CURRENT MICROBIOLOGY Vol. 13 (1986), pp. 237–242

Current Microbiology

Triple-layered Structure of Mycobacterial Cell Wall: Evidence for the Existence of a Polysaccharide-rich Outer Layer in 18 Mycobacterial Species

Nalin Rastogi,[1] Claude Frehel,[2] and Hugo L. David[1]

[1] Tuberculosis and Mycobacteriology Unit, Department of Bacteriology and Mycology, and [2] Electron Microscopy Unit, Department of Molecular Biology, Pasteur Institute, Paris, France

Abstract. An additional outer wall layer, composed mainly of acidic polysaccharides, was revealed in 18 species of mycobacteria by ruthenium red staining in electron microscopy. This report deals with the description of this additional layer. The ultrastructural implications at the level of the mycobacterial cell wall model and also the possible physiological role of this layer are briefly discussed.

Various models describing the mycobacterial wall architecture have been proposed [1, 5, 9] that consider only the ultrastructural data revealed by classic lead citrate staining [1, 6], chemical extractions and subsequent negative stainings [5], or freeze-fracture studies [10], which have been continued recently on various species of mycobacteria [2, 21]. Although the mycobacterial cell wall was proposed to be a triple-layered structure [5], composed of a basal peptidoglycan layer (PG), an intermediate electron-transparent layer (ETL) probably composed of arabinogalactan–mycolate complexes, and an outer-dense layer (ODL) that was supposed to be formed by slight heavy-metal deposits bordering the lipid-rich ETL [11, 16], none of these models took into consideration the existence of a true outer layer for mycobacteria in general.

While studying the colony-type variation in *Mycobacterium avium,* we revealed an additional polysaccharide outer layer (POL) by ruthernium red (RR) staining in the host-recycled SmT variants that was absent in the laboratory-maintained SmD variants [13]. This POL could be visualized only after the specific RR staining used to reveal surface acidic polysaccharides or glycocalyx [8], and was not detectable by the classic lead citrate staining. We later reported POL from armadillo-grown *M. leprae* [15, 17], and the rapidly growing mycobacteria *M. aurum* [3, 11]. Furthermore among the related mycolic-acid-containing microorganisms, *Nocardia asteroides* was POL positive whereas *Corynebacterium pseudotuberculosis* was POL negative [16, 17]. We recently reported POL in the mycolic-acid-containing bacterium "Gordona aurantiaca" also [18]. (The genus name "Gordona" does not appear in the approved list of bacterial names (*International Journal of Systematic Bacteriology* 30:225–420 1980) or the updated list (*International Journal of Systematic Bacteriology* 35: 375–376, 1985) and hence is enclosed in quotation marks.)

As far as the possible functions of POL are concerned, the POL-positive SmT colony-type variants of *M. avium* [13] were found to contain eight additional surface antigens as compared with the POL-negative SmD colony-type variants [23], and were also more negatively charged [11]. In phagocytized *M. avium,* an electron-transparent zone (ETZ) surrounded the bacteria inside phagosomes and protected them against phagolysosomal dissolution by lessening the diffusion of lysosomal enzymes [20]. This ETZ appeared in the first hours of phagocytosis around the bacterial surface in areas where the POL started to disappear [20], indicating that POL may be partly responsible for stimulating ETZ formation. Finally we showed recently that antibiotic colistin (polymyxin E) was active on *M. aurum,* not only because of its action on the cytoplasmic membrane, but also on the cell surface, as it completely disorganized the POL [3]. This gave preliminary support to a recent hypothetical model [9] according to which covalently bound mycolic

Address reprint requests to: Dr. Nalin Rastogi, Service de la Tuberculose et des Mycobactéries, Institut Pasteur, 25 Rue du Dr. Roux, F-75015 Paris, France.

Table 1. Summary of ultrastructural data after ruthenium red staining of mycobacteria

Mycobacterium	Localization of POL		Nature of POL			% Cells with POL		
	Distance from PG (ETL, nm)	Width of the RR positive material (nm)	Regular and continuous	Regular but broken	Regular but patchy	Less than 50%	50%–80%	80%–100%
1) *tuberculosis*	10.0 ± 0.5	8.0 ± 1.0	+	–	–	–	+	–
2) *bovis*	10.0 ± 0.5	7.0 ± 1.0	+	–	–	–	+	–
3) *africanum*	10.5 ± 0.5	6.5 ± 1.0	+	–	–	–	+	–
4) *avium* (SmT)[a]	10.0 ± 1.0	10.5 ± 1.5	+	–	–	–	–	+
5) *intracellulare*	10.0 ± 0.5	11.5 ± 1.0	–	–	+	–	+	–
6) *scrofulaceum*	11.0 ± 0.5	5.0 ± 0.5	+	–	–	–	+	–
7) *szulgai*	10.5 ± 0.5	6.0 ± 0.5	+	–	–	–	–	+
8) *xenopi*	10.5 ± 0.5	6.5 ± 0.5	+	–	–	–	+	–
9) *kansasii*	9.5 ± 0.5	5.0 ± 0.5	+	–	–	–	–	+
10) *simiae*	11.5 ± 0.5	5.5 ± 0.5	+	–	–	–	–	+
11) *fortuitum*	10.5 ± 0.5	10.0 ± 0.5	+	–	–	–	–	+
12) *chelonei*	11.0 ± 0.5	7.5 ± 1.0	–	+	–	+	–	–
13) *marinum*	11.5 ± 0.5	8.5 ± 0.5	+	–	–	–	–	+
14) *phlei*	11.0 ± 0.5	5.0 ± 0.5	+	–	–	–	–	+
15) *smegmatis*	10.5 ± 0.5	10.0 ± 0.5	+	–	–	–	–	+
16) *fallax*	10.0 ± 0.5	7.0 ± 0.5	+	–	–	–	–	+
17) *aurum*	9.5 ± 0.5	8.0 ± 0.5	+	–	–	–	–	+
18) *leprae*[b]	10.5 ± 0.5	10.0 ± 0.5	+	+	–	–	+	–

[a] Data on host-recycled, smooth, dome-shaped, transparent variant only, as a regular POL is absent from laboratory-maintained, smooth, dome-shaped, opaque variant (SmD), which contains few RR-positive residues only.
[b] In the case of armadillo-grown *M. leprae*, bacteria are in various stages of ultrastructural preservation; hence one can observe both a regular and continuous POL, as well as a broken POL in lysing bacilli.

acids on the mycobacterial cell surface must form a coherent outer monolayer and free complex lipids (possibly lipopolysaccharides or lipoproteins) might insert in it as sealers to give a functional bilayer.

All the above observations showed the importance of mycobacterial POL as antigenic determinants, and also for their intracellular survival, drug action, and various surface properties. For the above reasons, we decided to investigate the existence of POL in major representative strains from 18 mycobacterial species.

Materials and Methods

Organisms and growth. In this investigation, the type strains of the following species were used: *Mycobacterium tuberculosis* ATCC 27294, *M. bovis* ATCC 19210, *M. africanum* ATCC 25420, *M. avium* ATCC 15769, *M. intracellulare* ATCC 13950, *M. scrofulaceum* ATCC 19981, *M. szulgai* NCTC 10831, *M. xenopi* NCTC 11042, *M. kansasii* ATCC 12478, *M. simiae* ATCC 25275, *M. fortuitum* ATCC 6841, *M. chelonei* NCTC 946, *M. marinum* ATCC 927, *M. phlei* ATCC 11758, *M. smegmatis* ATCC 19420, and *M. fallax* ATCC 35219. *Mycobacterium aurum* CIPT 141210005 was not a type strain, but was included in this study as it was the most studied strain from our laboratory [3, 7, 12, 14].

Mycobacterium leprae was isolated and purified from liver and nodes of an experimentally infected armadillo WR 45 (kindly supplied by P. Draper, National Institute for Medical Research, Mill Hill, London) and also from liver tissues of experimentally infected armadillos AV and AP (provided through the *M. leprae* bank of the Pasteur Institute).

Mycobacterium avium was used both in its host-recycled SmT (smooth, dome shaped, and transparent) and laboratory-maintained SmD (smooth, dome shaped, and opaque) colony-type variants, which were obtained as reported earlier [13].

All the above organisms (except *M. leprae*) were grown as slants in the Lowenstein–Jensen medium at 37°C, except *M. marinum*, *M. chelonei*, and *M. fallax*, which grew better at 30°C. Tubes with 5 ml of the RVB10 medium [19] were inoculated with 0.1 ml of bacterial suspension containing about 10^6 viable cells, and the bacteria were harvested by centrifugation when the optical density (650 nm) of the bacterial culture reached about 0.2 as measured by a Coleman Junior II spectrophotometer.

Electron microscopy. The bacterial pellets were prefixed for 2 h at 4°C in a mixture of 2.5% (wt/vol) paraformaladehyde and 2.5% (wt/vol) glutaraldehyde prepared in 0.1 *M* cacodylate buffer (pH 7.2), containing 5 m*M* of $CaCl_2$ and $MgCl_2$, and 0.05% (wt/vol) of ruthenium red (RR). Bacteria were washed in the same buffer and fixed overnight at 4°C in 2.5% (wt/vol) glutaraldehyde and 0.05% (wt/vol) of RR stain in cacodylate buffer. Cells were then washed and postfixed in OsO_4 and uranyl acetate, concentrated in 2% (wt/vol) agar, dehydrated in acetone, and embedded in epon as reported earlier for mycobacteria [13, 15–18]. RR stain was present during the postfixation and dehydration steps also. Thin sections were cut on LKB Ultratome II, mounted on Formvar-coated copper grids, and observed under a Siemens 101 electron microscope.

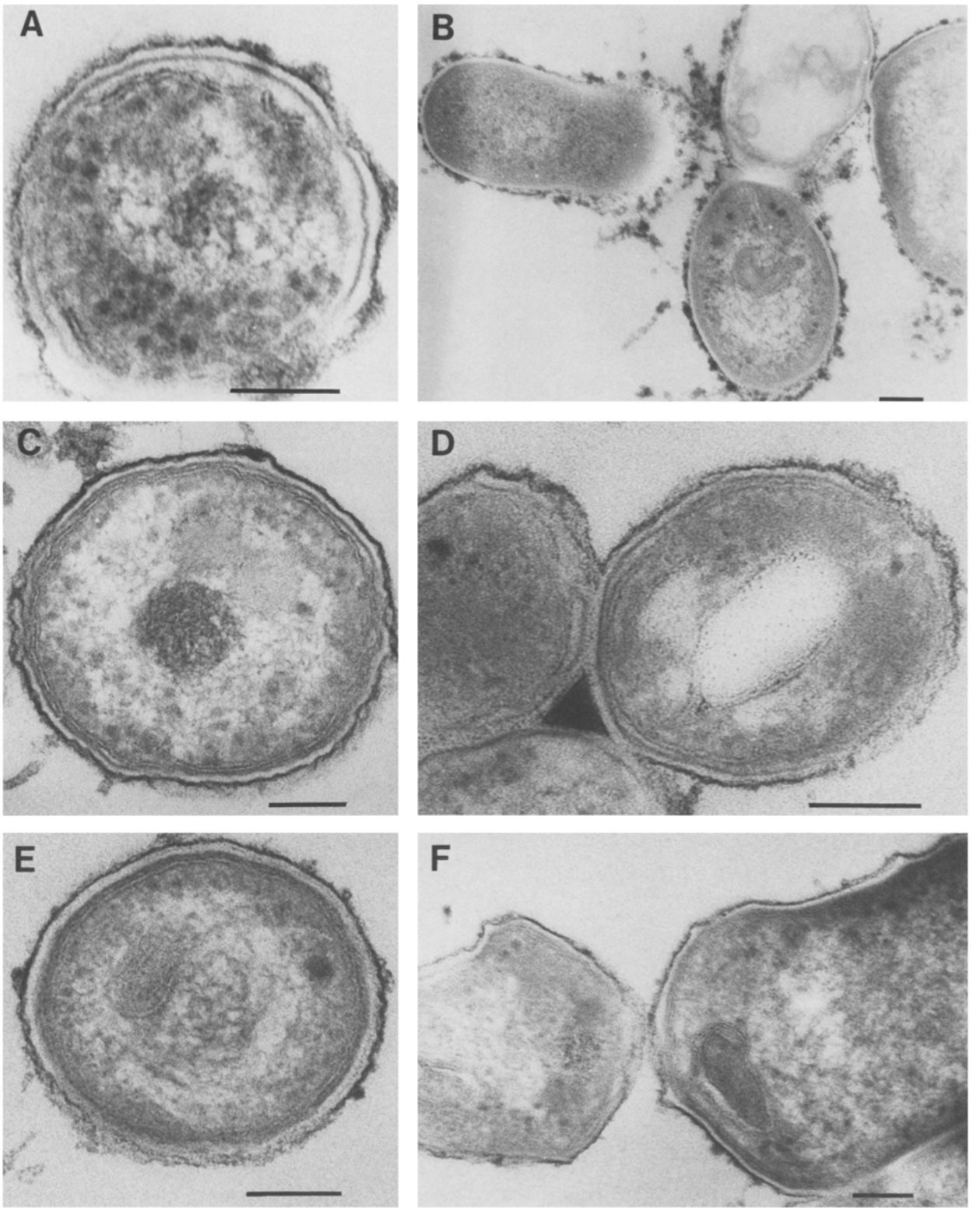

Fig. 1. Ultrastructural localization of polysaccharide outer layer (POL) by ruthenium red (RR) staining: (A) *Mycobacterium africanum*, (B) *M. intracellulare*, (C) *M. szulgai*, (D) *M. xenopi*, (E) *M. scrofulaceum*, and (F) *M. kansasii*. Bar marker = 100 nm.

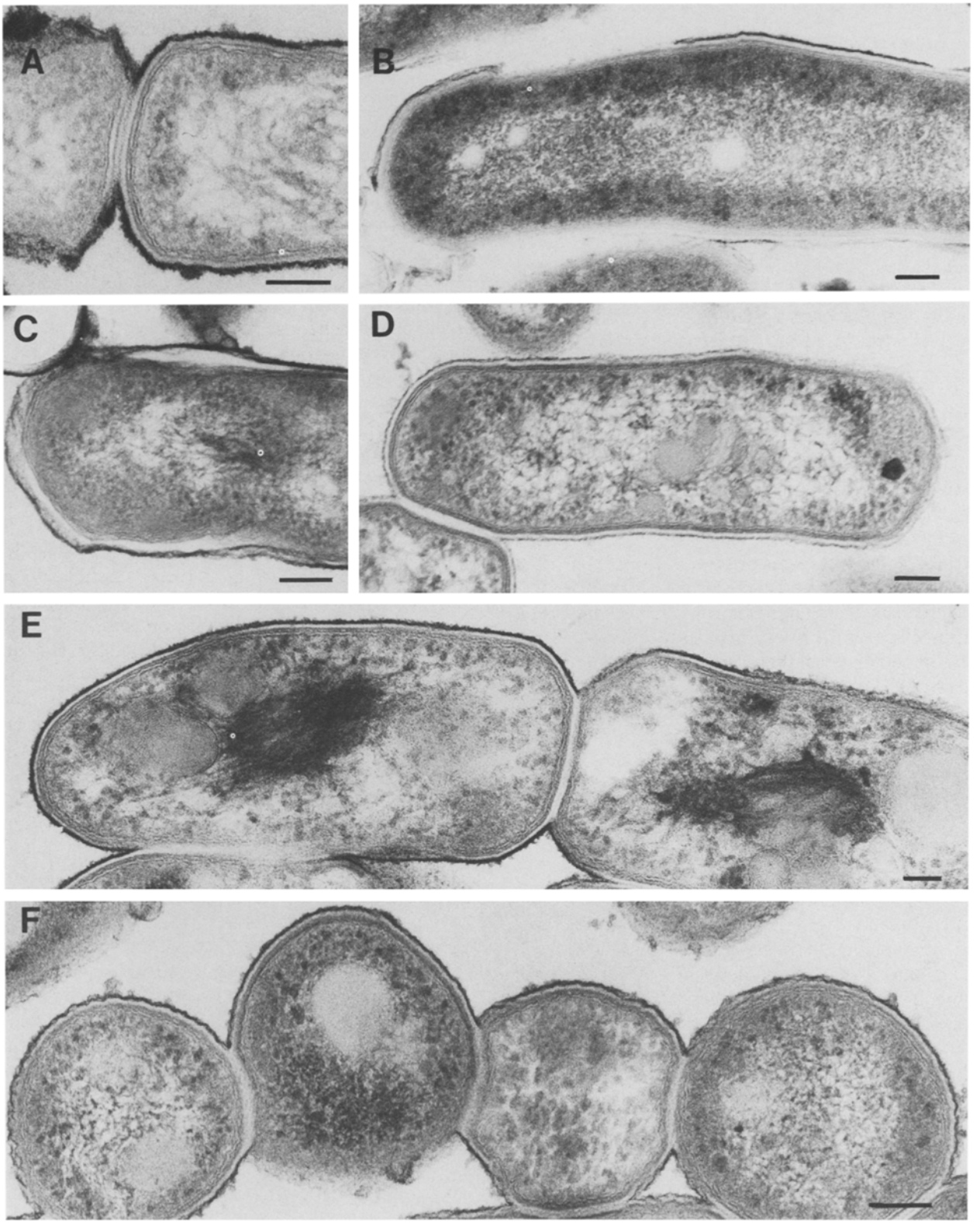

Fig. 2. Ultrastructural localization of polysaccharide outer layer (POL) by ruthenium red (RR) staining: (A) *Mycobacterium fortuitum*, (B) *M. chelonei*, (C) *M. marinum*, (D) *M. pheli*, (E) *M. smegmatis*, and (F) *M. fallax*. Bar marker = 100 nm.

Results

The ultrastructural data are summarized in Table 1, and a few representative electron micrographs are shown in Figs. 1 and 2. As shown in Table 1, all 18 mycobacterial species had a polysaccharide outer layer (POL), which was regular and continuous in a majority of species, except for *M. chelonei* (Fig. 2B) where it was often broken. In *M. intracellulare* (Fig. 1B), POL was regular but patchy. POL was absent in the SmD colony-type variants of *M. avium*, but many cells had rare RR-positive residues on their surface (Table 1). In armadillo-grown *M. leprae*, the bacteria isolated from infected tissues existed in various stages of ulstrastructural preservation [15], and consequently the POL was regular and continuous in morphologically intact, well-preserved bacilli, whereas it was often broken in lysing bacteria.

The width of the ETL that separated the POL from the basal peptidoglycan (PG) layer was almost constant for all the species and measured 9.5 ± 0.5 to 11.5 ± 0.5 nm whereas the width of POL varied, according to the species observed, from 5.0 ± 0.5 to 11.5 ± 1.0 nm (Table 1). The proportion of the POL-positive bacteria in our electron-microscopic preparations was always very high except for *M. chelonei*, in which less than 50% bacteria were POL positive (Table 1).

The electron micrographs of *M. avium*, *M. aurum*, and *M. leprae* are not included in Figs. 1 and 2, as detailed studies have been published [3, 11, 13, 15–18].

Discussion

Although the mycobacterial cell wall has often been described as a triple-layered structure [1, 5], there has been some controversy over whether the middle and outer wall layers described by Imaeda et al. [5] and the L_1 and L_2 layers described by Barksdale and Kim [1] exist as true mycobacterial wall layers. According to Draper [4], these layers observed in the electron microscope would correspond to the outermost electron-dense "debris" around the cells, appearing as true layers because of artifactual phenomena during processing for electron microscopy. Similar views were exposed earlier by Kölbel [6].

The present work showed the existence of a polysaccharde-rich outer layer (POL) in 18 different species of mycobacteria. This POL was not visualized earlier by classic lead citrate coloration, which only occasionally revealed a very thin deposit, as heavy metals sometimes demarcated the interface of ETL and POL. The width of ETL that separated POL from basal PG layer was almost constant (9.5 ± 0.5 to 11.5 ± 0.5 nm), but the width of RR-positive POL varied widely (5.0 ± 0.5 to 11.5 ± 1.0 nm) according to the species studied (Table 1). It is possible that the width of POL may vary according to the culture medium used or stage of growth. It would be interesting to verify whether different culture conditions could change the nature or the appearance of the POL.

The RR stain is performed on intact bacteria and does not penetrate the bacterial cell wall, therefore picking up only the surface acidic polysaccharides. In our case it reveals the POL as a true mycobacterial wall layer, but unfortunately it does not enable us to determine the precise chemical nature of these surface polysaccharides or other surface components that exist in association with them and are responsible for the differences observed in the surface properties of various mycobacteria [11].

Recently, the tannic acid staining method in electron microscopy [22] appeared to reveal an outer layer in *M. vaccae* that has the same location as the POL and probably corresponds to it. The RR stain is more specific, however, as it revealed the POL even when the lead citrate background staining was omitted (which we always used as a control), whereas the tannic acid method could not be used without background staining and consequently did not specifically reveal the POL.

Mycobacterial POL must have some important functions. Examples include the exclusion as a mechanism of drug resistance in *M. avium* [13], and the fate of phagocytized *M. avium* in mice bone-marrow-derived macrophages [20], where one could observe the formation of a protective electron-transparent zone (ETZ) around bacilli in areas of POL dissolution. The POL-positive *M. aurum* did not form a similar ETZ in these macrophages [20], however, and was consequently degraded. This implies that POL is a simplified ultrastructural representation of a highly complex chemical structure, and establishing the exact relationship between the two would require chemical extractions and subsequent specific cytochemical stainings.

ACKNOWLEDGMENTS

We thank M. C. Potar, R. Daty, and S. Cadou for excellent technical assistance, and are grateful to L. G. Chevance for electron-microscopic facilities and J. Lobrot for photographic illustrations.

Literature Cited

1. Barksdale L, Kim KS (1977) *Mycobacterium*. Bacteriol Rev 41:217–372
2. Benedetti EL, Dunia I, Ludosky MA, Van Man N, Trach DD, Rastogi N, David HL (1984) Freeze-etching and freeze-fracture structural features of cell envelopes in mycobacteria and leprosy-derived corynebacteria. Acta Leprol (Geneve) 2:237–248
3. David HL, Rastogi N (1985) Antibacterial action of colistin (polymyxin E) against *Mycobacterium aurum*. Antimicrob Agents Chemother 27:701–707
4. Draper P (1982) The anatomy of mycobacteria. In: Ratledge C, Stanford J (eds) The biology of myobacteria. London: Academic Press, pp 9–52
5. Imaeda T, Kanetsuna F, Galindo B (1968) Ultrastructure of cell walls of genus *Mycobacterium*. J Ultrastruct Res 25:46–63
6. Kölbel HK (1978) Anatomy of the mycobacterial cell. Ann Microbiol (Inst Pasteur) 129A:29–37
7. Lévy-Frébault V, David HL (1979) Mutations affecting pigment synthesis in *Mycobacterium aurum*. J Gen Microbiol 115:317–323
8. Luft JH (1971) Ruthenium red and violet. I. Chemistry, purification, methods of use for electron microscopy and mechanism of action. Anat Rec 171:347–368
9. Minnikin DE (1982) Lipids: complex lipids, their chemistry, biosynthesis and roles. In: Ratledge C, Stanford J (eds) The biology of mycobacteria. London: Academic Press, pp 95–184
10. Nguyen HT, Trach DD, Van Man N, Ngoan TH, Dunia I, Ludosky-Diawara MA, Benedetti EL (1979) Comparative ultrastructure of *Mycobacterium lepraemurium* cell envelopes. J Bacteriol 138:552–558
11. Picard B, Frehel C, Rastogi N (1984) Cytochemical characterization of mycobacterial outer surfaces. Acta Leprol (Geneve) 2:227–235
12. Rastogi N, David HL (1981) Ultrastructural and chemical studies on wall-deficient forms, spheroplasts and membrane vesicles from *Mycobacterium aurum*. J Gen Microbiol 124:71–79
13. Rastogi N, Frehel C, Ryter A, Ohayon H, Lesourd M, David HL (1981) Multiple drug resistance in *Mycobacterium avium*: is the wall architecture responsible for the exclusion of antimicrobial agents? Antimicrob Agents Chemother 20:666–677
14. Rastogi N, David HL, Rafidinarivo E (1983) Spheroplast fusion as a mode of genetic recombination in mycobacteria. J Gen Microbiol 129:1227–1237
15. Rastogi N, Frehel C, Ryter A, David HL (1982) Comparative ultrastructure of *Mycobacterium leprae* and *M. avium* grown in experimental hosts. Ann Microbiol (Inst Pasteur) 133B:109–128
16. Rastogi N, Frehel C, David HL (1984) Evidence for the taxonomic utility of periodic acid–thiocarbohydrazide–silver proteinate cytochemical staining for electron microscopy. Int J Syst Bacteriol 34:293–299
17. Rastogi N, Frehel C, David HL (1984) Cell envelope architectures of leprosy-derived corynebacteria. *Mycobacterium leprae* and related organisms: a comparative study. Curr Microbiol 11:23–30
18. Rastogi N, David HL, Frehel C (1985) Ultrastructure of "Gordona aurantiaca" Tsukamura 1971. Curr Microbiol 13:51–56
19. Redmond WB, Ward DM (1966) Media and methods for phage-typing mycobacteria. Bull WHO 35:563–568
20. Ryter A, Frehel C, Rastogi N, David HL (1984) Macrophage interaction with mycobacteria including *M. leprae*. Acta Leprol (Geneve) 2:211–226
21. Takeo K, Kimura K, Kuze F, Nakai E, Nonaka T, Nishiura M (1984) Freeze-fracture observations of the cell wall and peribacillary substances of various mycobacteria. J Gen Microbiol 130:1151–1159
22. Takade A, Takeya H, Taniguchi H, Mizuguchi Y (1983) Electron microscopic observations of cell division in *Mycobacterium vaccae* V_1. J Gen Microbiol 129:2315–2320
23. Thorel MF, David HL (1984) Specific surface antigens of SmT variants of *Mycobacterium avium*. Infect Immun 43:438–439