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# Thermodurant sandy desert soil *Streptomyces* from plant rhizosphere exposed to natural gas flare

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## ABSTRACT

About 450 isolates of aerobic, mesophilic and thermodurant streptomycetes were obtained from the rhizosphere of flowering plants: *Cyperus conglomeratus* Rottb., *Citrullus colocynthis* (L.) Schrad. and *Moltkiopsis ciliata* Forssk. These plants were growing in a sandy desert exposed to high temperature from natural gas flares in the Magwa oil fields of Kuwait. Of these isolates, 173 were identified as *Streptomyces albus*, 167 as *S. griseus* or *S. venezuelae*, 24 as *S. lavendulae*, 5 as *S. graminofaciens*, 11 as *S. flaveolus* and 67 as *S. diastaticus*. Thirteen isolates contained meso diaminopimelic acid in their whole-cell hydrolysate, hence they are not streptomycetes or streptoverticillia. Four hundred and thirty-five isolates were able to grow in the presence of 10–15% sodium chloride solution and 240 grew at 45°C, 187 exhibited antibacterial activity, and 93 showed antifungal activity.

## INTRODUCTION

With pressing environmental concern being expressed throughout the world, the ecology of microbial communities is gaining increasing attention. At the same time interest in the discovery of new secondary metabolites from microbial sources (Bu'Lock *et al.* 1982) continues. Therefore, we felt it would be important to study the occurrence of aerobic Actinomycetales in a sandy desert exposed to high temperatures throughout the year in Kuwait because this represented a unique habitat. Through the courtesy of Kuwait National Oil Company, we were able to study the occurrence of these aerobic Actinomycetales associated with the sparse vegetation at the Magwa Oil Field. This report represents the results of these studies.

## MATERIALS AND METHODS

### SAMPLING

The soil samples were obtained from the Magwa oil fields. The gas flares were shut off to allow sample collection. The samples, 1–5 g, were collected from under three dominant plants, *Cyperus conglomeratus*, *Citrullus colocynthis*, and *Moltkiopsis ciliata*

by digging out the loose sand adjacent to the roots (rhizosphere) at a depth of 5–15 cm and transferring it to sterile Petri dishes. All samples were collected in February 1978 under plants near the periphery of the flares.

## PREPARATION OF SAMPLES

The sample portions of one gram were added to Ringer's solution (1/4 strength) and shaken vigorously for 20–30 min. Dilutions from this were prepared in 1/4 strength Ringer's solution. Various dilutions of samples were made and 0·2 $cm^3$ of each dilution was added to the Petri dishes of appropriate media with and without antibiotics. The media used were: casein-starch agar (Küster & Williams 1964) and arginine-glycerol salts agar (El-Nakeeb & Lechevalier 1963). The antifungal agent, actidione, was added at 50 $\mu g/cm^3$ and the antibacterial agent, chlortetracycline, at 5–10 $\mu g/cm^3$ added to the various sterilised media at 46°C (Williams & Davis 1965). The plates were left in the incubator at 30°C overnight to remove water of condensation. Next day the plates were inoculated with suitable dilution of samples in duplicate sets and incubated at 30°, 45°, and 55°C for 4–10 days. The dishes were examined every day for appearance of typical *Streptomyces-Streptoverticillium* colonies. Such colonies were then picked up and streaked on all the nine International *Streptomyces* Project (ISP) media (Shirling & Gottlieb 1966). Triplicate streaks were made for each colony and each plate was incubated at 30°, 45°, and 55°C for periods of 4–10 days.

## PREPARATION OF INOCULA

Routinely, the inocula were prepared in tryptone-glucose-liver extract (TGLY) broth.

## CHARACTERIZATION OF ISOLATES

Each isolate was characterized following the methods of Shirling & Gottlieb (1966). For each isolate the following parameters were noted and recorded on data sheets.

*Chromogenicity.* Determination of melanin-like pigment (+chromogenicity) was carried out on TGLY as well as peptone-iron agar containing 0·1% yeast extract (Pridham & Lyons 1969).

*Diaminopimelic acid isomer analysis.* All cultures were grown in TGLY broth for 72 h in shake flasks. The cells were killed by addition of 0·1% formalin (1 $cm^3$), left overnight, collected by centrifugation and washed twice with distilled water. The cells were freeze-dried and stored at 0°C until analysed. The whole-cell hydrolyzates were prepared by the methods of Becker *et al.* (1964) and examined by thin layer chromatography on cellulose NM-300 plates.

*Colour determination.* Colour of aerial mycelia, vegetative mycelia, and diffusible pigments was determined on oatmeal agar (Küster 1972) after growth for 14 days at 30°C or for 5–7 days at 45°C using ISCC-NBS Color Names Charts Illustrated with Centroid Color Supplement to NBS Circular 553.

*Micromorphology of spore chains.* Each isolate was cultivated on several ISP media (Shirling & Gottlieb 1966) and the isolates were allocated to the appropriate sections of Pridham *et al.* (1958). Direct comparisons were made with the proposed type and neotype strains of each section (Pridham & Lyons 1969).

*Transmission electron microscopy of spore wall ornamentation.* Electron micrographs were made from Formvar-coated grids impressed on the aerial mycelium of 10–14 day old Petri dish cultures. The preparations were then examined in a transmission electron microscope at magnifications of 6000× to 20,000×. The isolates were categorized as smooth (Sm), spiny (Sp), warty (W), hairy (H), and knobby (Kn) spore surfaces (Lyons & Pridham 1971).

*Ability to use various carbon sources for growth.* Each isolate was cultivated on basal agar (Pridham & Gottlieb 1948) supplemented with individual carbon compounds (D-glucose, D-xylose, L-arabinose, rhamnose, D-fructose, D-galactose, raffinose, D-mannitol, I-inositol and salicin). Incubation was at 30°C for 10 days followed by 5 days at 45°C. Growth was estimated visually.

*Utilization of sucrose in static culture.* All cultures were examined for ability to utilize sucrose by inoculating a loopful of spores from an ISP2 agar plate to Czapeck's sucrose solution containing 1% sucrose. Cultures were incubated at 30°C and 45°C for 7–10 days and growth was estimated visually and assessed as excellent, fair or none.

*Tolerance to sodium chloride.* This characteristic was determined according to the method of Tresner *et al.* (1968). Incubations were carried out for 5–10 days at 30° and 45°C respectively. Growth was rated visually as excellent, fair or none.

*Thermotolerance.* A loopful of mature spores from ISP2 or ISP4 plates was streaked onto ISP2 or trypticase soy agar plates. The plates were wrapped in polyethylene bags and incubated at 45°C and 55°C for 4–7 days and examined for growth.

*Growth on crude petroleum.* Crude petroleum used in these experiments was obtained from one of the wells in the Magwa area. Weighed amount of crude petroleum (0·1–0·2%, w/v) was taken in individual conical flasks (250 $cm^3$), diethyl ether was added to sterilize the flasks and the flasks were left in a hood for 48 h to evaporate the ether. One hundred $cm^3$ of a sterile basal medium (Pridham & Gottlieb 1948) were added aseptically. Streptomycetes were inoculated and incubated at 30°C and 45°C on a shaker operated at 250 rpm and the growth examined visually.

*Antibiotic studies.* Three media were used for antibiotic studies: soybean meal glucose-yeast extract broth (Lyons & Pridham 1971), starch-nitrate broth, and AC-9 medium (Nazar *et al.* 1983). Mature spores from all isolates were used to inoculate the sterile liquid antibiotic production media. The flasks were incubated on a rotary shaker at 30°C or 45°C for 72–96 h. Culture filtrates were assayed by the paper disc method using six test microorganisms: *Bacillus subtilis* NRRL B-765, *Sarcina lutea* NRRL B-1018, *Escherichia coli* NRRL B-766, *Saccharomyces pastorianus* NRRL

Y-139, *Candida albicans* NRRL Y-477, and *Mucor ramannius* NRRL Y-1839. Inhibition zones from culture filtrates were measured in mm and recorded on data sheets.

## RESULTS AND DISCUSSION

Many media have been recommended in one way or another for isolation, cultivation or counting of actinomycetes. Actinomycete isolation agar (Difco), starch-asparagine-inorganic salts agar of Rao & Subrahmanyan (1929), and trypticase soy agar (Difco) have given the highest counts of aerobic, mesophilic and thermophilic actinomycetes (Pridham, personal communication). When these media were used for isolation from desert samples, fast growing bacterial colonies, presumably bacilli, covered the plates. Only a few *Streptomyces* or *Streptoverticillium* type colonies could be seen. Addition of antibacterial and antifungal antibiotics decreased the bacterial and fungal colonies on the plates but streptomycete colonies were hard to distinguish as most of them lacked the characteristic aerial mycelia.

After several trials we found that for these desert samples, best media for counting and isolation were: casein-starch agar (Küster & Williams 1964), and arginine-glycerol salts agar (El-Nakeeb & Lechevalier 1963). When used with the addition of both antibacterial and antifungal substances at appropriate concentrations, these two media gave clean isolations and interference from bacteria or fungi was minimal. Actinomycete colonies were easy to recognize due to abundant aerial mycelia. The counts of streptomycete/streptoverticillia were reasonably good (Williams & Davis 1965). The total counts of bacteria, molds and actinomycetes are shown in Table 1.

Unexpectedly, no streptomycetes or steptoverticillia could be isolated at 55°C and those isolated at 45°C were unable to grow at 55°C. However, the previous studies by Hashem & Diab (1974) and Diab & Al-Gounaim (1982) reported the isolation of a few actinomycetes at 55°C. Similarly, among the 400 *Streptomyces* species investigated by ISP workers, only six were able to grow at 45°C and two at 55°C (Kützner 1981). Studies by Tendler & Burkholder (1961) very clearly demonstrated that only

**Table 1.** Occurrence of mesophilic aerobic actinomycetales, bacteria and molds in Kuwait sandy desert soil. Total counts (number of colonies × $10^6$/g soil, fresh weight)

| Medium | Plant rhizosphere | Actinomycetales | Bacteria | Molds |
|---|---|---|---|---|
| Küster & Williams' | *Cyperus* | 1·50 | 0·65 | 0·39 |
| casein-starch agar | *Citrullus* | 0·78 | 0·45 | 0·45 |
| at 30°C | *Moltkiopsis* | 0·80 | 0·56 | 0·28 |
| Küster & Williams' | *Cyperus* | 0·65 | 0·003 | 0·004 |
| casein starch agar + actidione + | *Citrullus* | 0·57 | 0·006 | 0·003 |
| chlortetracycline at 45°C | *Moltkiopsis* | 0·62 | 0·000 | 0·000 |
| El-Nakeeb & Lechevalier's | *Cyperus* | 1·42 | 0·85 | 0·21 |
| agar at 30°C | *Citrullus* | 0·95 | 0·55 | 0·20 |
| | *Moltkiopsis* | 0·85 | 0·59 | 0·24 |
| El-Nakeeb & Lechevalier's | *Cyperus* | 0·95 | 0·007 | 0·002 |
| agar + actidione + chlortetracyline | *Citrullus* | 0·84 | 0·003 | 0·005 |
| at 45°C | *Moltkiopsis* | 0·76 | 0·006 | 0·008 |

a few thermophilic streptomycetes were recognized while *Thermoactinomyces* was of common occurrence.

The generic identity of each isolate was confirmed by the whole-cell hydrolyzate analysis for presence and kind of diaminopimelic acid isomer following the methods outlined by Becker *et al.* (1964). Four hundred and forty-five isolates contained LL-diaminopimelic acid and 13 contained meso diaminopimelic acid. The whole-cell sugar analysis (Becker *et. al.* 1965) and the lipid analysis of the 13 isolates indicated that they belonged to the genus *Nocardia* (Goodfellow & Minnikin 1981).

The most unique characteristic of these streptomycetes was their tolerance to high concentration of sodium chloride. Four hundred and thirty-five isolates were able to grow in the presence of salt (10–15%). Previous reports of Küster & Neumeirer (1979) indicated that only a limited number of terrestrial or marine strains tolerate high concentrations of salt. However, a strain of *Actinopolyspora halophila* (Gochnauer *et al.* 1975) can tolerate up to 25% sodium chloride.

## CHARACTERIZATION EVALUATION

Once the biochemical, morphological and physiological data had been completed, several of the most recently published keys for identification were followed to assure as accurate identifications as possible. Keys used were those of Hütter (1967), Küster (1972), Berd (1973), Pridham & Tresner (1974), Nonomura (1974), and Pridham (1976a, b). The detailed ISP descriptions of Shirling & Gottlieb (1968a, 1968b, 1969, 1972) were also consulted. Because of difficulties in arriving at the precise identification, the approach of Pridham (1976b) was relied upon to provide meaningful results. These are:

| | |
|---|---|
| *Streptomyces albus* (Rossi Doria) Waksman & Henrici | 173 isolates |
| *S. griseus* or *venezuelae* Waksman & Henrici | 165 isolates |
| *S. lavendulae* (Waksman & Curtis) | 24 isolates |
| *S. graminofaciens* (Charney *et al.*) | 5 isolates |
| *S. flaveolus* (Waksman) | 11 isolates |
| *S. diastaticus* (Krainsky) | 67 isolates |

The classification system of Hütter (1967) employs four criteria: colour of aerial mycelium, spore chain morphology, spore surface, and melanin formation. These characters together with data on halotolerance, thermotolerance, and antibiotic activities are presented in Table 2.

The classification of Pridham *et al.* (1958) which is based on morphological characteristics recognizes 3 sections: (1) Section *Rectus flexibilis* (RF) with straight, flexuous and fascicled chains of spores, (2) Section *Retinaculum apertum* (RA) with open loops, primitive coils and hooks, and (3) Section *Spira* (S) with open or closed coils. Correct assignment of morphology of each isolate was made by direct comparison with the proposed type and neotype strains of each section. However, for the sake of convenience and simplification only two morphological sections, i.e. the spiral and RF are included in Table 2 in line with Pridham & Tresner (1974).

Consideration of sugar utilization data and the four primary criteria of Hütter's classification suggested several names for the same isolate which were incompatible with the published antibiotic data. However, the precise information on the nature of antibiotics produced by each isolate requires much time and effort. Presently,

**Table 2.** Some characteristics of thermodurant *Streptomyces* species

| Colour series | Spore surface | | | | | Sporophores | | Growth | | Antibacterial species | Antifungal species |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sp | Sm | W | H | Kn | Spiral | RF | in 10–15% NaCl | at 45°C | | |
| White (133) | | | | | | | | | | | |
| Chromogenic | 8 | – | – | – | – | 8 | – | 8 | – | 8 | 7 |
| | – | 26 | – | – | – | 26 | – | 26 | 23 | 2 | 1 |
| | – | 10 | – | – | – | – | 10 | 10 | 9 | 4 | 3 |
| Non-chromogenic | 13 | – | – | – | – | 13 | – | 13 | 1 | 1 | – |
| | – | 49 | – | – | – | 49 | – | 49 | 14 | 29 | 4 |
| | – | 27 | – | – | – | – | 27 | 25 | 15 | 17 | 8 |
| Yellow (62) | | | | | | | | | | | |
| Chromogenic | – | 14 | – | – | – | – | 14 | 14 | 11 | 3 | – |
| | – | 6 | – | – | – | 6 | – | 6 | 6 | 2 | 2 |
| Non-chromogenic | – | 27 | – | – | – | – | 27 | 26 | 16 | 10 | 3 |
| | – | 15 | – | – | – | 15 | – | 15 | 6 | 4 | – |
| Gray (154) | | | | | | | | | | | |
| Chromogenic | – | – | 1 | – | – | – | – | – | 1 | – | 1 |
| | – | 13 | – | – | – | 13 | – | 8 | 1 | 7 | 1 |
| | – | 7 | – | – | – | – | 7 | 7 | 4 | 1 | – |
| | 6 | – | – | – | – | 6 | – | 6 | 2 | 3 | 1 |
| Non-chromogenic | – | – | 5 | – | – | 5 | – | 5 | 4 | 2 | 2 |
| | – | 87 | – | – | – | 87 | – | 82 | 51 | 28 | 24 |
| | – | 13 | – | – | – | – | – | 13 | 6 | 7 | 2 |
| | 22 | – | – | – | – | 22 | – | 21 | 13 | 9 | 7 |

**Table 2.** (*cont.*)

| Color series | Spore surface | | | | | Sporophores | | Growth | | Antibacterial species | Antifungal species |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Sp | Sm | W | H | Kn | Spiral | RF | in 10–15% NaCl | at 45°C | | |
| Blue (46) | | | | | | | | | | | |
| Chromogenic | – | – | – | 1 | – | 1 | – | 1 | 1 | 1 | 1 |
| | 26 | – | – | – | – | 26 | – | 26 | 14 | 18 | 6 |
| | – | 6 | – | – | – | 6 | – | 6 | 3 | 3 | – |
| | – | 1 | – | – | – | – | 1 | 1 | – | – | 1 |
| Non-chromogenic | 9 | – | – | – | – | 9 | – | 15 | 6 | 1 | 1 |
| | – | 3 | – | – | – | 3 | – | 3 | – | 2 | 2 |
| Red (50) | | | | | | | | | | | |
| Chromogenic | – | 8 | – | – | – | 8 | – | 7 | 4 | 5 | 3 |
| | – | 5 | – | – | – | – | 5 | 5 | 1 | 3 | 1 |
| | 1 | – | – | – | – | – | 1 | 1 | 1 | – | – |
| Non-chromogenic | – | 27 | – | – | – | 27 | – | 27 | 23 | 16 | 9 |
| | – | 9 | – | – | – | – | 9 | 9 | 3 | 3 | 4 |
| Total | 85 | 353 | 6 | 1 | – | 331 | 114 | 435 | 240 | 187 | 93 |

Sp = spiny, Sm = smooth, W = warty, H = hairy, Kn = knobby, – = zero.

individual isolates are under investigation for antibiotic potential at 45°C in our laboratory. It is of interest to mention that most of them elicit maximum antibiotic formation within 48 h. Surprisingly, none of the streptomycete isolates were able to utilize crude petroleum as a carbon source.

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# عزل بكتيريا ستربتومايسيز التي تتحمل درجات الحرارة العالية من المحيط الجذري لبعض النباتات التي تنمو في الصحراء الرملية المعرضة لشعلة الغاز الطبيعي

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پيوريا ، الينوي ، ٦١٦٠٤ ، الولايات المتحدة الامريكية

## خلاصة

لقد تم فصل حوالي ٤٥٠ عزلة من سلالة بكتيريا الستربتومايسيز الهوائية التي تعيش في درجة حرارة متوسطة وتتحمل درجات الحرارة العالية ، من المحيط الجذري لثلاثة نباتات زهرية هي : الثندي ، والحنظل (الشرى) ، والحماط . وتنمو هذه النباتات في صحراء رملية ، وتتعرض لدرجة حرارة عالية نتيجة لنموها بالقرب من مشاعل الغاز الطبيعي في حقل نفط المقوع في الكويت . ومن العزلات التي تم فصلها أمكن التعرف على ١٧٣ عزلة على انها ستربتومايسيز ألبس ، و ١٦٧ عزلة من ستربتومايسيز فريزيوس أوستربتومايسيز ڤنزويلي ، و ٢٤ عزلة من ستربتومايسيز لاڤنديولي ، و ٥ عزلات من ستربتومايسيز جرامينوفاشينز ، و ١١ عزلة من ستربتومايسيز فلاڤيولوس ، وأخيرا ٦٧ عزلة من ستربتومايسيز دياستاتيكاس . ولقد وجد أن ١٣ عزلة تحتوي على حمض ميزوداي أمينوپايمليك في ناتج التحليل الكلي لمحتوى الخلية ، وعليه فإنها ليست من مجموعة ستربتومايسيز أو من مجموعة ستربتوڤيرتيسيليا . ولقد وجد أن ٤٣٥ عزلة من هذه البكتريا لها قدرة على النمو في وجود محلول كلوريد الصوديوم تركيزه ١٠-١٥٪ ، و ٢٤٠ عزلة تمكنت من النمو عند درجة حرارة مقدارها ٤٥°م ، ١٨٧ عزلة تنتج مضادات حيوية ، وأخيرا ٩٣ عزلة تفرز مواد مضادة للفطريات .