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CLOSTRIDIUM LIMOSUM ANDRÉ IN PRÉVOT 1948, 165
AMENDED DESCRIPTION AND PATHOGENIC CHARACTERISTICS

Elizabeth P. Cato, C.S. Cummins and L. DS. Smith

Anaerobe Laboratory, Division of Basic Sciences
College of Agriculture, Virginia Polytechnic Institute, Blacksburg 24061

ABSTRACT. Seventeen strains of Clostridium limosum (Prévot) isolated from soil and fatal infections in cattle, swine, chickens, alligators, and from other sources are described. Strains of this species are proteolytic, nonsaccharolytic, slightly toxic, and pathogenic for laboratory animals, and produce acetic acid from peptone media as the only major acid. Large quantities of amines are also produced. Four strains representing variant types of the species could be differentiated from the others by serologic reactions, differences in cell wall composition and amine products.

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In 1948, Prévot published a description of a new species, Clostridium limosum (11). He attributed the description of the single strain, isolated from African soil (mud) and the name of the species to a co-worker, P. André. We have deposited the holotype strain (Prévot A46, VPI 2700) in the American Type Culture Collection (ATCC 25620). The characteristics of this strain agree with those originally reported, and with characteristics of 16 other strains, which also have been studied in this laboratory.

One of these strains was No. 666, the monotype strain of C. bubalorum, described by Prévot in 1938 (10). His description was based on studies of a culture isolated at the Institute Pasteur, Hanoi, from the long bone marrow of a mule. Prévot established a separate species for this organism and attributed its name to Kraneveld, presuming it to be the same organism that Kraneveld had described briefly in an extensive article on a disease of water buffalo published in 1930 (7). Kraneveld had repeatedly isolated cultures of a clostridium from the diseased tissue of water buffalo. He and other workers referred to the disease as "bacillary osteomyelitis bubalorum." Kraneveld's cultures were also examined by Zeissler and Rassveld (16) who felt that they were identical in all respects to Clostridium novyi type B [Bacillus gigas (Zeissler and Rassveld), Clostridium oedematiens type B (Oakley et al. 1947)] except in pathogenicity. The cultures from water buffalo were found to be non-pathogenic for test animals, and although the organism could be isolated from the blood or marrow of water buffalo that had been infected with it, disease could not be produced experimentally with pure cultures.

In 1947, Oakley et al. (9) examined two strains of the water buffalo organism and concluded that they were sufficiently like C. oedematiens (C. novyi) to be considered a member of this group. Oakley named the organism Clostridium oedematiens (C. novyi) type C. Thus it has been

assumed by many that Clostridium bubalorum of Prévot was identical to Clostridium novyi type C. It is evident from studies in this laboratory that the organism named C. bubalorum by Prévot is a distinct pathogenic species not related to C. novyi type C and therefore not the same as the "bacillary osteomyelitis bubalorum" organism. In this laboratory, its characteristics have been found to be essentially identical to those of Clostridium limosum.

Because the name C. bubalorum was given by Prévot in 1938 to strain 666 which he thought probably was the same as Kraneveld's organism, and because Kraneveld's organism has different characteristics, was not legally named by Kraneveld and is now regarded as C. novyi type C, we feel that C. bubalorum is a nomen confusum and the name C. limosum André in Prévot 1948, should be used to designate this species. We therefore request a Judicial Commission ruling to reject the name C. bubalorum Prévot 1938 and accept instead the name C. limosum André in Prévot 1948.

MATERIALS AND METHODS

Seventeen strains with similar characteristics are reported in the present study. They were isolated from soil and from animal infections as shown in Table 1.

Table 1. Sources of 17 strains of C. limosum

0658	bovine liver infarction, Montana	lesion similar to <u>C. haemolyticum</u>
1740	swine fetus, Colorado	same kind of organism isolated from "acute cattle deaths and decomposed swine fetuses"
1748-B	long bone of mule, Hanoi	labeled "#666, <u>C. bubalorum</u> variant"
1928	bovine heart, California	
1936-C	putrefied cat, France	labeled "571K, <u>C. bellonensis</u> variant"
1950	bovine liver infarction, Montana	conditions similar to #0658, different ranch
2153-A	duck, Malmo, Sweden	
2485	France, #5-7-Id	labeled " <u>C. bubalorum</u> ," probably derived from Prévot 666
2700	mud, Ebrie coastal lagoon, Ivory Coast	#A46X <u>C. limosum</u> , type culture
2709	alligator kidney, Florida	alligators dying with symptoms of paralysis
2710	alligator liver, Florida	alligators dying with symptoms of paralysis
2755	France	labeled "278, <u>P. capitovialis</u> variant"
2800	putrefied cat, France	labeled "5716, <u>P. capitovialis</u> variant"
3283	chicken, Arkansas	
4473	home preserved meat, France	labeled "1254A, <u>C. regulare</u> "
4479	soil, France	labeled "18/86-2A, <u>C. regulare</u> "
4480	France	labeled "1022B, <u>C. saprotoxicum</u> "

One strain labeled *C. bellonensis* (Prévot 571K) retained reactions similar to those observed by Prévot. He labeled it a variant of the species; however, it is not representative of the organism which Sacquepée named *C. bellonensis* in 1915 and described as saccharolytic (13). To our knowledge, none of Sacquepée's strains are extant, the descriptions are incomplete and *C. bellonensis* should probably be considered a *nomen dubium*.

Cultural characteristics were determined in pre-reduced media as described by Holdeman et al. (5).

Amines were determined from chopped meat medium according to the method of Holdeman and Brooks (4).

DNAase and RNAase formed by the organisms were tested for by a modification of the method of Jeffries et al. (6).

Cell walls were analyzed as follows:

1. Conditions of growth.

Strains were grown in 500 or 1000 ml Pyrex centrifuge bottles in peptone-yeast extract medium containing 1% glucose. Medium was prepared and sterilized as described by Moore and Cato (8). Each bottle contained a 1" x 3/8" teflon-coated magnetic stirring bar. The bottles were incubated at 37°C overnight to check sterility and warm the medium; they then were inoculated with 10 ml of an overnight culture in the same medium. After inoculation the original stopper was replaced by one carrying a glass tube surmounted by a 3.5" high chimney containing absorbent cotton wool soaked in alkaline pyrogallol. This acted as a safety valve so that the stopper was not forced out by gas pressure, and pressure did not build up inside the bottle. The pyrogallol served to prevent re-oxidation of the medium in the early stages of incubation.

Cultures were grown overnight (usually for 24 hrs), with stirring at 180-240 rpm, and then killed by adding formalin to a final concentration of 2%. After a further 24 hrs, the cells were centrifuged out, washed three times in distilled water and lyophilized. Yields were generally 500-600 mg dry wt of cells per liter of medium. Only an occasional spore was seen in cell suspensions from overnight cultures, and the number of spores present was judged to be too small to affect the results of cell wall analysis.

2. Preparation of cell walls.

The bacterial suspensions were disintegrated in a Braun Model MSK homogenizer, using 300 mg bacterial cells resuspended in 10-12 ml distilled water, and 10 ml glass beads (0.1 ml diam.) in a 50 ml container. The mixture was shaken (with cooling) for 3 minutes at 4000 cycles per minute, the beads removed by suction through a No. 2 porosity sintered glass filter, and the filtrate digested with pronase at pH 8.0 for 1.5-2 hrs at 56°C. The mixture was then centrifuged (29,000 G for 25-30 minutes), washed twice in distilled water, and a smear examined. Preparations showing any residual intact organisms were shaken again for 1 minute in the homogenizer and redigested as above. The final material was washed several times with distilled water, centrifuged at approximately 1500 x G to remove debris and dirt, and lyophilized. The yield of cell walls obtained from 300 mg of intact cells of *C. limosum* strains varied from 30-50 mg; i. e., from 10-16%.

3. Chromatographic Methods.

A) Amino Acids. 5 mg amounts of cell walls were suspended in 6 ml of 6 N HCl and hydrolysed in sealed tubes for 18 hrs at 100 to 105°C. After cooling, the hydrolysates were filtered, evaporated to dryness, and resuspended in 0.25-0.3 ml of distilled water.

Two-dimensional ascending chromatography was done on 8" squares of Whatman No. 1 paper in a Shandon DDH 8" Chromatank. Normally, 10 μ l of hydrolysate (= approximately 200 μ g of original cell wall) was applied at the origin. The first solvent was n-butanol/acetic acid/water (120:30:50) and the second solvent phenol/water (90:10) with 1 ml NH_4OH (sp. gr. 0.880) per 200 ml of solvent.

The spots were visualized by dipping the squares in ninhydrin (0.1% in 95% acetone) and heating at 80-100°C for 1-2 minutes. Permanent preparations were made by dipping the ninhydrin-treated papers in 5% nickel sulfate ($\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$), blotting and drying.

For determination of the isomers of diaminopimelic acid, 1-dimensional chromatograms were run on Schleicher and Schuell paper (No. 2043A) in the methanol/pyridine/HCl/water solvent of Rhuland et al. (12).

B) Sugars. 10 mg amounts of cell walls were hydrolyzed in 5 ml of 2 N H_2SO_4 for 2 hrs at 100°C and, when cooled, the acid was neutralized with solid BaCO_3 to a pH of approximately 7.0. The mixture was centrifuged, the barium sulfate deposit washed with 2 ml distilled water and the solution evaporated *in vacuo* over phosphorus pentoxide. When dry, the soluble material was taken up in 0.25-0.3 ml of distilled water and, if necessary, centrifuged to remove residual barium sulfate or other insoluble material. The sugars in hydrolysates were identified by descending chromatography on Whatman No. 1 paper using an ethyl acetate/pyridine/water solvent (80:20:10). The spots were visualized by the aniline hydrogen phthalate method or, in some cases, with alkaline silver (14, 15).

Cell wall agglutination tests were done as previously described by Cummins (1, 2).

The tests were incubated at 56°C for 2 hrs and allowed to stand on the bench overnight before being read.

RESULTS

All strains (including 2700) show characteristics in agreement with the original description as follows:

Morphology:

Rods with rounded ends, occurring singly or in pairs sometimes in short chains; oval, subterminal spores swelling the cell.

Original	VPI Results
0.7 x 3.0 μ	Size varies with medium, range - 0.3-1.6 x 1.3-16.0; median - 0.8-1.3 x 2.3-9
motile	15/17 strains motile, individual cells showing mono-, amphi-, or peritrichous flagellation.
Gram-positive	Decolorized cells frequent.

Physiology:

Strict anaerobe	Anaerobic; no strain grows on aerobic horse blood agar, but occasionally a strain (including 2700) will initiate growth from heavy inoculum in deep tubes of standard broth medium.
Good growth at 37°C, usually less growth at 45°C and 30°C, poor growth at 25°C	Three of the 17 strains fail to grow at 45°C
Resist heating 10 min at 100°C	Not tested - all strains resist heating 10 min at 80°C
Weakly reducing - neutral red partially reduced	In pre-reduced media, all strains reduce neutral red, none reduces resazurin.
No gas	In pre-reduced peptone-yeast extract-glucose (PYG) agar shake tubes, variable, sometimes large amounts of gas were detected.

Fetid odor; abundant growth in peptone water; heavy turbidity with sediment in glucose broth; no indol produced; nitrate not reduced; ammonia produced (from peptone, arginine, threonine, chopped meat); acetate produced from glucose (4 to 16 meq/100 ml peptone-yeast extract media with or without glucose); usually trace amounts of succinate (0-0.6 meq/100 ml) and trace to moderate amounts of lactate (0-1.9 meq/100 ml). Traces of propionic acid were occasionally detected by gas or liquid chromatography, alcohols were not.

All strains were β -hemolytic on horse blood agar. All produced ribonuclease and deoxyribonuclease; all but one (1928) produced collagenase; only strain 2709 produced elastase and only strain 2153A produced chitinase. All strains were proteolytic, digesting gelatin, milk and cooked meat. No strain hydrolyzed esculin or starch; none produced catalase or acetoin. Acid (below pH 6.0) was not produced from adonitol, amygdalin, L-arabinose, cellobiose, dextrin, dulcitol, DL-erythritol, esculin, D-fructose, D-galactose, glucose, glycerol, glycogen, inositol, inulin, lactose, D (+)-maltose, D-mannitol, D (+)-mannose, melezitose, melibiose, pectin, raffinose, rhamnose, D(-)-ribose, salicin, D-sorbitol, L-sorbose, starch, sucrose, trehalose, or D-xylose. All strains produced β -phenylethylamine and spermidine from peptone-yeast extract-glucose medium. Most of the strains grew at pH 9.0, although more slowly and less abundantly than at pH 7.0. All were inhibited by 6.5% NaCl or 2% bile. On egg-yolk agar, lecithinase could usually be demonstrated but lipase could not. Mucopolysaccharide amino acids of all strains appeared to be alanine, glutamic acid, and meso-diaminopimelic acid. The lecithinase produced by these strains was partially inhibited by antitoxin to *Clostridium perfringens* type A, *Clostridium novyi* type A, and *Clostridium novyi* type B indicating either a mixture of lecithinolytic enzymes or, less likely, a lecithinolytic enzyme bearing serological determinants in common with several serologically distinct lecithinases. The collagenase was not entirely inhibited by treatment with cysteine,

except possibly for strains 2153A and 1928. Citrate inhibited collagenase activity of about one-half of the strains.

Characteristics that are variable among strains are presented in Table 2. Cell walls were analyzed for their sugar content and agglutinating titers. From these data as shown in Table 3, the strains fall into three distinct groups with one strain (4479) an intermediate. Other phenotypic differences shown in Table 2 that can be correlated with the variation in cell wall composition are lack of motility and consistent production of formate from PYG in groups 2 and 3. In addition, both strains in group 2 consistently produced propionate from threonine. Neither produced di-n-butylamine from PYG, a characteristic they shared only with Prévot's type strain (VPI 2700).

Organisms in the first cell wall group generally produced more kinds of amines in larger amounts than those in the groups differing in cell wall composition (Table 4). Organism 2700 was unusual in that it produced relatively small quantities of amines.

Of sixteen strains tested, all were sensitive to aureomycin (5 mcg), bacitracin (2 units), chloromycetin (5 mcg), furadantin (100 mcg), oxycillin (1 mcg), penicillin (2 units), tetracycline (5 mcg), vancomycin (30 mcg), cephalothin (30 mcg), and ampicillin (2 mcg). All strains tested (13) were resistant to nystatin (100 mcg). Fifteen out of sixteen were resistant to polymyxin B (300 units) and triple sulfa (1 mcg). Resistance was variable to gantrisin (1 mg), kanamycin (30 mcg), lincomycin (2 mcg), neomycin (30 mcg), terramycin (5 mcg), oleandomycin (2 mcg), novobiocin (30 mcg), erythromycin (2 mcg), and dihydrostreptomycin (10 mcg).

Pathogenicity

Results of intraperitoneal injection of strains of *C. limosum* into mice and intramuscular inoculation into guinea pigs are shown in Table 5.

The pathogenic action of the virulent strains after intramuscular inoculation into guinea pigs seems to be associated with the action of the collagenase and the lecithinase. With the virulent strains, on the second or third day after inoculation, the muscle could be felt losing its normal turgor. On the fourth or fifth day, the skin was digested through and the hydrolyzed muscle drained to the outside. Death usually occurred two or three days after the digestion of the skin, possibly by secondary infection. Although the organism could be isolated from the heart blood, there was no evidence of generalized infection. The action of the lecithinase was evidenced by the many droplets of fat on the surface of the liquefied muscle and by the droplets on the surface of the heart blood. There was no evidence, however, of the marked intravascular hemolysis that one would expect if any appreciable amount of the lecithinase was picked up by the circulating blood. Intramuscular inoculation of non-virulent strains either with or without calcium chloride resulted only in a local lesion.

The organisms that *C. limosum* most closely resembles fall into two groups, the nonsaccharolytic clostridia and the lecithinase-producing clostridia. *C. limosum* differs from *C. hastiforme*, *C. histolyticum*, and *C. subterminale* in the nonsaccharolytic group in its ability to produce lecithinase. It also differs from *C. histolyticum* in not being aerotolerant, and from *C. subterminale* and *C. hastiforme* in producing only acetic acid as a major fermentation product. *C. limosum* differs from

Table 2. Variable Characteristics of Strains of *C. limosum*

Organism	motile	P + threonine	di-n-butyl amine	lecithinase	hipp. hyd.	H ₂ S	acid products from glucose	growth at pH 9.6	collagenase	RNAase	elastase
0658	+	-	++++	+	+	+	Asl	+	+++	+	-
1748	+	-	++++	+	+	+	AfsL	+	+++	+	-
1936 C	+	-	++++	+	+	+	AL	-	+++	++	-
1950	+	-	++++	+	+	+	AL	+	+	+	-
2153 A	+	-	++++	+	+	+	Asl	+	+++	+++	-
2485	+	-	++++	+	+	+	As	+	+	+	-
2700	+	-	-	+	+	+	AL	+	++	++	-
2709	+	-	++++	+	+	+	As	+	+	+	+
2710	+	-	++++	+	-	+	Asl	+	++	+	-
2800	+	-	++++	+	+	+	As	sl+	++++	+	-
3283	+	-	++++	+	+	+	Asl	+	+	+	-
4473	+	-	++++	+	+	+	As	-	+++	-	-
4480	+	-	++++	+	-	sl+	Asl	-		+	-
4479	+	-	++	-	+	+	Afs	+	+	-	+++
1740-1	-	+	-	+	-	-	AFSL	+	+++	+++	-
1928	-	+	-	+	-	sl+	Afsl	+	-	+++	-
2755	-	-	+	-	+	sl+	AfsL	+	+++	++	-

A = acetic; F = formic; S = succinic; L = lactic;

Capital letters = major acid products; lower case letters = minor acid products.

Table 3

Strain	CW Sugars				CW agglutinating* titres with sera to:			
	Galactose	Glucose	Mannose	Rhamnose	1936-C	2485	1928	2755
0658	+	+	±	±	640	160	<20	<20
1748	+	+	±	±	640	320	<20	<20
1936-C	+	+	±	±	640	320	<20	<20
1950	+	+	±	±	640	160	<20	<20
2153-A	+	+	±	±	640	320	<20	<20
2485	+	+	±	±	320	160	<20	<20
2700	+	+	±	±	320	320	<20	<20
2709	+	+	±	±	320	320	<20	<20
2710	+	+	±	±	320	160	<20	<20
2800	+	+	±	±	320	160	<20	<20
3283	+	+	±	±	160	160	<20	<20
4473	+	+	±	±	160	160	<20	<20
4480	+	+	±	±	160	320	<20	<20
4479	+	±	-	±	20	20	<20	<20
1740-1	+++	+	-	+	<20	<20	160	160
1928	+++	+	-	+	<20	<20	320	320
2755	+	-	-	-	<20	<20	<20	<20

* The results of individual tubes in cell wall agglutination tests were initially read as 4+, 3+, 2+, 1+, or + or Tr, and the titre was taken as the highest dilution showing a 1+ reaction. This was agglutination readily visible to the naked eye when the tube was compared with the control.

Table 4. Amine and Alcohol Products of Strains of C. limosum

	Ethyl-	n-propyl-	iso-butyl-	isoamyl-	pyrrolidine	hexyl-	di-n-butyl-	1, 3 diamino- propane	8 phenyl- ethyl-	cadaverine	trypt-	spermidine	spermine	methyl-	1 amino- 2 propanol	putrescine	trimethyl-	isoamyl alcohol	amyl alcohol	n-heptyl-
0658	+		+	+++			+++	+	+++	+		++	+							
1748		++	++	+++	+	++	+++	++	+++	++		++		+	+	+++				
1936 C	++			+++			+++		+++	++		++	+			+++				
1950			+	+++	+		+++	+	+++		++	+			+			+	+	++
2153 A	+			+++		+	+++		+++	++		+++				++				
2485	+	++		+++		+	+++	++	+++	++		+								
2700	+++	+	++	+					+			++	+		+					
2709		+++		+++			+++	++	+++	++		+	+							
2710	++		++	+++		+	+++	++	+++	+		+								
2800	+++	++	+	+++		++	+++	++	+++	++		+		+++		+				
3283	+	+		+++			+++		+++			+								
4473	++		+++	+++		++	+++		+++	+++	+	+	+	+			++			
4480	++		++	+++			+++		+++	++		+	+			+++	+			
4479			+	++			+		+++	++?		+	+			++	+			
1740-1	+	++	++	+				+	++		++	+								
1928	+	+	+						+++			+++	++							
2755			+	+	+	+	+		++			+	+					+		

TABLE 5
Pathogenicity

VPI #	Mice		Guinea pigs
	1 day culture	3 day culture	
0658	2/2*	1/2	0/2
1748	0/2	0/2	0/2
1936 C	0/2	0/2	0/2
1950	0/2	1/2	0/2
2153 A		2/2	0/2
2485	2/2		2/4
2700	0/2	2/2	0/2
2709	0/8	0/4	1/4 late
2710	0/10	0/2	2/7 (1 late)
2800	** $\begin{cases} 1/2 \\ 0/2 \end{cases}$	$\begin{cases} 1/2 \\ 0/2 \end{cases}$	2/6
3283	2/2	0/2	0/2
4473	0/2	0/2	0/1
4480	** $\begin{cases} 0/4 \\ 1/2 \end{cases}$	1/2	-
4479	0/2		0/1
1740-1	2/2	0/2	-
1928	2/2	0/2	0/2
2755	1/2	2/2	1/4

* # died/ # inoculated

** 2 separate trials

the lecithinase-forming strains of C. perfringens, C. litus-eburensis, and C. novyi in being nonfermentative. It also differs from C. perfringens in not producing theta toxin and in being motile, from C. novyi in not producing typical C. novyi toxins and in not fermenting lactate to produce propionate, and from C. litus-eburensis in producing acetic acid as the major fermentation product and in not producing spores in chains.

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