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The selective isolation of *Micromonospora* from soil by cesium chloride density gradient ultracentrifugation

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SUMMARY

A novel method is described for the selective isolation of *Micromonospora* from mixed microbial populations in soil. Microorganisms were released from soil by sonication, and the bulk of the soil debris was discarded by low-speed centrifugation. The supernatant microbial suspension was concentrated and applied to a continuous, linear (1.1–1.6 g/ml) gradient of CsCl which was then centrifuged at high speed. The gradient was fractionated, and each fraction was diluted and plated on a medium devoid of antimicrobial agents. *Micromonospora* were found in the 1.35–1.42 g/ml density band. Occasionally, *Bacillus* species were also obtained in this density range, but other nonfilamentous bacteria or actinomycetes were usually not observed. This technique allowed the isolation of portions of the soil *Micromonospora* population which were suppressed by conventional isolation techniques employing heat and antibiotics.

INTRODUCTION

Numerous methods have been developed to isolate members of the genus *Micromonospora* both to obtain them as sources of new antibiotics and to study their ecological significance. Among the actinomycetes, *Micromonospora* are second only to *Streptomyces* in the ability to produce antibiotics [3]. In the environment, micromonosporae are an important part of the aquatic ecosystem [7]. They appear to be metabolically active in lake-bottom sediments as well as in water-soaked surface soils and could play a role there in the cycling of nutrients [10].

Johnston and Cross [11] found that micromonosporae were the predominant actinomycete in lake water and sediments and could be isolated by simply plating samples on colloidal chitin agar containing cycloheximide. Although they are also quite common in soil [4], micromonosporae, however, make up only a small portion of the total soil bacterial population [1,13]. Selective techniques are therefore needed to isolate them from the numerically predominant streptomycetes and nonfilamentous bacteria. *Micromonospora* spores are more resistant to heat than are *Streptomyces* spores [12] and vegetative forms of soil bacteria [4] but are less resistant than *Bacillus* endospores. Micromonos-

porae can therefore be readily obtained by heating a soil suspension at 70°C for 30 min and plating on media which inhibits the outgrowth of *Bacillus* spores [6]. The antibiotics gentamicin [9], tunicamycin [23] and novobiocin [16] have also been used successfully to selectively isolate micromonosporae from soil. Combinations of heat treatments and antibiotics have been used in our laboratory to obtain soil dilution plates that often hold nothing but micromonosporae (unpublished data).

There is always a concern, however, when using such relatively harsh agents, that some fragile members of the target population might not survive. For instance, *M. megalomicea* NRRL 3718 does not grow in the presence of 25 μg/ml tunicamycin which was considered the optimum concentration of this antibiotic for isolating micromonosporae from soil [23]. Studies conducted by Kawamoto et al. [12] revealed that spores of *M. globosa* KCC A-0126 and *M. purpureochromogenes* ATCC 27007 were much more susceptible to killing by heat treatment (60°C, 20 min) than the majority of strains they tested.

This study describes an alternative, gentle method of separating micromonosporae from other soil microorganisms by density gradient ultracentrifugation.

MATERIALS AND METHODS

Soil samples. The A horizons of nine soils were selected for study. The origin, textural type, series (if known), and pH of each are listed in Table 1.

Release and concentration of cells. 2 g of soil were suspended in 15 ml of sterile saline and sonicated for 1 min using a Heat Systems/Ultrasonics Sonicator™ (model W-375) fitted with a standard microtip operating at 35 W acoustic energy. The heaviest soil particles were allowed to settle for 2 min, and approx. 12 ml of the upper layer were removed. This layer was centrifuged at 755 × *g* for 5 min at 4°C, the supernatant was decanted and retained, and the pellet was discarded. The supernatant was then centrifuged at 12 100 × *g* for 10 min at 4°C, the resulting supernatant was discarded, and the pellet, which was very small, was resuspended in 0.65 ml saline.

Preparation of density gradients. Biological grade CsCl was dissolved in pH 7 0.01 M phosphate buffer in saline to prepare 1.1 and 1.6 g/ml (w/v) solutions which were filter sterilized. With these two solutions as starting and ending concentrations, respectively, linear gradients were prepared using a Buchler gradient former (model No. 2-5102). Gradients having a final volume of 4.6 ml were prepared in 1/2 × 2 inch polyallomer centrifuge tubes. The gradient former and centrifuge tubes were rinsed with ethanol to minimize microbial contamination.

Ultracentrifugation and fractionation. 0.5 ml of soil cell concentrate was layered on the top of the CsCl gradient, and the tubes were centrifuged in a Beckman model L2-65B centrifuge with a Beckman SW 50.1 rotor at 173 500 × *g* for 17 h at 4°C. After

Table 1

Experimental soils

	Origin	Textural type	Series	pH
1	Luxembourg	Sandy loam	Not known	6.5
2	Michigan	Sandy loam	Ubly	6.8
3	Indiana	Muck	Edwards	6.2
4	Indiana	Sandy loam	Brady	7.0
5	Iowa	Loamy sand	Not known	7.2
6	Michigan	Loamy sand	Iosco	7.2
7	Michigan	Loamy sand	Montcalm	6.7
8	Michigan	Sandy loam	Emmet	7.1
9	Nebraska	Loamy sand	Not known	5.6

centrifugation, 0.3 ml fractions were removed sequentially from the top of the gradient column using a different, sterile 1.0 ml syringe for each fraction. The linearity of the gradient was determined with a Bausch and Lomb refractometer by measuring the refractive index of every fourth fraction. Refractive index was converted to density using a standard curve.

Plating. Density gradient fractions were decimally diluted in saline and portions were spread on a medium consisting of 0.1% soluble potato starch (J.T. Baker Chemical Co.), 0.01% yeast extract (Difco), 0.1% KNO_3, 0.05% NaCl, 0.05% $MgSO_4$, 0.05% KH_2PO_4, 1.0 ml of trace element solution per liter [8], and 1.5% agar in distilled water (pH 7.3 before autoclaving). When required, antibiotics were added to the sterilized molten agar medium before the plates were poured. Cycloheximide, nalidixic acid (neutralized with NaOH), and novobiocin were dissolved in methanol and added to give final concentrations of 50 μg/ml, 30 μg/ml, and 25 μg/ml, respectively. Nystatin was dissolved in methanol/dimethylsulfoxide 9:1 (v/v) and added to give a final concentration of 20 μg/ml. Plates were incubated at 30°C.

CsCl toxicity assessment. A 1.42 g/ml CsCl solution was prepared in pH 7 0.01 M phosphate-buffered saline. This solution was diluted to one-tenth strength in saline, and three-tenths ml portions were spread on plating media made without antibiotics. All solutions were sterile. Next, agar plate cultures of 43 morphologically different *Streptomyces*, 24 *Micromonospora,* 12 nocardioform actinomycetes, and 10 *Bacillus,* which had recently been isolated from soil, were individually suspended in sterile saline. The suspensions were transferred to standard 96-well microtiter trays, and a multipoint replicator was used to inoculate plating media spread with the CsCl dilution. Plates without CsCl were also inoculated. After 7 days incubation, growth on the CsCl supplemented plates was visually compared to growth on control plates.

Comparative treatments. Density gradient fractions containing micromonosporae from 2 g of soil were located by refractive index, pooled, and filtered through 0.2 μm polycarbonate filters (Nucleopore Corporation). The trapped cells were washed on the filter pad with a few ml of sterile saline to remove CsCl which might affect the cells during heat treatment. The filter was then placed in 3 ml of sterile saline, and the cells were removed from the filter by sonication for 20 s at 25 W acoustic energy. The resulting suspension was divided into three equal portions. One was not treated further, one was heated at 55°C for 6 min [19] and one was heated at 70°C for 30 min. All were decimally diluted in saline and plated. The untreated portion was plated on media containing no antibiotics. The portion heated at 55°C was plated on media supplemented with cycloheximide, nystatin, nalidixic acid and novobiocin. The portion heated at 70°C was plated on media containing only the two antifungals and nalidixic acid.

RESULTS

Microorganisms in the plated gradient fractions

Fig. 1 shows the results of a typical density gradient ultracentrifugation experiment. After centrifugation the gradient was essentially linear. Micromonosporae were virtually the only microorganisms in the 1.35–1.42 g/ml density band. Microscopic observation of this band revealed the presence of sporelike bodies. Filamentous forms were never observed in this fraction. The bacteria that concentrated in the 1.25–1.30 band were mostly endospore-forming rods typical of the genus *Bacillus.* Small numbers of other bacteria were encountered occasionally at all densities. They were both spherical and rod-shaped and had various colonial characteristics. No attempt was made to further characterize these cultures.

Although there were only a few streptomycetes in the fractions from the soil used in Fig. 1, these organisms were often observed in larger numbers in other soils. They do not form a distinct band but are usually spread out over the 1.15–1.35 gradient range. Fungi are only rarely obtained with this technique. They apparently remain with the bulk of the soil particles discarded in the first low-speed centrifugation in the release and concentration procedure [5].

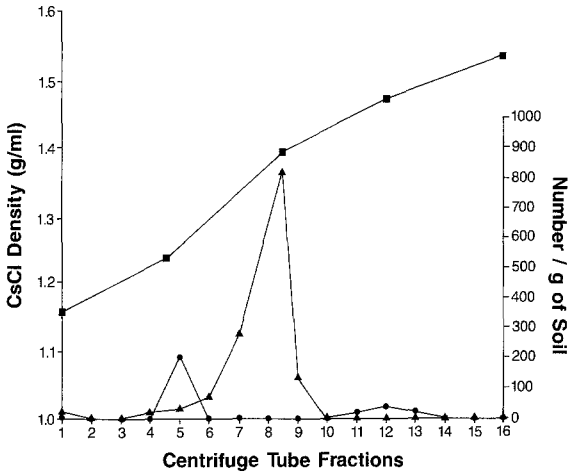

Fig. 1. Density gradient shape and plate counts of *Micromonospora* and nonfilamentous bacteria. ■, density of CsCl fractions; ▲, *Micromonospora*; ●, nonfilamentous bacteria.

CsCl toxicity assessment

It is possible that the CsCl spread onto plating media could itself be exerting a selective, toxic effect on soil microorganisms. To assess this, members of some of the major soil populations were inoculated onto plating media treated with the highest concentration of CsCl plated (1:10 dilution of a 1.42 g/ml solution) from the micromonosporae-containing density gradient band. All of the 43 *Streptomyces,* 24 *Micromonospora,* 12 nocardioform actinomycetes, and 10 *Bacillus* tested grew as well in the presence of CsCl as a control plates.

Comparative survival of micromonosporae

In order to determine whether CsCl density gradient ultracentrifugation has an advantage over other selective isolation methods, micromonosporae from the 1.35–1.42 g/ml density band from nine soils were treated three ways as detailed in Materials and Methods. Although the results vary from soil to soil, Table 2 shows that ultracentrifugation allows the recovery of segments of the micromonosporae population that are suppressed by methods involving heat and antibiotics from eight of the nine soils examined. Of the three methods tested, heating the organisms at 70°C for 30 min gave the poorest recovery in all cases but one.

Table 2

Comparative survival of *Micromonospora* recovered from the 1.35–1.42 g/ml CsCl fraction treated three ways

The treatments were
1: no heat/no antibiotics; 2: 55°C, 6 min/actidione, nystatin, nalidixic acid, novobiocin; 3: 70°C, 30 min/actidione, nystatin, nalidixic acid.

Soil	Treatment/ antibiotics	Number of *Micronomospora* ($\times 10^2$)/g soil after ultracentrifugation
1	1	30.0
	2	28.7
	3	6.0
2	1	10.7
	2	4.7
	3	3.5
3	1	35.0
	2	1.3
	3	0.3
4	1	12.3
	2	16.6
	3	7.7
5	1	9.2
	2	2.3
	3	0.8
6	1	2.2
	2	0.9
	3	0.8
7	1	4.3
	2	2.2
	3	2.0
8	1	9.0
	2	3.2
	3	0.9
9	1	24.5
	2	1.5
	3	2.6

DISCUSSION

Others have taken advantage of differences in buoyant densities to separate microorganisms in a mixture from each other and to separate microorganisms from nonmicrobial debris. Tamir and Gilvarg [21] used Renografin density gradient cen-

trifugation to separate spores and vegetative cells of *Bacillus megaterium*, and Prentice et al. [17] fractionated germinated and non-germinated spores of *B. subtilis* with both Renografin and sucrose. Martin et al. [15] used Renografin to separate *Thiobacillus ferroxidans* from *T. acidophilus*. Reibach et al. [18] used Percoll and Sutton and Mahoney [20] used sucrose to isolate *Rhizobium* from root nodules. Martin and MacDonalds [14] separated *Rhizobium* from root nodules and *Serratia marcescens* from soil with Percoll. Baker et al. [2] isolated *Frankia* from root nodules, for the first time in pure culture, with the aid of sucrose-density centrifugation.

A method is described here to separate micromonosporae from other microorganisms found in soil. Microorganisms released from soil particles by sonication are centrifuged through a CsCl density gradient column. Cells of different buoyant densities sediment to different positions in the gradient. *Bacillus* endospores, which are present in large numbers in many soils and often overgrow isolation plates, are less dense than micromonosporae. They concentrate in the 1.25–1.30 g/ml band which is in good agreement with Tisa et al. [22] who found that *Bacillus* endospores have buoyant densities that range from 1.26 to 1.35 g/ml, depending on the species, in Renografin and metrizamide. Other bacteria are present, usually in small numbers, throughout the density column but do not interfere with isolation because they do not swarm. *Streptomyces*, which are the numerically predominant actinomycete in most soils, are also less dense that micromonosporae. Fungi are removed in the initial clean-up procedure.

The separation of microorganisms from one another based on differences in bouyant density in a gradient is a gentle technique and therefore has an advantage over isolation methods using harsh agents. As the data in this study indicates, CsCl density gradient ultracentrifugation can be used to isolate members of the soil micromonosporae population that are sensitive to heat and antibiotics.

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