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Multiple Drug Resistance in *Mycobacterium avium*: Is the Wall Architecture Responsible for the Exclusion of Antimicrobial Agents?

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Whole cells of *Mycobacterium avium*, characterized by their negative response in the nine biochemical tests used for mycobacterial identification in our laboratory, turned positive for nitrate reductase, Tween-80 hydrolysis, β-glucosidase, acid phosphatase, alkaline phosphatase, penicillinase, and trehalase after their wall portion was removed to yield spheroplasts. This suggested that the negative results in most of the biochemical procedures were caused by the exclusion mechanism at the wall level. Preliminary transmission and scanning electron microscopic studies showed differences at wall level between laboratory-maintained opaque, dome-shaped (SmD) and host-recycled smooth, transparent (SmT) colony type variants of *M. avium* and suggested the presence of an outer regularly structured layer in SmT variants. Comparative ultrastructural studies utilizing different polysaccharide coloration methods confirmed the presence of an outer polysaccharide layer in SmT variants which was probably related to their enhanced pathogenicity for experimental animals and drug resistance as compared to that of SmD variants. These findings are discussed with respect to multiple drug resistance, virulence, and gene expression of *M. avium*.

The pathogenic mycobacteria, including *Mycobacterium avium*, are intracellular parasites which multiply within the host macrophage. Conditions in vitro are very different from those within the parasitized cell, since upon transfer of bacteria from host animals to in vitro culture conditions, a colony type transition can be observed. In fact, *M. avium* produces two types of colonies on 7H10 agar: the smooth transparent type (SmT) and the smooth, opaque, dome-shaped type (SmD) (64). SmT to SmD type transition has been shown to occur at a frequency of 10^{-4} to 10^{-5} (31), whereas SmD to SmT type transition occurs at a frequency of 10^{-6} to 10^{-7} (66). David (11) suggested that the ability of *M. avium* to survive in the tissues of humans and experimental animals might be related to expression of genes that specify the SmT type; since the expression of these genes would not be necessary for survival in artificial medium, they would be repressed upon transfer to laboratory culture conditions, resulting in predominance of SmD colonies. SmT variants have been shown to be more parasitic for chickens and mice (39, 49) and more resistant to various drugs used (21) than the SmD variants.

It was recently suggested that drug resistance in *M. avium* is probably due to a permeability barrier at the wall level (12; H. L. David, Rev. Infect. Dis., in press). Furthermore, the bacteria of *M. avium* complex give negative results in most of the biochemical procedures used in the identification of the mycobacteria (63, 64), and it is possible that these negative results are also caused by the same exclusion mechanism. We thought that the use of spheroplasts of *M. avium* might prove useful to examine the above hypothesis. In this report we present some preliminary data showing that spheroplasts of *M. avium*, in contrast to the intact cells, are permeable to various enzyme substrates as well as to the antibiotic tested (penicillin G).

Spheroplasts are wall-deficient forms which differ from protoplasts primarily by having external surfaces that contain some wall material (34, 37). There have been reports of spheroplast formation when myobacteria were grown in the presence of lysozyme and glycine (1, 3, 47, 48). Protoplasts and whole-cell ghosts have been prepared recently from *Mycobacterium smegmatis*, utilizing D-cycloserine and horse serum (43). Recently we have developed a method of spheroplast formation from *Mycobacterium aurum* and have proved our findings by chemical and ultra-

structural studies (42). In the present study, we employed the same technique and obtained successful formation of spheroplasts from the slow grower *M. avium*.

Preliminary transmission and scanning electron microscopic studies done on the transparent and opaque variants suggested the existence of a possible outer layer in the transparent variant, as well as a thicker peptidoglycan layer. A further detailed study of the two variants at the ultrastructural level, utilizing different polysaccharide staining techniques, led us to confirm the polysaccharide nature of this outer layer, found only in the host-recycled *M. avium* bacteria.

The above findings will be discussed in respect to the colony type variation, multiple drug resistance, pathogenicity, and gene expression of *M. avium* bacteria.

MATERIALS AND METHODS

Organism and growth. *M. avium* ATCC 15769 (serotype 2) maintained on Lowenstein-Jensen medium was used in these studies. Cells were grown in complete Middlebrook and Cohn 7H9 medium, which contained 4.7 g of 7H9 powder (Difco) and 0.5 ml of Tween-80 in 900 ml of distilled water. After sterilization, five ampoules of 20 ml each of Middlebrook ADC enrichment (Difco) were added in sterile conditions.

SmT variants isolated from rabbits were kindly supplied by M. F. Thorel. Rabbits were inoculated by intravenously injecting 1 ml of culture of *M. avium* ATCC (serotype 2) at 5 mg/ml. Rabbits were dead 14 to 15 days after inoculation. Lungs, liver, spleen, and kidneys were taken out, and the tissues were thoroughly ground with a mortar and pestle, treated with 4% (vol/vol) H_2SO_4 for 5 to 10 min, and then immediately neutralized with 6% (wt/vol) NaOH. The tissue suspension obtained was left standing to remove all tissue debris. From 3 to 6 drops of the turbid supernatant was inoculated into Lowenstein-Jensen tubes, and the bacteria grown after 21 days were used in the present investigation.

Preparation of *M. avium* spheroplasts. Spheroplasts were prepared from the laboratory-maintained SmD variant of *M. avium*. The method used was the same as described earlier for *M. aurum* (42), except that 60 μg of D-cycloserine per ml, 0.2 M glycine, 200 μg of lysozyme per ml, 2 mg of ethylenediaminetetraacetic acid per ml, and 1 mg of lithium chloride per ml were added after the cells had grown to logarithmic phase (7 to 9 days after inoculation; optical density of 0.1 to 0.15 at 650 nm). The period of treatment with the above spheroplast-inducing substances was 54 to 60 h (corresponding to three cell divisions) instead of the 18 h for *M. aurum*. After incubation, the cells were harvested by centrifugation, and our method was followed as reported earlier (42).

Biochemical tests. For the biochemical tests, the intact *M. avium* cells (both SmD and SmT variants gave the same results for the biochemical tests done) were taken from solid Lowenstein-Jensen medium, and spheroplasts prepared from the SmD variants were collected by centrifugation for these tests. The same quantities of both were used to perform the following tests: nitrate reductase (63), Tween-80 hydrolysis (28), urease (35), arylsulfatase (63), β-glucosidase (13), acid phosphatase (26), alkaline phosphatase (10), penicillinase (15), and trehalase (15).

β-Glucosidase and penicillinase substrate penetration in spheroplasts of *M. avium*. Spheroplasts were prepared in advance and were kept frozen in 2.5-ml amounts at −20°C in a tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride buffer (pH 8.0; 50 mM) containing 20% (vol/vol) saccharose at an optical density of 0.6 to 0.8 at 650 nm. Just before the experiment, the spheroplasts were thawed, rapidly centrifuged, and used in the following studies.

For the β-glucosidase test, the blank was prepared by adding 5 ml of Tris-hydrochloride (pH 7.0; 50 mM) to the spheroplast pellet, and for the test, volume was made up to 5 ml with substrate (prepared by dissolving 30 mg of *p*-nitrophenyl-β-D-glucoside in 10 ml of Tris-hydrochloride buffer, 50 mM, pH 7.0). Both the blank and the test were kept in optically matched tubes (16 by 125 mm) in a dry heating block at 37°C with shaking from time to time. At different time intervals, both blank and test solutions were centrifuged rapidly at room temperature, and the yellow color intensity was measured at 410 nm in a Coleman Junior II spectrophotometer. A standard curve for β-glucoside liberated was prepared with a solution of *m*-nitrophenol in Tris-hydrochloride buffer (50 mM; pH 8.0).

For the penicillinase test, spheroplasts were suspended in 5 ml of a 0.1 M solution of penicillin G in Tris-hydrochloride buffer (50 mM; pH 7.0), and the pH change due to penicilloic acid production was observed by utilizing a pH meter at 37°C.

Electron microscopic studies. (i) Negative staining. Appropriate dilutions of the glutaraldehyde-fixed bacteria (2.5%, wt/vol, in cacodylate buffer, pH 7.2, 0.1 M, overnight) were taken. Grids (200 mesh) were first passed into the diluted bacterial suspension and then directly into 4% (wt/vol) phosphotungstic acid, air dried, and examined on a Siemens 101 transmission electron microscope. All of the grids were carbon reinforced and Formvar coated before use.

(ii) Scanning electron microscopy. Three drops of a bacterial suspension with an optical density of 0.01 to 0.03 at 650 nm was absorbed on a 0.8-μm membrane filter (Millipore). The bacteria were prefixed with 2.5% (wt/vol) glutaraldehyde solution in cacodylate buffer (pH 7.2; 0.1 M) for 45 min, washed twice with the same buffer (0.2 M), postfixed with 1% (wt/vol) OsO_4 in cacodylate buffer, and dehydrated in a graded ethanol series. Filters after dehydration were critical-point dried, metallized utilizing a Polaron SEM E-5000 coating unit, and examined on a Cameca scanning electron microscope.

(iii) Transmission electron microscopy. Cells were prefixed overnight in 2.5% (wt/vol) glutaraldehyde in Michaelis-Veronal buffer containing 1 mM $CaCl_2$ and concentrated in 2.5% (wt/vol) agar. Small blocks were cut at this step, postfixed as described by Ryter and Kellenberger (46), and embedded in Epon 812. Thin sections were stained either by lead citrate or other polysaccharide stains as indicated and exam-

ined on a Siemens 101 transmission electron microscope.

Polysaccharide colorations used. (i) SP. Silver proteinate (SP) staining was used to localize the polysaccharides containing α1-2 glycoside bonds which were oxidized by periodic acid to give aldehydes, which in turn were detected by thiocarbohydrazide; the thio radicals were made visible by silver grain precipitates. The original method of Thiéry (61) was used with slight modifications as follows. Thin sections (silver to gold interference colors) mounted on plastic rings were stained by floating on drops of the following reagents at room temperature: H_2O_2, 10 volumes, 10 min; distilled water, three times for 10 s each and three times for 10 min each; 1% periodic acid, 20 min; distilled water, three times for 10 s each, three times for 10 min each; 0.2% thiocarbohydrazide in 20% acetic acid, 1 to 2 h; 10% acetic acid, three times for 10 s each; 5% acetic acid, three times for 10 s each; 2.5% acetic acid, three times for 10 s each; distilled water, three times for 10 s each and three times for 10 min each; 1% SP in double-distilled water, 30 min in the dark; distilled water, three times for 10 s each and three times for 10 min each. The SP solution was stored in the dark at 4°C and was stable for 3 to 4 weeks. The whole process can be written in a shortened form as PA_{20},TCH_{60-120}, SP_{30}, where PA is periodic acid and TCH is thiocarbohydrazide.

To be sure of no possible interference due to free aldehyde groups, in parallel control experiments the above method was applied without prior treatment with periodic acid so as to prevent the opening of α1-2 glycoside bonds. There were no silver grain precipitates in control experiments, however, and this excluded the possibility of any aldehyde contamination in test preparations. Thin sections obtained were mounted on the Formvar-coated copper grids for electron microscopic examination.

(ii) Phosphotungstic acid staining. Though not specific, this method has been shown to stain the peptidoglycan of the bacterial walls (45). Thin sections, mounted on plastic rings, were treated with a drop of 1% periodic acid for 10 min to remove excess osmium and then thoroughly rinsed with distilled water. Sections were stained by floating for 5 min on a drop of chromic-phosphotungstic mixture at a pH of 0.3 as reported earlier (41), rinsed rapidly in distilled water, and mounted on Formvar-coated copper grids for examination on a transmission electron microscope.

(iii) RR coloration. The ruthenium red (RR) stain does not penetrate intact bacteria and is used to locate outer polysaccharide layers. The originally glutaraldehyde-prefixed, nonembedded mycobacteria were postfixed for 2 h in a glutaraldehyde-RR stain (3.6% aqueous glutaraldehyde solution, 0.2 M cacodylate buffer, pH 7.3, and 0.15% RR in equal parts), washed three times in a 0.15 M cacodylate buffer for 10 min each time, and fixed overnight in a OsO_4-RR solution (5% aqueous OsO_4 solution, 0.2 M cacodylate buffer, pH 7.3, and 0.15% RR in equal parts). Bacteria at this stage were washed thoroughly in cacodylate buffer, postfixed with uranyl acetate in Michaelis buffer, and embedded in 2.5% (wt/vol) agar. Small blocks were cut at this step, dehydrated in acetone and embedded in Epon 812 as usual, and stained with lead citrate. All fixatives containing RR were prepared fresh due to their unstability.

RESULTS

Formation of *M. avium* spheroplasts. The present report shows the usefulness of the method of spheroplast preparation from mycobacteria as we described earlier (42). It proved useful not only for the fast grower *M. aurum*, but also for the slow grower *M. avium*. Figures 1A and B are transmission electron micrographs of spheroplasts obtained by this method. As compared to the ultrastructure of intact *M. avium* SmD variant (see Fig. 4A), the spheroplasts are swollen spherical structures lacking a distinct cell wall. Scanning electron micrographs show that the spheroplasts (Fig. 1C and D) have a tennis ball shape with wrinkled outer surfaces. Outside projections called "microspherules" are present. The density of these microspherules varies from one preparation to another, and their diameter varies from 50 to 150 nm. On the other hand, intact cells (SmD variants) are typically rod-shaped bacteria with smooth outer surfaces (see Fig. 3B).

Membrane permeability in *M. avium* spheroplasts. In the present investigation, spheroplasts of *M. avium* (SmD variant) gave positive results in seven of nine biochemical tests performed (Table 1), whereas the intact SmT or SmD variants remained negative for all nine tests. This suggested that intact cells were impermeable to various substrates at the wall level.

Figure 2 shows β-glucoside and penicillin G substrate hydrolysis in spheroplasts of *M. avium* as compared to that in intact cells. A very small pH change in intact cells is ascribed to a relatively slow rate of penicillin G diffusion through the wall (Fig. 2B), which would not be enough to produce a positive penicillinase test even after 18 h (Table 1). In contrast, a large pH change was produced by the spheroplast preparation within 3 h. Similarly, *M. avium* spheroplasts liberated nearly 400 nmol of β-glucoside per sample within 7 h, whereas intact *M. avium* cells did not liberate any detectable β-glucoside (Fig. 2A).

Transmission and scanning electron microscopic studies of the transparent and opaque variants of *M. avium*. The preliminary biochemical evidence suggested the importance of the wall architecture in the case of *M. avium*. Since the SmD and SmT variants show some pronounced differences in their pathogenicity and drug resistance (21, 39, 49), we tried in this study to look for the ultrastructural differences between these two variants which might account for these phenomena.

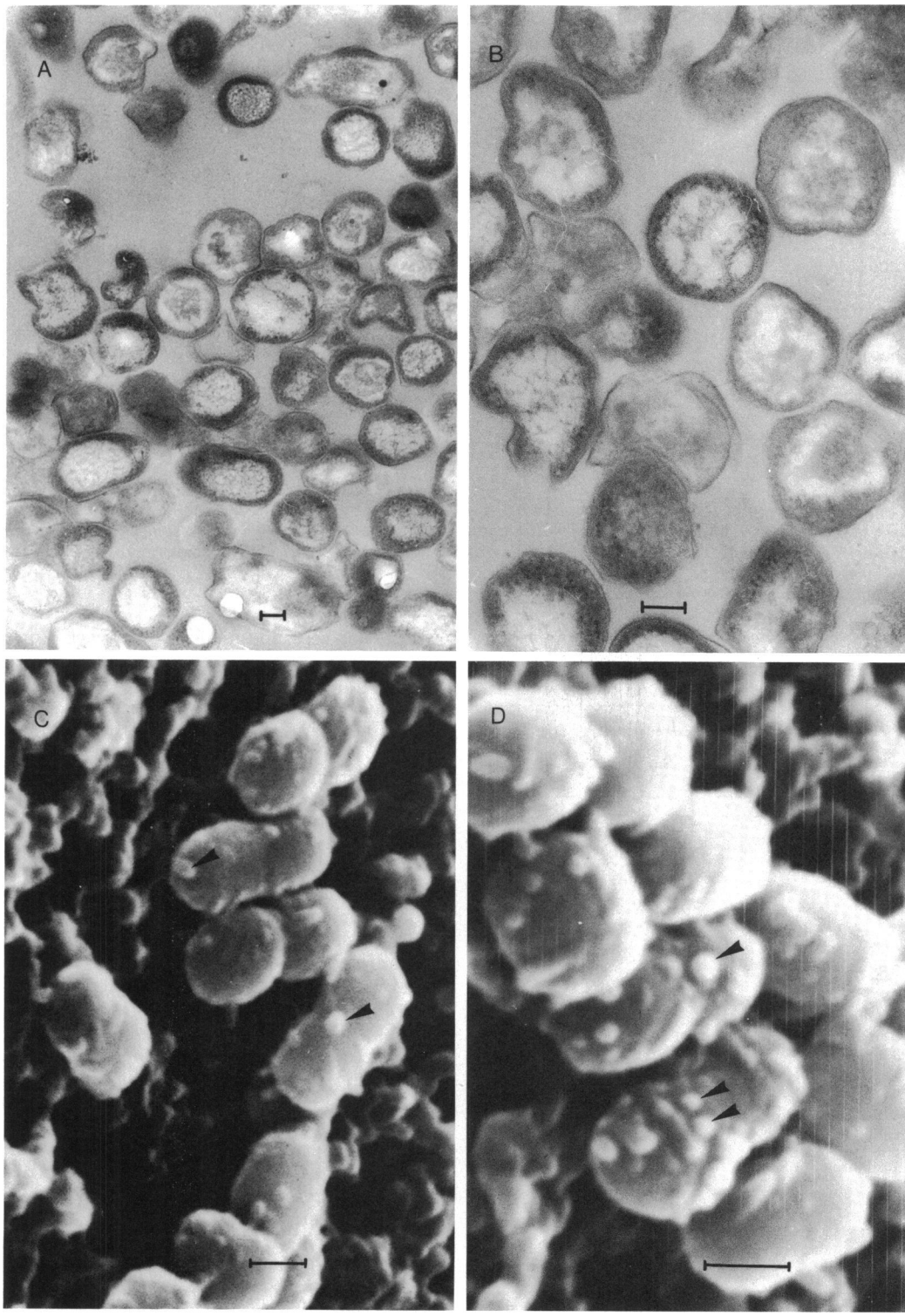

FIG. 1. *Spheroplasts obtained from M. avium SmD variant by transmission and scanning electron microscopy. (A) and (B) Transmission electron micrographs of ultrathin sections of M. avium spheroplasts, characterized as spherical, swollen forms lacking a distinct wall structure. (C) and (D) Scanning electron micrographs showing the tennis ball structure of these spheroplasts. Arrows indicate the outside globular elements called microspherules. These microspherules, 50 to 150 nm in diameter, indicate the physiological state of spheroplasts, and their number varies from one batch of spheroplast preparation to another. Bar, 100 nm.*

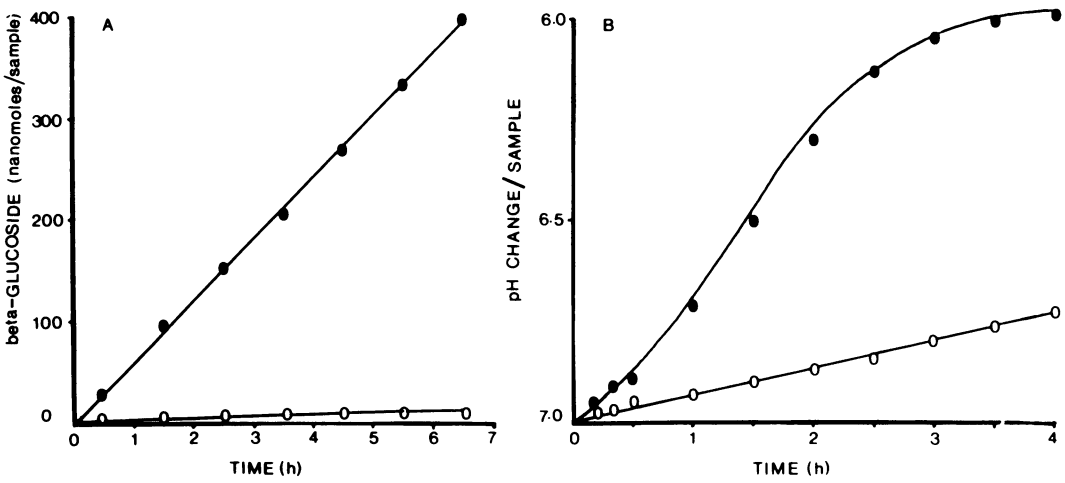

FIG. 2. *Penicillin G and β-glucoside substrate penetration in M. avium intact cells (SmD variants) and their spheroplasts. (A) Spheroplasts could liberate up to 400 nmol of β-glucoside per sample within 7 h, as compared to intact cells which could not liberate any β-glucoside. (B) Penicillin G substrate penetration was observed by the pH change produced due to penicilloic acid production by the M. avium penicillinase. Symbols: (○) intact cells; (●) spheroplasts.*

TABLE 1. *Routine biochemical tests performed for clinical identification of M. avium bacteria*[a]

Biochemical test	Test results with:	
	Intact cells (SmT or SmD variants)	Spheroplasts (SmD variants)
Nitrate reductase (2 h)	0	+
Tween-80 hydrolysis		
1 day	0	0
2 days	0	+
3 days	0	+++
Urease		
2 h	0	0
18 h	0	0
Arylsulfatase (3 days)	0	0
β-Glucosidase		
3 h	0	+
18 h	0	+++
Acid phosphatase (2 h)	0	+++
Alkaline phosphatase (2 h)	0	+++
Penicillinase		
5 h	0	+
18 h	0	+++
Trehalase		
5 h	0	+
18 h	0	+++

[a] Difference of permeability between intact *M. avium* bacteria and their spheroplasts.

Scanning electron micrographs show that the SmT variants have irregular outer surfaces (Fig. 3). Further subcultures of the SmT variants on 7H10 agar produced cells with only slightly irregular surfaces, and finally a SmD variant population was obtained which was characterized by smooth outer surfaces as observed by the scanning electron microscope (Fig. 3B). A few elongated filamentous forms of the *M. avium* bacteria were occasionally observed. We have recently studied such long forms and discussed their growth and cell division (N. Rastogi and H. L. David, J. Gen. Microbiol., in press).

When negatively stained by phosphotungstic acid, SmT variants (Fig. 3C) were medium-sized rods, frequently with a halo-like structure around each one. In contrast, SmD variants (Fig. 3E) were longer forms with no halo structures. Contrary to earlier reports (59, 60), we did not observe any fibrous outer surfaces in either case.

Preliminary transmission electron micrographs (Fig. 3D) suggested that SmT variants had a thicker electron-dense layer, probably corresponding to the cell wall peptidoglycan, followed by an electron-transparent zone of about 9 nm.

Fine structure of the SmT and SmD variants compared by different staining procedures. Figure 4 shows the results of different coloration methods on ultrathin sections of the SmD variant of *M. avium*, compared to the host-recycled SmT variants (Fig. 5). The following observations were made.

(i) Lead citrate. When stained with lead citrate (46), both variants showed ultrastructural details with mesosomes, cytoplasmic membrane, and the cell wall. However, in the SmT variant (Fig. 5A) the cell wall appeared much thicker. The electron-dense portion of the cell wall, probably corresponding to the peptidogly-

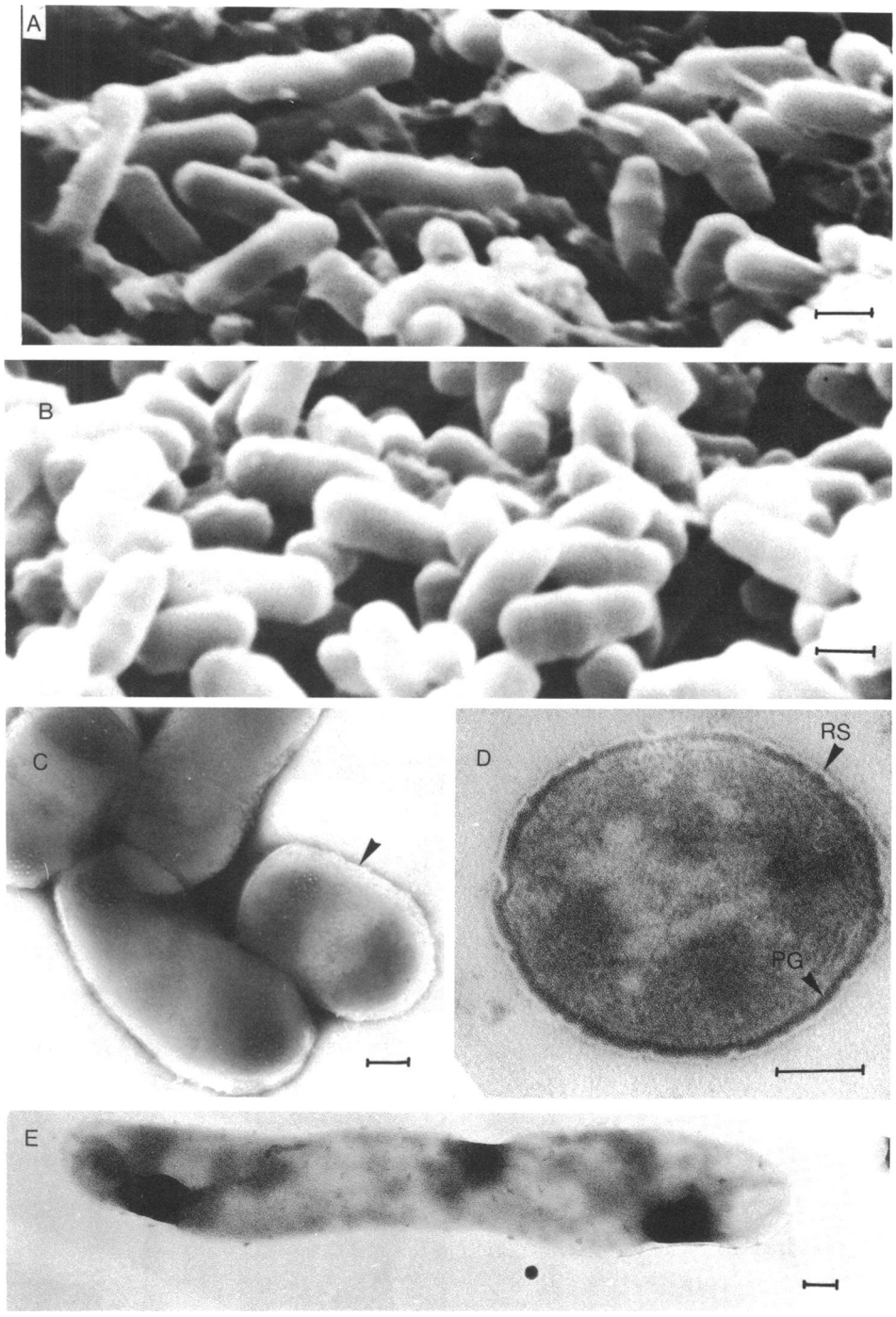

FIG. 3. *Preliminary electron microscopic studies indicating the presence of an outer regularly structured (RS) layer in SmT variants. Scanning electron micrographs show that SmT variants contain a rough, irregular outer surface (A), whereas the SmD variants have smooth outer surfaces without any irregularities (B). In transmission electron micrographs of the negatively stained material, halolike structures are seen around SmT variants (C; shown by an arrow), whereas SmD variants contain no such halolike structures (E). Finally, a transmission electron micrograph of an ultrathin section of an SmT variant shows a visible regularly structured layer around a thick electron-dense portion of the cell wall, probably representing the peptidoglycan (PG) layer (D). Similar regularly structured and thick electron-dense layers were absent in SmD variants. Bar, 100 nm.*

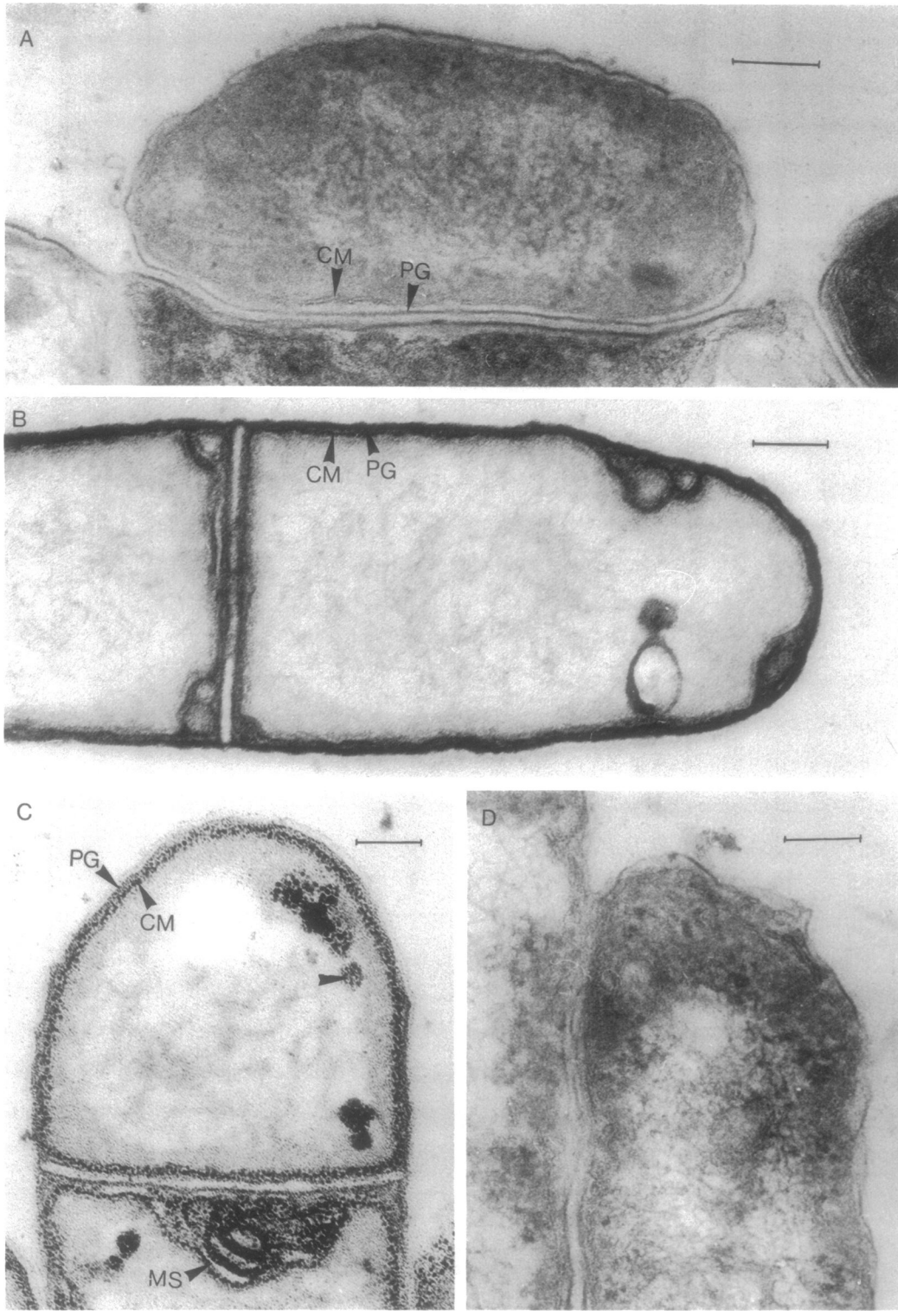

FIG. 4. *Fine structure of the opaque (SmD) variant of M. avium. (A) Bacteria stained by lead citrate present a normal mycobacterial cell wall structure with a rich cytoplasm. Cytoplasmic membrane (CM) and cell wall with a peptidoglycan layer (PG) are visible. (B) Stained by the method of Rambourg (41), the SmD variants show a continuous band all around the cell, representing the peptidoglycan layer. The outer layer of the cytoplasmic membrane is equally stained. (C) SP (61) stains the cytoplasmic membrane and mesosomes (MS). The peptidoglycan-containing layer is not stained by this method. (D) RR stain (30) does not show the presence of any polysaccharidic outer layer from SmD variants. Bar, 100 nm.*

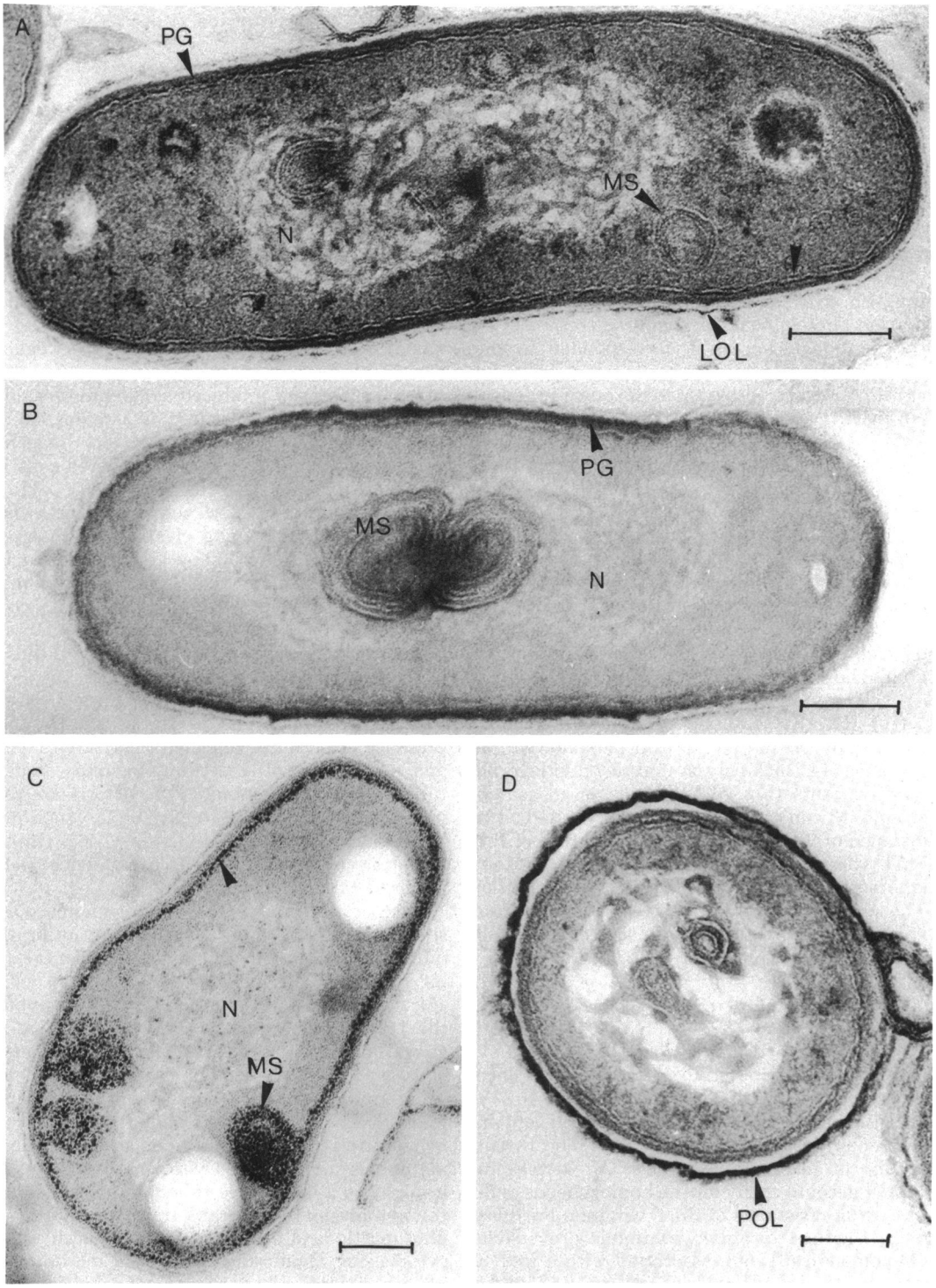

FIG. 5. *Fine structure of the host-recycled transparent (SmT) variant of M. avium. Stained by lead citrate, SmT variants show a thick peptidoglycan (PG) layer, well-developed mesosomes (MS), nucleus (N), and a lipid outer layer (LOL). (B) Colored by the method of Rambourg (41), SmT variants have a peptidoglycan layer about 14 nm thick, as well as well-developed mesosomes. (C) SP (61) stains the cytoplasmic membrane (CM) and mesosomes. (D) RR stain (30) shows the presence of a well-developed polysaccharidic outer layer (POL). This layer, about 13 nm thick, is situated at a distance of 9 to 12 nm from the cell wall of SmT variants. Bar, 100 nm.*

can layer, was about 14 nm in the SmT variant (Fig. 5A), but only 7 nm in the SmD variant (Fig. 4A). An electron-transparent zone was present in SmT variants, probably corresponding to the outer lipid layer of the typical mycobacterial cell wall (25). In SmD variants, however, this layer was difficult to distinguish because it was not limited on its external side by a thin dense layer as in SmT variants.

(ii) Phosphotungstic acid. With phosphotungstic acid stain (Fig. 4B and 5B) the wall portion that appears probably corresponds to the peptidoglycan, as it corresponded to the electron-dense layer observed earlier with lead citrate. An electron-transparent zone was apparent in SmT variants.

(iii) SP. SP coloration showed the same location of the silver grain precipitates in SmT and SmD variants (Fig. 4C and 5C), i.e., along the cytoplasmic membrane, in the periplasmic space, and inside mesosomes. The same silver grain pattern has been reported in *Mycobacterium phlei* (40) and is probably characteristic of the *Mycobacterium* species, in contrast to the *Bacillus* species (40, 45), in which no silver deposits are observed in the cell wall. The silver grains show the presence of polysaccharides with α1-2 glycoside bonds.

(iv) RR. RR staining performed on intact bacteria by the method of Luft (30) showed the presence of a thick polysaccharide outer layer in SmT variants (Fig. 5D). This layer measured around 13 nm in width and was located at a distance of 9 to 12 nm from the cell wall of the SmT variant. This outer layer was absent in laboratory-maintained SmD variants (Fig. 4D). The polysaccharide outer layer observed after RR coloration appears to lie approximately 3 nm outside the lipid outer layer.

DISCUSSION

The multiple drug resistance of *M. avium* could not be attributed to mutator effects since this strain was neither more susceptible to UV radiation than other bacteria, nor deficient in UV-repair mechanisms (9, 14, 32). The recent finding of plasmid DNA from *M. avium* (8) cannot account solely for the homogeneous multiple drug resistance of SmT variants. Furthermore, the SmT to SmD variation is a reversible phenomenon (31, 66), associated with a loss of virulence and multiple drug resistance (21, 49).

In contrast to *M. aurum*, the radiomimetic properties of the antibiotic mitomycin C and the effects of UV irradiation are not associated in *M. avium* (9; David, in press); a likely explanation is the impermeability of *M. avium* cell wall to this antibiotic.

Results of the present investigation confirmed this exclusion mechanism at the wall level: spheroplast preparations from *M. avium* SmD variant gave positive results in seven of nine biochemical tests performed (Table 1). This also suggested the crypticity of certain enzymes in *M. avium*, which was further confirmed by comparing penicillin G and β-glucoside substrate penetration between the intact SmD cells and their spheroplasts (Fig. 2). Our efforts to assay nitrate reductase were not fruitful; it was weakly positive, and a quantitative assay, measuring the red color intensity produced at 540 nm, was not possible. Trehalase measured by assaying liberated glucose by PGO-enzyme color reagent (Sigma) was highly active and appeared constitutive.

The spheroplasts of *M. avium* appear to be living, since by scanning electron microscopy (Fig. 1C and D) they show external globular projections called microspherules. Similar microspherules have been reported earlier in yeast protoplasts (36), and a relation between their presence and the physiological activity of these protoplasts has been observed.

Preliminary scanning and transmission electron microscopy of SmT variants suggested the presence of an outer regularly structured layer and a thicker wall structure. Regularly structured layers from a number of bacteria are reported to absorb iron strongly (6, 7), which inhibits normal leukocyte function (7, 65). Iron-chelating compounds from *M. avium* have been reported earlier (33).

Further transmission electron microscopic studies comparing the two strains by utilizing different coloration methods gave some interesting results. (i) In contrast to *Bacillus* sp. (40, 45), the SP method revealed in both variants the presence of a polysaccharide rich in α1-2 glycol bonds along the cytoplasmic membrane. This cytochemical reaction can reveal teichoic acids (45). Apparently no teichoic acids were found in the analysis of isolated cell walls of mycobacteria (4). However, since the stained material is not associated with the cell wall itself, it might be a better idea to look for this acid in the cytoplasmic membrane fraction. (ii) Phosphotungstic acid staining colored the cell wall, cytoplasmic membrane, and inside mesosomes in both variants. The material stained at the cell wall level probably reflects the presence of peptidoglycan since this layer did not react with SP stain. However, in contrast to *Bacillus* (40, 45), it can be concluded that the presence of carbo-

hydrates on the cytoplasmic membrane and mesosome reacting with both phosphotungstic acid and SP is peculiar to mycobacteria. (iii) Stained by RR, the SmT variant showed the presence of a thick layer of acidic polysaccharides which was totally absent in SmD variants. Thus the main ultrastructural difference between the SmD and SmT variants appears to be at the cell wall level.

We think that this outer layer might be responsible for causing the impermeability of SmT variants in part towards many substrates and drugs, which is implied by its virulence and host invasion capacities. Low resistance to antibiotics in *Escherichia coli* and *Neisseria gonorrohoeae* (50, 51) has been earlier explained to be caused at the wall level, since ethylenediaminetetraacetic acid-treated bacteria showed increased permeability to antibiotics. The same appears to be true for *M. avium* also. This exclusion at the wall level probably depends on a number of factors including molecular size, polarity and charges, etc.

The pronounced pathogenicity of SmT variants as compared to SmD variants might also reside in part in this outer polysaccharide layer. Laboratory studies have shown that purified bacterial capsular polysaccharides have no toxic effects as such on the host, but the hypothesis that these capsular polysaccharides are necessary if not sufficient for host invasion by bacteria is confirmed by existing experimental data. For example, uncapsulated bacteria are susceptible to the killing action of complement alone (29, 55), whereas their encapsulated counterparts are resistant to the direct effects of complement alone (22, 38, 44). Anderson et al. (2) proposed that some bacteria would exert a toleragenic effect by the release of their outer polysaccharidic sheath in vivo. Sutherland (56) proposed that pathogenic encapsulated bacteria are able to release their capsular polysaccharides readily.

Although this report shows for the first time that the outer polysaccharide layer is associated with colony type transition in M. avium, Draper and Rees (18, 19) showed the presence of an outer electron-transparent zone in *M. lepraemurium* inside host cells and proposed a hypothesis concerning its probable role in the mycobacterial defense mechanism. Similar evidence has accumulated concerning *M. leprae* bacilli in situ (20, 67). Studies on this electron-transparent zone, which is made up of parallel fibrils, show the presence of key amino acids and 6-deoxyhexoses (19). Draper later studied similar fibrillar material from an in vitro-cultivated strain of *M. avium* and considered it to be a C mycoside (17). Recently Barrow et al. (5) isolated a superficial cell wall sheath from bacteria of the *M. avium* complex grown in vitro on a 7H11 medium and showed its peptidoglycolipid nature.

The polysaccharide outer layer we found in host-recycled SmT variants of *M. avium* was absent in SmD variants of the same bacteria, obtained by successive subcultures of the original ATCC strain in the laboratory. This suggests that this outer layer probably is different from the superficial cell wall sheath isolated from the *M. avium* complex (5).

In our opinion, the presence of an outer layer associated with the colony type variation, differential drug susceptibility, and virulence of *M. avium* can be explained only at the level of genetic expression. An interesting model to explain this phenomenon could be a metastable gene controlled by an invertible promoter. In one of its orientations, this control gene would permit synthesis of a repressor of an unlinked gene responsible for one of the colony types, whereas in its inverted position the other colony type would be expressed. Recently, enough experimental evidence has accumulated to suggest the presence of such an invertible gene control phenomenon for the phase type variation in *Salmonella* (52–54), for *flu*$^+$ and *flu* transition states in *E. coli* K-12 (16), and for different host specificities of the bacteriophage Mu (62). Though such a possibility cannot be excluded for the colony type variation in *M. avium*, genetic confirmation would not be easy.

Apart from the outer layer, other factors might also be associated with colony type transition in *M. avium*. A rather similar colony type variation has been reported in gonococci (27), and some recent studies have shown important differences among the surface proteins isolated from opaque and transparent colonies of gonococci (23, 24, 57). The probable role of these surface proteins in the infective process at the level of adhesion and entry to host cells has been recently discussed (58). A similar investigation in *M. avium* might be fruitful.

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