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Comparative study of chemoattractants for the isolation of motile-spored actinoplanetes

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Four chemoattractants and three media were used to isolate actinomycetes from environmental samples. Each chemoattractant was evaluated for its ability to attract motile-spored actinoplanetes. The attractants compared were γ-collidine, D-xylose, vanillin and phosphate-buffered potassium chloride (bKCl). The method of Hayakawa *et al* for preparing the chemoattractants was combined with a modified chemotactic method. Of the chemoattractants tested, γ-collidine yielded a slightly greater number of motile-spored actinoplanetes than bKCl or D-xylose, and a significantly greater number than vanillin. bKCl attracted about the same number of organisms as D-xylose. By using several attractants and media with a variety of soils, distinctly different isolates were obtained with each combination.

Keywords: chemotaxis; chemoattractants; actinoplanetes; isolation

Introduction

High throughput natural product screening programs depend upon the isolation of diverse microorganisms in large quantities to discover unique bioactive compounds. In order to obtain a diverse microbial population, multiple isolation techniques must be used on a variety of environmental samples [14]. Motile-spored members of the actinoplanetes (eg *Actinoplanes, Ampullariella, Dactylosporangium*, etc) produce many types of novel antibiotics [13] and therefore are of special interest in natural product screening programs. However motile-spored actinoplanetes are isolated less frequently than other actinomycetes such as streptomycetes in routine isolation programs.

The isolation of motile-spored actinoplanetes was studied by Palleroni [11] using a chemotactic approach to separate motile from nonmotile actinomycete spores. Hayakawa *et al* [5] also investigated actinomycete chemotaxis using a variety of chemical attractants.

In this study we combined the methods of Palleroni [11] and Hayakawa *et al* [5] in the comparison of four chemoattractants, using soil samples as the source for all isolations. Three different media were also compared to determine which would yield the greatest number of motile-spored actinoplanetes.

Techniques for improved isolation of these so called 'rarer' actinomycetes include baiting [1], the Makkar and Cross [10] technique for enrichment of germinated swimming spores and the use of polyvalent phage [8] to eliminate streptomycetes and common eubacteria. It has been our experience that these techniques alone yield fewer numbers of unique isolates than chemotactic approaches.

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Materials and methods

Environmental samples

The nine environmentally and geographically diverse soil samples (Table 1) used for the isolations were frozen at −20 °C until prepared for isolation. Samples were air dried and rehydrated several times (Lechevalier MP, personal communication; [10]) to enrich for sporangia-forming actinomycetes. Soils were ground using a mortar and pestle.

Preparation of chemoattractants and buffer

The four attractants used were γ-collidine (Aldrich, Milwaukee, WI, USA), D-xylose (Aldrich), vanillin (Mallinckrodt, Chesterfield, MO, USA) and phosphate-buffered potassium chloride (bKCl). Potassium phosphate buffer (0.1 M) and sterile distilled water were used as controls to the chemoattractants. The bKCl was prepared as described by Palleroni [11], except that we increased the phosphate concentration to 0.05 M. The procedure of Hayakawa *et al* [5] was followed, preparing the three attractants at a concentration of 0.01 M and diluting them

Table 1 Environmental samples used

Sample	pH	Location, and soil type	No of isolates
J209	4.47	Bolivia, sand	6
J211	8.01	India, sand	102
J212	4.89	Cameroon, Mundemba; riverbank, sand	31
J218	7.71	Grand Cayman Island, Grand Cayman; under grass, sand	252
J223	5.69	Thailand, Sukhothai; paddyfield, clay	81
T285	6.47	Indonesia, Lombok; rainforest, loam	70
T287	7.95	USA, Oklahoma; wheat field, sandy loam	53
T288	8.16	USA, Connecticut; tidal flats, clay	13
T289	8.44	Netherlands Antilles, St Maarten; tidal pool, sand	3

with 0.1 M potassium phosphate buffer. Aqueous solutions of the attractants were adjusted to pH 7.0. Before use, γ-collidine was filter sterilized and D-xylose, vanillin and bKCl were autoclaved.

Media

The media used for the isolations were humic acid vitamin (HV) agar [6], powdered chitin (PC) agar [7] and casamino acids-yeast extract-glucose (YCED) agar [2]. All media contained 50 $\mu g\ ml^{-1}$ of both cycloheximide and Nystatin, and 15 $\mu g\ ml^{-1}$ of novobiocin to inhibit the growth of fungi and eubacteria.

Chemotaxis procedure

A modified chemotactic method [11] was used. Plexiglas chambers containing five sets of two connected wells each 2.5 cm in diameter and 1.3 cm in depth were manufactured by a local company. These were placed under UV light for 20 min for sterilization. Soil was placed in the bottom of each side of connecting wells. Sterile water was added to each well up to the channel level. Chambers were incubated for 1 h at 28° C. Sterile 1-μl Volupette capillary pipets (Baxter Diagnostics, McGaw Park, IL, USA) were filled with chemoattractants and placed evenly on 12-mm long and 3-mm wide channels which connected the wells. Water was added so that it just covered the capillary tubes. Chambers were again incubated for 1 h at 28° C. Capillary tubes were lifted from the chambers and gently rinsed with sterile water. Contents of the capillary tubes were expelled directly onto an agar plate and spread using a sterile glass spreader. The inoculated plates were incubated for 10–14 days at 28° C. Actinomycete colonies were transferred onto starch casein agar [9] for further growth and characterization.

Characterization and enumeration of isolates

Colonies of isolated strains were observed initially using a binocular dissecting microscope. Isolates determined to be unique on the basis of colony color, diameter, sporulation and mycelial pattern were enumerated for each medium and attractant combination, for each medium, for each attractant, and for each soil sample.

A total number of isolates for each soil sample was calculated by adding the individual numbers of unique isolates obtained for each attractant–medium combination. A ratio of unique isolates for a soil sample to the calculated total number of isolates for that soil was used to demonstrate the relative efficiency of the chemotactic isolation.

All isolates were then examined directly on agar plates using a light microscope with a 40× long working distance objective [3] to categorize isolates into broad presumptive groups [4] based on their morphological characteristics.

Results

Microbial colonies on original isolation plates were widely dispersed and readily isolated onto new media. We observed some contamination of the plates with eubacteria but no fungi. Mean numbers of isolates for each chemoattractant are shown in Figure 1a. Although a few actinomycete isolates were obtained from the control treatments, the mean numbers of isolates for these treatments approached zero and were excluded from the figures. γ-Collidine attracted a mean number of 9.0 isolates, a significantly higher yield than the mean number of 5.0 isolates attracted by vanillin (Figure 1a). There were no significant differences between the mean number of isolates attracted by D-xylose and bKCl, which yielded 7.1 and 6.3 isolates, respectively. Although there was a significant difference in the mean yields obtained with γ-collidine and vanillin, neither differed significantly from the mean yields of D-xylose or bKCl.

No significant differences were observed in the mean number of actinomycetes between the three isolation media (Figure 1b).

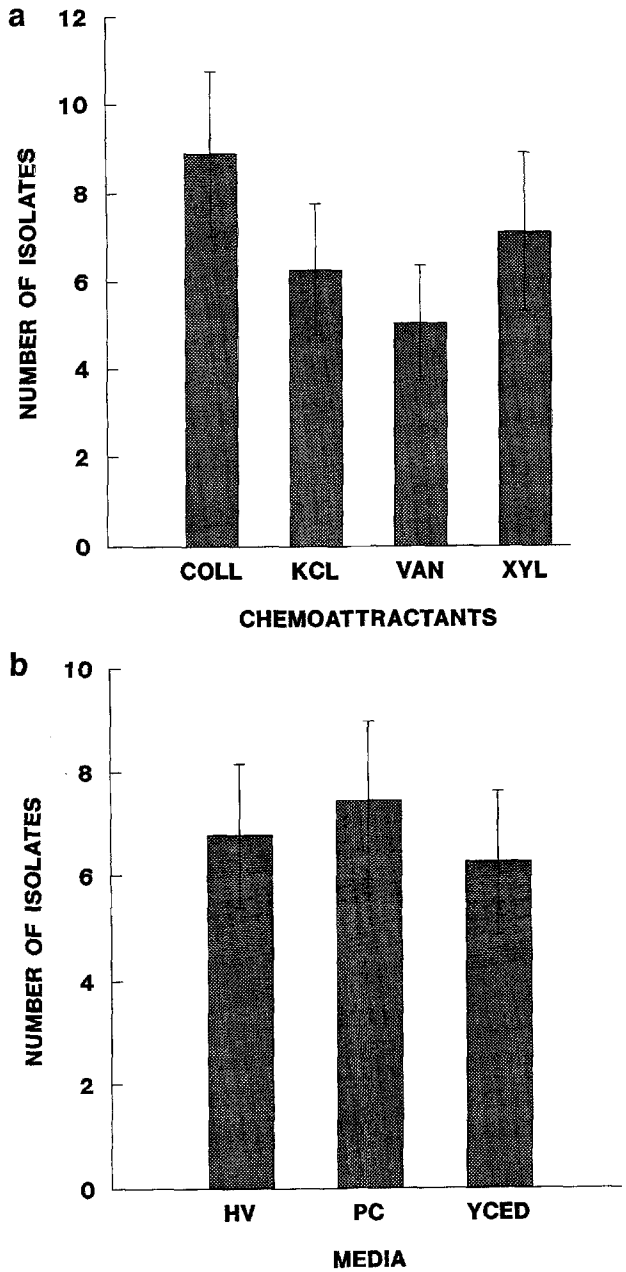

Figure 1 Mean number of isolates by chemoattractant (a) and media treatment (b)

The total number of isolates was slightly greater than the number of unique isolates due to replicates within an attractant or medium treatment. The ratio of unique isolates to total isolates ranged from 86% to 96% (Figure 2a, b).

The total number of unique organisms for the soil samples ranged from three isolates to 252 (Table 1). The mean number over all nine environmental samples was 73, with a mean of six duplicate organisms per sample.

The results from the phenotypic classification of the isolates show that presumptive motile-spored actinoplanetes were the dominant group isolated (Figure 3). Motile-spored actinoplanetes made up 71% of the organisms, with 12% of the organisms being classified as unknown due to a lack of sporulation or atypical hyphal growth pattern. The remaining isolates we obtained from these soils included *Micromonospora*, actinobacteria, nocardioforms, streptomycetes, and maduromycetes.

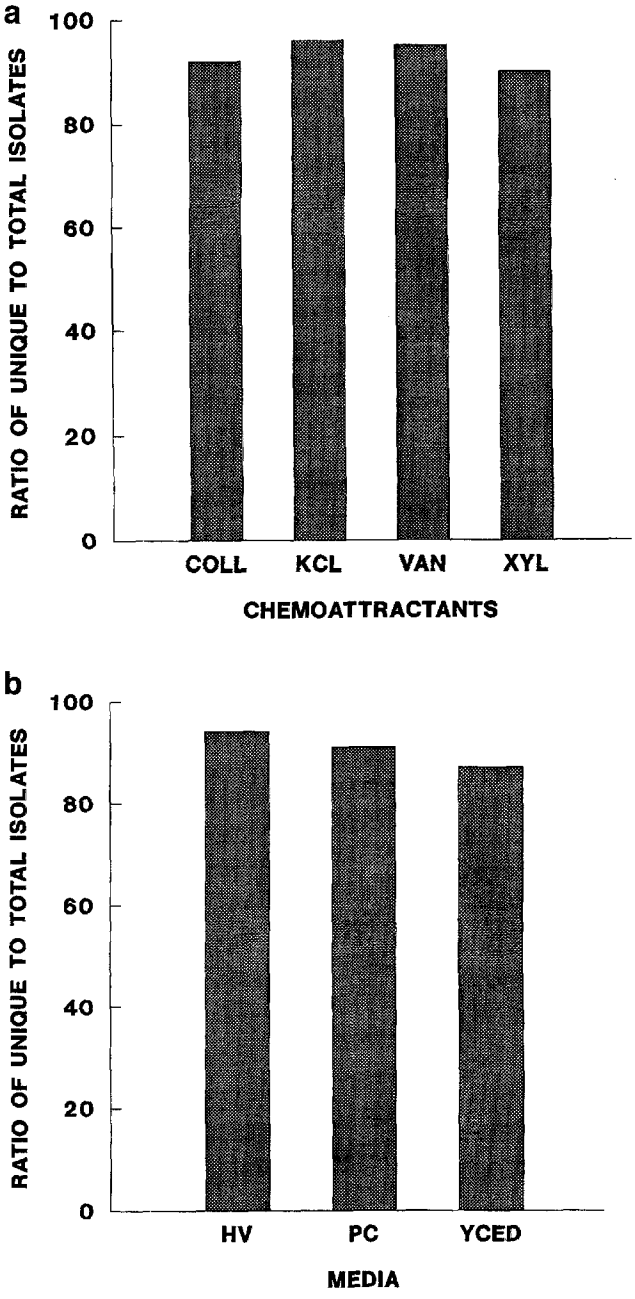

Figure 2 The ratio of unique isolates for the chemoattractants (a) and the medium treatments (b)

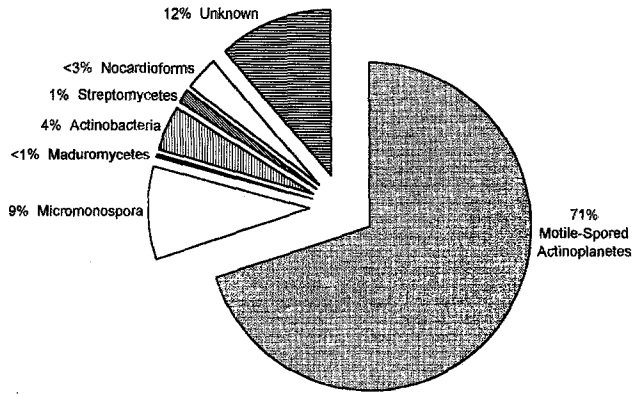

Figure 3 Phenotypic composition of unique isolates for all soil samples

Discussion

In deciding which chemoattractants to use for this study, γ-collidine, D-xylose and vanillin were selected because of the efficiency demonstrated by Hayakawa *et al* [5]. bKCl was included because of the success Palleroni [12] illustrated when using various concentrations of sea water.

The three media used in this study were selected for various reasons. HV agar was chosen because of the results it has provided in our laboratory for the isolation of actinomycetes. PC agar was included because few organisms other than actinomycetes can hydrolyze chitin as their carbohydrate, making fungi and eubacteria less likely to grow. Both HV and PC agar are complex media, so YCED medium was chosen because of its simplicity.

γ-Collidine did not attract significantly more motile-spored actinoplanetes than either D-xylose or bKCl, but vanillin was significantly less effective than γ-collidine. Hayakawa *et al* [5] may have found γ-collidine to be more efficient by using only two samples. None of the three media yielded a significantly different number of organisms.

There were few duplicate organisms between attractants and media, or within a total sample. The majority of organisms obtained were motile-spored actinoplanetes. These results suggest that the use of a combination of attractants and media is a very efficient method for the isolation of motile-spored actinoplanetes. Several samples should be used simultaneously to acquire an adequate number of organisms. However, because of the variability in the number of isolates that were obtained from a particular environmental sample our results showed less significance than those reported by Hayakawa *et al* [5]. It is possible that if Hayakawa *et al* [5] had used a larger number of diverse samples they also may have observed significant variation between samples.

The isolation of other actinomycetes by this chemotactic procedure may be explained through several hypotheses. Spores of actinomycetes or other bacteria might be pushed into the capillary tubes by motile spores. Another hypoth-

esis is that a small number of bacteria floating in the water may enter the capillary tube, or possibly even stick, and then are expelled along with motile spores. A third hypothesis is that the charge of a spore may be attracted by the opposite charge of the buffers used in the chemoattractants. It is still unclear exactly why nonmotile-spored actinomycetes are being isolated by this method, but the numbers of these isolates are low.

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