IDENTIFICATION OF A *MYCOBACTERIUM* AND A *BREVIBACTERIUM* PRODUCING ANTIBACTERIAL SUBSTANCES

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Abstract

Two strains, HG104 and AS50, were isolated from the soil of Tehran's suburbs. Taxonomic studies showed that HG104 and AS50 belong to *Mycobacterium* spp. and *Brevibacterium* spp. respectively, which produce antibacterial substances with relatively high activity against some important clinical Gram-positive and Gram-negative bacteria.

Introduction

Most of the producers of the known antibiotics have been isolated from various natural sources such as soil [1]. Soil is the best source for isolating antibiotic producing microorganisms, and nearly all have been isolated in various screening programs from this source [2]. The antibiotic producing ability of the microbial florae isolated from Iran's environment is not, however, well known. We therefore decided to screen different soil samples from various parts of Iran, with the aim of looking for new strains of antibiotic producing microorganisms. Results obtained from a part of these investigations are thus presented in this paper.

Materials and Methods

Standard microorganisms used for chemotaxonomic study and antibiogram testing were obtained from the PTCC (Persian type culture collection) and are as follows: *Streptomyces rimosus* PTCC : 1198, *Nocardia asteroides* PTCC : 1173, *Mycobacterium smegmatis* PTCC : 1307, *Bacillus subtilis* PTCC : 1253, *Staphylococcus aureus* PTCC: 1113, *Escherichia coli* PTCC: 1222, *Pseudomonas aeruginosa* PTCC : 1047, *Salmonella typhi* PTCC : 1232.

Keywords: Antibiotic; *Brevibacterium; Mycobacterium*

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Taxonomic Studies

Two strains, HG104 and AS50, which produce antibiotics, were isolated from a soil sample collected in Tehran, Iran. Methods and media described by the International Streptomyces Project [3] were used to determine most of the physiological characteristics of the strains. Their cell wall composition was analyzed according to the method of Becker *et al.* [4,5]. For diaminopimelic acids (DAPs), a 5 mg (dry weight) sample was hydrolyzed in 1 ml of 6N HCl in a sealed Pyrex tube in an oven at 100°C for 18 hours. The hydrolysate was filtered, evaporated to dryness three times over a boiling water bath to remove HCl, and taken up in 0.3 ml of distilled water. Descending chromatography was carried out on Whatman No. 1 paper with methanol-water-10N HCl and pyridine (80 : 17.5:2.5: 10, by volume) as eluent.

For amino acid detection [5], the paper was sprayed with acetonic ninhydrin (0.1%, w/v), followed by heating for 2 min at 100°C: DAP purchased from Sigma company was a mixture of LL-, DD- and *meso* isomers used as standard. In this system, *meso* -DAP travel more slowly than LL-DAP and present a $R_{LL.DAP}$ of 0.8 [4].

For determination of cell wall sugars, a 10 mg (dry weight) sample was hydrolyzed in 1 ml of 2N H_2SO_4 in a sealed Pyrex tube at 100°C for 2 hours. The hydrolysate was neutralized to pH 5.0 to 5.5 with saturated solution of $Ba(OH)_2$ with methyl red as an internal indicator. The precipitate of $BaSO_4$ was centrifuged at 3,000 rpm and the supernatant fluid was poured *in vacuo* over H_2SO_4. The

final product was redissolved in 0.4 ml of distilled water. An amount of 50 µl of the hydrolysate was spotted on Whatman No. 1 paper for descending paper chromatography in the same solvent system as was described above for amino acid. 10 µl of a solution containing galactose, glucose, arabinose, mannose and ribose, each at a concentration of 5 µg/µl, was spotted on the same paper for reference. After 24 hours elution, the paper was removed, dried at room temperature, and sprayed with aniline phthalate reagent [5].

The mycolic acids type and melting points were determined respectively by the methods of Minnikin *et al.* [6] and Kanetsuna *et al.* [7]. *M. smegmatis* and *N. asteroides* were used as reference strains for this purpose.

Fermentation

Loopfuls of culture from strains HG104 and AS50 were used separately to inoculate 500 ml flasks containing 100 ml of 1% yeast extract (Difco). The pH was adjusted to 7.2 before sterilization. The inoculated flasks were incubated on a rotary shaker (180 rpm) at 28-30°C for 72 hours. Aliquots of each culture (5 ml) were taken and centrifuged (6000 rpm) for 10 min. The biomass was suspended in strerilized distilled water and transferred into 500 ml flasks containing 100 ml of the different media which are described in the footnotes of Tables 1(a) and 1(b). The inoculated flasks were incubated on a rotary shaker (180 rpm) under the above-mentioned conditions 120 hours for HG104 and 192 hours for AS50. Supernatant from each culture was separated by centrifuge and used for antibiogram tests according to the classical diffusion methods [8].

Results and Discussion

Taxonomy of Strains HG104 and AS50

Chemotaxonomic and physiological studies of HG104 and AS50 are illustrated in Figure 1, Tables 2 and 3. Figure 1 shows paper chromatogram of diaminopimelic acid of the strains. DAP spots were olive-green fading to yellow [4] while amino acids present in the hydrolysate gave purple spots with ninhydrin reagent and moved faster than DAP [4]. The olive-green spot of the hydrolyzed sample of *S. rimosus* PTCC : 1198 (lane 1), which contains LL-DAP in its cell wall [4], standard DAP (lane 2), AS50 (lane 3) and HG104 (lane 4) are compared in Figure 1. DAP spots in lane 3 and 4 moved slower than LL-DAP. The $R_{LL\text{-}DAP}$ of lower spots in lane 3 and 4 were 0.8 as known for *meso*-DAP [4].

The results obtained from sugar identification and presented in Table 2 show that characteristic cell-wall carbohydrates of HG104 are arabinose and galactose. Only traces of ribose and glucose were detected for strain AS50.

Table 1(a). Results obtained from fermentation studies on strain AS50 120 hours after inoculation

Medium[b]	OD600	Inhibition zone against *B. subtilis* (mm)
1	0.5	-[a]
2	0.5	-
3	0.5	-
4	1.2	17
5	1.2	9
6	-	-
7	0.5	9
8	0.5	-
9	1.2	-
10	1.2	9
11	1.2	8
12	1.2	9
13	1.2	10

a: No inhibition zone was observed in antibiogram.

b: Medium 1) Glucose 5%, $(NH_4)_2SO_4$ 0.7%, K_2HPO_4 0.1%; 2) Glucose 5%, $(NH_4)_2SO_4$, 0.7%, K_2HPO_4 0.1%; 3) Glucose 5%, $(NH_4)_2SO_4$ 0.7%, K_2HPO_4 0.1%, Succinic acid 0.1%; 4) Yeast extract (Difco) 1%, Glucose 1%; 5) Nutrient broth (Difco) 1%, Glucose 1%; 6) Sabouraud Dextrose (Difco) 1%; 7) Peptone (Difco) 1%, Glucose 1%; 8) Tryptone (Difco) 1%, Glucose 1%; 9) Peptone (Difco) 1%, Meat extract 5%, Glucose 0.4% Sodium acetate 0.2%, Sodium citrate 0.2%, $CaSO_4$ 0.000175%, $FeSO_4$ 0.0015%, $MgSO_4$ 0.5%, KCl 0.05%, K_2HPO_4 0.1%, $NaNO_3$ 0.4%; 10) Yeast extract (Difco) 1%, Maltose 1%; 11) Yeast extract (Difco) 1%, Lactose 1%; 12) Yeast extract (Difco) 1%, Saccharose 1%; 13) Yeast extract (Difco) 1%, Fructose 1%

Table 1(b). Results obtained from fermentation studies on strain HG104 192 hours after inoculation

Medium[b]	OD600	Inhibition zone against *B. subtilis* (mm)
A	1.2	12
B	-	-[a]
C	1.3	16
D	1..2	12
E	0.7	-
F	0.9	25
G	0.7	13
H	1.2	17

a: No inhibition zone seen in antibiogram.

b: Medium: A) Peptone (Difco) 1%; Yeast extract (Difco) 0.4%, K_2HPO_4 0.2%; B) Peptone (Difco) 1%, $(NH_4)_2SO_4$ 0.4%, K_2HPO_4 0.2%; C) Yeast extract (Difco) 0.6%, Glucose 1%; E) Tryptic soy broth (Difco) 3%, Glucose 1%; F) Sabouraud dextrose broth (Difco) 3% in tap water; G) Nutrient broth (Difco) 1%, Glucose 1%; H) Nutrient broth (Difco) 1%, Glucose 1%.

Table 2. Chemotaxonomy of HG104 and AS50

Determinative molecules in cell hydrolysates	AS50	HG104	*M. smegmatis*	*N. asteroides*
meso-DAP	+	+	+	+
LL-DAP	Trace	-	-	-
Arabinose	-	+	+	+
Galactose	-	+	+	+
Mannose	-	-	-	-
Ribose	Trace	-	-	-
Xylose	-	-	-	-
Glucose	Trace	Trace	Trace	Trace
Mycolic acid	-	+	+	-
Nocardomycolic acid	-	-	-	+
Precipitate Melting point	-	47-51°C	57-60°C	80°C

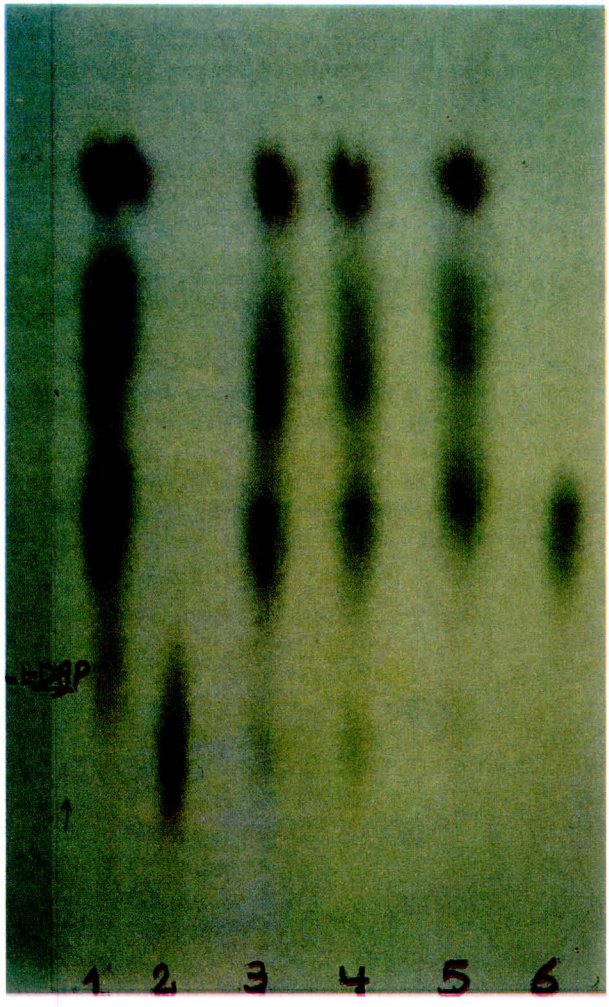

Figure 1. Paper chromatogram of representative strains. (1) *Streptomyces rimosus* (PTCC 1198); (2) DAP which was mixture of LL-, DD- and *meso* isomers used as standard; (3) Strain AS50; (4) Strain HG104; (5) Other isolated strains; (6) Lysine.

Lipid analysis [6] showed that whole cells of strain HG104 contained long chain mycolic acids, because alcohol treatment of the methanolysis products gave a large amount of a white precipitate exhibiting a melting point around 50°C, characteristic of the *Mycobacterium* genus, instead of the oily pellet obtained for *Nocardia* species. Moreover, the complex pattern observed on TLC for methanolysis products, compared to that of reference strains *M. smegmatis* and *N. asteroides*, was in agreement with this interpretation. HG104 has a cell wall type IV, i.e. it contains *meso*-DAP, arabinose, galactose and mycolic acid in its cell wall.

Strain AS50 has cell wall type III as it contains *meso*-DAP in its whole-cell hydrolysate, but it did not contain arabinose and mycolic acid. Some of the morphological, physiological and biochemical properties of this microorganism are the same as the *Brevibacterium* genus from the *Actinobacteria* group described in Bergey's manual [9]. Strain AS50 exhibited rod-coccus cycle and V-shaped elements after 24 hours of growth on yeast extract medium. Cultivation on malt extract medium (Difco), after 6 hours had elapsed, produced visible immature branched mycelium. No acid was produced from the glucose in the medium which also contained hydrolyzed peptone and gelatin and no growth under anaerobic conditions took place. The old culture of this strain was Gram-negative. AS50 did not produce endospore and did not form aerial mycelium.

Fermentation of Strains HG104 and AS50

Fermentation studies (Tables 1(a) and 1(b)) showed that the best media for antibiotic production were medium 4 for AS50, and medium F for HG104. The supernatant from medium 4, inoculated by AS50, inhibited the growth of *S. aureus*, *S. typhi* and *E. coli*. Medium F, which was inoculated with HG104, inhibited the growth of all the

Table 3. Physiological properties of two isolated strains, HG104 and AS50

	AS50	HG104
Gram stain	-[a]	+
Starch hydrolysate	-	-
Decomp. of casein	+	ND[b]
Urea hydrolysis	-	-
Gelatin liquification	-	+
Melanin pigment formation: ISP 7	+	-
Acid production from glucose in medium containing peptone	-	ND[b]
Anaerobic growth	-	-
Motility	-	-
Acid fastness	-	+
Endospore stain	-	-
Growth temp. (°C)		
25	+	+
30	+	+
37	+	+
45	-	-
50	-	-
Antibiotic production at temp. (°C)		
25	+	-
30	+	+
37	+	+
45	-	-
50	-	-
Carbon source utilization		
Mannitol	+	+
Maltose	+	+
Lactose	-	+
Glucose	+	+
Fructose	+	-
Saccharose	+	+
Glycerol	-	-
Arabinose	-	-
Galactose	+	-
Sorbitol	-	-
Starch	-	-
Sodium citrate	+	-
Sodium tartrate	-	-
Paraffin	-	-
Aerial mycelium formation		
Bennet Agar medium	-	-
Czapeks Agar medium	-	-
Nutrient Agar medium	-	-

a: In old culture
b: Not determined

mentioned test strains in addition to *P. aeruginosa*.

References

1. Schneider, W. C. Methods of screening, identification and determination of antibiotics. In *The chemistry and biology of antibiotics*, (ed. V. Betina), pp. 51-52. Elsevier Scientific Publishing Co., (1983).
2. Williams, S.T. and Wellington, E. M. H. Principles and problems of selective isolation of microbes. In *Bioactive microbial products: search and discovery*, (ed. J. D. Bu'Lock, L. J. Nisbet and D. J. Winstanley), pp. 9-26. Academic, London (1982).
3. Shirling, E.B. and Gottlieb, D. Methods for characterization of streptomyces species. *Int. J. Syst. Bacteriol.*, **16**, 313-340, (1966).
4. Becker, B., Lechevalier, M.P., Gorden, R.E. and Lechevalier, H.A. Rapid differentiation between nocardia and streptomyces by paper chromatography of whole-cell hydrolysate. *Applied Microbiol.*, **12**, 421-423, (1964).
5. Becker, B., Lechevalier, M.P. and Lechevalier, H. A. Chemical composition of cell wall preparations from strains of various forms - genera of aerobic actinomycetes. *Ibid.*, **13**, 236-213, (1965).
6. Minnikin, D.E., Alshamony, L. and Goodfellow, M. Differentiation of *Mycobacterium, Nocardia* and related taxa by thin-layer chromatographic analysis of whole organism methanolysates. *J. Gen. Microbiol.*, **88**, 200-204, (1975).
7. Kanetsuna, F. and Bartoli, A. A simple chemical method to differentiate *Mycobacterium* from *Nocardia*. *Ibid.*, **70**, 209-212, (1972).
8. Schneider, W. C. Methods of screening, identification and determination of antibiotics. In *The chemistry and biology of antibiotics*, (ed. V. Betina), pp. 60-62. Elsevier Scientific Publishing Co., (1983).
9. Jones, D. and Keddie, R.M. *Bergey's Manual of Systematic Bacteriology*, Vol. 4, (ed. S.T. Williams *et al.*) Williams and Willkins Co., (1989).