MM 42842, A NEW MEMBER OF THE MONOBACTAM FAMILY PRODUCED BY *PSEUDOMONAS COCOVENENANS*

I. IDENTIFICATION OF THE PRODUCING ORGANISM

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A bacterial soil isolate designated 326-32B produces a new member of the monobactam series of antibiotics, MM 42842, and the bulgecins. Identification studies show isolate 326-32B to be a strain of *Pseudomonas cocovenenans* which is a species previously noted for the production of toxoflavin. A description of *P. cocovenenans* does not appear to have been previously published and the identity of strain 326-32B was established by means of a direct comparison with the deposited organism *P. cocovenenans* NCIB 9450.

The properties of strain 326-32B, and *P. cocovenenans* NCIB 9450 were compared with those of the monobactam and bulgecin producing organisms *Pseudomonas acidophila* ATCC 31363 and *Pseudomonas mesoacidophila* ATCC 31433. The four organisms were found to share certain properties, including the ability to grow at pH 4.0.

In the course of screening for new antibiotics, a bacterial culture designated 326-32B was isolated from a soil sample and found to produce a new monocyclic β-lactam antibiotic, MM 42842.[1] Members of the bulgecin family[2] of antibiotics were detected in the same culture (S. J. BOX and S. R. SPEAR; unpublished data). The culture 326-32B also produces a yellow non-fluorescent diffusible pigment which is established in this paper to be toxoflavin. Toxoflavin has been reported previously as a product of *Pseudomonas cocovenenans*.[3] No description of *P. cocovenenans* appears to have been published, although the deposited strain NCIB 9450 was available for comparison with 326-32B.

MM 42842 is structurally related to the monocyclic β-lactam antibiotics sulfazecin and isosulfazecin which are products of the organisms *Pseudomonas acidophila* ATCC 31363[4,5] and *Pseudomonas mesoacidophila* ATCC 31433,[4,6] respectively. These two organisms, like culture 326-32B, have been reported to produce the bulgecin antibiotics.[2]

This paper describes identification studies on 326-32B, in which its properties are directly compared with *P. cocovenenans* NCIB 9450, *P. acidophila* ATCC 31363 and *P. mesoacidophila* ATCC 31433. The results show that culture 326-32B is a strain of *P. cocovenenans*, although there were also interesting similarities with *P. acidophila* ATCC 31363 and *P. mesoacidophila* ATCC 31433.

Materials and Methods

Abbreviations

The following abbreviations are used in this paper: ATCC, American Type Culture Collection, Rockville, Maryland, U.S.A.; NCIB, National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland, UK; NCTC, National Collection of Type Cultures, London NW9, England, UK; PHB, poly-β-hydroxybutyrate.

Bacterial Strains

Culture 326-32B was isolated from soil taken from beneath a *Diospyros kaki* tree, growing in the Medicinal Plant Garden, Hoshi University, Tokyo. *Pseudomonas maltophilia* 732 was a clinical isolate. All other cultures used in this study were obtained from respective culture collections. The cultures were stored as a suspension in 20% glycerol at −80°C. Culture 326-32B did not survive well on Nutrient agar slopes at room temperature.

Identification Methods

An incubation temperature of 30°C was used for all tests unless otherwise stated. Cellular morphology, flagella morphology, Gram reaction, motility, the oxidase and the catalase test were all performed on cells grown on Nutrient agar at pH 7.0. All cultural tests were read daily for up to 7 days unless otherwise stated.

The oxidase test was performed by rubbing cells on filter paper impregnated with a 1%-solution of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine. A dark purple reaction within 10 seconds was interpreted as a positive result. Flagella morphology was examined by use of a negative staining technique and transmission electron microscopy. The flagella index method[7] was used to determine number of flagella. Intracellular accumulation of PHB was determined on cells grown for 24 hours in a nitrogen deficient medium[8] with glucose as the sole carbon source. Hydrolysis of PHB was determined in a basal mineral medium as described below with the addition of PHB as a sole carbon source at a concentration of 0.25%. Tests for Tween 80 hydrolysis,[9] arginine dihydrolase[10] and urease activity[11] were performed using the media as originally described. Tests for denitrification, starch hydrolysis and lysine decarboxylase were performed as described by STOLP and GADKARI.[12] The Methyl red and Voges-Proskauer (MR-VP), indole and O-F tests were performed using media as described by COWAN.[13] Commercially available media were used for determining gelatin hydrolysis, DNase activity and levan production from sucrose. Ability to produce fluorescent pigments were determined on King A and King B media.

Growth at 41°C was determined in PY broth (peptone 1%, yeast extract 0.3%, pH 7.0). Ability to utilize various organic compounds as sole carbon sources was determined in a mineral medium composed of K_2HPO_4 0.7%, KH_2PO_4 0.2%, $MgSO_4$ 0.01% and $(NH_4)_2SO_4$ 0.1%, pH 7.0. Organic compounds were incorporated at a final concentration of 0.25%. pH requirements for growth were determined on Nutrient agar (Oxoid) with the pH adjusted after autoclaving with 1 M HCl or 1 M NaOH prior to dispensing into Petri dishes. A replicating device was used to inoculate the agar plates with a suspension of cells for the carbon utilization tests and to determine pH requirements for growth. The suspensions were of a density so as to give an inoculum of approximately 10^6 colony forming units per 'spot'.

The GC% base ratio was determined on DNA extracted from the cells by the method of MARMUR.[14] The GC% was determined by the melting point method.

Extraction of Yellow Pigment

Organism 326-32B was cultured with confluent growth for 2 days at 30°C on the surface of B3 agar, of composition glucose 1%, fructose 1%, Tryptone 0.5%, soya peptone 0.5% and agar 1.8%, pH 7.0 in 9-cm Petri dishes. The pigment was extracted from 2 litres of B3 agar using $CHCl_3$. The crude $CHCl_3$ extract was evaporated to dryness to yield a solid. The dried extract was chromatographed on a silica gel column (2.5×36 cm) using $CHCl_3$ - MeOH (9: 1). Fractions containing the yellow pigment were combined and final chromatography in $CHCl_3$ - EtOAc (10: 3) afforded the pure product as a yellow powder. $CHCl_3$ extracts of yellow pigment produced by *P. cocovenenans* NCIB 9450 and culture 326-32B were also made from the broth medium described by VAN DAMME *et al.*[3] for toxoflavin production.

Results

Chloroform extraction and silica gel chromatography of the yellow pigment produced by culture 326-32B on B3 agar, yielded 7 mg of pigment from 2 liters of agar. The electron impact mass spec-

trum (EI-MS) of the yellow pigment indicated a molecular weight of 193 and elemental analysis gave a molecular formula of $C_7H_7N_5O_2$. The UV, IR and ^1H NMR data obtained on this material were identical to those reported[8,15,16] for the aza-purine, toxoflavin (Fig. 1).

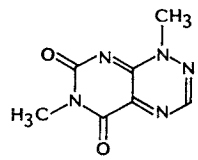
A ^{13}C NMR spectrum was also obtained and was consistent with the proposed structure; ^{13}C NMR ($CDCl_3$) δ 29.1, 43.3, 145.1, 145.4, 150.3, 154.1 and 158.4 ppm.

The yellow pigment produced by culture 326-32B on B3 agar, and established to be toxoflavin, was examined on silica gel TLC. Also examined were chloroform extracts of yellow pigments produced by culture 326-32B and *P. cocovenenans* NCIB 9450 in the toxoflavin production medium of VAN DAMME *et al.*[3] All three preparations of pigment had an Rf of 0.4 in chloroform - methanol (10 : 1).

Each of the four organisms tested were Gram-negative polarly flagellated rods. They were oxidative in the O-F test, strict aerobes, and catalase positive. Growth did not occur at pH 3.5, and ethanol was not oxidised to acetic acid on FRATEUR's ethanol medium.[17] Growth factors were not required and a variety of organic compounds were used as sole carbon sources as shown in Table 2. These properties are consistent with the four organisms, including culture 326-32B, belonging to the genus *Pseudomonas*.

Culture 326-32B was found to have properties very similar to *P. cocovenenans* NCIB 9450, as shown in Tables 1, 2 and 3. In particular, as described above, culture 326-32B was found to produce toxoflavin as a yellow pigment in the broth medium of VAN DAMME *et al.*[3] and on B3 agar. Production of this pigment was not observed however on Nutrient agar or on King A and King B media. Toxoflavin production by *P. cocovenenans* NCIB 9450 was also demonstrated in VAN DAMME's broth medium, as has been reported for this culture.[3] As shown in Table 2, culture 326-32B was able to utilize nine sole carbon sources which *P. cocovenenans* NCIB 9450 was unable to utilize. However, the two cultures had the same abilities with respect to the utilization of a further 81 sole carbon sources, and were similar with respect to all other tests carried out.

Fig. 1. The structure of toxoflavin.



Culture 326-32B and *P. cocovenenans* NCIB

Table 1. Characteristics of *Pseudomonas acidophila* ATCC 31363, *Pseudomonas mesoacidophila* ATCC 31433, culture 326-32B and *Pseudomonas cocovenenans* NCIB 9450.

	Culture			
	P. acidophila ATCC 31363	*P. mesoacidophila* ATCC 31433	326-32B	*P. cocovenenans* NCIB 9450
Toxoflavin production	−	−	+	+
Gelatin hydrolysis	−	+	+	+
Arginine dihydrolase	−	+	−	−
Growth at 37°C	−	+	+	+
Growth at 41°C	−	+	+	+
No. of flagella	1	>1	>1	>1
Base content (mol % G+C)	62.5	68.8	69.6	68.2

The four cultures have the following properties in common.

Positive reactions: Catalase test, Tween 80 hydrolysis, PHB accumulation, growth at pH 4.0, urease.

Negative reactions: Oxidase test, starch hydrolysis, lysine decarboxylase, growth at 4°C, denitrification, indole production, MR-VP tests, levan production on sucrose agar, DNase production, PHB hydrolysis.

Table 2. Carbon utilization.

Carbon source	*Pseudomonas acidophila* ATCC 31363	*Pseudomonas mesoacidophila* ATCC 31433	326-32B	*Pseudomonas cocovenenans* NCIB 9450
Acetamide, L-leucine, L(+)-rhamnose	+	−	−	−
Maltose, α-methyl-D-glucoside, sucrose	+	+	−	−
β-Alanine, dulcitol, ethanol, melibiose, *meso*-tartarate, D(−)-tartarate, mesaconate	−	−	+	+
L-Arabinose, *n*-butyrate, citrate, DL-isocitrate, fumarate, L-lysine, malonate, pelargonate, propionate	−	+	+	+
S-Aminovalerate, L-cystine, L-cysteine, glycine, levulinate, putrescine	−	+	−	−
Adipate, *p*-hydroxybenzoate, L-phenylalanine	+	+	+	−
Benzoate, hydroxy-L-proline, L-valine, quinate	−	+	+	−
Adonitol	+	−	+	−
L-Isoleucine	−	−	+	−

Utilized by all four strains: Acetate, L-alanine, L-arginine, L-asparagine, L-aspartate, betaine, D(+)-cellobiose, citraconate, D-fructose, α-D-fucose, D(+)-galactose, D-gluconate, D-glucose, L-glutamate, L-glutamine, glycerol, L-histidine, DL-β-hydroxybutyrate, *meso*-inositol, 2-ketogluconate, lactate, malic acid, mannitol, mannose, L-proline, D-ribose, saccharate, L-serine, sorbitol, succinate, L-tartarate, threonine, trehalose, L-tryptophan, L-tyrosine, D-xylose.

Utilized by neither strain: α-Amylamine, 2,3-butanediol, erythritol, geraniol, glycolic acid, *m*-hydroxybenzoate, lactose, maleic acid, methionine, norleucine, D-pantothenate, 1,2-propanediol, raffinose, starch, testosterone, tryptamine, D-tryptophan.

All carbons were at 0.25% in mineral medium containing ammonium sulfate as sole nitrogen source. Plates were incubated at 30°C and read after 7 days.

Table 3. pH requirements for growth of *Pseudomonas* sp.

	pH range of growth	
	2 days	7 days
Pseudomonas acidophila ATCC 31363	4.2~7.5	4.0~7.6
P. mesoacidophila ATCC 31433	4.0~8.7	3.8~8.8
Pseudomonas sp. 326-32B	3.8~8.0	3.8~8.1
P. cocovenenans NCIB 9450	3.9~8.2	3.8~8.4
P. acidovorans NCIB 9681	4.9~11.1	4.6~11.1
P. aeruginosa NCTC 10662	4.8~>11.3	4.4~>11.3
P. alcaligenes NCIB 9945	4.8~>11.3	4.6~>11.3
P. cepacia NCIB 9085	4.0~8.8	3.8~9.1
P. fluorescens NCIB 10586	4.9~10.9	4.6~11.1
P. lemoignei NCIB 9947	5.3~9.1	4.9~9.35
P. maltophilia 732	4.6~>11.3	4.6~>11.3
P. mendocina NCIB 10541	5.1~>11.3	5.1~>11.3
P. stutzeri NCIB 9721	5.5~>11.3	5.1~>11.3
P. pseudoalcaligenes NCIB 9946	5.5~>11.3	5.1~>11.3
P. testosteroni NCIB 8893	4.8~11.1	4.8~11.1

The medium used was Nutrient agar, adjusted with 1 M HCl or 1 M NaOH before dispensing. Plates were spot inoculated with approximately 10^6 cfu. Incubation was at 30°C for 7 days.

9450 were found to have certain properties in common with *P. mesoacidophila* ATCC 31433 and, to a lesser extent, with *P. acidophila* ATCC 31363, as shown in Tables 1, 2 and 3. The properties observed for *P. acidophila* ATCC 31363 and *P. mesoacidophila* ATCC 31433 agree well with information previously published on these organisms.[4~6] All four cultures gave a delayed, weak oxidase reaction

which was scored as negative. *P. acidophila* ATCC 31363 has been reported as oxidase negative.[4,5] *P. mesoacidophila* ATCC 31433 was originally reported as oxidase positive in the patent literature,[18] but more recently as oxidase negative.[4,6]

P. acidophila ATCC 31363 and *P. mesoacidophila* ATCC 31433 have been reported to have acidophilic properties.[4~6] The pH requirements reported for growth of these two organisms were confirmed, and found to be similar to culture 326-32B and *P. cocovenenans* NCIB 9450 (Table 3). When compared with a number of other members of the genus *Pseudomonas* it was observed that only *Pseudomonas cepacia* NCIB 9085 shared their ability to grow at pH 4.0. The cultures able to grow at pH 4.0 were also all less tolerant to alkaline conditions as compared to the other pseudomonads studied.

P. acidophila ATCC 31363 and *P. mesoacidophila* ATCC 31433 were not observed to produce toxoflavin, or any other pigment, in the media used, including VAN DAMME's broth medium.

Discussion

The data in this paper clearly indicate that culture 326-32B is a strain of *P. cocovenenans*, as judged by its close similarity, including toxoflavin production, to the deposited member of this species. This paper also seems to provide the first published description of this species. *P. cocovenenans* was previously reported in connection with the production of toxoflavin, a toxin implicated in episodes of fatal food poisoning when ingested with contaminated native coconut products.[3] Toxoflavin (xanthothricin) is also a product of a streptomycete.[19]

P. cocovenenans appears to have several distinctive properties in common with *P. acidophila* ATCC 31363 and *P. mesoacidophila* ATCC 31433. Most notably all four organisms studied were able to grow on Nutrient agar at pH 4.0.

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