The cell envelope of mycobacteria, which include the causative agents of tuberculosis and leprosy, is crucial for their success as pathogens. Despite a continued strong emphasis on identifying the multiple chemical components of this envelope, it has proven difficult to combine its components into a comprehensive structural model, primarily because the available ultrastructural data rely on conventional electron microscopy embedding and sectioning, which are known to induce artifacts. The existence of an outer membrane bilayer has long been postulated but has never been directly observed by electron microscopy of ultrathin sections. Here we have used cryo-electron microscopy of vitreous sections (CEMOVIS) to perform a detailed ultrastructural analysis of three species belonging to the Corynebacterineae suborder, namely, Mycobacterium bovis BCG, Mycobacterium smegmatis, and Corynebacterium glutamicum, in their native state. We provide new information that accurately describes the different layers of the mycobacterial cell envelope and challenges current models of the organization of its components. We show a direct visualization of an outer membrane, analogous to that found in gram-negative bacteria, in the three bacterial species examined. Furthermore, we demonstrate that mycolic acids, the hallmark of mycobacteria and related genera, are essential for the formation of this outer membrane. In addition, a granular layer and a low-density zone typifying the periplasmic space of gram-positive bacteria are apparent in CEMOVIS images of mycobacteria and corynebacteria. Based on our observations, a model of the organization of the lipids in the outer membrane is proposed. The architecture we describe should serve as a reference for future studies to relate the structure of the mycobacterial cell envelope to its function.

The suborder of Corynebacterineae is a distinct group of gram-positive bacteria and comprises mycobacteria and other genera such as Corynebacterium, Rhodococcus, and Nocardioides. The medical importance of the group is enormous; it includes the causative agents of human diseases such as tuberculosis and leprosy, Mycobacterium tuberculosis and Mycobacterium leprae, respectively. The structure of the cell envelope of these bacteria has been the subject of numerous studies because it is already clear that the powerful biological activities of known wall components contribute significantly to the disease process. Indeed, lipids isolated from the cell envelope can elicit responses by the host immune system very similar to the responses generated by M. tuberculosis infection (e.g., granuloma formation) (22).

Schematically, the envelope of this bacterial group is composed of a typical plasma membrane (PM) surrounded by a cell wall core, which, in turn, is surrounded by an outer layer (OL) called the capsule in the case of pathogenic mycobacterial species (Fig. 1). The cell wall core consists of peptidoglycan covalently bound to arabinogalactan, which itself is covalently bound to mycolic acids (very-long-chain, from C30 to C90, α-alkyl-β-hydroxy fatty acids) (9). This envelope is unusual in that it is very rich in lipids, and unlike other gram-positive microorganisms, Corynebacterineae possess an outer permeability barrier. It has been postulated that this barrier is formed by a lipid bilayer analogous to the outer membrane (OM) of gram-negative bacteria (33). The arrangement of the lipids in this hypothetical OM has been long debated (26, 32, 34, 43, 44). Based on the chemical structures of the main cell envelope constituents, several models of the cell envelope, in particular, the hypothetical OM bilayer of mycobacteria, were developed (26, 32, 33, 43, 44). According to these models, the innermost
leaflet consists mainly of the mycolic acids, which are, at least in part, covalently linked to the cell wall arabinogalactan. The outermost leaflet is proposed to be composed of various glycolipids, including trehalose monomycolate and trehalose dimycolate; of phospholipids; and of species-specific lipids such as glycopeptidolipids (GPL), phthiocerol dimycocerosate, and sulfolipids (8, 26, 33, 44). The presence of por-forming proteins in low numbers in mycobacteria relative to Escherichia coli may explain both the limited permeability of mycobacterial cell envelopes and the generally rather low susceptibility of these bacteria to toxic agents (6, 9, 36).

Nevertheless, not only is the precise organization of the OM bilayer debated, but even its existence can be questioned since there are no direct observations of this structure. On the one hand, the bilayer model is supported by data from freeze fracture electron microscopy which have clearly shown the occurrence of a major fracture plane in the outer part of the envelope, in addition to the expected plane that typifies the PM (5, 7, 33, 35, 43).

On the other hand, no sign of an OM bilayer was seen in ultrathin sections of Corynebacterineae (Fig. 1) (9, 11, 43), questioning its existence. Importantly, all electron microscopy analyses of ultrathin sections were done with specimens from which water had been removed, a prerequisite for electron microscopy observation at room temperature. We call this technique the conventional preparation method. Even when dehydration has been performed at low temperature by freeze-substitution in order to better preserve biological structures, no OM bilayer was seen in Corynebacterineae (9, 28, 39, 40). This is possibly due to the fact that, during dehydration, water-soluble molecules tend to aggregate and lipid molecules may be prone to extraction or rearrangement by organic solvents (14).

In an attempt to resolve the question of the existence of an OM bilayer in Corynebacterineae, we addressed the native structure of both mycobacteria and the closely related corynebacteria, whose cell envelope resembles that of mycobacteria after conventional electron microscopy (43). We focused our study on Mycobacterium smegmatis, Mycobacterium bovis, and Corynebacterium glutamicum by cryo-electron microscopy (cryoEM) of vitreous sections (CEMOVIS). In the CEMOVIS technique, specimens are vitrified by high-pressure freezing (i.e., cooled to liquid nitrogen temperature without water crystallization). The vitreous specimens are then cryosectioned and imaged in a cryo-electron microscope in their fully hydrated, native state. The artifacts of aggregation and lipid extraction are therefore prevented (1). Importantly, this is the only sectioning technique for electron microscopy involving freezing where the vitreous state can be unambiguously confirmed, by electron diffraction. With this approach, we provide detailed insights into the structure of the mycobacterial cell envelope in its native state and a direct visualization of the mycobacterial OM. Furthermore, we demonstrate that mycolates are essential constituents of this structure through the use of a wild-type and a mycolate-free strain of C. glutamicum (41).
with a LaB6 cathode, a tungsten cathode, and a field emission gun, respectively. The accelerating voltages were 100, 120, and 200 kV, respectively. Specimens were irradiated with a low electron dose. Electron diffraction was used to check whether water was vitreous or crystalline. Crystalline sections were discarded. Images were recorded with a TemCam-F224HD charge-coupled device camera (Tietz Video and Image Processing Systems, Munich, Germany) at magnifications of 22,500, 33,000, and 53,000. No image processing other than that described in the figure legends was performed.

Whole-mount plunge-frozen cells were transferred to a Gatan cryoholder and imaged at a magnification of 50,000 in a CM200-PEG (FEI) operated at an accelerating voltage of 200 kV. Images were recorded on Kodak SO-163 plates and scanned at a pixel size of 0.5 nm. For conventional electron microscopy, C. glutamicum (CGL2020) and M. smegmatis mc2155 were grown, prepared, and imaged as previously described (16, 43).

Quantitative measurements. Pixel size was calibrated by using a two-dimensional crystal of catalase (Agar Scientific). At magnifications of 22,500, 33,000, and 53,000, the pixel sizes are 0.63, 0.50, and 0.31 nm, respectively. During cryosectioning, material is compressed along the cutting direction. Nevertheless, it has been shown that compression does not affect the dimensions measured perpendicularly to the cutting direction (13). Dimensions were measured accordingly on average density profiles calculated along rectangular selections with the software ImageJ (NIH, Bethesda, MD). The width of selection was measured perpendicularly to the cutting direction (13). Dimensions were measured accordingly on average density profiles calculated along rectangular selections with the software ImageJ (NIH, Bethesda, MD). The width of selection was measured perpendicularly to the cutting direction.

The density of OsO4-stained cells was measured with the software EMMENU (Tietz Video and Image Processing Systems) in raw image files acquired at 120 kV at a defocus between −1.7 and −2.3 μm. The density of the OM was normalized to the density of the background.

RESULTS

Vitreous sections—general considerations. We investigated the structure of the cell envelopes of two species of mycobacteria with CEMOVIS, namely, M. smegmatis, a fast-growing nonpathogenic species, and M. bovis BCG, a slow-growing vaccine strain belonging to the same complex as M. tuberculosis and showing more than 99.9% genome sequence identity with this pathogen (19). Henceforth, M. smegmatis refers to strain mc2155 unless mentioned differently. A low-magnification micrograph of M. smegmatis is shown in Fig. 2. Its quality is representative of the majority of the sections that we observed. It does not contain chatter or crevasses, which are cutting artifacts that complicate image interpretation because they produce irregular distortions (inhomogeneities) (2). Knife marks (arrows) and compression along the cutting direction cannot be prevented but are homogeneous and therefore do not hinder image interpretation.

Structure of the cell envelopes of M. bovis BCG and M. smegmatis. The cell envelopes of M. smegmatis and M. bovis BCG are structurally similar in CEMOVIS (Fig. 3A to F; Table 1). They are composed of a PM, a granular layer (GL), an inner wall zone (IWZ) of low density, a medial wall zone (MWZ) of intermediate density, and an OM of higher density. The bilayer aspect of the OM can be best visualized in micrographs recorded with a relatively small defocus value (Fig. 3A and D) and in the corresponding density profiles (Fig. 3C and F), whereas the organization of the GL, the IWZ, and the MWZ is best seen in micrographs recorded with a larger defocus value (Fig. 3B and E). The GL is found next to the PM, in the IWZ. The appearance of the mycobacterial GL and IWZ is similar to that of the GL and the IWZ of typical gram-positive bacteria (e.g., S. gordonii) visualized by CEMOVIS (58) (Fig. 3G and H; Table 1). The MWZ of mycobacteria is topologically identical to the gram-positive peptidoglycan layer (outer wall zone) but is thinner. On images recorded with a relatively large defocus, the MWZ seems separated from the OM by a low-density gap (Fig. 3F). However, on images recorded very close to focus, the gap is strongly reduced (Fig. 3C) or absent (data not shown), whereas the leaflets of the OM are distinct. The gap is thus a phase-contrast artifact that can lead to a slight underestimation of the MWZ thickness.

There is no structure similar to the mycobacterial OM in classical gram-positive bacteria (e.g., S. gordonii), but the mycobacterial OM is structurally analogous to the OM of gram-negative bacteria (e.g., E. coli) (Fig. 3I and J). The thickness of the mycobacterial OM is similar to the published thickness of the gram-negative OM visualized by CEMOVIS (Table 1) (29). Even though an OM in mycobacteria has long been suggested and supported by freeze fracture electron microscopy, here we report a direct observation of such a bilayer in native mycobacteria. Together, these comparisons indicate the following three points: (i) the IWZ occupies the position shown to be a periplasmic space in other gram-positive bacteria (30, 31); (ii) the MWZ is likely formed, at least in part, of peptidoglycan; and (iii) the OM is made of molecules specific to mycobacteria and not ubiquitous in gram-positive bacteria. Because these molecules form a bilayer, they are likely to contain a hydrophobic moiety. OsO4 is considered to label predominantly lipids (56). We thus fixed M. smegmatis cells with glutaraldehyde, postfixed them with OsO4, and subsequently processed them for CEMOVIS. In a negative control, cells were processed for CEMOVIS directly after glutaraldehyde fixation. The density of the OM is, on average, 39% higher in OsO4-stained cells than in control cells (P < 0.01, Fig. 4). These data suggest that the OM is mainly made of lipids. Besides, the bilayer aspect of the OM is lost after OsO4 staining in most of the images, an observation that requires further investigation. Mycolic acids, which are the hallmark lipids of Corynebacterium and Mycobacterium, of which a fraction is covalently bound to peptidoglycan via arabinogalactan, are likely to be a major constituent of the OM.

In order to test our hypothesis that the OM of mycobacteria is made at least in part of mycolic acids, we wanted to observe mutant mycobacteria that would be devoid of mycolic acid. However, this was impossible because mycobac-
teria cannot survive in the absence of this compound (3, 18, 41, 42, 53). For the same reason, treating cells with drugs that inhibit the synthesis of mycolic acids (e.g., isoniazid) proved useless. Indeed, at the highest sublethal isoniazid concentration, the amount of mycolic acid per cell is only reduced by 20% (4). Furthermore, probes such as gold-coupled antibodies could not be used because they are too large to diffuse through the cell envelope. And since speci-

![Cell envelope of mycobacteria and gram-positive and gram-negative bacteria by CEMOVIS.](image)

TABLE 1. Dimension of cell envelope structures based on CEMOVIS analysis

<table>
<thead>
<tr>
<th>Organism</th>
<th>Avg position (nm) ± SD (no. of measurements)</th>
<th>Avg thickness (nm) ± SD (no. of measurements)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GL</td>
<td>OM</td>
</tr>
<tr>
<td><em>M. smegmatis</em></td>
<td>8.4 ± 1.2 (8)</td>
<td>35.2 ± 1.9 (8)</td>
</tr>
<tr>
<td><em>M. bovis</em> BCG</td>
<td>9.0 ± 1.3 (8)</td>
<td>31.5 ± 1.9 (8)</td>
</tr>
<tr>
<td>Wild-type <em>C. glutamicum</em></td>
<td>8.7 ± 0.7 (9)</td>
<td>44.5 ± 7.5 (9)</td>
</tr>
<tr>
<td><em>C. glutamicum</em> Δpks13</td>
<td>8.4 ± 1.4 (6)</td>
<td>NA^c</td>
</tr>
<tr>
<td><em>E. coli</em> K-12^d</td>
<td>NA</td>
<td>27.3</td>
</tr>
<tr>
<td><em>S. gordonii</em> Challis^e</td>
<td>8.3 ± 0.5 (10)</td>
<td>NA</td>
</tr>
</tbody>
</table>

^a Distance between the center of the PM and the center of the designated structure.

^b In the case of bilayers, the thickness corresponds to the total width of the structure and not to the peak-to-peak distance.

^c NA, not applicable.

^d Adapted from reference 29. Where no standard deviation is given, values were calculated from data in Table 1 or measured on Fig. 6 in reference 29.

^e Adapted from reference 58. Half the thickness of the PM was subtracted from the thickness of the IZW and the cell envelope (58), respectively, in order to fit the measurement methods used in the present paper.
mencs must be kept frozen, immunolabeling of cryosections is impossible.

Nevertheless, we studied *M. smegmatis* tmptB, a mutant devoid of GPL and which forms very large cell aggregates (49). GPL are considered to be an important constituent of the OM outer leaflet in several mycobacterial species (26), and their disruption may therefore affect the OM. CEMOVIS revealed the structure of the contact zone between tmptB cells within an aggregate: all of the layers of the cell envelope are present, except the OM outer leaflet, and cells within the aggregate are interacting via their OM inner leaflet (see Fig. S1A in the supplemental material). This assertion is confirmed by images showing that, at the edge of the contact zone, the OM outer leaflet is highly curved and is continuous between the two cells involved in the contact, whereas this is not the case for the inner leaflet (see Fig. S1B in the supplemental material). Contacts between wild-type cells also exist, and their structure is identical to that of contacts seen between tmptB cells (see Fig. S1C in the supplemental material). However, the deletion of GPL generates an increase in the frequency of such contacts and, accordingly, the surface ratio of the OM outer leaflet versus the inner leaflet is reduced, which is in agreement with the expected localization of GPL in the OM outer leaflet and reinforces the hypothesis that the bilayer that we have observed is indeed the long-sought-for mycobacterial OM. The lipidic nature of the OM is further supported by the fact that the OM inner leaflet was never observed in direct contact with the aqueous environment but is always coated with either the OM outer leaflet or a cutting artifact, we examined whole-mount plunge-frozen wild-type *C. glutamicum* cells by cryoEM (13). The cytoplasm appears completely featureless due to the large thickness of the sample (data not shown), but both the PM and the OM can readily be observed. The OM appears smoother than in CEMOVIS micrographs, indicating that this structure is particularly prone to cutting artifacts (Fig. 5D). Furthermore, after Gaussian filtering of whole-mount cell micrographs, a bilayer aspect can be distinguished in many places of the OM but its dimensions are close to the resolution limit due to specimen thickness (Fig. 5D and E). Altogether, our data suggest that the corynebacterial OM is a bilayer.

Importantly, the cell envelope of mycolate-free *C. glutamicum Δpks13* lacks an OM (Fig. 5F to H). The MWZ thickness does not significantly differ between wild-type *C. glutamicum* and *C. glutamicum Δpks13*. This indicates that mycolic acids are not present in the MWZ and that the OM found in wild-type *C. glutamicum* is formed of mycolic acids and possibly of other lipids that interact with them. Furthermore, the extracellular medium of *C. glutamicum Δpks13* contains a large amount of filaments. These are 3.9 ± 0.2 nm thick, which is thinner than the OM found in wild-type *C. glutamicum*. Thus, they possibly represent lipids that normally interact with mycolic acids to form the outer leaflet of the OM. In the absence of mycolic acid, they would still be exported but would not be linked to the cell wall anymore and would be released in the outer medium. This interpretation needs to be tested by further analysis of the released material.
DISCUSSION

Insights into the native structure of mycobacterial cell envelope and comparison with images of conventional preparations. A variety of electron microscopy techniques have been used to decipher the unusual architecture of mycobacteria and related microorganisms. These include ultrathin sectioning of both conventionally processed and freeze-substituted samples, negative staining, and freeze fracture. Although the impression prevails from all of the methods that the cell envelope is layered, different techniques give different pictures of the layering. Consequently, the assignment of the known chemical components of the wall to ultrastructurally defined layers is not straightforward (9).

In ultrathin conventional sections, the cell envelope of mycobacteria is seen as being composed of an asymmetric PM with a thin inner leaflet and a thick outer leaflet (10, 47, 48); a thick, electron-dense layer (EDL); an electron-transparent layer (ETL); and an OL of variable density and thickness (Fig. 1). Because of the ETL’s transparency and the disappearance of this layer after removal of lipids by alkaline hydrolysis (12), the ETL is assumed to contain lipids, mainly mycolic acids. The density of the EDL makes it likely to contain the peptidoglycan to which the arabinogalactan is attached at many sites. In mycobacteria, but not in corynebacteria, the PM is separated from the EDL by a space that may correspond to a periplasm (Fig. 1) (9, 43). Based on electron microscopy data, notably those from freeze fracture (5, 7, 33, 35, 43), and on the chemical structures of the main cell envelope constituents, a model of the envelope was developed in 1982 by Minnikin (33), followed by several improvements and modifications (26, 32, 34, 43, 44). Nevertheless, discrepancies remained between the images provided by various ultrastructural techniques and chemical knowledge.

Application of CEMOVIS to representatives of pathogenic and slow-growing, as well as saprophytic and rapid-growing, mycobacterial species, i.e., *M. bovis* BCG and *M. smegmatis*, respectively, resulted in images that differ from those previously obtained by conventional and freeze-substitution electron microscopy in the following important points. (i) The PM has its typical bilayer aspect, with a thickness similar to that observed in other bacterial cells (Table 1 and Fig. 6) (29–31, 58). The inner leaflet has the same density as the outer one, in contrast to the asymmetrical appearance often reported by thin conventional sections of mycobacteria, that was speculated to be due to the dehydration process involved in sample preparation, which could cause the collapse of the GL against the outer leaflet of the PM. (ii) A compartment similar to the periplasmic space of both gram-positive and gram-negative bacteria (29–31, 58) is apparent in CEMOVIS images not only in mycobacteria, as it was predicted from conventional electron microscopy (9), but also in corynebacteria. This could provide cells with a space where enzymatic reactions involved in cell envelope maintenance can take place. As mentioned, the GL lies in the mycobacterial and corynebacterial IWZ. This layer has been observed in gram-positive bacteria but not in gram-negative bacteria (58). Thus, its presence in both mycobacteria and corynebacteria is consistent with the fact that these bacteria belong to the gram-positive group. Recent cryo-electron tomography-based reports of a similar structure in cell wall-free *Mycoplasma*
The thickness of the OM is 4 to 5 nm in C. glutamicum. The presence of this layer in conventional preparation of mycolate-free corynebacteria (i.e., Corynebacterium amycolatum [43]) indicates that the layer either represents a technical artifact or contains lipids other than mycolates. Most importantly, both M. bovis and M. smegmatis cells treated by CEMOVIS unambiguously show the presence of an outer bilayer, a key point of validation of the common basic feature of the current cell envelope models.

The density of the OM is strongly increased after fixation with OsO₄, a chemical that stains mainly lipids (56). In cells devoid of GPL, which in the OM are considered to be mainly present in the outer leaflet (26), the OM inner leaflet has not been seen uncoated and in direct contact with the aqueous outer medium. Yet, the reduction in the surface area of the OM outer leaflet is correlated with an increase in the contact surface between the OM inner leaflets of two different cells forming an aggregate. These observations strongly support the hydrophobic and lipidic nature of the leaflets composing the OM.

To firmly establish the involvement of mycolates in the OM, we used corynebacteria as a surrogate model for studying essential mycobacterial compounds, such as mycolic acids, which are dispensable in corynebacteria (3, 18, 41, 42, 53). We compared the architecture of a wild-type strain and a mycolate-free mutant of C. glutamicum (41). Our data clearly show that whereas the wild-type strain had a distinct OM, the pks13 knockout mutant is devoid of this structure, making a compelling argument that the OM is a real structure that contains mycolic acids.

**Challenges to the current models of the mycobacterial OM.**

The thickness of the OM is 4 to 5 nm in C. glutamicum and 7 to 8 nm in mycobacteria. This observation is consistent with the presence of mycolyl residues in the structure and the shorter chain lengths of corynomycolic acids (32 to 36 carbons) compared to those of mycobacteria (70 to 90 carbons). The separation of the density peaks of the PM bilayer (3.9 ± 0.4 nm in M. bovis BCG) corresponds to the separation measured by cryoEM and X-ray scattering in liposomes made of phosphatidylcholine with acyl chain lengths of 16 to 18 carbons (25, 52), which is consistent with the lengths of the main fatty acid constituents of the PM (11). On the other hand, the mycobacterial OM is only slightly thicker than the PM (Table 1 and Fig. 6), whereas the main (meromycolic) chain of the mycolic acids is much longer (49 to 61 carbons in M. bovis BCG [50] and 35 to 58 carbons in M. smegmatis [57]). Similarly, both chains of C. glutamicum mycolic acids are made of 16 to 18 carbons (8), like the main constituents of the PM, but the corynebacterial OM is considerably thinner than the PM. It therefore appears that to accommodate the limited thickness of the OM, the lipids facing the arabinogalactan-bound mycolic acids must be intercalated between mycolic acid chains, resulting in a zipper-like structure (Fig. 7). These lipids could be represented by extractable lipids (i.e., noncovalently bound to arabinogalactan) of a ubiquitous nature (e.g., trehalose mycolate, phospholipids).
and of species-specific types (e.g., sulfolipid, phthiocerol dimerococerosate). Such a structure was originally proposed by Min-

ninkin (33). The models proposed by Rastogi (44) and Liu et al. (26), in which the extractable lipids form a totally distinct monolayer rather than intercalating with the nonextractable mycolic acids, seems unlikely because they result in an OM that would be much thicker than what we see by CEMOVIS. Villeneuve et al. have recently proposed a novel conforma-
tional model of mycolic acids where the meromycoly chain is folded upon itself to create a compact structure (54, 55). In this case, the thickness of a monolayer of mycolic acids corresponds to the length of mycolic acid short arm, which is unfolded (20 to 26 carbons). Although this model does not contradict our CEMOVIS data, it is not sufficient to fully explain them. In-

deed, the short arm of M. smegmatis mycolic acids is 22 carbons long (57), i.e., 20 to 30% longer than phospholipids forming the PM, whereas the thickness of the PM equals that of the OM in our data. Likewise, both chains of corynebacterial mycolic acids should be unfolded, as suggested by the model of Villeneuve et al.; as mentioned above, these chains have the same length as the PM phospholipids (16 to 18 carbons), whereas the OM is thinner than the PM. Thus, even if the mycobacterial meromycoly chain is folded and compact, both the mycobacterial and corynebacterial arabinogalactan-bound mycolic acids forming the inner leaflet of the OM have to be intercalated to a certain extent with the longest chains of free lipids (e.g., trehalose mycolate) forming the OM outer leaflet (Fig. 7).

Since we submitted this work for publication, a different laboratory has published CEMOVIS images and cryo-electron tomograms of M. smegmatis, M. bovis BCG, and C. glutamicum (21). Their results are essentially similar to ours, but the models of the OM of mycobacteria that they propose significantly differ from ours in that the mycolic acids are unfolded. As we have explained above, we think that the unfolded meromycoly chain is too long to fit in the OM. Hoffmann and colleagues have not proposed any model for the OM of corynebacteria.

**Future directions and conclusion.** In contrast to pictures from conventional (Fig. 1) and freeze-substitution (39, 40) techniques, no OL/capsule was seen at the surface of either mycobacteria or corynebacteria. This situation may be due to the fact that the components of such a layer would have the same density as the cryoprotection medium and would therefore be indistinguishable from this medium in CEMOVIS images. On the other hand, in mycobacteria, the capsular constituents are known to be loosely attached to the cell wall and most of them are found released in the culture fluids of in vitro-grown bacteria (23, 24, 37, 38), potentially explaining why they are not visible in our images. When mycobacteria grow intracellularly, these constituents are confined around the bacteria by the phagosomal membrane (9). Techniques were developed to maintain these constituents around in vitro-grown cells and visualize them by conventional transmission electron microscopy (17). Further studies are warranted to address the issue of the structure of the mycobacterial OL and capsule by CEMOVIS.

In conclusion, our study brings a new reference structure of the cell envelope of mycobacteria and corynebacteria. This should serve as a framework for building new models of the organization of chemical components in the cell envelope of mycobacteria. It also provides a basis for analyzing whether the cell envelope becomes significantly modified when mycobacteria are enclosed within phagosomes in macrophages, a ques-
tion we are currently addressing.

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