***Mycobacterium obuense*, a Rapidly Growing Scotochromogenic Mycobacterium Capable of Forming a Black Product from *p*-Aminosalicylate and Salicylate**

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SUMMARY

Five strains of rapidly growing scotochromogenic mycobacteria were shown to have a capacity to degrade *p*-aminosalicylate and salicylate, forming a black product(s). The organisms differ distinctly from known mycobacterial species that have this capacity, *Mycobacterium fortuitum*, *M. abscessus* and *M. borstelense*, and from other mycobacteria. The organisms are considered to belong to a new species of the genus *Mycobacterium* and are named *Mycobacterium obuense* sp. nov.

INTRODUCTION

Penso, Cattaneo, Morellini & Vicari (1955) observed that *Mycobacterium piscium*, *M. thamnopheos* and *M. friedmanni* darkened media containing *p*-aminosalicylate (PAS) and thought that the PAS was changed, first, to *m*-aminophenol and then to a dark product. When a group of mycobacteria from soil sources grew on Ogawa egg medium containing 0.1 or 0.2% (w/v) PAS the medium became black and catechol was considered an intermediate (Tsukamura, 1961). Tarshis (1964), Medina, Meissner & Bönicke (1964), and Tsukamura (1965*a*) reported that PAS degradation was characteristic for *M. fortuitum*, which also converted salicylate to a black product (Tsukamura, 1965*a*) via catechol as an intermediate (Tsukamura, 1965*b*). More recently, *M. abscessus* (*chelonei*) and *M. borstelense* have also been found to degrade PAS and salicylate (Tsukamura, Mizuno & Tsukamura, 1968; Tsukamura, 1970). All these mycobacteria are rapidly growing, non-photochromogenic mycobacteria. Until now, no organism showing this capacity was found among scotochromogenic mycobacteria. In the course of a co-operative study of Japanese national sanatoria, a rapidly growing scotochromogenic mycobacterium was found to have this ability. Similar organisms were subsequently isolated from the soil. These mycobacteria were shown to belong to a new species.

METHODS

Strains. Five strains of rapidly growing, scotochromogenic mycobacteria capable of degrading PAS and salicylate have so far been isolated: no. 4388 from the sputum of a patient with pulmonary disease, and nos. 5434, 5435, 5436 and 5437 from soil. The method used for the isolation from soil was as described previously (Tsukamura & Tsukamura, 1967), but without selection through mouse body.

Tests. One hundred and two tests were used. Ninety-four of the 95 tests described previously (Tsukamura, 1967) were used, omitting a test for tolerance to 8-azaguanine. To these 94 the following eight tests were added: arrangement of bacterial cells when observed microscopically after the Ziehl-Neelsen stain (compact grouping state or dispersed state); peroxidase; tolerance to 5 µg. ethambutol/ml. in Ogawa egg medium; tolerance to 0.1%

(w/v) and 0.2 % (w/v) NaNO_2 in Sauton agar; tolerance to 1 % (w/v) Tween 80 in Sauton agar; α -esterase activity; β -esterase activity.

Composition of Ogawa egg medium was described previously (Tsukamura, 1967). The composition of Sauton agar is: sodium glutamate, 4.0 g.; KH_2PO_4 , 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; citric acid, 2.0 g.; ferric ammonium citrate, 0.05 g.; glycerol, 30 ml.; purified agar, 30.0 g.; distilled water, 970 ml., with pH to 7.0 by 10 % (w/v) KOH.

The methods of these tests were described previously (Tsukamura, 1966; Tsukamura & Tsukamura, 1968). α -Esterase and β -esterase activities were tested by the method of Kppler (1965). The presence of free naphthylamines in excess of 10 $\mu\text{g.}/\text{ml.}$ was expressed as positive reaction.

Estimation of the S-value (similarity coefficient) between two strains was made as described previously (Tsukamura, 1966). The hypothetical mean organism (HMO) (Tsukamura & Mizuno, 1968) was used to express a mean characterization of a species.

Virulence for mice, guinea pigs, rabbits and chickens was tested by the method previously described (Tsukamura & Tsukamura, 1967).

RESULTS AND DISCUSSION

Biological and biochemical characters of PAS- and salicylate-degrading, scotochromogenic mycobacteria

Gram-positive; acid-fast; rods (approx. 3 $\mu\text{m.}$ by 0.5 to 0.7 $\mu\text{m.}$); no compact grouping; smooth, yellow or orange colonies after 3 days on Ogawa egg medium and on Sauton agar; scotochromogenic; catalase-positive; peroxidase-negative; NO_3^- not reduced; 3 day arylsulphatase-negative; 2 week arylsulphatase-positive; PAS and salicylate degraded to black products; growth on 0.2 % (w/v) PAS in Ogawa egg medium; growth at 28° and 37° but not at 45°.

Urease, nicotinamidase, pyrazinamidase and allantoinase activities present but acetamidase, benzamidase, isonicotinamidase, salicylamidase, succinamidase and malonamidase absent.

Acid from glucose, mannose, arabinose, mannitol and sorbitol within 2 weeks at 28°; acid usually from inositol (3 out of 5); acid not formed from galactose, xylose, rhamnose and trehalose.

Glycerol, glucose, mannose, galactose, arabinose, xylose (4 out of 5), trehalose, mannitol, sorbitol, fructose, sucrose, ethanol, propan-1-ol, 1,2-propanediol, 2,3-butanediol, acetate, succinate, malate, pyruvate and fumarate utilized as sole source of carbon within 2 weeks at 37°; inositol utilized by 3 of 5; rhamnose, 1,3-butanediol, 1,4-butanediol, citrate, benzoate and malonate not utilized.

Glutamate, glucosamine, ethanolamine (by 3 of 5) and trimethylene diamine utilized as simultaneous nitrogen and carbon source; serine, acetamide and benzamide not utilized.

Glutamate, serine, urea, succinamide and NO_3^- utilized as the sole source of nitrogen; methionine, benzamide and NO_2^- not utilized; acetamide, pyrazinamide, isonicotinamide, nicotinamide variable.

Tolerant to: 0.05 % and 0.1 % (w/v) salicylate; 5 $\mu\text{g.}$ ethambutol/ml.; 10 $\mu\text{g.}$ thiophen-2-carboxylic acid hydrazide/ml.; 0.1 % and 0.2 % (w/v) NaNO_2 ; 1 % (w/v) Tween 80, 0.1 % and 0.2 % (w/v) picric acid and 62.5 $\mu\text{g.}$ but not 125 $\mu\text{g.}$ $\text{NH}_2\text{OH} \cdot \text{HCl}/\text{ml.}$ α -esterase-negative; β -esterase-positive.

Two strains, no. 4388 and 5435, were avirulent when tested in mice, guinea pigs, rabbits and chickens.

The most important characters of these strains are the abilities to degrade PAS and salicylate, abilities considered characteristic for *Mycobacterium fortuitum*, *M. abscessus* and *M. borstelense* (Tsukamura, Mizuno & Tsukamura, 1968; Tsukamura, 1970), although these three organisms are non-photochromogenic whereas the strains in the present study are scotochromogenic. The S-value between these strains ranged from 85 to 97 % (Table 1) and the strains are suggested as belonging to a species.

Table 1. *S-Value table for rapidly growing scotochromogenic mycobacteria degrading PAS and salicylate*

Strain no.	S-Value (%)			
	—	—	—	—
4388	—	—	—	—
5434	93	—	—	—
5435	85	90	—	—
5436	87	92	93	—
5437	88	93	96	97

Table 2. *Comparison between a PAS- and salicylate-degrading scotochromogenic Mycobacterium (Mycobacterium obuense) and other mycobacteria*

Species	(Intraspecies mean S-value) ± (Standard deviation)† (%)	'Permissible low limit' for a species‡ (%)	S-Value between the HMOs of <i>M. obuense</i> and other species (%)
<i>M. tuberculosis</i>	98.0 ± 1.20 (n = 90)	95.6	45
<i>M. bovis</i>	99.2 ± 1.02 (n = 20)	97.2	43
<i>M. kansasii</i>	97.0 ± 2.09 (n = 12)	92.8	56
<i>M. avium</i>	94.4 ± 2.11 (n = 26)	90.2	57
<i>M. intracellulare</i>	93.0 ± 2.31 (n = 76)	88.4	59
<i>M. nonchromogenicum</i>	96.2 ± 2.71 (n = 93)	90.8	57
<i>M. gastri</i>	97.3 ± 2.95 (n = 8)	91.3	60
<i>M. scrofulaceum</i>	95.3 ± 2.12 (n = 26)	91.1	64
<i>M. gordonae</i> (tap-water scoto- chromogens)	97.7 ± 1.36 (n = 49)	95.0	61
<i>M. marinum</i>	95.1 ± 4.56 (n = 10)	86.0	60
<i>M. thermoresistibile</i>	92.8 ± 2.86 (n = 39)	87.1	68
<i>M. chitae</i>	97.5 ± 1.73 (n = 4)	94.0	66
<i>M. borstelense</i>	93.0 ± 2.10 (n = 6)	88.8	59
<i>M. abscessus</i>	95.5 ± 5.01 (n = 24)	85.5	72
<i>M. fortuitum</i>	90.0 ± 3.74 (n = 77)	82.5	76
<i>M. phlei</i>	94.0 ± 2.63 (n = 21)	88.7	75
<i>M. aurum</i> *	92.0 ± 5.05 (n = 34)	81.9	83
<i>M. parafortuitum</i> *	92.5 ± 2.51 (n = 6)	87.5	81
<i>M. vaccae</i> *	94.7 ± 2.19 (n = 12)	90.3	69
<i>M. rhodesiae</i> *	96.2 ± 2.16 (n = 16)	91.9	79
<i>M. smegmatis</i>	96.7 ± 1.78 (n = 26)	93.1	72

* The HMO of these species were prepared using the characters shown in the text. The HMO of the other species were prepared as written previously (Tsukamura & Mizuno, 1968).

† The intraspecies mean S-value is a mean S-value for the membership of a species to the HMO of itself. The numbers in parentheses are the numbers of strains used for preparation of the HMO.

‡ (Intraspecies mean S-value) - 2 × (standard deviation).

Comparison between the PAS- and salicylate-degrading scotochromogenic mycobacteria and other mycobacteria

The S-values between the HMO of the PAS- and salicylate-degrading scotochromogenic mycobacteria and the HMO of the other mycobacteria are much lower than the 'permissible

Table 3. Comparison between a PAS- and salicylate-degrading scotochromogenic *Mycobacterium* (*Mycobacterium obuense*) and *M. aurum*

	Mean S-value* (%) to the HMO of	
	<i>M. obuense</i>	<i>M. aurum</i>
<i>M. obuense</i>	95.00 ± 3.94† (n = 5)	80.00 ± 4.30 (n = 5)
<i>M. aurum</i>	81.41 ± 2.38 (n = 34)	92.00 ± 5.05 (n = 34)

* (Mean S-value) ± (standard deviation). The number in parentheses is the number of test strains.

† Intraspecies mean S-value.

N.B. There is a significant difference between the intraspecies mean S-value and the interspecies mean S-value (*P* less than 0.1 % by the 't'-test).

low limit' (Tsukamura, 1969) for the known mycobacterial species (Table 2). The only exception is *Mycobacterium aurum*. This preliminary comparison indicated that the PAS- and salicylate-degrading organisms were distinct from all known species except *M. aurum*. The S-value between the HMO of these organisms and the HMO of *M. aurum* is 83 %, which is slightly higher than the permissible low limit of 81.9 % for *M. aurum*. However, the value is much lower than the intraspecies mean S-value of 92.0 % for *M. aurum* (Table 2). Thus, even though these two taxons may overlap in their characters, they are probably different from each other.

A numerical comparison of these two taxa (Table 3) showed that they are separable from each other with significant difference between the intraspecies and the interspecies mean S-values.

If, as proposed by Runyon (1965), any taxon differentiable from other taxa is regarded as a species, the taxon consisting of PAS- and salicylate-degrading strains should also be regarded as an independent species. Thus the taxon was named as *Mycobacterium obuense* nov. sp. The origin of the name is a locality (Obu) where four of five strains have been isolated.

Type strain of *Mycobacterium obuense* is strain no. 4388 and it has been deposited in the National Collection of Type Cultures, London (NCTC 10778) and in the American Type Culture Collection, Rockville, Maryland, U.S.A. (ATCC 27023).

Characters useful for differentiation of Mycobacterium obuense from other mycobacterial species

The following characters are considered to be important for identification of this species.

(1) Degradation of PAS at 0.2 % (w/v) in the Ogawa egg medium and of salicylate at 0.1 % (w/v) in the Sauton agar with the production of a black product. One loopful of the test organism is used to inoculate the Ogawa egg medium (the Löwenstein-Jensen medium) containing 0.2 % (w/v) sodium *p*-aminosalicylate (PAS) or the Sauton agar containing 0.1 % (w/v) sodium salicylate and the tubes are incubated at 37° for 7 days. Degradation of PAS or salicylate is shown as a black colouring.

(2) Growth within 3 days on Ogawa egg medium (or Löwenstein-Jensen medium) and on the Sauton agar.

(3) Yellow, wet, smooth colonies on the Sauton agar and on egg media.

(4) NO₃⁻ not reduced.

(5) Urease, nicotinamidase, pyrazinamidase and allantoinase are positive among the 10 amidase tests of Bönicke (1962).

Table 4. *Differentiation of Mycobacterium obuense* from *M. fortuitum*, *M. abscessus* and *M. borstelense*

Character	Percentage of strains showing a positive reaction			
	<i>M. obuense</i>	<i>M. fortuitum</i>	<i>M. abscessus</i>	<i>M. borstelense</i>
Number of strains tested	5	77	24	34
Colonial pigmentation	100	0	0	0
Three day arylsulphatase	0	94	79	100
Growth on NH ₂ OH medium (0.25 mg./ml.)	0	100	100	100
Acid from arabinose	100	0	0	0
Acid from sorbitol	100	4	0	0
L-Serine (as N & C source)	0	52	100	97
Trimethylene diamine (as N & C source)	100	98	92	0
Nitrate reduction	0	95	25	0
Allantoinase	100	96	4	0
Ethanol (as C source)	100	91	13	0
Propan-1-ol (as C source)	100	98	13	0

Table 5. *Differentiation of Mycobacterium obuense* from other rapidly growing scotochromogenic mycobacteria

Character	Percentage of strains showing a positive reaction							
	<i>M. obuense</i>	<i>M. rhodesiae</i>	<i>M. para-fortuitum</i> *	<i>M. aurum</i>	<i>M. vaccae</i>	<i>M. phlei</i>	<i>M. thermo-resistibile</i>	<i>M. flavescens</i>
Number of strains tested	5	16	6	34	12	21	39	5
Growth at 45°	0	0	0	0	0	100	100	0
Growth at 52°	0	0	0	0	0	86	98	0
Nitrate reduction	0	0	67	29	0	100	92	100
Salicylate degradation	80	0	0	0	0	0	0	0
PAS degradation	100	0	0	0	0	0	0	0
Acetamidase	0	0	83	62	100	33	0	0
Acid from arabinose	100	0	67	68	0	33	0	0
Acid from xylose	0	100	100	79	0	33	0	0
Acid from rhamnose	0	0	17	56	100	0	0	0
Acid from mannitol	100	100	100	88	100	71	0	0
Acid from sorbitol	100	0	0	0	0	95	0	0
Glucosamine (as N & C source)	100	100	100	100	0	5	0	0
Acetamide (as N & C source)	0	0	0	53	0	100	0	0
Monoethanolamine (as N & C source)	60	0	0	94	0	0	0	0
Trimethylene diamine (as N & C source)	100	100	100	91	0	95	0	0
Malonate (as C source)	0	0	0	0	0	95	0	0

* Some strains grew at 45° immediately after isolation from soils.

(6) Glutamate, glucosamine and trimethylene diamine utilized as simultaneous nitrogen and carbon source.

(7) Acid formed from glucose, mannose, arabinose, inositol (variable), mannitol and sorbitol.

Until now, the capacity to degrade PAS and salicylate, forming a black product, has been considered characteristic of three non-photochromogenic mycobacteria, *Mycobacterium*

fortuitum, *M. abscessus* and *M. borstelense* (Tsukamura, Mizuno & Tsukamura, 1968; Tsukamura, 1970). *Mycobacterium obuense* is the fourth organism that has this capacity. Several characters differentiate *M. obuense* from these three mycobacteria (Table 4). Differentiation from other rapidly growing scotochromogenic mycobacteria is given in Table 5.

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