In addition, precursors of epothilones (for examples, 5,13-dihydroxy-2,4,6,14-tetramethyl-15-(2-methyl-4-thiazolyl)-10,14-pentadecadien-3-one, 11-hydroxy-4,12-dimethyl-13-(2-methyl-4-thiazolyl)-8,12-tridecadien-3-one, and 3,5-dihydroxy-6-methyl-7-(2-methyl-4-thiazolyl)-6-heptenoic acid), were frequently detected. Meanwhile, icumazole was rarely ob-

Table 1 Antimicrobial activities of isolates against seven indicator microorganisms, and known natural products produced by isolates

Strain No.	Antimicrobial activity against							Chemical name
	MI[a]	Cg[b]	Gr[c]	Ca[d]	Wa[e]	Mh[f]	An[g]	
58	-	-	-	-	-	-	-	Disorazole I
113	-	-	-	-	-	-	-	Soraphen E, Epothilone D or G2
124	-	-	-	-	-	-	-	Spirodienal, Ambruticin VS2, Ripostatin
128	-	-	-	-	-	-	-	Spirodienal, Sorazolone E2
150	-	-	-	-	-	-	-	
165	-	-	-	-	-	-	-	
169	-	-	-	-	-	-	-	Spirodienal, Ambruticin VS2
192	-	-	-	-	-	-	-	Ambruticin VS2
205	-	-	-	-	-	-	-	Spirodienal, Microsclerodermin M
206	-	-	-	-	-	-	-	
214	-	-	-	-	-	-	-	Ambruticin VS2, Jerangolide D
221	-	-	-	-	-	-	-	Pellasoren A
223	-	-	-	-	-	-	-	
236	-	-	-	-	-	-	-	
239	-	-	-	-	-	-	-	
240	-	-	-	-	-	-	-	
245	-	-	-	-	-	-	-	
247	-	-	-	-	-	-	-	Tuscoron B, Phoxalone, Epothilone N, Disorazole B2
248	-	-	-	-	-	-	-	Spirodienal, Ambruticin VS2
259	-	-	-	-	-	-	-	Disorazole F2
262	-	-	-	-	-	-	-	
272	-	-	-	-	-	-	-	
303	-	-	-	-	-	-	-	Pellasoren A
319	-	-	-	-	-	-	-	Tuscoron B, Phoxalone
321	-	-	-	-	-	-	-	Soraphen E, Disorazole F2
322	-	-	-	-	-	-	-	Tuscoron B
327	-	-	-	-	-	-	-	Ambruticin VS2, Epothilone D or G2
339	-	-	-	-	-	-	-	Spirodienal
373	-	-	-	-	-	-	-	
175	+	-	-	-	-	-	-	Spirodienal, Microsclerodermin M
249	-	+	-	-	-	-	-	Spirodienal, Disorazole A2
130	-	-	+	-	-	-	-	Microsclerodermin M
155	-	-	+	-	-	-	-	Disorazole A2
313	-	-	+	-	-	-	-	Spirodienal, Pellasoren A
127	-	-	-	-	+	-	-	Ambruticin VS3
135	-	-	-	-	+	-	-	Microsclerodermin M
151	-	-	-	-	+	-	-	Spirodienal, Ambruticin VS2
157	-	-	-	-	+	-	-	Epothilone B
357	-	-	-	-	+	-	-	Spirodienal, Chivorazole E, Disorazole F2
233	-	-	-	-	-	+	-	Spirodienal, Soraphen E
243	-	-	-	-	-	+	-	Ambruticin VS3
104	-	-	-	-	-	-	+	Spirodienal, Microsclerodermin M
105	-	-	-	-	-	-	+	
156	-	-	-	-	-	-	+	Disorazole A
199	-	-	-	-	-	-	+	Tuscoron B, Chivorazole A
201	-	-	-	-	-	-	+	
207	-	-	-	-	-	-	+	Microsclerodermin M
217	-	-	-	-	-	-	+	Spirodienal
220	-	-	-	-	-	-	+	Chivorazole A
224	-	-	-	-	-	-	+	
235	-	-	-	-	-	-	+	
238	-	-	-	-	-	-	+	Pellasoren A, Soraphen A
252	-	-	-	-	-	-	+	Ambruticin VS2
266	-	-	-	-	-	-	+	Pellasoren A
278	-	-	-	-	-	-	+	Spirangien B
291	-	-	-	-	-	-	+	
305	-	-	-	-	-	-	+	Pellasoren A
311	-	-	-	-	-	-	+	Ambruticin VS2, Tuscoron B
342	-	-	-	-	-	-	+	Spirangien Q
258	+	+	-	-	-	-	-	Phoxalone, Epothilone A1 or Chlorotonil A
283	+	-	-	-	-	-	+	Spirodienal
299	-	+	-	-	-	+	-	Spirodienal, Phoxalone
164	-	+	-	-	-	-	+	

Table 1 Continued

Strain No.	Antimicrobial activity against							Chemical name
	MI[a]	Cg[b]	Gr[c]	Ca[d]	Wa[e]	Mh[f]	An[g]	
154	-	-	+	-	-	-	+	
181	-	-	+	-	-	-	+	
200	-	-	+	-	-	-	+	Spirangien A, Spirangien B
213	-	-	+	-	-	-	+	Disorazole A
329	-	-	+	-	-	-	+	Spirodienal, Tuscoron B, Soraphen E, Epothilone D or G2
371	-	-	+	-	-	-	+	Epothilone E or A9 or M, Phoxalone, Ambruticin VS1
273	-	-	-	+	-	-	+	Spirangien A, Spirangien B
123	-	-	-	-	+	+	-	Spirangien A, Spirangien B
57	-	-	-	-	+	-	+	Ambruticin VS2, Disorazole F2, Soraphen E, Chivosazole E
136	-	-	-	-	+	-	+	Pellasoren A
161	-	-	-	-	+	-	+	Ambruticin VS2, Disorazole I
50	-	-	-	-	-	+	+	Ambruticin VS5
107	-	-	-	-	-	+	+	Pellasoren A
125	-	-	-	-	-	+	+	Disorazole A, Pellasoren A
129	-	-	-	-	-	+	+	Disorazole A, Pellasoren A
131	-	-	-	-	-	+	+	Phoxalone, Chivorazole A
144	-	-	-	-	-	+	+	Disorazole A
145	-	-	-	-	-	+	+	Disorazole A
166	-	-	-	-	-	+	+	Pellasoren A
212	-	-	-	-	-	+	+	Spirodienal, Pellasoren A, Microsclerodermin M
215	-	-	-	-	-	+	+	Disorazole A
264	-	-	-	-	-	+	+	Spirodienal, Ambruticin VS2, Tuscoron B, Epothilone D or G2, Epothilone N
328	-	-	-	-	-	+	+	Ambruticin VS2, Soraphen E
121	+	-	+	-	-	+	-	Ambruticin VS3
115	+	-	-	-	-	+	+	Ambruticin VS2, Epothilone D or G2
148	-	+	+	-	-	-	+	Spirodienal, Ambruticin VS2, Tuscoron B, Phoxalone, Spirangien Q
211	-	+	-	+	-	-	+	Spirodienal, Ambruticin VS2, Microsclerodermin M
142	-	+	-	-	-	+	+	Pellasoren A
158	-	-	+	+	-	-	+	Ambruticin VS2, Tuscoron B
114	-	-	+	-	+	-	+	Spirodienal, Ambruticin VS2, Disorazole F2
216	-	-	+	-	-	+	+	Spirodienal, Soraphen E
307	-	-	+	-	-	+	+	Spirangien Q
103	-	-	-	-	+	+	+	Spirodienal, Ambruticin VS2, Disorazole F2, Microsclerodermin M, Chivosazole E
116	-	-	-	-	+	+	+	Disorazole A
126	-	-	-	-	+	+	+	Disorazole A, Pellasoren A
139	-	-	-	-	+	+	+	Spirodienal, Soraphen E, Epothilone E or A9 or M, Chivorazole E
172	-	-	-	-	+	+	+	
230	-	-	-	-	+	+	+	Spirodienal, Ambruticin VS2, Disorazole F2, Epothilone D or G2
254	-	-	-	-	+	+	+	Spirodienal, Disorazole F2, Soraphen E, Microsclerodermin M
286	-	-	-	-	+	+	+	Spirodienal, Disorazole F2, Spirangien Q, Microsclerodermin M
304	-	-	-	-	+	+	+	Pellasoren A
225	-	+	-	-	+	+	+	Disorazole A, Phoxalone, Chivorazole A, Tuscoron B
295	-	-	+	+	+	-	+	Icumazole A
138	-	-	+	-	+	+	+	Spirodienal, Sorazolone E2, Disorazole F2, Epothilone D or G2
141	-	-	+	-	+	+	+	Spirodienal, Disorazole A
237	-	-	+	-	+	+	+	Spirodienal, Ambruticin VS2, Disorazole F2, Epothilone E or A9 or M, Spirangien Q
101	-	-	-	+	+	+	+	Spirodienal
118	-	-	-	+	+	+	+	Spirodienal, Chivosazole E, Tuscoron B, Microsclerodermin M
203	-	-	-	+	+	+	+	Spirodienal, Soraphen E, Disorazole A2
102	+	+	+	-	+	-	+	Spirodienal, Ambruticin VS2, Chivosazole E, Microsclerodermin M
250	+	+	+	-	+	-	+	Spirodienal, Ambruticin VS2
301	+	+	+	-	+	-	+	Pellasoren A, Spirangien A, Spirangien B, Tuscoron A or Epothilone I_3
202	-	+	+	-	+	+	+	Spirodienal, Ambruticin VS2
162	-	-	+	+	+	+	+	Tuscoron B, Chivotriene, Eliamid, Epothilone D or G2, Microsclerodermin M
280	-	-	+	+	+	+	+	Epothilone E or A9 or M
396	-	-	+	+	+	+	+	Spirodienal, Epothilone E or A9 or M, Microsclerodermin M
290	+	+	+	+	+	+	+	Spirodienal, Ambruticin VS2, Ambruticin VS5, Soraphen E, Secoepothilone A Me ester or Soraphen M
No. of active strains against indicator strains								
	9	13	27	11	35	42	75	

[a]*M. luteus*; [b]*C. glutamicum*; [c]*G. rubripertincta*; [d]*C. albicans*; [e]*W. anomalus*; [f]*M. hiemalis*; [g] *A. niger*. +: active against indicator microorganism; -: non-active against indicator microorganism

Table 2 Known natural products produced by isolated strains

$(M+H)^+$ m/z	RT[a] min	Chemical name	No. of strains	$(M+H)^+$ m/z	RT[a] min	Chemical name	No. of strains
552	7.2	Spirodienal	39	530	6.4	Ambruticin VS2	25
433	4.5	Pellasoren A	16	887	7.4	Microsclerodermin M	15
553	4.4	Tuscoron B	12	538	6.8	Soraphen E	11
730	5.2	Disorazole F2	11	759	16.5	Disorazole A	10
425	3.8	Phoxalone	8	491	6.0	Epothilone D or G2	8
826	8.5	Chivosazole E	6	511	8.6	Epothilone E or A9 or M	5
708	4.3	Spirangien Q	5	717	20.7	Spirangien B	5
703	18.9	Spirangien A	4	866	6.6	Chivosazole A	4
502	15.5	Ambruticin VS3	3	746	3.3	Disorazole A2	3
422	4.9	Sorazolone E2	2	489	11.0	Ambruticin VS5	2
546	3.5	Epothilone N	2	758	5.2	Disorazole I	2
355	10.1	Chivotriene	1	360	3.3	Jerangolide D	1
390	17.0	Eliamid	1	496	4.6	Ripostatin	1
512	5.6	Soraphen A	1	517	11.3	Ambruticin VS 1	1
568	20.0	Icumazole A	1	639	6.6	Epothilone B	1
780	4.4	Disorazole B2	1	480	3.5	Epothilone A1 or Chlorotonil A	1
526	3.2	Secoepothilone A Me ester or soraphen M	1	535	10.0	Tuscoron A or Epothilone I_3	1

[a]retention time

Table 3 Major unidentified products from isolates

$(M+H)^+$ m/z	RT[a] min	Strain No.
255	6.9	105, 125, 127, 142, 144, 172, 175, 200, 213, 238, 245, 266, 272, 273, 278, 301, 303, 305
273	8.5	124, 138, 203, 249
282	8.0	57, 250, 291, 301, 371
329	8.7	57, 103, 114, 286
330	8.4	57, 101, 103, 203, 249, 280, 286, 342, 357
374	4.4	57, 101, 102, 103, 104, 114, 124, 128, 129, 130, 131, 138, 139, 141, 151, 154, 157, 158, 161, 175, 192, 199, 203, 205, 206, 207, 212, 217, 220, 223, 225, 230, 233, 235, 236, 237, 239, 249, 250, 252, 254, 262, 283, 286, 290, 311, 321, 327, 329, 342, 357, 396
394	3.9	212, 235, 238, 252
400	5.1	125, 131, 166, 213, 220, 238, 313
417	3.9	57, 102, 103, 104, 113, 114, 118, 124, 138, 139, 141, 151, 158, 202, 203, 216, 220, 230, 233, 237, 247, 250, 254, 258, 259, 280, 286, 291, 299, 307, 313, 319, 321, 329, 342, 371, 373, 396
439	5.7	103, 113, 114, 115, 139, 158, 161, 192, 216, 237, 248, 254, 258, 259, 264, 283, 286, 290, 321, 328, 339, 371
459	4.9	50, 113, 115, 118, 151, 158, 162, 169, 199, 202, 203, 216, 247, 249, 259, 280, 286, 291, 307, 313, 319, 321, 357, 373, 396
560	3.4	114, 151, 328
572	5.8	128, 158, 252, 264, 373
652	4.5	113, 115, 130, 145, 148, 166, 192, 202, 203, 225, 235, 252, 259, 264, 299, 327, 328, 329, 339
659	3.4	162, 166, 169, 207, 216, 223, 225, 286, 291, 311, 327, 328, 339, 357
698	4.8	131, 162, 175, 225, 239, 327
741	5.1	138, 139, 216, 237, 254
747	7.9	114, 135, 148, 150, 157, 175
755	6.3	118, 130, 139, 199, 207, 214, 216, 224, 240, 254, 286, 328, 329, 339, 357
821	6.8	148, 192, 202, 207, 211, 252, 264
889	6.2	135, 139, 157, 158, 165, 206, 207, 211, 214, 216, 237, 295, 311, 329, 339, 357
997	4.0	104, 175, 235, 259, 327, 329
1000	8.6	220, 252, 299
1124	6.2	130, 211, 311, 339

[a]retention time

served. Frequently, genus *Sorangium* strains produce several chemically completely different compounds (Gerth *et al*., 2003). Six strains (No. 103, 148, 162, 237, 264, and 290) and 12 strains (No. 57, 102, 118, 138, 139, 225, 230, 247, 254, 286, 301, and 329) isolated from soil in Japan produced five and four different known compounds, respectively (Table 1). Most of them exhibited antimicrobial activity against multiple indicator microorganisms. On the other hand, 18 isolates without antimicrobial activity produced known compounds, suggesting that these isolates may have antimicrobial activity as they only produced low amounts of known compounds in the cultures.

In the analysis, many products, which did not match the molecular weights of known products from the genus *Sorangium*, were found in the 120 isolate cultures, including some showing protonated ions at *m/z* 255, 374, 417, 439, 459, and 652 that were frequently found (Table 3).

Twenty-two purified cellulolytic myxobacteria strains were used for 16S rRNA gene sequence analysis. For genomic DNA extraction, fruiting bodies on the filter paper from 22 isolated strains were transferred into 1.5-m*l* tubes, and 50 *μl* of 50 mM NaOH was added. After incubation at 80℃ for 5 min, 11 *μl* of 1 M Tris-HCl (pH 7.0) was added, and the tubes were centrifuged at 12,000 rpm for 3 min. The supernatant was used as template for 16S rRNA gene sequence analysis. The 16S rRNA gene from isolates was amplified via PCR using the universal primer set 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGCTACCTTGTTACGACTT-3'). PCR products were purified by PEG precipitation. The sequencing reaction was performed with the same primers using BigDye Terminator v3.1 Cycle Se-

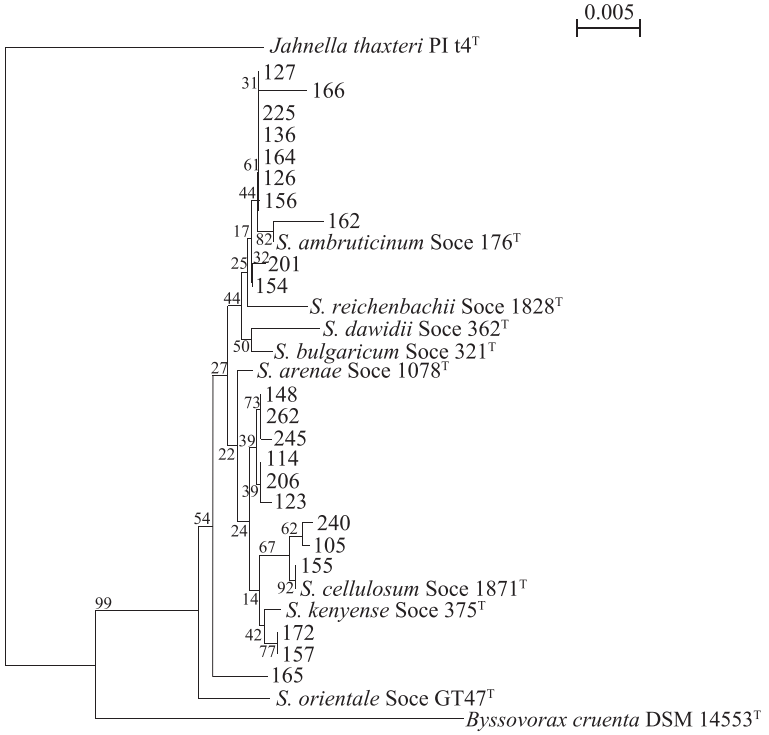

Fig. 1 Neighbor-joining tree based on partial 16S rRNA gene sequences (1,300 bp) showing relationships among 22 isolated strains, 8 *Sorangium* strains, and *Byssovorax cruenta*. *Jahnella thaxteri* (*Sorangiineae*, *Polyangiaceae*) was used to root the tree. Bar, 0.05 substitutions per nucleotide position. Numbers at the forks represent bootstrap values (%) out of 1,000 replicates.

Table 4 Highest sequence similarities between isolates and reference strains in 16S rRNA gene sequences, and known products and antimicrobial activities of isolates

Strain No.	Closest species	% of similarity	Known products	Antimicrobial activity[a]
126	*S. ambruticinum*	99.86	Disorazole A, Pellasoren A	Y, M
127	*S. ambruticinum*	99.91	Ambruticin VS3	Y
136	*S. ambruticinum*	99.86	Pellasoren A	Y, M
154	*S. ambruticinum*	99.36	None	A, M
156	*S. ambruticinum*	99.86	Disorazole A	M
162	*S. ambruticinum*	99.62	Tuscoron B, Epothilone D/G2, Chivotriene, Microsclerodermin M, Eliamid	A, Y, M
164	*S. ambruticinum*	99.86	None	B, M
166	*S. ambruticinum*	99.52	Pellasoren A	M
201	*S. ambruticinum*	99.70	None	M
225	*S. ambruticinum*	99.86	Disorazole A, Tuscoron B, Phoxalone, Chivosazole A	B, Y, M
114	*S. arenae*	99.78	Spirodienal, Ambruticin VS2, Disorazole F2	A, Y, M
123	*S. arenae*	99.72	Spirangien A, Spirangien B	Y
148	*S. arenae*	99.72	Spirodienal, Ambruticin VS2, Tuscoron B, Phoxalone, Spirangien Q	B, A, M
206	*S. arenae*	99.79	None	None
245	*S. arenae*	99.64	None	None
262	*S. arenae*	99.59	None	None
105	*S. cellulosum*[b]	99.65	None	M
155	*S. cellulosum*[c]	99.78	Disorazole A2	A
240	*S. cellulosum*[b]	99.77	None	None
157	*S. kenyense*	99.79	Epothilone B	Y
165	*S. kenyense*	99.41	None	None
172	*S. kenyense*	99.51	None	Y, M

[a]Antimicrobial activity against A: actinomycete, B: bacterium, Y: yeast, M: mold. [b]*S. cellulosum* Soce 56. [c]*S. cellulosum* Soce 1871^T

quencing Kit (Applied Biosystems).

Phylogenetic tree was constructed by the neighbor-joining method (Saitou & Nei, 1987) using the ClustalX program, and the resulting neighbor-joining tree based on 16S rRNA gene sequences is shown in Fig. 1. The 16S rRNA gene sequences (approximately 1,300–1,400 bp in length) were analyzed using an EzBioCloud database. The 16S rRNA gene sequences of all strains showed more than 99% sequence identity with the cellulose-decomposing genus *Sorangium*. As shown in Table 4, 10 out of the 22 strains had the highest 16S rRNA gene similarity with *Sorangium ambruticinum* (Soce 176^T), and the other strains had the highest similarity with *Sorangium arenae* (Soce 1078^T), *S. cellulosum,* (Soce 56 or Soce 1871^T) or *Sorangium kenyense* (Soce 375^T) (Mohr *et al*., 2018). The isolates with the highest similarity to *S. ambruticinum* produced mainly pellasoren A and disorazole A, and had antifungal activities.

CONCLUSION

We isolated cellulolytic myxobacteria from soils in Japan, and these strains could be isolated from approximately 20% of soil samples. Purification of the isolates was mainly performed by heating and drying the fruiting bodies. Approximately 80% of isolates were found to produce metabolites with antimicrobial activity, and they had mainly antifungal activity. Based on LC-MS analysis of extracts from 120 isolates, 34 known natural products of the genus *Sorangium* were produced by 100 isolates.

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REFERENCES

Bode, H.B. & Müller, R. 2006. Analysis of myxobacterial secondary metabolism goes molecular. J. Ind. Microbiol. Biotechnol. **33**: 577–

588.

Dawid, W. 2000. Biology and global distribution of myxobacteria in soils. FEMS Microbiol. Rev. **24**: 403–427.

Dworkin, M. 1996. Recent advances in the social and developmental biology of the myxobacteria. Microbiol. Rev. **60**: 70–102.

Gaspari, F., Paitan, Y., Mainini, D., Losi, D., Ron, E.Z. & Marinelli, F. 2005. Myxobacteria isolated in Israel as potential source of new anti-infectives. J. Appl. Microbiol. **98**: 429–439.

Gerth, K., Pradella, S., Perlova, O., Beyer, S. & Müller, R. 2003. Myxobacteria: proficient producers of novel natural products with various biological activities-past and future biotechnological aspects with the focus on the genus *Sorangium*. J. Biotechnol. **106**: 233–253.

Gerth, K., Trowitzsch, W., Piehl, G., Schultze, R. & Lehmann, J. 1984. Inexpensive media for mass cultivation of myxobacteria. Appl. Microbiol. Biotechnol. **19**: 23–28.

Han, K., Li, Z., Peng, R., Zhu, L., Zhou, T., Wang, L., Li, S., Zhang, X., Hu, W., Wu, Z., Qin, N. & Li Y. 2013. Extraordinary expansion of a *Sorangium cellulosum* genome from an alkaline milieu. Sci. Rep. **3**: 2101. doi:10.1038/srep02101.

Komaki, H., Fudou, R., Iizuka, T., Nakajima, D., Okazaki, K., Shibata, D., Ojika, M. & Harayama, S. 2008. PCR detection of type I polyketide synthase genes in myxobacteria. Appl. Environ. Microbiol. **74**: 5571–5574.

Mohr, K.I., Wolf, C., Nübel, U., Szafranska, A.K., Steglich, M., Hennessen, F. Gemperlein, K., Kampfer, P., Martin, K., Müller, R. & Wink, J. 2018. A polyphasic approach leads to seven new species of the cellulose-decomposing genus *Sorangium*, *Sorangium ambruticinum* sp. nov., *Sorangium arenae* sp. nov., *Sorangium bulgaricum* sp. nov., *Sorangium dawidii* sp. nov., *Sorangium kenyense* sp. nov., *Sorangium orientale* sp. nov. and *Sorangium reichenbachii* sp. nov. Int. J. Syst. Evol. Microbiol. **68**: 3576–3586.

Reichenbach, H. 1999. The ecology of the myxobacteria. Environ. Microbiol. **1**: 15–21.

Reichenbach, H. 2001. Myxobacteria, producers of novel bioactive substances. J. Ind. Microbiol. Biotechnol. **27**: 149–156.

Reichenbach, H. & Dworkin, M. 1992. The myxobacteria, *In* Trüper, H.G., Dworkin, M., Harder, W. & Schleifer, K.-H. (eds.), The Prokaryote, second edition. p. 3418–3487, Spriger-Verlag, Berlin.

Reichenbach, H., Lang, E., Schumann, P. & Spröer, C. 2006. *Byssovorax cruenta* gen. nov., sp. nov., nom. rev., a cellulose-degrading myxobacterium: rediscovery of *'Myxococcus cruentus'* Thaxter 1897. Int. J. Syst. Evol. Microbiol. **56**: 2357–2363.

Saitou, N. & Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4**: 406–425.

Schneiker, S., Perlova, O., Kaiser, O., Gerth, K., Alici, A., Altmeyer, M.O., Bartels, D., Bekel, T., Beyer, S., Bode, E. & other 49 authors 2007. Complete genome sequence of the myxobacterium *Sorangium cellulosum*. Nat. Biotechnol. **25**: 1281–1289.

Shimkets, L.J., Dworkin, M. & Reichenbach, H. 2006. The myxobacteria, *In* Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H. & Stackebrandt, E. (eds.), The Prokaryotes vol. 7, p. 31–115, Springer, Berlin.

Stanier, R.Y. 1942. The cytophaga group: a contribution to the biology of myxobacteria. Bacteriol. Rev. **6**: 143–196.

日本の土壌から分離されたセルロース分解性粘液細菌の抗菌活性と天然物質

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セルロース分解性粘液細菌は細菌のなかで最も大きなゲノムを有し，新規な有用物質を生産すると考えられるが，日本ではほとんど分離されていない．日本の土壌を 2000 ヵ所から採取し，ろ紙を置いた培地に接種後，室温で 30 日程度培養すると 2 割程度のプレートでセルロース分解性粘液細菌の子実体が観察され，主に 60℃の高温処理と室温での乾燥により純化を行った．純化菌 22 株の 16S rRNA の塩基配列を決定すると，*Sorangium ambruticinum, S. arenae, S. cellulosum* や *S. kenyense* と 99%以上の相同性が見られた．120 株の純化菌を XAD-16 樹脂を含む P 培地と E 培地に 30 日ほど振とう培養後，生産物をアセトン抽出し，微生物の生育阻害を見たところ，約 6 割の抽出物において *Aspergillus niger* の生育阻害が見られ，分離株は主に真菌に対する抗菌物質を生産していた．また，120 株の抽出物の LC/MS 解析より spirodienal, ambruticin, disorazole および epothilone など 34 の *Sorangium* 属由来の既知化合物が 100 株の抽出物より同定されたが，*Sorangium* 属由来の既知物質と分子量が異なる化合物も多く見出され，未知の有益物質を生産している可能性が示唆された．