Taxonomic Consideration of *Microbacterium lacticum*, *Microbacterium flavum*, and *Microbacterium thermosphactum*

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Classification of the microbacteria in a separate genus, *Microbacterium* Orla-Jensen, has been rejected by some authors. In this study three strains of *Microbacterium lacticum* Orla-Jensen, the type species of the genus, one strain of *Microbacterium flavum* Orla-Jensen, and two strains of *Microbacterium thermosphactum* McLean and Sulzbacher were examined with the hope that the somewhat precarious taxonomic position of these species might be clarified. Included in this examination were: the determination of deoxyribonucleic acid (DNA) base composition by the thermal denaturation technique, the development of protein and esterase profiles by polyacrylamide gel electrophoresis, and selected enzyme assays associated with the Embden-Meyerhof, hexosemonophosphate, and tricarboxylic acid pathways. The DNA base ratio studies showed *M. thermosphactum* to have a per cent guanine plus cytosine content of 36 compared with 58 for *M. flavum* and 64 for *M. lacticum*. The enzyme assays also indicated heterogeneity. All possessed enzymes associated with the Embden-Meyerhof and the hexosemonophosphate pathways, but *M. thermosphactum* alone failed to yield enzymatic evidence for an operational tricarboxylic acid cycle. Also, only *M. flavum* showed evidence of a glyoxylate shunt. Protein profiles and esterase patterns were also sufficiently different to suggest a separation of *M. thermosphactum* from *M. lacticum* and *M. flavum*. These studies furnish additional evidence that *M. flavum* be placed in the genus *Corynebacterium* Lehmann and Neumann 1896. *M. thermosphactum* is an unusual organism which does not appear to be assignable to any of the presently recognized families of bacteria. The placement of *M. lacticum* in the genus *Corynebacterium* Lehmann and Neumann 1896 would render *Microbacterium* Orla-Jensen 1919 a later, subjective synonym of *Corynebacterium*. *M. lacticum* does possess characteristics not unlike those of the plant pathogenic corynebacteria.

The validity of the genus *Microbacterium* has been questioned ever since its origination by Orla-Jensen (26) in 1919. The grouping of the microbacteria by Orla-Jensen on the basis of size and heat resistance has never been a satisfactory one. The validity of this genus was initially questioned by Wittern (38). A major portion of her investigation dealt with the taxonomic position of *M. mesentericum* Orla-Jensen, which she assigned to the genus *Mycobacterium* Lehmann and Neumann. Jensen (13) concluded that *M. lacticum* Orla-Jensen and *M. liquefaciens* Orla-Jensen be placed in the genus *Corynebacterium* Lehmann

and Neumann. Studies by Jensen (14) with *M. flavum* Orla-Jensen led him to the conclusion that this organism was actually a mycobacterium. Work by Speck (34), Orla-Jensen (27), and Doetsch and Pelczar (9) indicated a very close relationship of the microbacteria with the genera *Corynebacterium*, *Propionibacterium*, and *Lactobacillus*. The genus *Microbacterium* has been placed in several different families and currently is in the family *Corynebacteriaceae* (3). With the isolation of *M. thermosphactum* by McLean and Sulzbacher (22), further confusion was added to the taxonomy of the microbacteria. Several authors have tried to

evaluate or justify the present location of this genus and have added much information in attempts to clarify the present situation (5-8, 30, 33, 35).

It is apparent that conflicting opinions exist concerning the systematic position of these organisms. In this study deoxyribonucleic acid (DNA) base ratios have been applied because such values are considered useful taxonomic aids. Comparative biochemical pathways, protein patterns, and esterase patterns have also been utilized to present data that may be of some significance in evaluating the taxonomic position of the genus *Microbacterium*.

MATERIALS AND METHODS

Strains used. The following organisms were used: *M. thermosphactum* ATCC 11509 and I24 (isolated in this laboratory); *M. flavum* ATCC 10340 and 0J8; *M. lacticum* ATCC 8180, CC10 and 359-M1. Cultures of strains 0J8, CC10, and 359-M1 were kindly provided by J. J. Iandolo.

These organisms were cultured in Trypticase soy broth (TSB, Baltimore Biological Laboratory) at either 22 C (*M. thermosphactum*) or 32 C (*M. flavum* and *M. lacticum*). Stock cultures (1% inoculum) were maintained in TSB at -20 C. For the study of tricarboxylic acid cycle enzymes and the glyoxylate shunt system, the organisms were grown in a medium of the same composition as TSB except that glucose was replaced by 0.2% succinate.

Preparation of cells and cell-free extracts. All cultures were harvested in the late logarithmic phase of growth by centrifugation at 2 C, washed twice with 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.2), and resuspended in the same buffer. Cell-free extracts were prepared by multiple passes through a French pressure cell at 8,000 psi. The cell debris was removed by centrifugation at 15,000 × *g* for 30 min at 2 C. The cell-free extracts were either used immediately or stored at -20 C.

Isolation of DNA and determination of melting curves. The isolation of DNA was achieved by the method of Marmur (23). To obtain complete lysis with the sodium lauryl sulfate treatment, strains of *M. lacticum* and *M. thermosphactum* were heated for 20 min at 65 C and those of *M. flavum* were heated for 15 min at 60 C. After isolation and purification of the DNA, the purified material was dissolved in a solution containing 0.15 M sodium chloride and 0.15 M sodium citrate (SSC) or in a 1:10 dilution of the SSC solution depending on the expected melting point. The melting curves for the DNA were obtained by using a Bausch & Lomb Spectronic 505 spectrophotometer. Temperature was controlled by a Lauda K2 thermostat circulator connected to the cell holder. Thermocouples were used to measure rise in temperature. The content of DNA was regulated to give a starting optical density of 0.50 at 260 nm. The adjustment procedure (when using 1:10 dilution of SSC) to standardize all values to the SSC level was used (10).

Polyacrylamide gel electrophoresis for protein and esterase staining. The gel electrophoresis procedure was previously described by Collins-Thompson et al. (4) except that the gel contained 0.08% *N, N, N', N'*-tetramethyl ethylenediamine and 0.08% ammonium persulfate.

Protein profiles. Protein fractions in the gel were stained for 30 min in an aqueous solution containing 50% methyl alcohol, 1.0% glacial acetic acid, and 0.05% amido black. Destaining of the gel was achieved by repeated washing in 10% glacial acetic acid on a shaker. The finished gels were scanned at 600 nm with a Gilford gel scanning attachment to a Beckman DU spectrophotometer converted with a photomultiplier connected to a Sargent model SR recorder.

Esterase patterns. The zymogram technique of Hunter and Markert (12) and Allen (1) was used for esterase activity, except that the staining buffer was replaced by 0.12 M Tris-hydrochloride (pH 7.2) and 3% acetone was used to aid in the staining procedure. Four naphthyl esters were used: acetate, propionate, butyrate, and caproate. Staining was allowed to proceed for 1 hr, whereupon the gels were washed in water and scanned at 600 nm in a manner similar to that used for the protein stains.

TABLE 1. Bibliography of procedures used for the assay of selected enzymes

Enzyme	Reference
Hexokinase (EC 2.7.1.1)	(20)
Fructose diphosphate aldolase (EC 4.1.2.13)	(20)
Glucose-6-phosphate dehydrogenase (EC 1.1.1.49)	(20)
Phosphogluconate dehydrogenase (EC 1.1.1.43)	(20)
Lactate dehydrogenase (EC 1.1.1.27)	(17)
Reduced NAD oxidase (EC 1.6.99.3)	(36)
Reduced NADP oxidase (EC 1.6.99.1)	(36)
Citrate synthetase (EC 4.1.3.7)	(37)
Aconitate hydratase (EC 4.2.1.3)	(2)
Isocitrate dehydrogenase (NADP) (EC 1.1.1.42)	(25)
Threo-D-isocitrate glyoxylate lyase (EC 4.1.3.1)	(21)
Oxoglutarate dehydrogenase (EC 1.2.4.2)	(29)
Succinyl-CoA synthetase (EC 6.2.1.5)	(28)
Succinate dehydrogenase (EC 1.3.99.1)	(19)
Fumarate hydratase (EC 4.2.1.2)	(11)
Malate dehydrogenase (EC 1.1.1.37)	(16)

Enzyme assays. Whenever possible, enzyme activities were determined spectrophotometrically by using a pyridine nucleotide-dependent system and a total volume of 3 ml in each cuvette. A unit of enzyme activity was defined as that amount of enzyme catalyzing the formation of 1 μ mole of product per min. Specific activity was expressed as units of enzyme per milligram of protein. The protein contents of cell-free extracts were determined by the biuret method (31). Crystalline serum albumin was used for the standard curve. The enzymes assayed and

the references to the procedures used are listed in Table 1.

RESULTS AND DISCUSSION

The DNA base compositions, expressed as moles per cent of guanine plus cytosine of the moles of total base (% GC), were determined for the test organisms by the 50% denaturation

TABLE 2. *Thermal denaturation of DNA from species of Microbacterium*

Organism	T _m (SSC) ^a	T _m (0.1 SSC)	% GC
<i>M. lacticum</i> CC10	95.6 ^b	80.6	64.1
<i>M. lacticum</i> ATCC 8180	95.1 ^b	80.1	62.9
<i>M. flavum</i> ATCC 10340	93.2 ^b	78.2	58.2
<i>M. thermosphactum</i> I 24	84.1		36.1
<i>M. thermosphactum</i> ATCC 11509	84.1		36.1
<i>Escherichia coli</i> K-12	89.7		49.8
<i>Propionibacterium</i> <i>shermanii</i>	96.5 ^c	81.5	66.3

^a T_m(SSC): value for 50% melting of DNA in 0.15 M sodium chloride, 0.15 M sodium citrate.

^b Projected values based on the *E. coli* standard.

^c This value taken from the literature.

TABLE 3. *Specific activities of selected enzymes assayed from cell-free extracts of three species of Microbacterium*

Enzyme	Specific activity ^a		
	<i>M.</i> <i>lacticum</i> CC10	<i>M.</i> <i>flavum</i> OJ8	<i>M.</i> <i>thermosphactum</i> I24
Hexokinase ^b	0.026	0.041	0.396
Fructose diphosphate aldolase ^b	0.014	0.046	0.389
Glucose-6-phosphate dehydrogenase ^b	0.010	0.010	0.115
6-phosphogluconate dehydrogenase ^b	0.067	0.019	0.279
Lactate dehydrogenase ^b	0.050	0.037	0.433
Reduced NAD oxidase	0.073	0.092	0.010
Reduced NADP oxidase	0	0.01	0
Citrate synthetase	0.030	0.054	0
Aconitate hydratase	0.059	0.109	0
Isocitrate dehydrogenase	0.114	0	0
Isocitrate lyase (EC 4.1.3.1)	0	0.320	0
Oxoglutarate dehydrogenase	0	0	0
Succinyl-CoA synthetase	0.035	0	0
Succinate dehydrogenase	0.010	0.050	0
Fumarate hydratase	0.072	0.075	0.002
Malate dehydrogenase	0.314	0.478	0.002

^a Expressed as micromoles of substrate transformed per minute per milligram of protein.

^b Cells grown in TSB (Trypticase soy broth). Otherwise grown in a medium of the same composition as TSB except glucose was replaced by 0.2% succinate.

point (T_m) method. These results are given in Table 2. Included as reference standards are the values for *Escherichia coli* and *Propionibacterium shermanii*, which agree within 1% with values in the literature. The heterogeneity of the genus *Microbacterium* is certainly indicated by the % GC values obtained. *M. thermosphactum*, with a % GC of 36, coincides with the streptococci (% GC: 34-42%); *M. lacticum*, with a % GC of 64, corresponds with the arthrobacters (% GC: 60-64%) and is just slightly different from the corynebacteria (% GC: 48-59%); *M. flavum*, with a % GC of 58, corresponds with the corynebacteria.

The specific activities of selected enzymes

from cultures of the test organisms are given in Table 3. The most noticeable results in Table 3 are the general similarity of the enzyme complement in *M. lacticum* and *M. flavum*, which are quite different in this respect from *M. thermosphactum*. *M. thermosphactum* showed a high activity in the catabolism of glucose to lactic acid, a characteristic of the lactic acid bacteria. Also, those enzymes associated with the tricarboxylic acid cycle were almost totally absent. Both *M. lacticum* and *M. flavum* showed lower activity in glucose catabolism to lactic acid, but the tricarboxylic acid cycle enzymes were present. The presence and high activity of isocitrate lyase in *M. flavum*

FIG. 1. Protein profiles of cell-free extracts of *Microbacterium flavum* and *M. lacticum* after separation by polyacrylamide gel electrophoresis and staining with amido black.

indicated a glyoxylate shunt pathway, which has also been shown to be present in the corynebacteria (18). The absence of oxoglutarate dehydrogenase activity in these organisms is a condition that is similar to that found in many bacteria that degrade sugar without the formation of carbon dioxide.

It has been suggested (24) that the protein and enzyme profiles developed from electrophoretic separation in gels might show promise in distinguishing bacterial species. Protein profiles of *M. lacticum* and *M. flavum* are given in Fig. 1, and those of *M. thermosphactum* and *Lactobacillus brevis* are given in Fig. 2. *L. brevis* was included simply as a standard for our separation method, but it does provide the profile of an organism not in the genus being investigated.

It was hoped that the protein profiles obtained in this investigation would (i) be easily

interpreted, (ii) show no significant difference between strains of a given species, (iii) show a definite but modest difference between the closely related species, *M. lacticum* and *M. flavum*, and (iv) show a substantial difference between *M. thermosphactum* and the other two species of *Microbacterium*. None of these hopes was very conclusively realized except that the *M. thermosphactum* strains had a protein profile substantially different from that of either *M. lacticum* or *M. flavum*, which were also substantially different from one another. The protein profiles did, however, show the heterogeneity of these *Microbacterium* species.

Comparison of the esterases of *M. lacticum*, *M. thermosphactum*, and *M. flavum* (*L. brevis* was again included as a standard for the separation procedure) on four substrates is given in Table 4. Both strains and species varied in both the location of esterase bands and in

FIG. 2. Protein profiles of cell-free extracts of *Microbacterium thermosphactum* and *Lactobacillus brevis* after separation by polyacrylamide gel electrophoresis and staining with amido black.

TABLE 4. Migration distance and intensity of esterase bands developed by polyacrylamide gel electrophoresis of cell-free extracts of strains of three species of *Microbacterium* and one of *Lactobacillus*

Strain	Distance travelled (mm)	Band intensity ^a of α -naphthylester of:			
		Acetate	Propionate	Butyrate	Caproate
<i>Lactobacillus brevis</i> X2	18	+	++	+++	—
	24	++	++	+++	+
	41	—	+	+	+
	45	—	—	—	+
<i>Microbacterium flavum</i> ATCC 10340	17	++	+	+	—
	36	+	+	+	—
	49	+	+	+	—
	53	+	+	+	—
<i>M. thermosphactum</i> ATCC 11159	13	++	*	*	*
	20	+++	*	*	*
	32	+++	*	*	*
	44	++	*	*	*
<i>M. thermosphactum</i> 124	12	+	+	—	—
	34	—	++	+	+
	46	+++	+++	+++	+
	67	+	+	—	—
<i>M. lacticum</i> ATCC 8180	35	+	++	+	—
	42	+++	+++	+++	+
	45	+++	+++	+	—
	62	+	+++	—	—
<i>M. lacticum</i> 359 M-1	10	+	+	—	—
	22	+	+	+	+
	42	+++	++	+	+
	45	+++	++	+	+
	51	++	+	+	+

^a Intensity of bands: +++ very strong, ++ strong, + weak; * not performed.

their intensity. Again the esterase profiles serve only to show the heterogeneity of this group.

Apparently it has been proposed that the genus *Microbacterium* be eliminated from the forthcoming edition of *Bergey's Manual*. *M. flavum* will then be transferred to the genus *Corynebacterium* and *M. thermosphactum* will be assigned to an appendix group of organisms which are not readily classifiable (M. Rogosa, personal communication).

Both the DNA base ratio and the metabolism of *M. flavum* correspond with those of corynebacteria. This additional information and the well established similarities already reported in the literature (30) substantiate the transfer of *M. flavum* to the genus *Corynebacterium*.

M. lacticum has an unfamiliar cell wall murein pattern like that of the plant pathogenic corynebacteria (32). The GC percentage obtained in this study, 63, is somewhat higher

than that for the genus *Corynebacterium* (48-59%). Another source reports a *M. lacticum* strain with a % GC of 69.3 (39); however, both strains in the present study were consistently lower. Both *M. lacticum* and *M. flavum* come from dairy sources, and, although *M. lacticum* is the more heat-resistant, the heat resistance of *M. flavum* far exceeds that of most bacteria (3, 6). Both have a deficient tricarboxylic acid cycle but deviate in the glyoxylate shunt, and with minor differences they coincide in other classical characteristics (3, 6). It is open to question whether *M. lacticum* should also be classified in the genus *Corynebacterium*, but this arrangement seems just as valid as to maintain it in the convenient genus *Microbacterium*. The transfer of *M. lacticum*, the type species of the genus *Microbacterium*, to the genus *Corynebacterium* would render *Microbacterium* Orla-Jensen 1919 a later, subjective synonym of *Corynebacterium* Lehmann and Neumann 1896.

Taxonomically speaking, there is little compelling need to keep *M. thermosphactum* together with *M. lacticum* and *M. flavum*. The former is primarily associated with meat products, not dairy products, is not thermoduric, and grows readily at refrigeration temperatures. The % GC of *M. thermosphactum* coincides with that of the lactic acid bacteria, particularly the streptococci, and is substantially below that of the corynebacteria. However, *M. thermosphactum* is catalase-positive, and Shaw and Stead (33) reported that the lipid composition is incompatible with that of the *Lactobacillaceae*. The presence of DL-diaminopimelic acid in the cell walls of *M. thermosphactum* (32) suggested a greater similarity with the *Corynebacteriaceae* than with the *Lactobacillaceae*. Thus, it appears that *M. thermosphactum* cannot be comfortably placed in any of the presently accepted families of bacteria.

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