Reclassification of *Brevibacterium leucinophagum* Kinney and Werkman as a Gram-Negative Organism, Probably in the Genus *Acinetobacter*

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A study of the tricarboxylic acid cycle enzymes of *Brevibacterium leucinophagum* ATCC 13809, the type strain of this species, together with morphological, physiological, and electron microscopy studies indicated that, contrary to its present classification, *B. leucinophagum* is gram negative and probably is better classified in the genus *Acinetobacter*.

A previous study of 50 strains of bacteria, including gram-negative and gram-positive species, indicated a clear correlation between their taxonomic grouping and the regulatory properties of their citrate synthases (13). Citrate synthases from gram-negative bacteria were inhibited by reduced nicotinamide adenine dinucleotide (NADH), and in strictly aerobic species this inhibition was overcome by adenosine 5'-monophosphate (AMP). Subsequent studies (12) of the molecular sizes of these different enzymes showed citrate synthases of gram-negative bacteria to be "large" (molecular weight 200,000 to 300,000), whereas the enzymes from gram-positive bacteria were "small" (molecular weight < 100,000). Investigations on other enzymes of the tricarboxylic acid cycle have shown that, in acinetobacters and certain other gram-negative aerobes, pyruvate dehydrogenase, isocitrate dehydrogenase, α -ketoglutarate dehydrogenase and succinate thiokinase participate with citrate synthase in an extensive "multipoint" control of the activity of the cycle by the adenylate system (P. D. J. Weitzman, in press).

During an investigation of the genus *Brevibacterium*, we examined ATCC 13809, the type strain of *Brevibacterium leucinophagum* Kinney and Werkman (4), which was described by these authors as a gram-positive bacterium which frequently becomes gram-negative after 12 to 13 h. However, our studies of the citrate synthase of *B. leucinophagum* suggested that this organism is not gram positive and prompted us to examine the other tricarboxylic acid cycle enzymes. In addition we rechecked the cultural, morphological, and physiological characters of this organism.

MATERIALS AND METHODS

Strains. The following organisms were used: *Brevibacterium leucinophagum* ATCC 13809; *Brevibacterium linens* ATCC 9174; *Acinetobacter lwoffii* NCTC 5867; *A. lwoffii* strain 4B, identified by S. P. Lapage; *Acinetobacter anitratus* NCTC 8102; and *Pseudomonas aeruginosa* NCIB 8295. All cultures were maintained on nutrient agar (Oxoid) at 30 C and were preserved by freeze-drying.

Tests. Cultural, morphological, and physiological tests with *B. leucinophagum* were performed by the methods of Kinney and Werkman (4) and Thornley (8). Sensitivity to penicillin was determined by lawning a 24-h culture of *B. leucinophagum* onto blood agar base medium (Oxoid) and immediately placing on the plate a high-potency penicillin disk (Sentests, Evans Medical Ltd., Liverpool). The test was read after incubation for 24 h and 48 h at 30 C.

Preparation of cells and cell-free extracts. All cultures were grown aerobically in nutrient broth (Oxoid) for 24 h at 30 C. The cells were collected by centrifugation at $25,000 \times g$ for 10 min, washed with buffer of composition 20 mM tris(hydroxymethyl)aminomethane-hydrochloride, pH 8.0, 10 mM $MgCl_2$, and 1 mM ethylenediaminetetraacetic acid, and disrupted by ultrasonic treatment in an M.S.E. 100-W sonicator for 2 min at full power with cooling. After recentrifugation, the supernatant solutions were used without further purification.

Enzyme studies. Measurements of citrate synthase activity and of the effects of NADH and AMP were carried out spectrophotometrically at 412 nm as previously described (13). The dehydrogenases were assayed spectrophotometrically at 340 nm under conditions previously reported: isocitrate dehydrogenase (5), α -ketoglutarate dehydrogenase, and pyruvate dehydrogenase (11), using 1 mM α -ketoglutarate or 1 mM pyruvate, respectively. The three dehydrogenases were tested for AMP stimulation by including 1 mM AMP in the assays. Succinate thiokinase was assayed by a polarographic procedure

(10) which monitors the appearance of coenzyme A at a dropping mercury electrode at -0.3 V. Reaction mixtures contained 0.1 M phosphate buffer, pH 8.0, 10 mM $MgCl_2$, and 0.1 mM succinyl-coenzyme A, and the adenosine diphosphate (ADP) concentration was varied to obtain an approximate K_m value for this substrate. K_m values could be classified as either "high" (~ 1 mM) or "low" (~ 0.02 mM).

The molecular sizes of citrate synthases were determined by gel filtration on Sephadex G-200 using catalase (beef liver, $2\times$ crystallized, Sigma Chemical Co., Kingston-upon-Thames, England) and lactate dehydrogenase (rabbit muscle, The Boehringer Corp., Ltd., London) as marker proteins (12). Large citrate synthases were eluted before catalase and small enzymes were eluted after lactate dehydrogenase.

Electron microscopy. ATCC 13809 was grown in 500 ml of nutrient broth (Oxoid) for 48 h at $30^\circ C$. The growth was harvested by centrifugation at $25,000 \times g$ for 10 min. The pellet was resuspended in 20 ml of growth medium and prefixed in glutaraldehyde, fixed with osmium tetroxide, embedded in agar, and finally dehydrated in ethanol and embedded in araldite as described by Glauert and Thornley (2). Thin sections were cut on an LKB ultratome equipped with a glass knife. These sections were collected on 300 -mesh copper grids coated with Formvar, stained for 30 min with the undiluted lead citrate stain of Reynolds (7), and examined in a Siemens Elmiskop 1A electron microscope.

RESULTS AND DISCUSSION

The results of the examination of the tricarboxylic acid cycle enzymes are presented in Table 1. For comparative purposes, the results obtained with *A. lwoffii*, *P. aeruginosa*,

and *B. linens* are included. The citrate synthase of *B. leucinophagum* is of the large type and is inhibited by NADH; this inhibition is overcome by AMP. These results indicate that *B. leucinophagum* is an aerobic, gram-negative bacterium (cf. Weitzman and Jones [13]). Furthermore, the reactions of other tricarboxylic acid cycle enzymes listed in Table 1 suggest that *B. leucinophagum* is very similar to *A. lwoffii* (5, 11).

The results of the cultural, morphological, and physiological tests with *B. leucinophagum* were in good agreement with the findings of Kinney and Werkman (4), who first described this organism, except for the reaction to the Gram stain, the ability to utilize lactose and mannose oxidatively, and the slow urease activity. In our hands this organism was never found to be frankly gram positive but was gram negative with traces of gram-positive staining. Consistent with the abovementioned results, *B. leucinophagum* was found to be resistant to the action of penicillin (Table 2). Additionally, an electron micrograph of a thin section of the cell wall indicated a gram-negative structure; that is, the cell contained an outer membrane, a dense intermediate layer, and a plasma membrane (Fig. 1).

The reaction to the Gram stain is usually unequivocal, and most bacteria can be easily assigned to one of the two major divisions. However, there are bacteria which give equivocal Gram staining reactions and, because of this, are often placed in an unsuitable genus. For example, ATCC 15716 was placed in the genus

TABLE 1. Characteristics of selected enzymes from *Brevibacterium leucinophagum* ATCC 13809 and certain other bacteria

Characteristic	<i>Brevibacterium leucinophagum</i> ATCC 13809	<i>Acinetobacter</i> spp. ^a	<i>Pseudomonas aeruginosa</i> NCIB 8295	<i>Brevibacterium linens</i> ATCC 9174
Pyruvate dehydrogenase				
Stimulation by AMP	+	+	—	—
Citrate synthase				
Inhibition by NADH	+	+	+	—
Reactivation by AMP	+	+	+	—
Molecular size	Large	Large	Large	Small
Isocitrate dehydrogenase				
Stimulation by AMP	+	+	—	—
α -Ketoglutarate dehydrogenase				
Stimulation by AMP	+	+	—	—
Succinate thiokinase				
Apparent K_m for ADP	High	High	Low	Low

^a Three strains, *Acinetobacter lwoffii* NCTC 5867; *A. lwoffii* strain 4B; *Acinetobacter anitratus* NCTC 8102.

^b Symbols: +, positive response; —, no response.

TABLE 2. *Characterization of Brevibacterium leucinophagum* ATCC 13809 and comparison with *Acinetobacter phenon* 2 of Thornley.

Characteristic	<i>Brevibacterium leucinophagum</i> ATCC 13809		<i>Acinetobacter phenon</i> 2 of Thornley (8)
	Present study	Kinney and Werkman (4)	
Colonial morphology (nutrient agar)	Small (1-2 mm diam) colonies, iridescent	white to buff-colored colonies by reflected light	Opaque colonies
Cell morphology	Gram-variable coccoid rods	Gram-positive coccoid rods becoming negative after 12-16 h	Gram-variable coccoid rods
Motility	— ^a	—	—
Growth at:			
10 C	+	+	•
30 C	+	+	+
37 C	+	+	+
45 C	—	—	•
Oxidase	—	•	—
Catalase	+	•	+
Fermentation of glucose (acid/gas)	—	—	—
Oxidative acid production from:			
Arabinose	+	•	+
Fructose	—	—	—
Galactose	+	•	+
Glucose	+	+	+
Glycerol	—	—	—
Lactose	+	—	+
Maltose	—	—	•
Mannitol	—	—	•
Mannose	+	—	•
Raffinose	—	—	•
Salicin	—	—	•
Sorbitol	—	—	•
Sucrose	—	—	—
Xylose	+	+ ^b	+
Methyl red	—	—	•
Acetyl methyl carbinol	—	—	•
Utilization of:			
Acetate	+	+	•
Citrate	+	+	+
α -Ketoglutarate	+	+	•
Malate	+	+	•
Succinate	+	+	•
Leucine	+	+	•
NO ₃ reduction	—	—	—
NH ₃ from peptone	—	—	•
Urease	+ ^c	—	•
Gelatin liquefaction	—	—	—
Cellulose	—	•	•
Casein	—	•	—
Starch	—	—	•
Indole production	—	—	•
Penicillin sensitivity (5 iu disks)	—	•	—

^a Symbols: +, positive; —, negative; •, not done.^b Kinney and Werkman found that the ability to produce acid oxidatively from xylose was lost after subculturing.^c NH₃ produced from urea only after 7 to 10 days of incubation at 30 C.

FIG. 1. ATCC 13809, section through cell; $\times 55,000$. Abbreviations: OM, outer membrane of cell wall; D, dense intermediate layer of cell wall; PM, plasma membrane. The scale mark represents 0.1 μm .

Achromobacter by Tulecke, Orenski, Taggart, and Colavito (9) as *A. liquefaciens*, but further studies on this organism suggested that it was gram positive (8). Studies on the citrate synthase enzyme agreed with these findings (3). In most cases, electron micrographs of sections of the cell wall resolve equivocal Gram stain reactions. However, in *Haemophilus vaginalis* not only is the interpretation of the Gram stain the subject of controversy, but also electron micrographs of thin sections of the cell wall are interpreted differently by different workers. Reyn, Birch-Anderson, and Lapage (6) reported a cell wall structure typical of gram-positive bacteria whereas the results of Criswell, Stenback, Black, and Gardner (1) led them to suggest that the cell wall is of the gram-negative type.

The ease with which the activity of citrate synthase can be assayed and its molecular size determined in crude bacterial extracts makes it an ideal test for resolving taxonomic difficulties in bacteria which possess the enzymes of the tricarboxylic acid cycle and which give equivocal results with the Gram stain. With gram-negative bacteria, further information pertinent to

their taxonomic grouping can be obtained from the study of other tricarboxylic acid cycle enzymes. These tests are far simpler and quicker to carry out than electron micrographs of thin sections of the cell wall.

In conclusion, enzyme studies on *B. leucinophagum* ATCC 13809, the type strain of this species, indicate that this bacterium is a gram-negative organism. These observations are supported by electron microscopy and penicillin sensitivity. The results of physiological and biochemical tests, together with the multipoint control of the tricarboxylic acid cycle, suggest that *B. leucinophagum* may be better placed in the genus *Acinetobacter*. Furthermore, the results listed in Table 2 indicate a close relationship of this organism to *Acinetobacter* phenon 2 of Thornley (8).

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REPRINT REQUESTS

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