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Chemical Characterization of Organisms Isolated from Leprosy Patients

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Chemical analyses of the cell walls of organisms isolated in various parts of the world from cases of lepromatous and tuberculoid leprosy make possible their assignment to one of the three genera: *Corynebacterium*, *Mycobacterium*, or *Propionibacterium*. One, bacterium 22M, remains unassigned. The combined chemical and enzymatic properties attributed to leprosy bacilli freshly harvested from lepromata are found collectively, but not individually, in these three genera.

Since 1880, numerous microbiologists have reported the isolation of two classes of bacteria from cases of leprosy. The organisms most commonly isolated were pleiomorphic, gram-positive bacilli often referred to as "diphtheroids," whereas less frequently gram-positive, acid-fast rods, presumably belonging to the genus *Mycobacterium*, were grown. A very useful review of this aspect of the microbiology of leprosy is that by Wolbach and Honeij (61). Historically, bacteria isolated from cases of leprosy have been regarded as "contaminants." In 1960, C. C. Shepard succeeded in transferring the leprosy bacilli-generating-system from man to the mouse (54, 55). He used noncultivability as one identifying characteristic for concluding that the acid-fast bacilli he observed were indeed leprosy bacilli. He stated that "... only nonacid-fast organisms grew and these were the expected varieties of surface contaminants" (54).

In 1965, we became concerned with the nature of these "contaminants." From our own efforts and from laboratories in other countries we have accumulated representatives of organisms isolated from patients with both tuberculoid and lepromatous leprosy. At first we examined the ultrastructure of these leprosy-derived organisms (LDO) and found that on anatomical grounds they belonged to two, perhaps three, recognizable groups (27). A reinvestigation of deep lepromatous tissues revealed that one could often demonstrate in them two distinct kinds of bacilli (K.-S. Kim and L. Barksdale, Tenth International Leprosy Congress, Bergen, Norway. Paper no. 8/66, 1973). In addition, it has been found that the acid-fastness of Hansen's bacilli differs chemically from the acid-fastness of mycobacteria, including *Mycobacterium lepraemurium* (25, 26). This fact now seems to be well established (13; J. Convit and M. E. Pinardi, Tenth International Leprosy Congress, Bergen, Norway. Paper no. 10/69, 1973). It appeared to us, then, that the preconceived notion that leprosy bacilli must be only mycobacteria was open to question. Etémadi has found mycolic acids in the bound lipids from human lepromin (24), suggesting that among Hansen's bacilli there must be some mycobacteria. In contrast, Prabhakaran and Kirchheimer demonstrated a unique phenolase in leprosy bacilli harvested from the spleens of persons dying with lepromatous leprosy (45-47). No such phenolase has ever been demonstrated in mycobacteria, corynebacteria, or nocardias, but L. Beaman did find similar phenolase activity in propionibacteria isolated from cases of leprosy (6). It is clear, then, that there is a need to fully characterize LDO because they may have a role in leprosy.

This report uses the now established, classical methods of microbial chemistry for identifying the cell wall mureins (peptidoglycans) and their associated lipids and peptidolipids from the LDO (19, 28, 34, 52).

MATERIALS AND METHODS

Bacterial strains: (i) corynebacteria. *Corynebacterium diphtheriae* strain HF is a nontoxinogenic derivative of the Halifax strain (4, 40); *Corynebacte-*

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rium sp. 2628 LB was originally isolated from lepromata from a Carville patient as 2628 (27); *Corynebacterium* sp. 2628 T60, an aerotolerant mutant of 2628 LB, was originally selected as a large colony on Tween 60 agar (27); *Corynebacterium* sp. Medalle X was obtained from C. Reich and D. Power (51).

(ii) Mycobacteria. The mycobacteria used were: *Mycobacterium avium* ATCC 19421; *M. intracellulare* ATCC 13950 (the original strain of Cuttino and McCabe [20]); *Mycobacterium* sp. ATCC 607; *Mycobacterium* sp. ICRC (Indian Cancer Research Center; supplied by C. V. Bapat by arrangement with K. J. Ranadive [48]); *Mycobacterium* sp. NQ (isolated from hamster passage of inocula from human leprosy by C. Binford; stock was received from J. Wierserma [9, 60]; and *Mycobacterium* sp. 1081, 1285, and 1582 (obtained by J. Convit from hamsters inoculated with homogenates of human lepromata [11, 12]).

(iii) Nocardias. *Nocardia asteroides* strains 92 and 96 and *N. corallina* strain 305 were obtained from S. G. Bradley (30); *N. corynebacterioides* (*C. rubrum*, smooth) was obtained from J. A. Serrano (53).

(iv) Propionibacteria. Propionibacteria used were: *Propionibacterium* (*Corynebacterium*) *acnes* ATCC 11827, *Propionibacterium* sp. S.F. (27), *Propionibacterium* sp. 2629 LT (isolated from a Carville patient as 2629 [27]), *Propionibacterium* sp. 29, and *Propionibacterium* sp. 2 (6).

(v) Other. Bacterium 22M was obtained from C. Reich (33).

Cultivation of organisms. All cultures were maintained on Butter Agar (27). Cells to be collected for cell wall analysis were transferred to 250 ml-flasks containing 50 ml of liquid medium. Strains SF, 2629LT, 2, 29, and *P. acnes* 11827 were grown anaerobically for 72 h at 35 C in Isolation and Maintenance Medium for Actinomyces of Pine and Watson (43) and Pochi and Strauss (44). Strains 2628LB, 2628T60, 22M, Medalle X, 1081, 1285, NQ, ICRC, 1582, *C. diphtheriae* HF, *Mycobacterium avium*, *Mycobacterium* sp. 607, *M. intracellulare*, *N. asteroides*, *N. corallina*, and *N. corynebacterioides* were all grown in RVB liquid medium of Redmond (49) at 35 C in an increased CO_2 atmosphere with moderate agitation. All mycobacterial strains were incubated for 10 days, whereas the other organisms were grown for three days. Actively growing seed cultures were transferred to two-liter flasks containing from 300 to 400 ml of RVB broth and 1,500 ml of Isolation and Maintenance Medium for Actinomyces broth. The final yields of cells used in this study were obtained from these larger volumes of media. Cell growth was followed by checking the increase in optical density at 590 nm. Purity of cultures was checked with the Gram stain and the Ziehl-Neelsen stain, by using phase-contrast microscopy of wet mounts, and by observing growth characteristics established for each strain. Formalin was added to all harvested cultures to give a final concentration of 0.5% (vol/vol) prior to storage at 4 C. The cells were collected at 6,000 $\times$ *g*, washed three times in 0.85% saline, and frozen until needed.

Preparation of cell walls for chemical analysis. The standard methods established by Cummins (17), Salton (52), DeWeese (22), Martin (36), and others were used. The collected cells were washed three times in 0.05 M sodium phosphate buffer (pH 7.5). The cell pellets were weighed and suspended in buffer (1 g/5 ml of buffer) and homogenized for 5 min in a Virtis blender. The resulting bacterial suspensions were passed through a Ribi cell fractionator, first at 15,000 to 20,000 lb/in^2 (effluent temperature of 5 C) and then at 35,000 to 40,000 lb/in^2 (effluent temperature of 5 to 10 C) to break the cells open. The extent of cell breakage was determined with the phase-contrast microscope. The broken-cell suspension was centrifuged at 20,000 $\times$ *g* for 45 min. Three layers were formed in the pellet: an upper membrane layer, a thick cell wall layer, and a small bottom layer of unbroken cells. The middle layer was collected, suspended in buffer at 4 C, and repeatedly washed until no membrane or whole cells could be detected. The crude cell walls were then suspended in 1 M NaCl in buffer (1 g of cell wall/10 ml of NaCl) for 12 h at 4 C. These were centrifuged (15,000 $\times$ *g*) for 20 min and washed three times in buffer. The cell walls were suspended in trypsin (2 mg/ml of phosphate buffer at pH 8.0) at a concentration of 1 g/15 ml (vol/vol). These were placed in a water bath-incubator (37 C) with mild agitation for 2 h, and then Formalin (0.1 ml/20 ml of suspension) was added to prevent the growth of bacterial contaminants. The cell wall-trypsin mixture was incubated for an additional 16 h. The cell walls were centrifuged (12,000 $\times$ *g* for 20 min), and the upper translucent layer was collected. This layer was suspended and washed four times as described above. The cell wall purity was determined by phase-contrast microscopy, the Gram stain, the Ziehl-Neelsen stain, electron microscopy, and spectrophotometrically at wavelengths from 205 to 300 nm. The purified walls were divided into two portions, one being added to preweighed tubes and lyophilized, whereas the other portion was suspended in pepsin (crystallized two times; 2 mg/ml in 0.2 N of HCl) at a concentration of 0.5 g/15 ml and placed in a 35 C water bath with mild agitation for 18 h. The trypsin-pepsin-treated cell walls were centrifuged (12,000 $\times$ *g* for 20 min), suspended in distilled water, washed four times, distributed in preweighed tubes, and lyophilized.

Removal of lipids. Trypsin-pepsin-digested cell walls were extracted with KOH-ethanol (0.5% wt/vol) as described by Cummins (17) and Beaman et al. (7). We found that this procedure did not adequately remove the more firmly bound lipids of the mycobacterial cell walls. Therefore, we used the more rigorous method of extraction described by Kanetsuna and others for extracting the mycolic acids from mycobacteria (31, 58). Trypsin-pepsin cell walls were suspended in 2.5% KOH (wt/vol)-methanol-benzene (1:1) and refluxed at 60 C for 24 h. The cell wall material was allowed to settle, washed three times in methanol-benzene, washed four times over a period of 24 h with diethyl ether, dried, suspended in distilled water, and washed three additional times in order to obtain a uniform suspension. Samples were assessed with the electron microscope. The walls were then

placed in preweighed tubes and lyophilized.

Qualitative amino acid analysis. Trypsin-treated cell walls were hydrolyzed in 6 N HCl as described by Cummins et al. (18, 19). The hydrolysates were spotted on Whatman no. 1 filter paper and two-dimensional descending chromatography was used with development first in pyridine-water (4:1) for 16 h, followed by butanol-acetic acid-water (3:1:1) for 16 h. The chromatograms were sprayed with 0.5% (wt/vol) ninhydrin in butanol, heated at 105 C for 5 min, and compared with standards.

Amino sugar assay. Trypsin-treated cell walls were hydrolyzed in 6 N HCl for 4 h at 110 C and prepared as described for amino acid analysis. The chromatograms were sprayed as described by Partridge (42) and by Crumpton (17).

Determination of diaminopimelic acid. The cell walls were prepared as described for amino acids and assayed as described by Becker et al. (8).

Analysis of cell wall sugars. The trypsin-treated cell walls were hydrolyzed in 2 N H_2SO_4 as described by Cummins and Harris (18). Two-dimensional descending chromatography was done on Whatman no. 1 filter paper with development first in butanol-pyridine-water (6:4:3) followed by butanol-acetic acid-water (3:1:1) for 16 h in each solvent. The chromatograms were sprayed with oxalate as described by Murray and Proctor (41) and compared with standards.

Quantitative amino acid analysis. Trypsin-, trypsin-pepsin-treated, trypsin-pepsin-KOH-ethanol-, and trypsin-pepsin-KOH-methanol-benzene-ether-extracted cell walls were each hydrolyzed in 6 N HCl in evacuated tubes for either 4 h or 22 h at 110 C. The hydrolysates were dried in a rotary flash evaporator and resuspended in citrate buffer (pH 2.2) as described for amino acid analysis by Beckman Instruments manual. All samples were analyzed on a Beckman 120 C amino acid analyzer and the amino acid amounts were determined by the width times height method as described by the Beckman manual. All samples were analyzed in duplicate, and each experiment was repeated. During prolonged hydrolysis with 6 N HCl, the amino sugars underwent hydrolytic degradation with corresponding increases in ammonia. Therefore, we have attempted to correct for the hydrolytic loss of these amino sugars by extrapolation of the results obtained at different lengths of hydrolysis. At the same time we have adjusted the values obtained for ammonia to correspond as nearly as possible to those released from non-amino sugar components of the cell wall.

Analysis for lipids. Harvested cells were washed once with distilled water, and the pellets were then treated with 5% KOH (wt/vol) in methanol containing 1 to 2 ml of benzene (total volume of 10 ml). This suspension was refluxed overnight. The methanol-benzene mixture was evaporated by heating in vacuo, and the residue was taken up in ether-water (1:1) and acidified with H_2SO_4 (20% vol/vol). The acidified mixture was agitated vigorously, and the ether layer was removed and washed with distilled water until the pH was approximately neutral. The ether layer was then dried by the addition of sodium sulfate and filtered through Whatman no. 10 filter paper, and the filtrate was evaporated to dryness. This was the fatty acid residue (FAR). Methylation of the FARs was carried out as follows. Diazomethane was prepared in a hood by a slow addition (over 15 min) of 1 g (total) of nitrosomethyl-urea to a constantly agitated vessel (in an ice bath) containing 3 ml of KOH, and stored at 4 C. The life of each preparation was no more than 8 days. The FARs were methylated by combination with diazomethane in ether. The instant reaction was signaled by the evolution of nitrogen into the ether. Completion of the reaction was indicated by cessation of bubbling. Ether and excess diazomethane were evaporated from the methylated FAR, and the latter was then analyzed.

Analysis by thin-layer chromatography. Preparative thin layers were made with silica gel P.F. 254 Merck. The methylated FARs were spotted and developed on these, by using petroleum ether-diethyl ether (8:2), and detected with Rhodamine B. The hydroxylated ester zones were scraped from the plates and eluted with ether; for analytical thin-layer chromatography, petroleum ether-diethyl ether (7:3) was used.

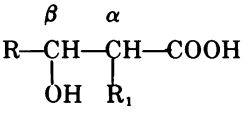
Gas chromatography. Zones from the thin-layer chromatography were analyzed by using the gas chromatograph FM model 400 with a flame ionization detector. Two columns (designed by Applied Science Laboratories, State College, Pa.) of phase SE 30 (10%) on chromasorb W (60 to 80 mesh; acid-washed) were used. Column *a* (6 feet [182.88 cm] long, stainless steel) served for the pyrolytic cleavage at 305 C from a flash heater (temperature program ranged from 217 to 330 C). Column *b* (2 feet [60.96 cm] long, copper, and prepared as above) was used for the analysis of trimethylsilyl-ether derivatives of the corynomycolates. Analyses were isothermal at 310 C, and the derivatives were prepared according to the method of Sweeley and associates and others (24, 57; M. A. Lanéelle-Carrieu, thesis no. A.O. 3193, University of Toulouse, France, 1969).

Mass spectrometry. The spectral data for nocardomycolic acids of *N. corynebacterioides* and the new corynomycolic acids reported here from the human leprosy-derived corynebacteria were obtained by B. C. Das, Mass Spectrometry Service, Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France.

RESULTS

Composite qualitative data from amino acid and amino sugar analyses of the cell walls of the LDO being investigated are recorded in Table 1. Clearly, three of the isolates have mureinic backbones and associated saccharides characteristic of *Corynebacterium*; five exhibit those typical of *Mycobacterium*; and four lie outside the *Corynebacterium-Mycobacterium-Nocardia* (CMN) group, having the backbones and associated sugars found in *Propionibacterium* as exemplified by *P. acnes* (16, 29, 59). For the singular organism, bacterium 22M, no generic assignment is suggested.

Members of the CMN group are characterized by having associated with their outer envelopes α-branched, β-hydroxylated, long-chain fatty acids of the general formula for mycolic acids (1-3):



These occur in ester linkage, e.g., esterified to the arabinogalactan of the cell walls of corynebacteria, mycobacteria, and nocardias (35, 37) or to trehalose in the dimycolates, which are called cord factors (10, 32). Methyl esters of mycolic acids on pyrolysis yield an ester moiety and an aldehyde moiety. Analysis by pyrolytic chromatography (24; M. A. Lanéelle-Carrieu, thesis) of methylated corynomycolates from silica gel plates at once transforms them into trimethyl-silyl derivatives cleaved into esters (α chains) and aldehydes (β chains) of the parent molecules.

The results of analyses of corynomycolic acids from *C. diphtheriae* and the LD-corynebacteria comprise Tables 2 and 3. It is clear that the LD-corynebacteria show a wider spread of carbon numbers in their corynomycolate homologues than do those of the strain of *C. diphtheriae* (control) examined. Furthermore, a new corynomycolate, $C_{37}H_{70}O_3$, is the major component of the LD-corynebacteria. This

TABLE 1. *Composite data from qualitative analyses of the cell walls of bacteria isolated from cases of human leprosy*[a]

Compound	*Corynebacterium* sp. 2628LB, 2628T60, and Medalle X	*Mycobacterium* sp. ICRC, NQ, 1081, 1285, and 1582	*Propionibacterium* sp. 2629LT, SF, 2, and 29	Bacterium 22M
LL-diaminopimelic acid	−	−	++	−
Meso-diaminopimelic acid	++	++	−	++
Glutamic acid	++	++	++	++
Alanine	++	++	++	++
Glycine	T	T − +	++	T
Lysine	−	T	T − +	−
Histidine	−	−	+	−
Aspartic acid	T	+	T	T
Muramic acid	++	++	++	++
Glucosamine	++	++	++	++
Galactosamine	−	T	+	+
Galactose	++	++	++	++
Glucose	T	−	++	−
Mannose	+	−	++	−
Arabinose	++	++	−	++
Xylose	−	−	−	−
Rhamnose	−	+	−	+
Unidentified compounds	−	−	−	−
Characteristic lipid found	C_{37} Corynomycoldienic acid	C_{60}-C_{85} Mycolic acids	Branched methyl, penta-, and hepta-decanoates	Palmitic acids

[a] Intensity of chromatographic spots ranges from trace (T) to ++++.

TABLE 2. *Pyrolysis gas chromatography of trimethyl-silyl-ether derivatives of corynomycolic acid esters*

Bacterial strain	Carbon no.					
	28	30	32[a]	32	34	36
Corynebacterium diphtheriae gravis strain HF	3.9	17.7	5.4	72.8		
Corynebacterium sp. Medalle X	3.1	7.3		17.3	20.3	51.9
Corynebacterium sp. 2628T60	5.5	10.0		25.5	18.0	40.0
Corynebacterium sp. 2628LB	4.4	11.0		22.5	18.6	43.3

[a] May contain one double bond.

TABLE 3. *Analyses for corynomycolic acids*[a]

Bacterial strain	Carbon chain length: no. of double bonds[b]		Inferred number of carbons and double bonds prior to pyrolysis
	Aldehyde	Ester	
C. diphtheriae gravis strain HF	16:0	16:0	32:0
Corynebacterium sp. 2628LB,[c]	18:1	18:1	36:2
2628T60, and	16:0	18:1	34:1
Medalle X (composite data)	14:0	18:1	31:1

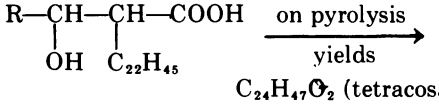
[a] Analyses were by gas chromatography as described in Materials and Methods.

[b] Found in trimethyl-silylether derivatives of corynomycolic acid esters on pyrolysis.

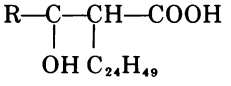
[c] Mass spectrometric analysis of corynomycolate methyl esters from this strain carried out by B. C. Das (see Materials and Methods) indicate the major component to have the empirical formula $C_{37}H_{70}O_3$.

should serve as a marker in further studies. The degrees of unsaturation found in the LDO-corynomycolates also are distinctive.

The structure of the mycolic acids of mycobacteria are more complex than are the coryno- and nocardomycolic acids. The ketone, hydroxyl, cyclopropane, carboxyl, etc. groupings are always localized on the main chain (i.e., the aldehyde of pyrolysis), whereas the ramified end (i.e., the ester of pyrolysis) is saturated and contains 22 or 24 carbon atoms. Thus, for the majority of mycobacteria other than *M. tuberculosis*,



whereas human strains of *M. tuberculosis*, having the general formula



yield $C_{26}H_{51}O_2$. Mycolic acids of all of the LDO-mycobacteria gave methyl tetracosanoates after pyrolytic cleavage.

From the lipid fractions of the LD-propionibacteria were recovered branched methyl-heptadecanoates and methyl-pentadecanoates (23, 38, 39). The major lipid components of strain 22M were palmitic acids.

Comparative data on the total amino acid composition of the cell walls of the corynebacteria are given in Table 4, that of the mycobacteria are given in Table 5, and those for the propionibacteria comprise Table 6.

TABLE 4. *Amino acid and amino sugar composition of trypsin-pepsin-treated cell walls of known corynebacteria and LD-corynebacteria*

Amino acids and amino sugars	Molar ratios			
	Corynebacterium diphtheriae HF	*C.* sp. 2628LB	*C.* sp. 2628T60	*C.* sp. Medalle X
Glutamic acid	1.00	1.00	1.00	1.00
Alanine	1.68	1.65	1.69	1.70
Meso-diaminopimelic acid	0.94	0.82	0.80	0.90
Glycine	0.11	0.19	0.20	0.19
Muramic acid[a]	0.75	0.78	0.73	0.82
Glucosamine[a]	0.68	0.76	0.69	0.71
Galactosamine[a]	Trace	ND[b]	ND	ND
Ammonia[a]	1.40	1.35	1.41	1.23
Aspartic acid	0.05	0.09	0.13	0.05
Threonine	0.02	0.05	0.07	0.03
Serine	0.11	0.16	0.11	0.06
Proline	0.01	0.03	0.06	0.02
Valine	0.03	0.06	0.09	0.02
Methionine	0.02	0.06	Trace	0.01
Isoleucine	0.02	0.05	0.08	0.04
Leucine	0.04	0.08	0.14	0.04
Tyrosine	0.01	ND	0.01	ND
Phenylalanine	0.09	0.12	0.09	0.11
Lysine	0.03	0.04	0.06	0.06
Histidine	0.01	0.02	0.02	Trace
Arginine	0.01	0.02	0.03	Trace

[a] Adjusted for hydrolytic loss: ammonia corrected for degradation of amino sugars. Molar ratios are expressed with respect to glutamic acid.

[b] ND, Not detected.

The cell wall analyses of the nocardial strains used as "nocardial standards" gave amino sugar and amino acid patterns somewhat in between those of the corynebacteria and mycobacteria. Nocardomycolic acids were detected in each strain, and a new nocardomycolic acid was found to be synthesized by *N. corynebacterioides*. Because none of the LDO had cell wall compositions similar to those of the nocardias, further data on the latter are not included in this paper.

Electron micrographs of cell walls of corynebacteria and mycobacteria with and without their outer peptidolipid envelopes are shown in Fig. 1. A comparative analysis of such walls for amino acids reveals a difference. Those amino acids associated with the peptidolipid are lost on extraction of the walls with alkaline ethanol (7). However, it is important to note that more drastic treatment was required to remove the peptidolipids from the mycobacterial cell walls than was required for corynebacteria and nocardias. Data on the molar ratios of certain amino acids found in standard (trypsin-pepsin-digested) cell walls and in standard wall prepa-

TABLE 5. *Amino acid and amino sugar composition of trypsin-pepsin-treated cell walls of known mycobacteria and LD-mycobacteria*

Amino acids and amino sugars	Molar ratios							
	M. intracellulare	*M. avium*	*M.* sp. 607	*M.* sp. ICRC	*M.* sp. NQ	*M.* sp. 1081	*M.* sp. 1285	*M.* sp. 1582
Glutamic acid	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Alanine	1.49	1.38	1.46	1.39	1.45	1.40	1.41	1.43
Meso-diaminopimelic acid	0.74	0.71	0.80	0.64	0.84	0.74	0.89	0.63
Glycine	0.30	0.16	0.29	0.36	0.16	0.51	0.17	0.50
Muramic acid[a]	0.76	0.71	0.91	0.73	0.64	0.74	0.87	0.67
Glucosamine[a]	0.67	0.68	0.77	0.56	0.55	0.59	0.68	0.54
Galactosamine[a]	0.17	0.08	Trace	0.21	0.26	0.24	0.24	0.12
Ammonia[a]	1.53	1.53	1.50	1.80	1.67	1.39	1.39	1.47
Aspartic acid	0.14	0.08	0.16	0.31	0.11	0.16	0.10	0.32
Threonine	0.16	0.05	0.13	0.23	0.10	0.14	0.13	0.24
Serine	0.15	0.09	0.08	0.19	0.09	0.36	0.08	0.28
Proline	0.10	0.06	0.09	0.20	0.10	0.13	0.06	0.16
Valine	0.11	0.03	0.10	0.30	0.09	0.09	0.09	0.24
Methionine	0.06	0.03	Trace	0.10	0.05	Trace	0.07	0.09
Isoleucine	0.09	0.03	0.05	0.15	0.05	0.07	0.06	0.14
Leucine	0.14	0.07	0.13	0.31	0.10	0.12	0.10	0.34
Tyrosine	0.02	0.03	0.03	Trace	0.02	0.03	Trace	0.08
Phenylalanine	0.12	0.04	0.10	0.06	0.13	0.07	0.03	0.18
Lysine	0.05	0.02	0.03	0.13	0.04	0.05	0.04	0.11
Histidine	0.01	0.01	0.01	0.07	0.01	0.08	0.02	0.05
Arginine	0.06	0.02	0.04	0.19	0.04	0.04	0.04	0.18

[a] Adjusted for hydrolytic loss: ammonia corrected for degradation of amino sugars. Molar ratios are expressed with respect to glutamic acid.

TABLE 6. *Amino acid and amino sugar composition of cell walls of known propionibacterium and LD-propionibacteria*

Amino acids and amino sugars	Molar ratios[a]				
	P. acnes 11827	*P.* sp. SF	*P.* sp. 2629LT	*P.* sp. 29	*P.* sp. 2
Glutamic acid	1.00	1.00	1.00	1.00	1.00
Alanine	1.35	1.48	1.77	1.96	1.57
LL-diaminopimelic acid	0.97	1.01	1.09	1.10	0.97
Glycine	0.94	1.01	1.07	1.01	1.00
Muramic acid[b]	0.61	0.45	0.63	0.62	0.61
Glucosamine[b]	0.71	0.60	0.86	0.65	0.70
Galactosamine[b]	0.56	0.45	0.39	0.54	0.98
Ammonia[b]	2.89	4.65	3.39	3.01	1.24
Aspartic acid	0.03	0.03	0.02	0.02	0.02
Threonine	0.05	0.05	0.02	0.01	0.03
Serine	0.04	0.04	0.02	0.02	0.07
Proline	ND	ND	ND	ND	ND
Valine	0.04	ND	ND	ND	ND
Methionine	ND	ND	ND	ND	ND
Isoleucine	0.05	0.02	0.01	Trace	0.01
Leucine	0.04	0.02	0.01	0.01	0.02
Tyrosine	ND	ND	ND	ND	ND
Phenylalanine	ND	ND	ND	ND	ND
Lysine	0.07	0.14	0.05	0.07	0.34
Histidine	0.34	0.30	0.35	0.30	0.33
Arginine	0.02	0.02	ND	0.01	0.01

[a] Molar ratios are expressed with respect to glutamic acid.
[b] Adjusted for hydrolytic loss: ammonia corrected for hydrolytic degradation of amino sugars.
[c] ND, Not detected.

rations further extracted with either alkaline-ethanol or KOH-methanol-benzene-ether prior to being subjected to hydrolysis are presented in Table 7. Inspection of Table 7 reveals differences in the amino acids associated with the peptidolipids of corynebacteria and those of mycobacteria. The strains listed in this table are representative of all the strains studied. Serine, threonine, and valine seem to be a part of the peptidolipids of *Corynebacterium* sp. 2628LB. Phenylalanine appears to be an important amino acid of the peptidolipids of all three organisms shown in Table 7. Glycine, on the other hand, is not removed with the lipid material from any of these strains. Serine, proline, valine, and leucine are consistently present in the cell walls of mycobacteria, and removal of the lipid from these walls does not greatly alter the content of these amino acids within the wall.

DISCUSSION

On the basis of cell wall analysis, all but one of the LDOs can be assigned to one of three bacterial genera (*Corynebacterium*, *Mycobacterium*, or *Propionibacterium*). For the first time in 100 years, it is possible to know that from human leprous lesions different microbiologists have cultivated one or the other of these

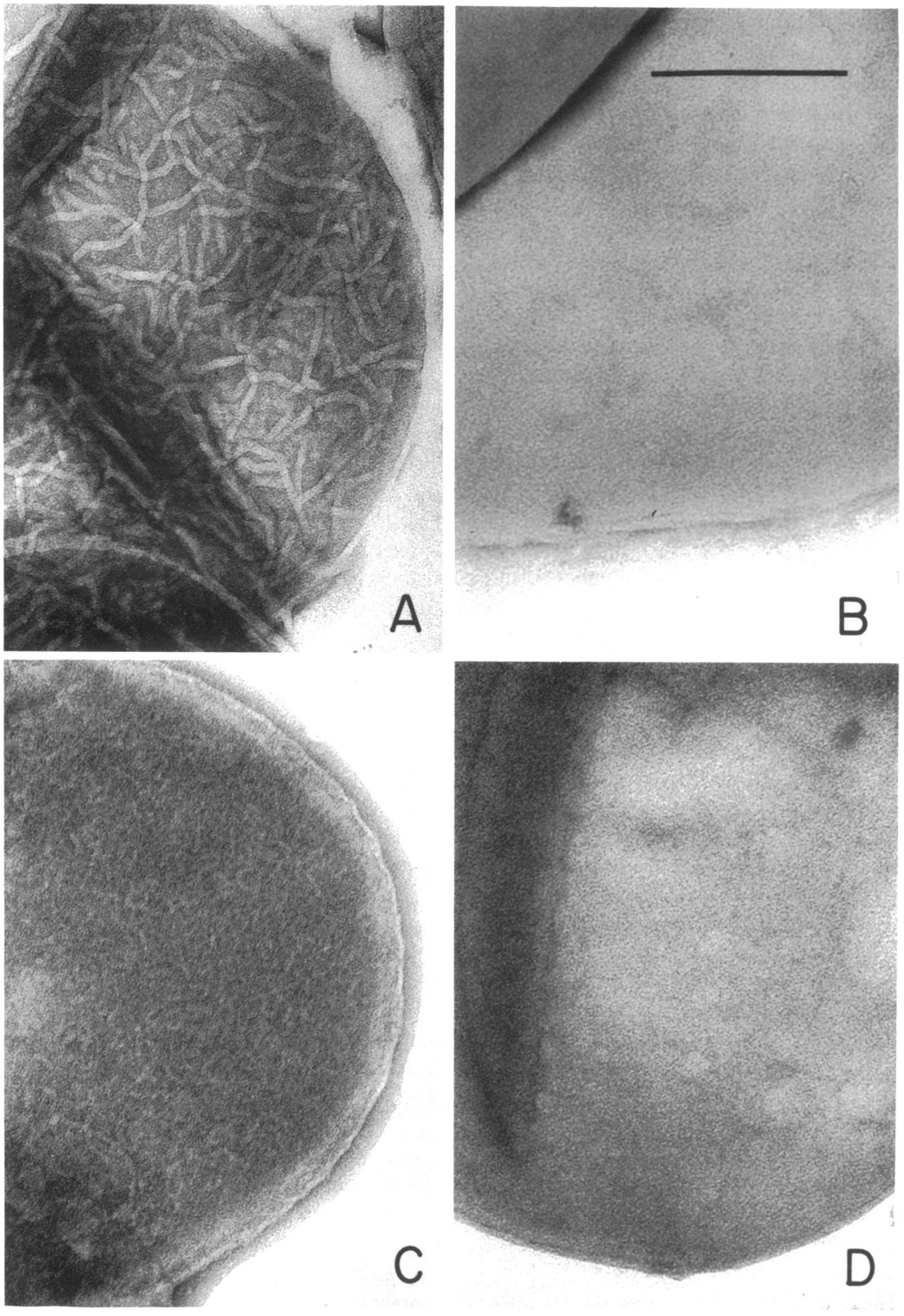


FIG. 1. *(A) Trypsin-pepsin-digested cell wall of LDO-mycobacterium, strain NQ (all other LDO-mycobacteria had similar appearing cell walls). (B) As in (A), plus extraction with KOH-methanol-benzene-ether as described in Materials and Methods. (C) Trypsin-pepsin-digested cell wall of LDO-corynebacterium, T60, (D) As in (C), plus extraction with KOH-ethanol. (See text). All micrographs ×150,000. Bar, 0.2 μm.*

TABLE 7. *Amino acid loss from trypsin-pepsin-treated cell walls during lipid extraction*[a]

Amino acid	Molar ratios[a]					
	Corynebacterium sp. 2628LB		*Mycobacterium intracellulare*		*Mycobacterium* sp. NQ	
	A[b]	B	A	B	A	B
Glycine	0.19	0.15	0.30	0.24	0.16	0.27
Threonine	0.05	ND	0.16	0.01	0.10	0.01
Serine	*0.16*	*ND*	0.15	0.13	0.09	0.09
Proline	0.03	ND	0.10	0.04	0.10	0.07
Valine	*0.06*	*ND*	0.11	0.04	0.09	0.06
Leucine	0.08	0.03	0.14	0.06	0.10	0.05
Tyrosine	ND	ND	0.02	ND	0.02	ND
Phenylalanine	0.12	ND	0.12	ND	0.13	0.01
Histidine	0.02	0.01	0.02	0.02	0.02	0.02

[a] Figures expressed taking glutamic acid as 1.

[b] A, Hydrolysates of trypsin-pepsin-treated cell walls; B, Hydrolysates of trypsin-pepsin-treated cell walls after removal of lipids (see Materials and Methods); ND, no detection of compound. See text for discussion of differences.

bacteria. From the data of Shepard (54, 55) and Rees (50), it is not known whether any of these bacteria have been recovered from mice inoculated with homogenates of human lepromata. However, it is known from the work of Binford (9, 60) and Convit (11, 12) with hamsters, and Ranadive with mice (48), that none of the LD-mycobacteria examined here causes a leprosy-like infection in animals. It is also known from Fisher, Barksdale, and Pollice (unpublished data) that neither the LD-propionibacteria nor the LD-corynebacteria produce in mice the kind of infection Shepard has described as developing after the inoculation of mice with homogenates from human lepromata (55, 56). We take this to mean that not one of these organisms per se is the etiologic agent of leprosy. This paper, then, sets the record straight as to what is the real nature of the "diphtheroids," coryneform bacteria, and acid-fast bacteria isolated from cases of human leprosy.

Wolbach and Honeij have suggested that it is very unlikely that more than one organism is involved in the etiology of leprosy (61). This seems a reasonable assumption. However, the possibility of mixed etiology has never been ruled out. At present, leprosy is a clinical rather than an etiologic entity. It may have a compound etiology, and the integral agents may always be the same or they may be different. The morphological hallmark of leprosy was early understood to be the globus: "characteristic colonies" of acid-fast bacilli "growing within an as yet unidentified restricting membrane" (14, 21). More recently it has been shown that these bacilli differ from mycobacteria not only on the basis of their occurrence within a unique membrane, but also in their acid-fastness which, unlike that of mycobacteria, can be extracted with pyridine (25, 26). The association of these two unique properties within one organism makes leprosy bacilli very different from any other acid-fast bacilli. It is now known that there can be cultivated from untreated cases of active lepromatous and tuberculoid leprosy spheroidal bodies of leprosy (SPBL) capable of differentiating into globi (5). The development of globi is abortive when the SPBL are in pure culture, but in mixed culture the globi are filled with acid-fast bacilli and the acid-fastness is extractable with pyridine. The bacteria reported to function effectively in mixed culture, "helper bacteria," were corynebacteria of the sort examined in this paper. It remains to be seen whether or not mycobacteria and propionibacteria can function as helper bacteria when growing with SPBL and whether or not such combinations produce leprosy-like infection in experimental animals.

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