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A method for the selective isolation of *Myxococcus* directly from soil

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A new method is described for the selective isolation of species of *Myxococcus* directly from soil by dilution plating. The method involves suppression of competing microorganisms with antibiotics combined with air drying and wet heat treatment of soils. Fungi were eliminated by supplementing the plating medium with cycloheximide and nystatin. Non-sporulating bacteria were controlled by air drying soils and then heating aqueous soil dilutions for 10 min at 56°C. The predominant sporulating bacteria in soil, *Streptomyces* and *Bacillus*, were suppressed by adding either tiacumicin B, ristocetin or vancomycin to the medium. Swarming of *Myxococcus* colonies was controlled with a casein digest-yeast extract plating medium (CY-C10 agar). Ultrasound treatment of soil suspensions gave the highest number of *Myxococcus* colonies in the soils studied, but these cultures could be recovered without ultrasound. Strains of *Myxococccus fulvus*, *M. xanthus*, *M. coralloides*, *M. stipitatus* and *M. virescens* were isolated from soil using this technique. Soils examined yielded one or two *Myxococcus* species per sample.

Keywords: myxobacteria; *Myxococcus*; soil; selective isolation; direct isolation method

Introduction

Myxobacteria are prolific producers of a variety of bioactive secondary metabolites including antibacterial, antifungal, antiviral and antitumor compounds [16]. Remarkably, Reichenbach found that 60–80% of the myxobacteria tested had antibacterial or antifungal activity [12]. About 50 basic structures and 300 structural variants have been described from these cultures, and most appear to be unique to myxobacteria [14]. Even though these cultures are attractive sources of new compounds, they have not received wide attention in pharmaceutical bioactive metabolite screening programs primarily because they are difficult to isolate. In fact, most of the new compounds described were obtained from myxobacteria isolated in one laboratory by Reichenbach and associates [14].

In general, the isolation of myxobacteria of all types can be a time-consuming and often tedious process. They are usually obtained from herbivore dung, decaying plant material and bark of living and dead trees by placing samples of these materials in a moist chamber [13] and observing them frequently for the development of characteristic fruiting bodies. They can also be recovered from soil by baiting with herbivore dung [4] and by placing soil on streaks of bacterial or yeast cells on agar plates [17]. The fruiting bodies that form are picked and streaked in the hope of obtaining a pure culture. Often, however, the fruiting bodies are contaminated with other bacteria, and an involved purification process is usually necessary [13].

Soil is the preferred source for obtaining myxobacteria in our laboratory, as it is in many pharmaceutical screening laboratories, because a wide variety of soils are easy to obtain, and large numbers are collected, stored and used to recover different microorganisms. Singh [17] estimated that myxobacteria of various kinds were present at 2×10^3 to 76.4×10^3 per g of soil. McCurdy [7] found that the *Myxococcus* populations alone ranged from 1×10^3 to 45×10^5 per g in some soils. Although they are present in relatively high numbers, myxobacteria are not usually seen on soil dilution plates for several reasons: (1) they grow slowly and are overgrown by other soil microorganisms; (2) on the lean media most frequently used in soil isolation studies, they form delicate spreading swarms that are easily overlooked; (3) they produce a slime matrix which does not readily disperse in aqueous diluent [13]. The purpose of the current study was to solve these problems and develop a rapid dilution plating method for selectively isolating one genus of myxobacteria, *Myxococcus*, directly from soils to obtain large numbers for high throughput metabolite screening. We focused on *Myxococcus* because, in our experience, members of this genus were easier to cultivate than other myxobacteria. In addition, they are rich sources of secondary metabolites. Reichenbach and Höfle pointed out in a recent review [14] that, of 24 published bioactive compounds isolated from myxobacteria, 13 were produced by *Myxococcus (Corallococcus)* species.

Materials and methods

Antibiotics and cultures

Bacitracin, chloramphenicol, chlortetracycline hydrochloride, cycloheximide, gramicidin D, leucomycin V, monensin (sodium salt), neomycin sulfate, novobiocin (sodium salt), nystatin, penicillin G, rifampicin, thiostrepton and vancomycin hydrochloride were purchased from Sigma Chemical Co (St Louis, MO, USA). Sulfanilamide was obtained from JT Baker Chemical Co (Phillipsburg, NJ, USA). Chelocardin, clarithromycin, difloxacin, erythromycin, ristocetin,

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tiacumicin B and spectinomycin were obtained from Abbott Laboratories (Abbott Park, IL, USA). Tiacumicin B is a new antibiotic discovered in our screening program [3,19]. The cultures tested in antibiotic sensitivity studies are listed in Table 1. The myxobacteria and *Bacillus* strains were obtained from the American Type Culture Collection (ATCC) or the National Center for Agricultural Utilization Research (NRRL) as indicated by their accession numbers. The *Streptomyces* strains used were isolated in our own program. Cultures with different mature spore mass colors were selected to ensure variety.

Antibiotic sensitivity studies

All cultures were grown for antibiotic sensitivity studies on basal medium CY-C10 agar which consisted of (g L^{-1} in distilled water): Casitone (Difco, Detroit, MI, USA), 10; yeast extract (Difco), 1; $CaCl_2 \cdot 2H_2O$, 1; and agar 15. This medium was supplemented with 50 μg ml^{-1} each of cycloheximide and nystatin unless noted otherwise. CY-C10 medium is a modification of CY agar [11]. Test antibiotics were dissolved in appropriate solvents and added to the sterilized, molten agar after it had cooled to about 55°C. The molten agar was dispensed into standard 100-mm diameter petri plates. Bacitracin, penicillin G, ristocetin (with sufficient 1 N HCl to dissolve it) and vancomycin were dissolved in water. Thiostrepton was dissolved in dimethyl sulfoxide and nystatin in methanol/dimethyl sulfoxide 4 : 1. The remainder were dissolved in methanol. Solutions of the antibiotics soluble in water were sterilized by filtration. The others were added without filtration. Each antibiotic was tested at 1, 5, 10, 20 and 40 μg ml^{-1}.

Myxococcus strains to be inoculated onto antibiotic test plates were grown at 30°C for 4–5 days on CY-C10 agar without antifungals. Colonies were macerated using Radnoti Econo-Grind Micro Tissue Grinders (Radnoti Glass Technology, Monrovia, CA, USA, catalog No. 440613), and dense suspensions were dispensed in sterile 96-well microplates. *Streptomyces* isolates were grown at 28°C on ATCC 172 agar [2] until they were well sporulated. The *Bacillus* strains were grown overnight at 28°C on nutrient agar supplemented with (g L^{-1}): yeast extract (Difco), 5; $CaCl_2 \cdot 2H_2O$, 0.1; $MgCl_2 \cdot 6H_2O$, 0.2; and $MnCl_2 \cdot 4H_2O$, 0.01. Spore suspensions of the *Streptomyces* strains and cell suspensions of the *Bacillus* strains were prepared in sterile distilled water and also placed in microplates. A sterile multipoint inoculator was used to spot all culture suspensions onto antibiotic-amended agar petri plates. The inoculated plates were incubated at 30°C. Growth was assessed after overnight incubation for *Bacillus* strains and after 7 days for *Myxococcus* and *Streptomyces*.

Table 1 Microorganisms used in antibiotic sensitivity studies

1) *Angiococcus (Myxococcus) disciformis* ATCC 33172
2) *Chondrococcus (Myxococcus) macrosporus* ATCC 29039
3) *Myxococcus fulvus* ATCC 25199
4) *Myxococcus virescens* ATCC 25203
5) *Myxococcus xanthus* ATCC 25232
6) *Bacillus brevis* ATCC 8185
7) *Bacillus cereus* ATCC 9139
8) *Bacillus circulans* NRRL B-1359
9) *Bacillus megaterium* ATCC 9885
10) *Bacillus subtilis* ATCC 6633
11) *Streptomyces* sp 1
12) *Streptomyces* sp 2
13) *Streptomyces* sp 5
14) *Streptomyces* sp 7
15) *Streptomyces* sp 8
16) *Streptomyces* sp 9
17) *Streptomyces* sp 10

Soil treatment, media and plating

Soils were air dried at room temperature overnight if they were not from an arid climate and dry already. In the new procedure described here, 0.1-g portions of dry soil were suspended in 0.9 ml of sterile distilled water, and these were treated with ultrasound for 1 min using a Heat Systems/Ultrasonics Sonicator model W-375 (Farmingdale, NY, USA) fitted with a cup horn at a dial setting of 2. Soil suspensions were diluted in sterile distilled water, and 10^{-2} and 10^{-3} dilutions were placed in a water bath at 56°C for 10 min. These dilutions were then plated on CY-C10 agar containing 10 μg ml^{-1} ristocetin, tiacumicin B or vancomycin and 50 μg ml^{-1} each of cycloheximide and nystatin.

Comparative studies were also performed using SP agar [6] and ACE agar [7]. SP agar was supplemented with 10 μg ml^{-1} each of sulfanilamide and neomycin and 50 μg ml^{-1} each of cycloheximide and nystatin. Soil dilutions plated on this medium were also treated with ultrasound and wet heat as indicated above. ACE agar was prepared wtih 5 g L^{-1} dried whole yeast cells (Red Star Nutritional Yeast, Universal Foods Corporation, Milwaukee, WI, USA), 1 g L^{-1} yeast extract (Difco), 10 μg ml^{-1} each of sulfanilamide and neomycin and 100 μg ml^{-1} of cycloheximide. Soil suspensions were dispersed briefly using a vortex mixer without ultrasound or heat treatment when plated on ACE agar. All plates were incubated at 30°C in sealed polyethylene bags and examined periodically. Colonies were usually picked from soil spread plates after 7 days incubation.

Culture identification

To determine how many different species of *Myxococcus* were isolated by this approach, 24 soil samples from various sources were plated on CY-C10 agar supplemented with 10 μg ml^{-1} tiacumicin B using the method described here. Typical swarming colonies were transferred from soil spread plates to SP (casitone-Mg^{2+}) agar plates [6] for identification. These isolates were incubated at 30°C under continuous fluorescent light until fruiting bodies formed, typically in 7 days. Many of the differential features recommended by McCurdy [8] to distinguish *Myxococcus* species were then used to identify these isolates. Fruiting bodies were examined using a stereomicroscope. Wet mounts were prepared from crushed fruiting bodies, and the size, shape and refractility of myxospores were determined using phase contrast microscopy. Plates were also exposed to long wavelength ultraviolet light (360 nm) to determine if the fruiting bodies fluoresced.

Results

Antibiotic sensitivity studies

In an initial study, the five *Myxococcus* strains shown in Table 1 were tested for their resistance to 19 antibacterial compounds. Most of them were sensitive to low levels of nine of these compounds. An antibiotic was selected for further study if not more than one myxobacterium strain was sensitive to it. The ten antibiotics picked on this basis are shown in Table 2. Seven *Streptomyces* and five *Bacillus* strains (Table 1) were then examined for their sensitivity to these ten compounds. The results of both of these studies are summarized in Table 2. At relatively low levels, ristocetin, tiacumicin B and vancomycin inhibited most of the *Streptomyces* and *Bacillus* strains while allowing growth of all of the *Myxococcus* strains. These three antibiotics were then used in soil plating experiments.

Direct isolation comparing ristocetin, tiacumicin B and vancomycin

Table 3 shows the results of plating eight soil samples on CY-C10 agar containing 10 μg ml^{-1} of either ristocetin, tia-

Table 2 Sensitivity of five *Myxococcus* spp, five *Bacillus* spp and seven *Streptomyces* spp to ten antibiotics

Antibiotic	Level (μg ml^{-1})	Test Cultures[a] 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Bacitracin	40	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+
	20	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+
	10	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+
	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Erythromycin	40	−	−	−	+	−	−	−	−	−	−	−	−	−	−	+	−	+
	20	−	−	+	+	−	−	−	−	−	−	−	−	−	−	+	−	+
	10	−	−	+	+	+	−	−	−	−	−	+	+	−	+	+	−	+
	5	−	−	+	+	+	−	−	−	−	−	+	+	−	+	+	−	+
	1	−	−	+	+	+	−	−	−	−	−	+	+	−	+	+	+	+
Gramicidin D	40	−	+	+	+	+	+	−	−	−	−	+	+	+	+	−	−	+
	20	−	+	+	+	+	+	−	−	−	−	+	+	+	+	+	−	+
	10	−	+	+	+	+	+	−	−	−	+	+	+	+	+	+	−	+
	5	−	+	+	+	+	+	−	−	−	+	+	+	+	+	+	−	+
	1	−	+	+	+	+	+	−	−	−	+	+	+	+	+	+	−	+
Monensin	40	+	−	−	+	−	−	−	−	−	−	+	+	−	+	+	−	−
	20	+	+	−	+	+	−	−	−	−	−	+	+	−	+	+	−	−
	10	+	+	+	+	+	−	−	−	−	+	+	+	−	+	+	−	+
	5	+	+	+	+	+	−	−	−	−	+	+	+	+	+	+	+	+
	1	+	+	+	+	+	+	+	−	−	+	+	+	+	+	+	+	+
Penicillin G	40	−	+	+	−	−	−	+	−	−	−	+	−	−	−	−	−	+
	20	−	+	+	−	+	−	+	−	−	−	+	−	+	+	+	−	+
	10	−	+	+	+	+	−	+	−	−	−	+	−	+	+	+	−	+
	5	−	+	+	+	+	−	+	−	−	−	+	+	+	+	+	−	+
	1	−	+	+	+	+	−	+	+	−	−	+	+	+	+	+	+	+
Ristocetin	40	+	+	−	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	20	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	10	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	5	+	+	+	+	+	−	−	−	−	−	+	−	−	+	−	−	−
	1	+	+	+	+	+	−	−	−	−	−	+	+	−	+	−	−	+
Spectinomycin	40	−	+	+	+	+	−	+	−	−	−	+	+	+	−	+	−	+
	20	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	−	+
	10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+
	5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sulfanilamide	40	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tiacumicin B	40	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	20	+	+	+	+	+	+	−	−	−	+	−	−	−	−	−	−	−
	10	+	+	+	+	+	+	−	−	−	+	−	−	−	−	−	−	−
	5	+	+	+	+	+	+	−	−	+	+	+	−	−	−	−	−	+
	1	+	+	+	+	+	+	+	+	−	−	+	+	−	+	−	+	+
Vancomycin	40	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	20	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	10	+	+	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−
	5	+	+	+	+	+	−	−	−	−	−	+	−	−	−	−	−	−
	1	+	+	+	+	+	−	+	−	−	−	+	+	−	+	+	−	−
Control		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

[a]Test culture numbers follow the sequence in Table 1.
Key for Table: + = growth; − = no growth.

Table 3 Comparison of ristocetin, tiacumicin B and vancomycin for isolating *Myxococcus* directly from soil

Soil	Source	Antibiotic (10 μg ml^{-1})								
		Ristocetin			Tiacumicin B			Vancomycin		
		Myxococcus[a]	Actinomycetes[b]	Other bacteria[b]	*Myxococcus*	Actinomycetes	Other bacteria	*Myxococcus*	Actinomycetes	Other bacteria
A	California	30	6	70	31	1	95	25	5	63
B	New Mexico	9	5	55	9	2	137	11	8	45
C	Sri Lanka	5	1	54	4	0	35	9	1	47
D	Ethiopia	4	3	94	4	1	71	8	0	49
E	Ethiopia	13	6	100	24	1	93	19	1	98
F	California	5	2	22	8	1	62	8	12	26
G	California	1	1	67	0	0	70	0	0	43
H	Texas	6	7	80	9	0	64	3	3	55
Total		73			89			83		
Average			3.3	68		0.8	78		3.8	53

[a]*Myxococcus* reported as the sum of all colonies observed on four plates (two plates each at 10^{-3} and 10^{-4} dilutions).
[b]Actinomycetes and other bacteria reported ($\times 10^4$) per g of soil.

cumicin B or vancomycin. Because of the low numbers of *Myxococcus* in some soils, counts from four soil spread plates were summed. Tiacumicin B was selected for further study, but, as shown in Table 3, there was little difference among the three antibiotics.

Comparison of direct isolation methods

The 11 soils shown in Table 4 were diluted and spread, without additional treatment, on ACE agar containing sulfanilamide, neomycin and cycloheximide. These plates were completely overgrown with spreading bacteria. This medium was then modified in an attempt to recover *Myxococcus*. The yeast cells and yeast extract were removed from ACE agar because yeast extract enhances swarming in some bacteria [1]. ACE agar without yeast cells and yeast extract is SP agar [7]. The same 11 soil samples were treated with ultrasound and mild heat and plated on this modified medium. Table 4 shows the results of plating these soil samples on SP agar with sulfanilamide and neomycin compared to plating them on CY-C10 agar amended with tiacumicin B. Even with modifications, the SP agar gave less than a third of the *Myxococcus* recovered using CY-C10 agar with tiacumicin B.

Effects of various combinations of tiacumicin B, ultrasound and wet heat

To determine how important antibiotic supplementation, wet heat treatment and ultrasound were for recovering *Myxococcus*, three soils were plated using every combination of these variables. The soils were plated on CY-

Table 4 Comparison of two methods for isolating *Myxococcus* from soil

Soil	Source	Method					
		CY-C10 agar/Tiacumicin B (US and MH[a])			SP agar/Sulfanilamide and Neomycin (US and MH)		
		Myxococcus[b]	Actinomycetes[b]	Other bacteria[c]	*Myxococcus*	Actinomycetes	Other bacteria
I	California	16	0	~50	11	8	*
J	New Mexico	7	0	~50	0	~50	*
K	Sri Lanka	11	3	~50	0	1	*
L	Ethiopia	5	4	~50	0	30	*
M	Ethiopia	18	3	~50	3	3	~100
N	Philippines	0	~50	~100	1	~50	*
O	Australia	1	29	~50	0	7	*
P	Spain	2	0	~100	2	~50	*
Q	New Mexico	0	0	~100	1	0	~100
R	Mexico	4	0	10	0	0	9
S	California	0	31	41	1	1	33
Totals		64			19		

*Plates covered with swarming bacteria.
[a]US – Soil suspension treated with ultrasound for 1 min prior to plating; MH – Soil suspension treated with wet heat at 56°C for 10 min prior to plating.
[b]*Myxococcus* and actinomycetes reported as the sum of all colonies observed on four plates (two plates each at 10^{-3} and 10^{-4} dilutions).
[c]Other bacteria reported ($\times 10^4$) per g of soil.

C10 agar. Table 5 shows that the combination of antibiotic supplementation and wet heat treatment are critical to isolate *Myxococcus*. Ultrasound increased the number of *Myxococcus*, but in two of the three soils examined in this study, it also significantly increased the number of interfering bacteria.

Identification of isolates

We identified 33 of the 34 swarming isolates obtained from 24 soil samples. The results of this study are shown in Table 6. Based on morphology and color, most soils yielded one colony type, and only one species was identified in these cases. Seven soils (8, 9, 10, 12, 17, 18 and 24) yielded two colony types, and, in each of these samples, two different species were identified. In sample 19, the sizes of the fruiting bodies were quite different in two isolates, but all of their features indicated that they were both *M. stipitatus*. Isolate 4-1 might also be a strain of *M. stipitatus*, but it could not be identified with certainty. The fruiting bodies fluoresced as is characteristic of *M. stipitatus*, but they did not form stalks. The most common species isolated from the 24 soils examined here was *M. fulvus* which was found 14 times. In addition, ten strains of *M. xanthus* were obtained, six of *M. coralloides*, two strains of *M. stipitatus* and one of *M. virescens*.

Discussion

Although *Myxococcus* spp have proven to be a rich source of novel metabolites, methods for isolating them are indirect, slow and tedious. In addition, strains recovered from soil are often contaminated with other bacteria. Only one dilution plating technique for isolating them directly from soil has hitherto been published [7], but this method did not yield any *Myxococcus* isolates in our hands. In the study reported here, we describe a new selective dilution plating method to readily recover pure cultures of *Myxococcus* directly from soil. The method involves suppression of fungi and competing bacteria with antibiotics, air drying and mild wet heat treatment of soil, controlling myxococcal colony spreading with a rich medium and dispersal with ultrasound.

In order to isolate members of a slow-growing, minor soil population such as *Myxococcus*, it is essential to suppress the rapidly growing and numerically predominant microorganisms found there. Different approaches are needed for each of the major groups of soil cultures: fungi, sporulating bacteria and non-sporulating bacteria.

The combination of cycloheximide and nystatin, which has been used for a long time to isolate actinomycetes from soil [20], provided excellent control of fungi on CY-C10 agar. Two physical treatments of soil that are also widely used in the isolation of actinomycetes are drying and mild wet heat. Many non-sporulating bacteria are killed when soil is dried or treated with mild wet heat (50–55°C) for a short time [5,15,21]. Fortunately, *Myxococcus* spores (myxospores) are resistant to desiccation and mild wet heat (56–58°C) [13,18]. All soils that were not already dry were routinely air dried in these studies. The value of wet heat for controlling competing bacteria is shown in Table 5.

The Gram-positive *Streptomyces* and *Bacillus* are the most numerous spore-forming bacteria in soil [9]. We evaluated 19 Gram-positive and broad spectrum antibiotics to inhibit them and found that ristocetin, tiacumicin B and vancomycin were about equally effective (Table 3) in suppressing these populations while permitting growth of *Myxococcus* which are Gram-negative. McCurdy [7] mentioned that some myxobacteria were resistant to ristocetin, but the effect of the other two antibiotics on the growth of these cultures apparently has never been examined before.

Myxobacteria colonies at times are not easily recognized because they form thin swarms that can cover an entire plate on lean media or can be compact and not swarm on rich media [13]. In initial studies with these organisms, CY-

Table 5 Effect of various combinations of tiacumicin B-amended media, wet heat pretreatment and ultrasound on the isolation of *Myxococcus* from soil samples

Variable[a]			Soil/Source								
1	2	3	T/California			U/New Mexico			V/Ethiopia		
			Myxo-coccus[b]	Actino-mycetes[c]	Other bacteria[c]	*Myxo-coccus*	Actino-mycetes	Other bacteria	*Myxo-coccus*	Actino-mycetes	Other bacteria
+	+	+	62	0	82	20	0	39	25	0	65
+	−	−	1	7	86	0	0	101	0	0	TNTC
−	+	−	0	19	*	0	9	*	0	4	*
−	−	+	0	16	*	0	7	TNTC[d]	0	0	TNTC
+	+	−	52	0	14	15	0	56	22	0	5
+	−	+	2	1	57	0	0	TNTC	0	5	TNTC
−	+	+	0	12	*	0	5	*	0	3	TNTC
−	−	−	0	7	*	0	6	TNTC	0	0	TNTC

[a]Variables: (1) plating media amended with 10 μg ml^{-1} tiacumicin B; (2) soil suspension treated with wet heat at 56°C for 10 min prior to plating; (3) soil suspension treated with ultrasound for 1 min prior to plating.
[b]*Myxococcus* reported as the sum of all colonies observed on four plates (two plates each at 10^{-3} and 10^{-4} dilutions).
[c]Actinomycetes and other bacteria reported ($\times 10^4$) per g of soil.
[d]TNTC = Too numerous to count.
*Indicates that plates were overgrown with swarming bacteria.

Table 6 Identification of fruiting, swarming bacteria isolated from 24 soil samples

Soil – isolate	Soil source	Stalk	Fruiting body size dia. (μm)	Fruiting body fluorescent	Vegetative cell mass color	Fruiting body shape	Myxospore diameter	Identification
1 – 1	Philippines	+	80–100	–	Yellow	Spherical	< 2.5 μm	*Myxococcus fulvus*
2 – 1	Philippines	–	100–200	–	Yellow	Spherical	≥ 2.5 μm	*M. xanthus*
3 – 1	Philippines	–	100–400	–	Yellow	Irregular	< 2.5 μm	*M. coralloides*
4 – 1	Sri Lanka	–	200–300	+	Reddish-orange	Irregular	< 2.5 μm	
5 – 1	New Mexico	+	80–300	–	Yellow	Spherical	< 2.5 μm	*M. fulvus*
6 – 1	Texas	–	100–300	–	Reddish-orange	Irregular	< 2.5 μm	*M. coralloides*
7 – 1	California	–	100–400	–	Flesh-colored	Spherical	≥ 2.5 μm	*M. xanthus*
8 – 1	California	–	100–500	–	Yellow	Irregular	< 2.5 μm	*M. coralloides*
8 – 2	California	+	80–200	–	Yellow-orange	Spherical	< 2.5 μm	*M. fulvus*
9 – 1	Colorado	–	150–300	–	Yellow-orange	Spherical	≥ 2.5 μm	*M. xanthus*
9 – 2	Colorado	+	100–300	–	Yellow-orange	Spherical	< 2.5 μm	*M. fulvus*
10 – 1	Colorado	–	100–250	–	Yellow	Spherical	≥ 2.5 μm	*M. xanthus*
10 – 2	Colorado	+	100–400	–	Buff	Spherical	< 2.5 μm	*M. fulvus*
11 – 1	Colorado	+	150–300	–	Flesh-colored	Spherical	< 2.5 μm	*M. fulvus*
12 – 1	California	–	100–700	–	Yellow	Irregular	< 2.5 μm	*M. coralloides*
12 – 2	California	–	100–300	–	Yellow-orange	Spherical	≥ 2.5 μm	*M. xanthus*
13 – 1	New Mexico	–	100–150	–	Yellow	Spherical	≥ 2.5 μm	*M. xanthus*
14 – 1	Spain	–	100–300	–	Yellow	Spherical	≥ 2.5 μm	*M. xanthus*
15 – 1	Sri Lanka	–	40–100	–	Reddish-orange	Irregular	< 2.5 μm	*M. coralloides*
16 – 1	Nigeria	+	100–500	–	Orange	Spherical	< 2.5 μm	*M. fulvus*
17 – 1	California	+	50–200	–	Yellow	Spherical	< 2.5 μm	*M. fulvus*
17 – 2	California	–	150–250	–	Yellow-orange	Spherical	≥ 2.5 μm	*M. xanthus*
17 – 3	California	–	100–150	–	Yellow-orange	Spherical	≥ 2.5 μm	*M. xanthus*
18 – 1	Texas	+	50–300	–	Yellow	Spherical	< 2.5 μm	*M. fulvus*
18 – 2	Texas	–	100–300	–	Yellow	Spherical	≥ 2.5 μm	*M. xanthus*
18 – 3	Texas	+	50–200	–	Yellow	Spherical	< 2.5 μm	*M. fulvus*
19 – 1	Kansas	+	100–600	+	Reddish-orange	Spherical	< 2.5 μm	*M. stipitatus*
19 – 2	Kansas	+	100–300	+	Reddish-orange	Spherical	< 2.5 μm	*M. stipitatus*
20 – 1	Pennsylvania	–	50–100	–	Yellow	Spherical	≥ 2.5 μm	*M. virescens*[a]
21 – 1	Pennsylvania	+	100–200	–	Yellow-orange	Spherical	< 2.5 μm	*M. fulvus*
22 – 1	Utah	+	200–300	–	Flesh-colored	Spherical	≥ 2.5 μm	*M. fulvus*
23 – 1	Arizona	+	100–200	–	Reddish-orange	Spherical	≥ 2.5 μm	*M. fulvus*
24 – 1	Wisconsin	–	40–150	–	Yellow	Spherical	< 2.5 μm	*M. fulvus*
24 – 2	Wisconsin	–	100–400	–	Orange	Irregular	< 2.5 μm	*M. coralloides*

[a]Produces a diffusible green pigment.

C10 medium seemed to strike a balance between these extremes since colonies were relatively thick and swarmed but grew to only 1–2 cm in diameter. This medium allowed ready selection of presumptive myxobacteria colonies and was used throughout the work reported here.

Reichenbach and Dworkin [13] pointed out that myxobacteria do not disperse readily in aqueous diluents because of their slimė matrix and therefore are often not seen with the usual plating techniques. One method that is used to disperse microorganisms in soil is ultrasound [10,22]. Myxospores are resistant to ultrasound [18], which has been used to disperse myxospores in pure culture [13]. Table 5 indicates that, as expected, ultrasound treatment of soil gave the highest counts of *Myxococcus* strains on isolation plates, but, surprisingly, reasonable numbers were obtained even without it. In some soils the numbers of interfering bacteria also increased with this treatment.

McCurdy described a method for enumerating and isolating *Myxococcus* spp from soil using ACE agar supplemented with neomycin, sulfanilamide and cyclohexamide [7]. We observed no *Myxococcus* from soils spread on ACE agar, and all plates were overgrown with bacteria. McCurdy noted that soil spread plates were extensively overrun with contaminants, and he isolated relatively few pure cultures using this method. *Myxococcus* colonies were observed, however, when we modified this technique by eliminating yeast from ACE agar (SP agar) and treating soil samples with mild heat and ultrasound (Table 4). Even then counts were much lower than on CY-C10 plating medium using our new procedure, and it was difficult to recover pure cultures because of overgrowth by spreading competitors.

We isolated representatives of five of the six *Myxococcus* species recognized in Bergey's Manual of Systematic Bacteriology [8] using the new isolation method described here. In the group of 24 soil samples examined, *M. flavus* was most often recovered followed by *M. xanthus*, *M. coralloides*, *M. stipitatus* and *M. virescens*. No strains of *M. macrosporus*, which is not very common [8], were isolated. Seven of the soils studied yielded two *Myxococcus* species, but the majority gave only one. Most strains were pure when picked directly from soil spread plates and could be used immediately in our screening program.

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