SYNONYMY OF EUBACTERIUM LIMOSUM AND
BUTYRIBACTERIUM RETTGERI:
BUTYRIBACTERIUM LIMOSUM COMB. NOV.

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SUMMARY. Eubacterium limosum (Eggerth 1935) Prévot 1938 and Butyribacterium rettgeri Barker and Haas 1944 have been shown to be synonymous. The name Butyribacterium limosum comb. nov. is proposed for this species. ATCC strain 8486 is designated as the type strain.

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Eggerth (1935) isolated and described a single strain of a new species of Gram-positive rods which he named Bacteroides limosus. He recognized that the generic position was temporary. The species has since been placed in the genus Eubacterium (Prévot, 1938; Breed et al., 1947). Lewis and Rettger (1940) described strains of anaerobic Gram-positive rods, one of which was subsequently redescribed and named Butyribacterium rettgeri (Barker and Haas, 1944). Fortunately, original strains of both organisms are still extant. Results reported here show that the nominal species E. limosum and B. rettgeri are synonymous and exhibit less variation than is usually observed between strains of the same species. It is proposed that the species is properly placed in the genus Butyribacterium established by Barker and Haas (1944) and that it retain the specific epithet limosum Eggerth (1935) by rule of priority.

METHODS

Cultures: American Type Culture Collection strain 8486 of Eubacterium limosum deposited by Eggerth, and ATCC 10825 of Butyribacterium rettgeri (strain 32 of Lewis and Rettger 1940), deposited by Barker, and strain 32 of Butyribacterium rettgeri obtained directly from H. A. Barker were compared in this study.

Media: Preparation of media was similar to that described by Moore and Cato (1962). Unless otherwise specified the media contained 2% peptone, 1% yeast extract and 1% carbohydrate or other substrate. One ml of resazurin solution (25 mg/100 ml of water) and 10 ml of a salt solution were added to 250 ml of all media (exceptions noted). The salt solution had the following composition: in grams/liter; K_2HPO_4 - 1.0, KH_2PO_4 - 1.0, $NaHCO_3$ - 10.0, $NaCl$ - 2.0, $CaCl_2 \cdot 2H_2O$ - 0.25, $MgSO_4$ - 0.20, $CoCl_2 \cdot 6H_2O$ - trace, $NaMoO_4 \cdot 2H_2O$ - trace. All ingredients except $CaCl_2$ and $MgSO_4$ were dissolved in a large volume of water. To prevent precipitation these last two were dissolved separately in less than 5 ml of boiling H_2O each and then added. Media were boiled in a 300 ml Erlenmeyer flask fitted with an inverted 250 ml flask with a small hole in the bottom as a chimney. Boiling was continued until the media were colorless to resazurin. They were then cooled under bubbling CO_2 which had been passed through hot reduced copper turnings. When cool, 0.125 g of cysteine was added and the pH adjusted to 7.0 (exceptions: arabinose, ribose and xylose to pH 6.7) with 2N NaOH. The media were kept anaerobic under bubbling O_2 -free N_2 and transferred to 18 x 150 mm plain rim test tubes by a spring actuated syringe through a 6-inch hypodermic needle. The media delivery needle was attached to a second 6-inch hypodermic needle through which O_2 -free N_2 gas was passed. As the N_2 gas bubbled the media to the rim, each tube was stoppered. Stoppered tubes were sterilized at 121°C for 15 min. in outside rows of wire racks, with rubber mesh matting above and below. A second wire rack was held over the stoppers with hooks made of coat hanger wire. Autoclave pressure was exhausted rapidly and all media were cooled quickly. All culture inoculations were made under jets of O_2 -free CO_2 by the methods of Hungate (1950) and of Bryant and Robinson (1961). An automatic dropper used to inoculate large numbers of tubes with each culture is to be described elsewhere.

Motility: Broth cultures first showing visible growth were examined in wet mounts.

Optimum temperature: The relative amount of visible growth was determined at 1 to 3 days or longer if growth was slow, in peptone-yeast-glucose (PYG) broth at 25°, 30°, 37° and 45° C.

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pH: At 1, 2 and 3 weeks in PYG and in all media at 3 weeks, terminal pH was determined with 6-inch glass and calomel electrodes directly in the culture tubes.

Aerobic growth: Visible growth was determined in PYG broth (without resazurin, cysteine or boiling) tubed two inches deep in 16 mm screw cap tubes.

Gelatin liquefaction: Liquefaction was determined in 12% gelatin, 0.5% peptone, 0.1% glucose, 1% yeast extract, with salt solution, cysteine and resazurin. The powdered gelatin was added to each tube followed by the reduced broth portion. After sterilization, inoculation and incubation for 3 weeks at optimum temperature, liquefaction was read after 30 minutes of refrigeration. The time required for liquefaction at room temperature was compared with uninoculated controls. Tubes showing complete liquefaction failed to harden during refrigeration.

PY broth: Growth was determined by visible turbidity in anaerobic media containing 1% peptone, 1% yeast extract, salt solutions and resazurin (PY).

Nutrient broth and litmus milk: Commercial media (Difco) were used. Both were prepared anaerobically by boiling with resazurin without salt solutions. Cysteine was added when cooled.

Indol production: Indol was determined with Kovac's reagent after 3-week incubation in 2% tryptone, 1% yeast extract with salts, cysteine and no resazurin.

Aesculin hydrolysis: After 3-week incubation hydrolysis was determined by adding 1% ferric ammonium citrate solution to PY broth containing 0.5% aesculin. Positive hydrolysis was indicated by the immediate appearance of a black color.

Substrate concentration: The following substrates were added to basal PY broth at 1% concentrations: arabinose, cellobiose, dextrin, fructose, galactose, glucose, inulin, lactate, lactose, maltose, mannose, pectin, pyruvate, raffinose, rhamnose, salicin, sucrose, starch, xylose, dulcitol, glycerol, inositol, mannitol, sorbitol and sodium hippurate.

The following substrates were added at 0.5% concentrations: aesculin, amygdalin, glycogen, melibiose, melezitose, ribose, trehalose, adonitol, and erythritol.

Cellulose digestion: The medium contained 0.05% each of glucose, maltose, starch, peptone, yeast extract, $(\text{NH}_4)_2\text{SO}_4$ and cysteine (added after cooling) with 50% of the salts solu-

tion and 20% of centrifuged clarified rumen fluid, 1 ml of resazurin solution/250 ml (medium E) and a strip of patapar parchment cellulose paper (Paterson Parchment Paper Co., Bristol, Pennsylvania) in each tube. The parchment was observed for visible disintegration.

Orotic acid digestion: Orotic acid digestion is usually determined during this series of observations, and although results are not reported for the present strains, the method is included because further reports will include the observation. Digestion was determined under conditions similar to those described by Wachsman and Barker (1954). The medium contained 0.5% tryptone, 0.05% yeast extract, 0.2% orotic acid and 12% (vol/vol) of a salts solution containing 0.05M K_2HPO_4 , 0.02% $MgSO_4 \cdot 7H_2O$, 0.0005% $FeSO_4 \cdot 2H_2O$, 0.0005% $MnSO_4 \cdot 4H_2O$ and 0.0005% $NaMoO_4 \cdot 2H_2O$. The medium was boiled without resazurin, cooled, 0.05% cysteine was added and the pH adjusted to 7.4 before anaerobic tubing. Orotic acid digestion was determined by disappearance of the orotic crystals and by change in O.D. at 280 m μ against a blank of the same medium without orotic acid. Ammonia production from these media was determined with Nessler's reagent in spot plate depressions. Positive controls with Zymobacterium oroticum were run with each set of analyses.

Catalase production: Determination was made on surface growth produced on anaerobic slants of PYG agar medium using 3% H_2O_2 .

Nitrate reduction: Nitrite was determined in PYG broth containing 0.1% KNO_3 and no resazurin with sulfanilic acid and dimethylamino-benzaldehyde.

Hippurate hydrolysis: The appearance of crystals after the addition of 0.5 ml of 50% H_2SO_4 to 2 ml of culture indicated positive hydrolysis.

Arginine hydrolysis: Ammonia was determined by the addition of 2 drops of Nessler's reagent to 2 drops of culture in spot plate depressions. The medium contained 0.5% yeast extract, 0.5% tryptone, 0.2% K_2HPO_4 , 0.05% glucose, 0.3% arginine, resazurin, salt solution and cysteine. The control medium was the same without arginine.

Heat resistance: A 24 to 48 hr PYG culture was suspended in an 80°C bath until the temperature in an unstoppered blank tube of liquid reached 79°; and then for an additional 20 minutes. The stoppers were wired in place until heating was complete at which time transfers were made to PYG to determine survival.

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Growth at pH 9.6 and in 6.5% NaCl: Visible turbidity was observed in suitably modified PYG media.

Growth in thioglycollate: Fluid thioglycollate medium (Difco) was observed for visible growth and the growth pattern.

Gas production: Gas was reported from any medium. It was detected by the appearance of splits in agar, bubbles in broth, or in Durham tubes of the "aerobic" glucose tubes.

Shape and arrangement: Gram-stained preparations were used for verbal descriptions and photomicrographs.

Size: Negative-stained preparations were used.

Colony type: Colonies were observed in shake tubes of PYG agar medium.

Fermentation products: Glucose, lactate, pyruvate and PY broth cultures were analyzed by the temperature-controlled chromatographic column of Bruno and Moore (1962) and by gas chromatography of ether extracts using a 1/4" six foot resoflex LAC-1-R-296 packing (Burrell Corp., Pittsburgh, Pa.) at 160°C under 20 lbs of He and a thermal conductivity detector at 200 milliamps.

RESULTS

A description of the strains observed in this study is reported in Tables 1 and 2 and Figures 1-4. In Table 1 the first 3 columns of data are those produced with the above methods. Data reported by Eggerth (1935) and by Lewis and Rettger (1940) and Barker and Haas (1944) are in the last 2 columns (see footnote b). Eggerth, and Barker and Haas also reported the organism to be H₂S negative. Eggerth reported no action on coagulated egg white, no hemolysis and no pathogenicity for white mice and rabbits. Barker and Haas reported the organism to be nonproteolytic. Both laboratories reported the source to be the intestinal tract of man or animals.

In Figure 3 small amounts of succinic acid are evident in each culture. However, only trace amounts were produced by the cultures; the medium itself contained succinate.

Table 1. Characteristics of Eubacterium limosum and Butyribacterium rettgeri.

	<u>Butyribacterium</u> <u>rettgeri</u> A 10825	<u>Butyribacterium</u> <u>rettgeri</u> S31	<u>Eubacterium</u> <u>limosum</u> A 8486	<u>Bacteroides</u> <u>limosus</u> Eggerth, 1935	<u>Butyribacterium</u> <u>rettgeri</u> Barker and Haas, 1944
Motility	-	-	-	-	-
Opt. temp. 25°	-	+++	++++	-	+
3 days 30°	++++	++++	++++	-	+
37°	++++	++++	++++	-	++++
45°	++++	++	+++	-	+
Term. pH 1 wk	4.6	4.6	4.4	-	-
Glucose 2 wk	4.8	4.6	4.5	-	-
3 wk	4.7	4.6	4.5	4.8	ag ^b
Aerobic grow.	1 wk	1 wk	1 wk	-	-
Gelatin liq.	comp.	sl	vsl	sl	- ^b
PY broth	5.9	6.0	6.0	-	-
Nutr. broth	(6.1)	6.3	6.4	-	-
Milk	nc	nc	nc	nc	nc ^a
Indol prod.	-	-	-	-	-
Aesculin hyd.	+	+	+	-	-
acid	-	-	-	6.0	-
Amygdalin acid	-	-	-	-	-
Arabinose acid	5.3	-	5.3	-	- ^b
Cellobiose acid	-	-	-	6.0	-
Cellulose acid	(-)	-	-	-	-
Dextrin acid	5.5	-	-	ag	-
Fructose acid	4.8	4.4	4.8	ag	-
Galactose acid	5.2	5.5	-	-	-
Glycogen acid	-	-	-	6.0	-
Inulin acid	-	-	-	6.0	-
Lactose acid	-	-	-	-	- ^b
Maltose acid	5.4	-	-	6.0	ab ^b
Mannose acid	-	-	-	6.0	-
Melibiose acid	-	-	-	-	-
Melezitose acid	-	-	-	-	-
Pectin acid	5.1	5.2	5.2	-	-
Raffinose acid	-	-	-	6.0	-
Rhamnose acid	-	-	-	-	- ^b
Ribose acid	5.0	4.9	5.3	-	-
Salicin acid	5.2	-	-	6.0	-
Sorbose acid	-	-	-	-	-
Sucrose acid	-	-	-	6.0	- ^b
Starch acid	-	-	-	6.0	-
Trehalose acid	-	-	-	ag	- ^b
Xylose acid	5.3	5.5	5.5	-	- ^b

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Table 1. (continued)

	<u>Butyribacterium</u> <u>rettgeri A 10825</u>	<u>Butyribacterium</u> <u>rettgeri S 31</u>	<u>Eubacterium</u> <u>limosum A 8486</u>	<u>Bacteroides</u> <u>limosus</u> <u>Eggerth, 1935</u>	<u>Butyribacterium</u> <u>rettgeri Barker</u> <u>and Haas, 1944</u>
Adonitol acid	5.0	5.0	5.2	ag	
Dulcitol acid	-	-	-	-	-
Erythritol acid	4.9	4.8	5.1	ag	
Glycerol acid	-	-	-	-	-
Inositol acid	-	-	-	-	-
Mannitol acid	5.1	4.8	4.9	ag	- ^b
Sorbitol acid	-	-	-	-	-
NO ₃ red	-	-	-	-	-
Catalase	-	-	-	-	-
Orotic acid					
NH ₃ prod.					
Arginine hyd.	-	-	-		
Hippurate hyd.	-	-	-		
80° 20 min	-	-	-		60°/30m+ ^b
pH 9.6 grow.	++++	+++	++++		
6.5% NaCl grow.	(-)	(-)	(-)		
Thiogly grow.	++++	++++	++++		
Gas prod.	-	+	+	+	sl

- = negative result, ++++ = greatest turbidity of growth, numbers = terminal pH at 3 weeks (in fermentation media only where pH was 5.5 or below), 1 wk = 1 week, comp. = complete, sl = slight, vsl = very slight, () = results in parenthesis indicate no visible growth of the cultures in that medium, nc = no change, ag = acid and gas.

^a = result reported by Lewis and Rettger 1940

^b = result reported both by Lewis and Rettger 1940 and Barker and Haas 1944.

Table 2. Characteristics of Eubacterium limosum and Butyrivacterium rettgeri.

Organism	Shape	Size	Colony type	Broth growth	Products of: (in meq/100 ml of culture)									
					acet-	buty-	lact-	isobuty-	isoval-	capro-	succ-	BuOH-	EtOH	
<u>B. rettgeri</u> A 10825	pleo. rods pointed, rounded or bifid ends	0.5-0.8 x 1.7-3.3 μ	pinpoint white	turbid sediment	glucose lactate pyruvate aerobic	1.6 4.0 2.8 lactate	0.7 3.2 1.7 no growth	1.3 trace trace no growth			trace trace trace			pres. ^a
<u>B. rettgeri</u> S31 Barker	pleo. rods pointed, rounded or bifid clubs, swellings	0.9-1.3 x 1.6-6.5 μ	pinpoint to 1 mm white lenticular	turbid sediment slimy	glucose lactate pyruvate aerobic	3.5 pres pres lactate	1.1 pres pres no growth	1.7 trace trace no growth			trace trace			trace trace
<u>Eubacterium limosum</u> A 8486	pleo. rods pointed, rounded or bifid clubs, swellings	0.6 x 1.6-4.8 μ	pinpoint white	turbid ropy sediment slimy	glucose lactate pyruvate aerobic	1.5 4.4 3.0 lactate	0.7 3.7 1.4 no growth	1.3 trace trace no growth			trace trace			trace trace
<u>Bacteroides limosus</u> Eggerth	pleo. short ovals. Wedge, curve hook, and bifid forms. No granules	0.5-1.5 x 1.0-5.0 μ	(surface) 2.0-4.0 mm raised cream- colored, mucoid sediment	diffuse cloudiness heavy, mucoid										
<u>B. rettgeri</u> Barker and Haas	Slightly bent, noncapsulated rods. Singles, pairs short chains. Some clubs and swollen forms No branches ^b	0.7 x 2.0-3.0 μ ^b	(surface) circular, entire or irreg. Translucent Gray-white to yellowish, Glistening, smooth ^b becoming umbonate 1.5-2.0 mm	turbid sediment ^b	glucose maltose lactate	pres pres 43.7	pres pres 28.6							CO ₂ pres CO ₂ pres CO ₂ 40.1

^a pres. = present

Figure 1. Gas chromatograms of ether extracts of lactate broth cultures incubated 3 weeks.

Figure 2. Gas chromatograms of ether extracts of glucose broth cultures incubated 3 weeks.

- A. Aerobic deep glucose broth tube E. limosum ATCC 8486.
- B. Anaerobic glucose broth E. limosum ATCC 8486.
- C. Anaerobic glucose broth B. rettgeri ATCC 10825.
- D. Anaerobic glucose broth B. rettgeri Barker S31.

Figure 3. Column chromatograms of glucose broth cultures.

A. E. limosum ATCC 8486.

B. B. rettgeri Barker S31.

C. B. rettgeri ATCC 10825.

Figure 4. Photomicrographs of E. limosum 8486 (a) and B. rettgeri 10825 (b).

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DISCUSSION

Butyribacterium rettgeri strain Barker-31 shared 83.9% of 58 positive characteristics, and 93% of 144 characteristics when both positive and negative characteristics were compared, with Eubacterium limosum ATCC strain 8486. B. rettgeri ATCC 10825 shared 75.8% of its positive characteristics with B. rettgeri B-31 and likewise 75.8% of its positive characteristics with E. limosum ATCC strain 8486.

This species is properly placed in the genus Butyribacterium established by Barker (1944) in the family Propionibacteriaceae. While acetate and butyrate are the major products, small amounts of lactic acid are produced from glucose. When lactate is the substrate under anaerobic conditions it is completely destroyed. This apparent discrepancy may result from differences in pH which may be expected to affect product equilibrium. With sodium lactate as the substrate the terminal pH is about 6.0 while with glucose the terminal pH is about 4.5.

Eggerth named Bacteroides limosus in 1935 and stated that a single strain was isolated. He deposited strain 8486 (Eubacterium limosum Prévot 1938) in the American Type Culture Collection. It is assumed that it is the monotype of Eggerth's species. This strain is here designated as the type strain of Butyribacterium limosum comb. nov. Other strains have been reported by Lewis and Rettger in 1940 and more recently from abscessed tissue by Prévot (personal communication).

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