

Antimicrobial activity of rare actinomycetes isolated from natural habitats in KwaZulu-Natal, South Africa

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This paper reports the presence of new antibiotic-producing organisms in the relatively underinvestigated region of KwaZulu-Natal, South Africa. Using a modified agar-streak method and selective isolation media during the primary screening phase, eighty isolates showing antimicrobial activity were isolated from soil samples of various habitats in the KwaZulu-Natal Midlands. The use of selective isolation media, with antibiotic incorporation and/or heat pretreatment, enhanced the isolation of certain rare actinomycete colonies. The number of culturable antibiotic-producing microorganisms constituted about 3% (on average) of the total microbial population in the different samples studied. The highest percentage of antimicrobially active isolates came from a forest soil site whereas the lowest percentage was present in a riparian soil. One of the isolates, N8, tentatively identified as an *Intrasporangium* species, was isolated from barnyard soil at a poultry farm. It produced at least one broad-spectrum antibiotic active against both Gram-positive and Gram-negative bacteria and fungi and also inhibited the growth of all seven test organisms, especially *Pseudomonas fluorescens* and *Xanthomonas campestris* pv *campestris* at minimum inhibitory concentrations (MIC) of 0.0625 µg/ml and 0.0025 µg/ml, respectively. To our knowledge, members of this actinomycete genus have not been associated previously with antibiotic production. These data confirm that KwaZulu-Natal soils harbour rare actinomycetes that inhibit the growth of *Pseudomonas fluorescens* and *Xanthomonas campestris* pv *campestris*, representatives of two genera which are notoriously difficult to contain in the field. Such antibiotic producers should become more commercially important if the current trend towards the use of biocontrol agents rather than chemical treatments of plant diseases persists.

Introduction

For the last five decades, antibiotics have revolutionized medicine by providing cures for life-threatening infections.¹ Lately, however, there has been a growing concern about the emergence and management of antibiotic-resistant bacteria.^{2,3} Most of the commonly used antibiotics in our hospitals have become ineffective due to these resistant bacteria. The need to discover and develop more-effective antibiotics with unique modes of action is greater than ever.⁴

KwaZulu-Natal province of South Africa is a potentially rich field for the discovery of new antibiotic-producing microorganisms.⁵ For example, the organism, *Streptomyces natalensis*, an actinomycete first used in the industrial production of pimaricin, was originally isolated from KwaZulu-Natal soil.⁶ However, interest waned in this area as the number of secondary metabolites identified as 'novel' decreased. Unsurprisingly, researchers had concentrated on the compounds produced by

members of the *Streptomyces* genus and thus the probability of finding novel compounds became increasingly remote.

Recently, however, taxonomists have shown increasing interest in the rarer actinomycetes as potential sources of novel bioactive metabolites,⁷⁻¹⁰ and methods designed to isolate¹¹⁻¹⁴ and identify^{9,15,16} a wide variety of such actinomycetes have been developed. Modern isolation methods involve five steps: choice of a substrate,^{17,18} composition of the isolation medium,^{19,20} pre-treatment and incubation conditions,¹⁴ colony selection²¹⁻²³ and, finally, purification.^{21,24} Of these factors, composition of the isolation medium, pre-treatment and incubation conditions are the most important, since they determine which organism will develop on the isolation plates.²²

Actinomycetes are mainly aerobic soil inhabitants and are widespread in nature. The term 'rare actinomycetes' or 'rare actinos' relates to those genera less easily detected than *Streptomyces*. No clear-cut habitat preference can be established for the different genera of rare actinomycetes; however, some, such as *Micromonospora* or *Actinoplanes*, are more abundant in decaying plant material or muddy soils on freshwater shores. Thermophilic species are often found in natural warm habitats, such as compost or hay mounds.²⁵

The aim of the study reported here was to continue the search for new antibiotic producers in KwaZulu-Natal by targeting rare actinomycetes using improved isolation methods. Members of the genus *Streptomyces* were excluded from the search, because they have been extensively studied in the past.²⁶⁻²⁸

Materials and methods

Bacterial isolation

Soil samples were collected at depths of 3–5 cm below the surface from various sites in the KwaZulu-Natal Midlands (Table 1). The samples were placed in sterile polyethylene bags, closed tightly and stored at 4°C until required.

Each sample was prepared, first, by vigorous hand-shaking in an autoclaved 1-litre beaker. All stones or debris present were subsequently removed. An approximately 1 g portion of each sample was suspended in 10 ml sterile distilled water from which three dilutions (10⁻² to 10⁻⁴) were prepared. Each dilution was divided into four equal aliquots, which were subjected to different treatments before plating on appropriate isolation media. Some samples were heat-pretreated in a water-bath at 70°C for 10 minutes²⁹ and incorporated with selective antibiotics into the isolation media. In others, there was no heat-pretreatment but a selective antibiotic was added to the media, while in some, there was neither pretreatment of the samples nor antibiotic incorporation into the media.

Distilled water was used for all media preparation. The following selective media were used: Czapek's medium²⁹ supplemented with 25 µg/ml novobiocin and 50 µg/ml cycloheximide; Kosmachev's medium,³⁰ M3 agar;³¹ MGA medium³² modified by omitting soil extract and vitamin solution and made more selective by the addition of 10 µg/ml polymyxin B and 12 µg/ml oxytetracycline; modified Czapek's agar³³ further modified by addition of 50 µg/ml cycloheximide and 4 µg/ml thiamine-HCl; Winogradsky's nitrite medium;³⁴ and Yeast extract agar [International *Streptomyces* Project (ISP) medium 2]^{35,36} modified with gentamicin added to the autoclaved and cooled medium

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to give a final concentration of 50 µg/ml. The cycloheximide was used to inhibit development of invasive fungi. These antibiotics were chosen on the basis of good results obtained by previous investigators on the selective isolation of rare actinomycetes.^{20,23,27} The plates were incubated at 30°C for 21 days, and all colonies were examined directly by light microscopy to detect the isolates.

Primary screening

The media used for primary screening of isolates were Iso-sensitest agar (ISTA) [Oxoid] or Mueller-Hinton (MH) agar (Oxoid). The test organisms, namely, the bacteria *Escherichia coli*, *Pseudomonas fluorescens*, *Serratia marcescens*, *Staphylococcus aureus*, *Streptococcus faecalis* and *Xanthomonas campestris* pv *campestris*, and the yeast *Candida utilis* were all from the culture collection in the Discipline of Microbiology, University of KwaZulu-Natal, and included both plant and human pathogens. The pathogens were selected on the basis of our 1999 survey on microbial infections most commonly encountered at public hospitals in the KwaZulu-Natal Midlands and in our university vegetable gardens.

Bioassay of isolates for inhibitory action was carried out using a modified cross-streak method. The test organisms were streaked at right angles to the line of growth of the producer isolate inoculated previously and grown for six days at 30°C. The inoculated plates were re-incubated overnight at 30°C and the extent of inhibition of the various test organisms measured in millimetres.

All isolates showing antimicrobial activity were purified using the three-way streak method. Purified isolates were streaked separately across the upper third of a plate of either Iso-sensitest agar or Mueller-Hinton agar and the same test organisms cross-streaked to confirm the original screening results.³⁷

Secondary screening

Fourteen isolates were selected for secondary screening on the basis of their inhibition of at least two or more of the test organisms. An exception was made in the case of isolates that inhibited only *Pseudomonas* or *Xanthomonas* spp., since members of these genera are resistant to many antimicrobial agents used in the field.

Growth from a mature slant culture of the producer isolate was inoculated aseptically into 250-ml Erlenmeyer flasks each containing 100 ml of seed medium NB (meat extract 1 g/l, yeast extract 2 g/l, peptone 5 g/l and sodium chloride 8 g/l, pH 7.1 after sterilization) or Oatmeal broth (ISP media 3 and 2, respectively, from which agar had been omitted)³⁵ and incubated in a rotary shaker at 30°C for 2 days. The seed culture (10 ml) was transferred into 200 ml of the same medium in a 500-ml flask and incubated at 30°C for 14 days under agitation at 250 rpm. After incubation, the cultures were centrifuged in a Heraeus Labofuge 200 at 9500 × g for 15 minutes. Aliquots of the supernatant (5 ml) were filter-sterilized (Cameo 25 AS acetate membrane) into sterile screw-capped glass vials. The filtration procedure was repeated to ensure that only cell-free supernatant (culture filtrate) was used for the antimicrobial activity bioassay.

Samples of the culture filtrates were assayed for antimicrobial activity using a modification of the agar well-diffusion method of Paik and Glatz.²⁴ After aseptically pouring the agar (5 mm deep), plates were incubated overnight at room temperature before a well (7 mm diameter) was cut in the centre of each plate. The plates were dried by further incubation at 37°C for 2 hours to facilitate sample diffusion through the agar. Culture filtrate (50 µl) was added into the well with a micropipette and the test organisms streaked radially outwards, starting 1 mm from the edge of the well. The plates were incubated at 30°C for 24 hours and observed for growth inhibition zones. All assays were carried out in triplicate. Plates in which the wells were filled with uninoculated culture medium served as controls.

Some samples were also tested using the agar disk-diffusion method of Eckwall and Schottel.³⁸ Commercial paper disks were spotted with a 50 µl aliquot of culture filtrate and allowed to dry before placing on the surface of Petri plates containing MH medium freshly seeded with the test organism. The plates were incubated at 30°C for 24 hours before the diameters of growth inhibition zones were measured.

Minimum inhibitory concentration (MIC) was determined using the broth micro-dilution method as recommended by the Swedish reference group for antibiotics (SRGA)³⁹ and its sub-committee on methodology and as per standard protocol of Hancock's doubling dilution method.⁴⁰ The indicator test organisms were prepared by growing them in NB to an optical density (OD) of 0.5 at 420 nm, whereafter X µl cell suspension

was added to 1 ml NB. The value of X was calculated using the equation:⁴⁰ $X = 10/OD$.

Prior to testing, either 100 µl or 50 µl of the cell suspension was diluted in 10 ml or 5 ml NB, respectively. Dilutions of the antibiotic-producer culture filtrate (stock solution) were prepared in a micro-titre plate (10 × 10 wells). From the stock solution, nine dilutions were made using the growth medium as diluent. A step-wise dilution factor of 2 (1.0, 0.5, 0.25, etc.) was used throughout the dilution procedure. Finally, 100 µl of the prepared suspension of the indicator test organism were added to each of the wells in the micro-titre plate and incubated at 30°C. The plates were examined for growth after 12 h, 24 h and 48 h incubation. The highest dilution showing no visible growth, i.e. a clear medium, was regarded as the MIC.

Taxonomy

Physiological, morphological, and chemical studies on eight selected isolates were carried out. Selection was based on sizes of inhibition zones greater than 3 mm on at least two or more test organisms, especially *Pseudomonas fluorescens* and *Xanthomonas campestris* pv *campestris* that are resistant to many commonly used antibiotics. Morphological studies were carried out with a Carl Zeiss light microscope and a Hitachi scanning electron microscope. The diaminopimelic acid (DAP) isomer in the cell wall was determined by the method of Becker *et al.*⁴¹ Carbon source requirements were studied by the method of Nitsch and Kutzner.⁴² The whole cell sugar pattern (WCSP) was obtained by a combination of the methods of Murray and Procter⁴³ and Staneck and Roberts.¹⁶

Results

The number of antibiotic-producing isolates varied with the sample source and the isolation pretreatment applied. Eighty of the approximately 2600 isolates screened, showed some antimicrobial activity. The highest percentage of active isolates came from the forest site, while the lowest percentage came from the riverine soil sample. Calculation of the number of antimicrobially active isolates in the total number of isolates screened from the various samples showed that the number of culturable antibiotic-producing microorganisms constituted only about 3% of the total overall microbial population of the samples studied (Table 1).

The average inhibition zone size was sometimes different for the secondary screening stage, but the spectrum of activity of the respective isolates remained unchanged from those recorded in the primary screening phase. Some of the isolates lost a substantial part of their activity on extended incubation. Isolated colonies, which grew within an inhibition zone, were recorded as resistant variants of the strain (Table 2 and Fig. 1A). Isolate N8 inhibited all the test organisms in the primary screen on both NA (Fig. 1A) and ISTA plates (not shown). This broad-spectrum inhibition was verified during secondary screening on MH medium (Fig. 1B), with uninoculated MH medium in the wells serving as a control (Fig. 1C). Surprisingly, this isolate showed no visible activity against the test organisms during secondary screening using the agar-well diffusion method. However, the disk-diffusion method, with *Serratia marcescens* as the test organism, gave positive results (Fig. 1D).

Isolate N2 showed highest activity against *P. fluorescens* with an MIC of 0.0039 µg/ml, whereas the MIC for *X. campestris* pv *campestris* was 0.25 µg/ml (Table 3). Isolate N8 showed good antimicrobial activity against all the test organisms used, especially the *Pseudomonas* and *Xanthomonas* species, with MICs of 0.0625 µg/ml and 0.0025 µg/ml, respectively. The MIC results for isolates N33 and N35 were also impressive, with values of 0.002 µg/ml and 0.0039 µg/ml against *P. fluorescens* and *X. campestris* pv *campestris*, respectively.

The physiological, morphological, and chemical characteristics of all isolates showing antimicrobial action are presented in

Table 4. Isolate N8, which had the broadest spectrum of activity, formed colonies with no aerial mycelium on NA. When young, the organism produced branched substrate mycelium, but on maturing, dark brown to grey round to oval dome-like bodies or vesicles were formed (Fig. 2A). The smooth-surfaced spores were produced in dense clusters contained in a thick sheath of extracellular material or inside the vesicles (Fig. 2B). In broth culture, the hyphae merged together to form synnemata, but when dried, extensive branching fragments were observed. The isolate could not utilize raffinose and rhamnose as carbon sources and no diffusible pigments were formed in either solid or liquid media. The cells were non-acid fast and catalase-positive. Optimum growth occurred between 27°C and 30°C.

High-performance amino-acid analysis⁴¹ (Beckman 6300 analyser) showed that N8 contained substantial amounts of L- or meso-DAP. None of the diagnostic sugars was present. Environmental scanning electron microscopy revealed that N8 spores are enclosed in a thick slimy layer which, when disrupted, exposes the spore mass. Scanning electron microscopy (SEM) revealed the smooth-surfaced, oval to rod-shaped spores.

A characteristic morphological feature of isolate N35 was the angular arrangement of bacillary elements as revealed by SEM (Fig. 3A) with some of the rod-like elements measuring up to 4 µm in diameter (Fig. 3B).

Discussion

Enumeration of the exact number of colonies isolated in this investigation is not possible, since many of the organisms that were obviously non-actinomycetes were disregarded. However, a rough estimate of the scale of the isolation programme may be obtained by considering the number of plates used. For each of 10 samples investigated, three dilutions (10⁻² to 10⁻⁴) were prepared and divided into four equal aliquots and each aliquot was subjected to a different treatment before plating on triplicate plates of at least seven appropriate isolation media. There were four treatment conditions as shown in Table 1. Thus, a total of 10 × 3 × 4 × 3 × 7 = 2520 isolation plates were used in this investigation.

Heat pretreatment of the samples was beneficial as it eliminated most of the unwanted Gram-negative bacteria that produce mucoid, spreading colonies on soil dilution plates,³⁴ thereby facilitating the isolation of actinomycetous organisms. The addition of antibiotic antibiotics such as gentamicin, streptomycin and novobiocin to the isolation media also enhanced the selection of members of the actinomycetales. The combined treatment of heat and antibiotic incorporation

Table 1. Percentage of the total number^a of isolates active against the test organisms* on primary screening.

Sample source	No. isolates per pre-treatment	Total no. isolates screened	No. isolates active	%
Chicken manure [CM]	H + A	38		
	H + NA	46		
	NH + A	94		
	NH + NA	166	354	12
Chicken litter [CL]	H + A	34		
	H + NA	61		
	NH + A	123		
	NH + NA	205	423	18
Cow manure [COW]	H + A	43		
	H + NA	55		
	NH + A	58		
	NH + NA	157	313	11
Compost soil [CS]	NH + A	45		
	NH + NA	76	121	2
Uncultivated farm soil [US]	NH + A	56		
	H + A	23		
	NH + NA	103	182	4
	H + A	36	94	0
River bank soil [RB]	NH + A	97		
	NH + NA	167		
	H + A	66		
	H + NA	105	435	23
Creosote-contaminated soil [CCS]	NH + NA	5	5	1
	NH + A	71		
Sweet waters (dry soil) [SW]	NH + NA	128		
	H + A	61	260	2
	NH + A	101		
	NH + NA	169		
Ilovo sugarcane field [CSF]	H + A	56		
	H + NA	60	386	7
	NH + A	101		
	NH + NA	169		
Grand total		2573	80	3.11

**Escherichia coli*, *Pseudomonas fluorescens*, *Serratia marcescens*, *Staphylococcus aureus*, *Streptococcus faecalis*, *Xanthomonas campestris* pv *campestris* and *Candida utilis*; NH, non-heated; H, heated; A, antibiotics added; NA, no antibiotics added.

^a Number based on the average colony count of 10⁻² to 10⁻⁴ dilutions of the sample.

[†]South African Paper and Pulp Industries.

Table 2. Average inhibition zone sizes (mm) of isolates* against test organisms on primary screening.

Isolate*	Source	Test organisms						
		Sa	Ec	Pf	Sm	Sf	Cu	Xc
N2	CL	13	10	20	21	–	20	15
N3	CM	25	20	25	–	–	nt	–
N4a	SW	15	–	30	20	–	nt	–
N4b	SW	–	30	15	–	–	–	–
N8	CM	20	25	20	23	22	27	24
N12	CSF (NH+A)	3	7	8	–	–	–	5
N15	CL	20	21	–	2	–	30	–
N16	CL	25	5	14	–	–	Rs	–
N19	SF (NH+A)	3	4	10	–	–	–	5
N25	CL	2	30	–	–	–	30	–
N30	CSF (H)	5	–	20	–	–	–	–
N33	SF (NH)	–	3	10	–	–	–	–
N35	SF (NH)	–	3	–	–	–	–	10
N48	CS	18	30	–	–Dc	–	21	19

*Sa, *Staphylococcus aureus*; Ec, *Escherichia coli*; Pf, *Pseudomonas fluorescens*; Sm, *Serratia marcescens*; Sf, *Streptococcus faecalis*; Cu, *Candida utilis*; Xc, *Xanthomonas campestris* pv *campestris*; Rs, resistant strain; CL, chicken litter; CM, chicken manure; CS, compost soil; SW, sweet waters (dry soil); nt, not tested; –Dc, discoloration; –, no inhibition; H, heated; NH, non-heated; A, antibiotics added; SF, SAPPI forest; CSF, sugarcane field; *selected for secondary screening.

Table 3. MICs (µg/ml) of the selected antimicrobially active isolates against the test organisms.

Isolate	Test organisms						
	Sa	Ec	Pf	Sm	Sf	Cu	Xc
N2	0.0078	0.0156	0.0039	uns*	uns	nt	0.25
N8	0.0078	0.0039	0.0625	0.125	0.25	0.002	0.0025
N12	nt	nt	0.25	uns	uns	uns	nt
N16	0.125	0.0039	0.0039	uns	uns	uns	0.002
N19	nt	nt	0.125	uns	uns	0.25	0.25
N30	nt	uns	0.0625	uns	uns	uns	uns
N33	uns	nt	0.002	uns	uns	uns	uns
N35	uns	0.031	uns	uns	uns	uns	0.0039

*uns, unsusceptible; nt, not tested; Sa, *Staphylococcus aureus*; Ec, *Escherichia coli*; Pf, *Pseudomonas fluorescens*; Sm, *Serratia marcescens*; Sf, *Streptococcus faecalis*; Cu, *Candida utilis*; Xc, *Xanthomonas campestris* pv *campestris*.

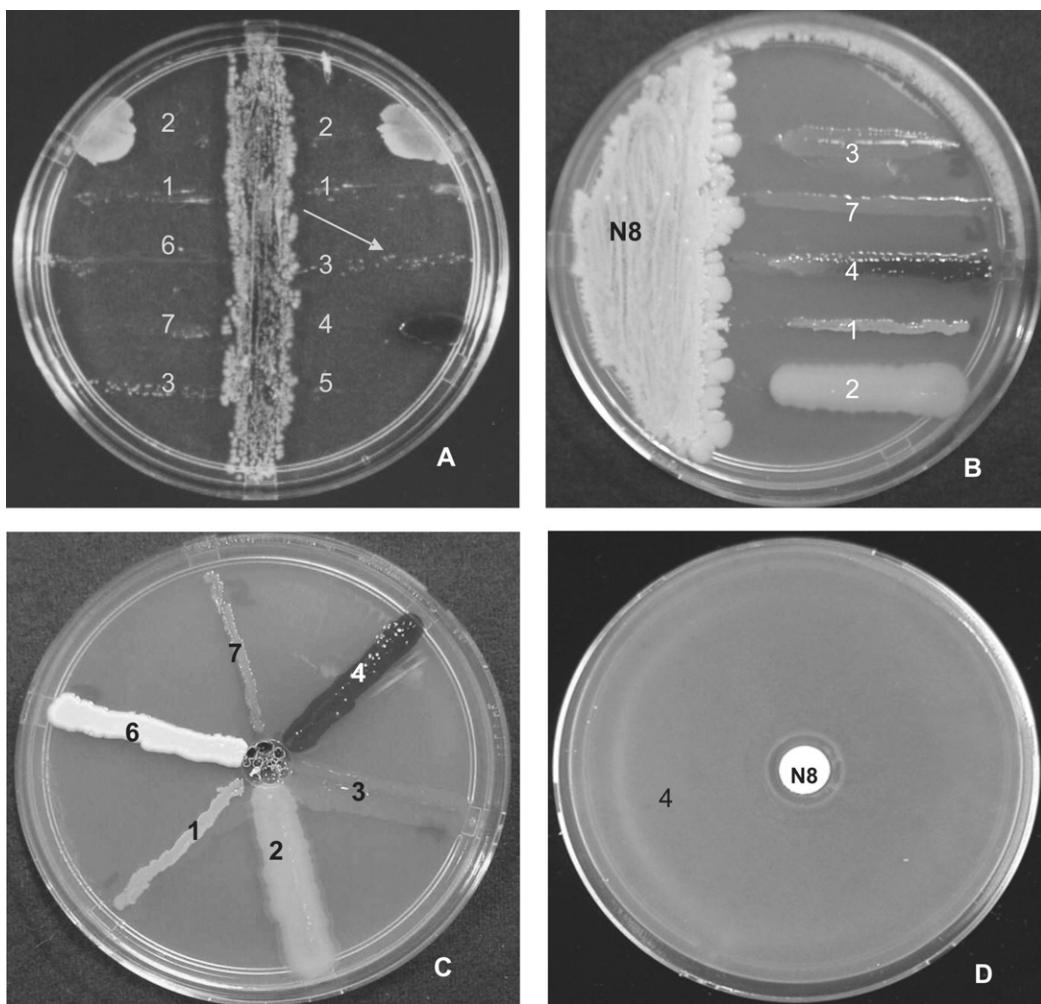


Fig. 1. Inhibition of test organisms by isolate N8: **A**, Inhibition of test organisms on primary screening (arrow shows resistant variants); **B**, confirmation of inhibition on MH agar; **C**, control showing uninhibited growth of test organisms on MH agar; **D**, inhibition of *Serratia marcescens* by N8 purified extract on secondary screening. Test organisms include: *Staphylococcus aureus* (1), *Escherichia coli* (2), *Pseudomonas fluorescens* (3), *Serratia marcescens* (4), *Streptococcus faecalis* (5), *Candida utilis* (6) and *Xanthomonas campestris* pv *campestris* (7).

Table 4. Physiological, morphological and chemical characteristics of antimicrobially active isolates against the test organisms.

Characteristic	Isolate							
	N2	N8	N12	N16	N19	N30	N33	N35
Physiological								
Gram reaction	+	+	Variable	+	+	Variable	nt	+
Catalase	+	+	-	+	+	-	-	+
Oxidase	-	-	+	-	+	+	+	+
Acid-fastness	-	-	-	-	- ^a	-	-	-
Spore production	+	+	+ ^b	+	-	-	-	+
V-formation	-	-	-	-	+	+	-	-
Motility	-	-	-	-	-	-	-	-
Morphological								
Aerial mycelium	Pale white orange	Pale white orange	Primary	Primary	NA	NA	NA	NA
Substrate mycelium	*YBR	*YBR	-	*YBR	Extensive	-	-	-
Cells/spores shape	Oval spores	Oval spores	Rod-shaped cells	Oval spores	Rod-shaped cells ^c	Drumsticks/Hammer	Rods	Rod-shaped cells
Spore surfaces	Smooth	Smooth	-	Smooth	-	-	-	-
Pellicle formation	+	+	-	+	nt	nt	-	nt
Chemical								
Diaminopimelic acid (DAP) isomer	Meso-DAP	L- or meso-DAP	Meso-DAP	L-DAP	L- or meso-DAP	None	L- or meso-DAP	None
Diagnostic sugar	NC	NC	Ara + Gal	Ara + Gal	Ara + Gal	Gal	Ara + Gal	Gal
Cell wall chemotype	I, III	I, III	IV	I	VI sensu	VI	NA	VI
WCSP type	C	C	A	A sensu	A	NA	A	NA
Glycine	+	++	nt	+	+++	nt	++	nt
Melanin pigment	nt	+	nt	+	nt	-	-	-

^a, Positive; ⁻, negative; nt, not tested; NA, not applicable; WCSP, whole-cell sugar pattern; Ara, arabinose; Gal, galactose; NC, no characteristic sugar; *YBR, yellow-brown-red; ^bbinds and retains fuscin; ^cendospores; ^dappear as doublets with tapered ends; ^espores linked by threadlike structures; ^fdoubtful.

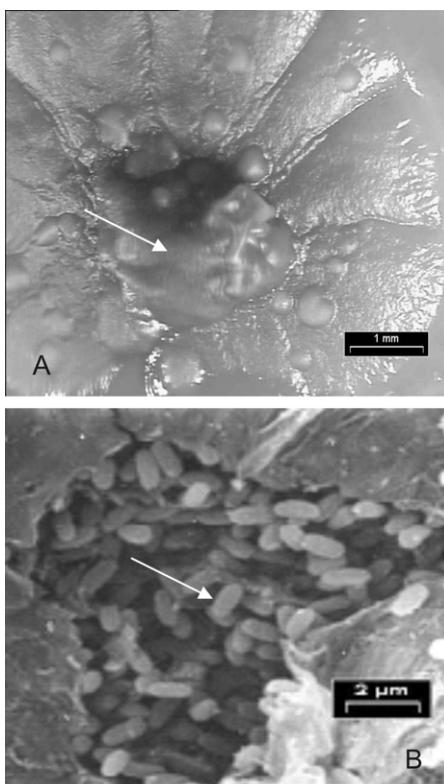


Fig. 2. Growth of N8 culture on Nutrient agar: **A**, Mature mycelium (arrow shows dome-like body or spore vesicles); **B**, scanning electron micrograph of mycelium (arrow shows oval-shaped spores exposed inside a broken vesicle).

into the media eliminated most of the unwanted fungi, yeasts and eubacteria and consequently reduced the number of colonies developing on the plates.

The disadvantage of adding antibiotic(s) to the isolation media is that potent antibiotic producers, sensitive to the antibiotic added, might be inhibited and hence remain undetected. On the other hand, the antibiotic(s) makes the medium more selective by limiting the types of organisms developing on the plates. Different members of the actinomycetales differ significantly in their sensitivity toward antibacterial antibiotics. Streptomycetes fortunately belong to the antibiotic-sensitive group,^{23,44} making it easier to target the rarer actinomycetales.

Some of the isolation media used, namely Winogradsky's nitrite medium,⁴⁵ M3 medium,³¹ and Kosmachev's medium,³⁰ were found to be selective for actinomycetes without heat pre-treatment and/or antibiotic incorporation, although a few colonies of other bacteria did occasionally develop on the plates. Thus, the selectivity of some of the so-called selective media used in this study is questionable.

Although the media used in this investigation were not compared directly, some differences were nonetheless evident. Modified Czapek's agar³³ appeared to enhance pigment-producing strains of actinomycetes, most probably the Nocardioform group. MGA³² supported growth of mainly filamentous organisms. Of all the media used, Kosmachev's medium³⁰ appeared to be the most selective, as most of the plates contained virtually pure cultures of non-streptomycetous actinomycetes. This medium has been reported to be selective for *Excellospora* and related genera.³⁰

Our screening results indicate that forest sites, with their usually high organic content and acidic nature, may offer a good hunting ground for antibiotic-producing microorganisms, especially in KwaZulu-Natal. Waksman^{46,47} has reported the presence of antibiotic-producing microorganisms in acidic forest soils.

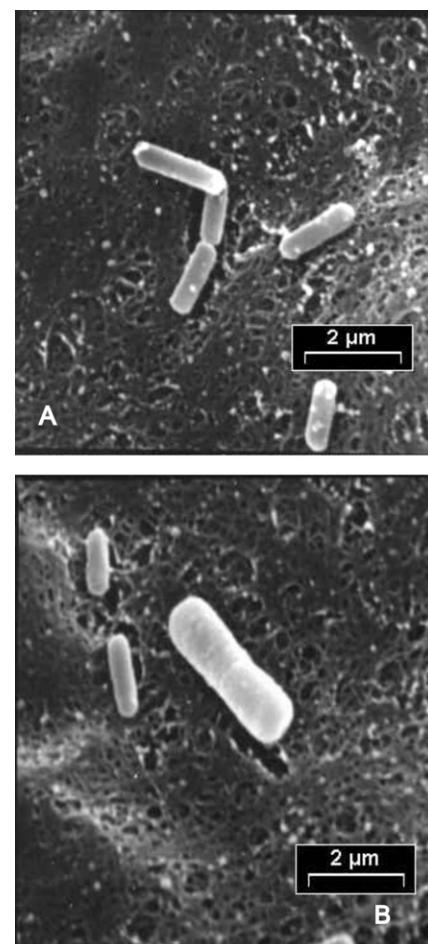


Fig. 3. Scanning electron micrograph of isolate N35: **A**, angular arrangement of bacillary elements; **B**, enlarged cell (up to 4 μm).

The soil samples from the riverine site were waterlogged and hence devoid of oxygen, thus making them unfavourable for growth of aerobic antibiotic-producing actinomycetes. Our negative results with this soil may also reflect an inability of the medium used to support growth and/or antibiotic production by the riparian microbiota.

Many of the other samples screened, such as compost soil, cow and chicken manure, and sugar-cane field soil, were rich in organic matter and contained a large variety of actinomycetes. As could be expected, the dry, uncultivated soil sample also contained many actinomycete isolates, since it has been reported that most arid soils usually have an alkaline pH and contain a number of the rare actinomycetes.¹² Kizuka *et al.*¹² reported on the distribution of actinomycetes in South African soils in 1997. Apart from this report, current quantitative data on the geographical and ecological distribution of actinomycetes in South Africa appears to be scarce in the scientific literature.

Our results showed that it is erroneous to compare one antibiotic agent against another on the basis of inhibition zone size, since the size and position of the growth inhibition zone are a consequence of many dynamic systems, including diffusion rate, proceeding simultaneously.⁴⁸ The observed discolouration of *Serratia marcescens* was not the result of metabolic interference, as incubation at a lower temperature restored the characteristic red colour. The resistance of *Streptococcus faecalis* to most of the isolates tested may have been a medium effect, or the strain of *Streptococcus faecalis* used may produce an enzyme, which inactivated any antibiotic produced.

The loss of some activity during secondary screening using the

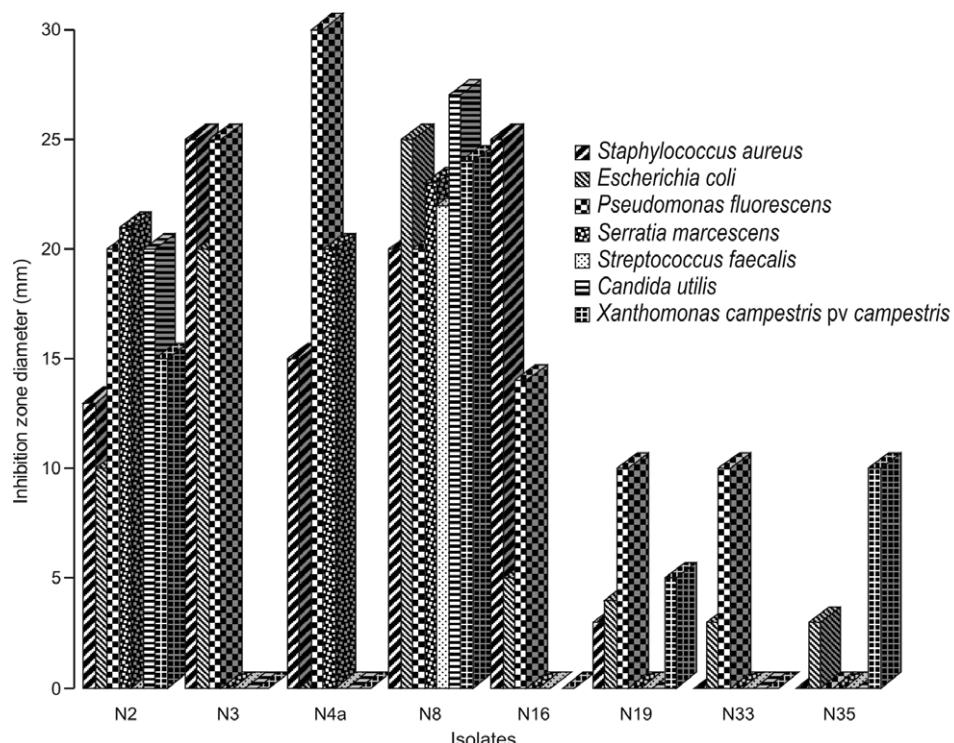


Fig. 4. Comparison of antimicrobial spectra of the active isolates selected for secondary screening.

agar plug method could be ascribed to procedural effect, including preparation of the culture filtrate, media composition, and incubation conditions. Another explanation may be that in the primary screens actively growing cells (with ongoing antibiotic production) were used, whereas cell-free filtrates were used in the secondary screening protocol and a large amount of antibiotic or antibiotic activity may have been lost.

The MIC values shown in Table 3 serve as an index of the relative antimicrobial activity and the antimicrobial spectra of the isolates. The spectra of activity of the isolates showed that N2, N8 and N16 were active against both Gram-positive and Gram-negative bacteria (Fig. 4). Isolate N8 produced a broad-spectrum antibiotic(s) active against both bacteria and fungi (yeasts), whereas N35 was inactive against Gram-positive bacteria. Isolate N8 was selected for further detailed description because it exhibited activity against all the test organisms used in this investigation.

Comparisons with descriptions of actinomycetales genera in *Berger's Manual of Systematic Bacteriology*⁴⁹ showed N8 to closely resemble members of the *Nocardioides* group. However, unlike N8, *Nocardioides* produce aerial mycelium that ultimately fragments. Hence, the isolate is unlikely to belong in this group. The absence of diagnostic sugars in whole-cell hydrolysates (WCSP C) of isolate N8, the presence of L-DAP (wall chemotype I), the formation of substrate mycelium with round to oval spore-like bodies and synnemata, together with other cultural and physiological properties, placed N8 in the genus *Intrasporangium*.

Similar approaches to those described above were used tentatively to identify isolates N2, N12, N16, N19 and N35 as *Thermomonospora* sp., *Saccharopolyspora* sp., *Nocardia* sp., *Corynebacterium* sp. and *Promicromonospora* sp., respectively. A molecular approach is currently under way to identify all isolates at the species level.

Conclusions

Our preliminary findings confirm that KwaZulu-Natal soils harbour antibiotic-producing microorganisms, as shown by

previous investigators.^{5,6,12,27} and that it could be beneficial to undertake a more comprehensive search for rare forms of antibiotic-producing organisms in this province of South Africa.

Of all the isolates assayed in the present investigation, N8, tentatively identified as an *Intrasporangium* species, appears to have the greatest potential. The results obtained from screening and MIC determinations showed it to be active against all the test organisms used, especially the *Pseudomonas* and *Xanthomonas* species, with MICs of 0.0625 µg/ml and 0.0025 µg/ml, respectively. However, specificity of action by antibiotics is often favoured by medical science.⁴⁴ It may be that the substance(s) produced by isolate N8 is/are toxic to many additional microorganisms. To ascertain this, further tests involving many more test organisms will be undertaken. The activity of the compound will be tested also for agricultural application against a range of phytopathogenic bacteria and fungi, since a major issue at present is the development of biocontrol agents to replace chemical treatments of plant diseases.^{8,50} The chemical nature of the antimicrobial compound(s) produced by N8 is/are currently being investigated. The indications are that the main antimicrobial substance is a polypeptide.

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