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Crystalline Cell Surface Layer of *Mycobacterium bovis* BCG

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A paracrystalline surface layer (S layer) was found as the outermost layer of the cell wall of five *Mycobacterium bovis* BCG strains. An oblique arrangement of the subunits in the S layer was only clearly seen in thin-sectioned and shadowed preparations, and the unit constant was about 5.5 nm.

A crystalline cell surface layer (S layer) has been described in some but not many pathogens, e.g., *Aeromonas salmonicida*, *Bacillus anthracis*, *Corynebacterium diphtheriae*, and many *Clostridium* species (14). In all those species, the arrangement of the subunits is hexagonal or tetragonal, and so far no pathogenic species with oblique periodicity in its S layer has been found (15). Recently this type of S layer has been described in an oral opportunistic pathogen—an anaerobic gram-negative bacterium, *Bacteroides forsythus* (8, 16).

The function of S layers is not clear, but in *A. salmonicida* one functional property can be described, because mutants devoid of the S layer are unable to multiply in fish (7). Thus, it is obvious that this type of protein, even in some other bacteria, can act as a virulence factor, like protein M in *Streptococcus sanguis* (4) and the yersinial outer membrane protein in *Yersinia enterocolitica* (2).

Highly immunogenic cell wall proteins have been described for many *Mycobacterium* species, e.g., *M. bovis* (12, 17) and *M. leprae* (5, 6, 9, 11). The localization of these proteins in the cell wall has not been done, but obviously they are located in the outermost part of the cell wall. In this paper we describe for the first time the occurrence of an S layer in *M. bovis* strains and also discuss the possible relationship between this periodic protein structure and the immunogenic proteins in this (1) and other *Mycobacterium* species.

Five strains were examined: the Glaxo strain, one Russian strain (obtained from M. Slosárek, Institute of Hygiene and Epidemiology, Prague, Czechoslovakia), and three Finnish strains isolated from patients (with BCG complications) vaccinated with the Glaxo BCG vaccine. The strains were cultivated in liquid Dubos medium for 2 weeks before preparation of the electron microscopic specimens. For thin sections, the cells were prefixed with 3% glutaraldehyde and prepared as described previously (10). For freeze-etching, the cells were suspended to form a thick suspension and frozen in liquid Freon 22 cooled by liquid nitrogen. The fracturing in freeze-etching unit BAF 400T (Balzers, Balzers, Liechtenstein) was performed at −100°C. After 1 min of etching, the platinum shadowing was done at an angle of 40°.

The cells were negatively stained with 2% (wt/vol) phosphotungstic acid (pH 6.5), and the metal shadowing was performed with platinum at an angle of 15° for 5 s without rotation, followed by 5 s of rotational shadowing (10).

The electron micrographs were taken with a JEM-100CX (negative staining) and JEM-1200EX (the others) transmission electron microscopes operating at 60 kV.

In the thin-sectioned cells of both the Russian strain (Fig. 1) and the Glaxo strain (not shown) as well as the Finnish isolates (not shown), an electron-dense layer was seen as the outermost layer of the cell wall. The clustered cells were surrounded by this layer, which was about 10 nm from the other layers of the cell wall. This layer was characterized earlier as a "pseudolayer," and unfortunately thin sectioning is not the technique of choice to visualize it completely. Similar difficulties were found in the negative staining (not shown), where no real image of the layer could be seen. The shadowing techniques, however, gave a very interesting structure of this layer. In the freeze-etched cells, a periodic structure was seen on the surface of many but not all cells (Fig. 2). A very similar image of the surface layer was observed in the platinum-shadowed preparation, where isolated surface layer fragments could be seen (not shown). The oblique periodicity of the surface array was clearly visible, and the unit constant of the lattice was about 5.5 nm.

This layer has also been described as a ruthenium red-stainable layer (13), which thus must have acidic polysaccharide material. This also explains why the periodic structure was not always seen; the polysaccharides and glycolipids of the cell wall could cover the periodic structure. This was easily observed in the freeze-etched cells (Fig. 2) and also in the metal-shadowed specimen, where the sheet of the periodic array and the aperiodic material were seen together. Thus, these covering amphiphilic glycolipids could form a matrix in which proteins were embedded (3).

The close contact of this periodic protein layer and the aperiodic polysaccharide material earlier led to the interpretation that these layers are the so-called mycosides, mainly composed of sugar material.

The oblique arrangement of the subunits of this array indicates that the units are formed by two molecules, and the periodic structure indicates that this layer was formed by a protein(s), because the other cell wall components (polysaccharides, glycolipids, etc.) are not known to form this type of periodic structure. In addition, the very immunogenic mycobacterial cell wall proteins, 46,000 and 22,000 to 23,000 M_r in *M. bovis* (17) and 65,000 M_r in *M. leprae* (5), are known to be dimers, which fits very well with the linear periodicity of this S layer. Thus, it is tempting to speculate that those mycobacterial cell wall antigens which possess good antigenic properties and give an immunogenic response are located in the S layer.

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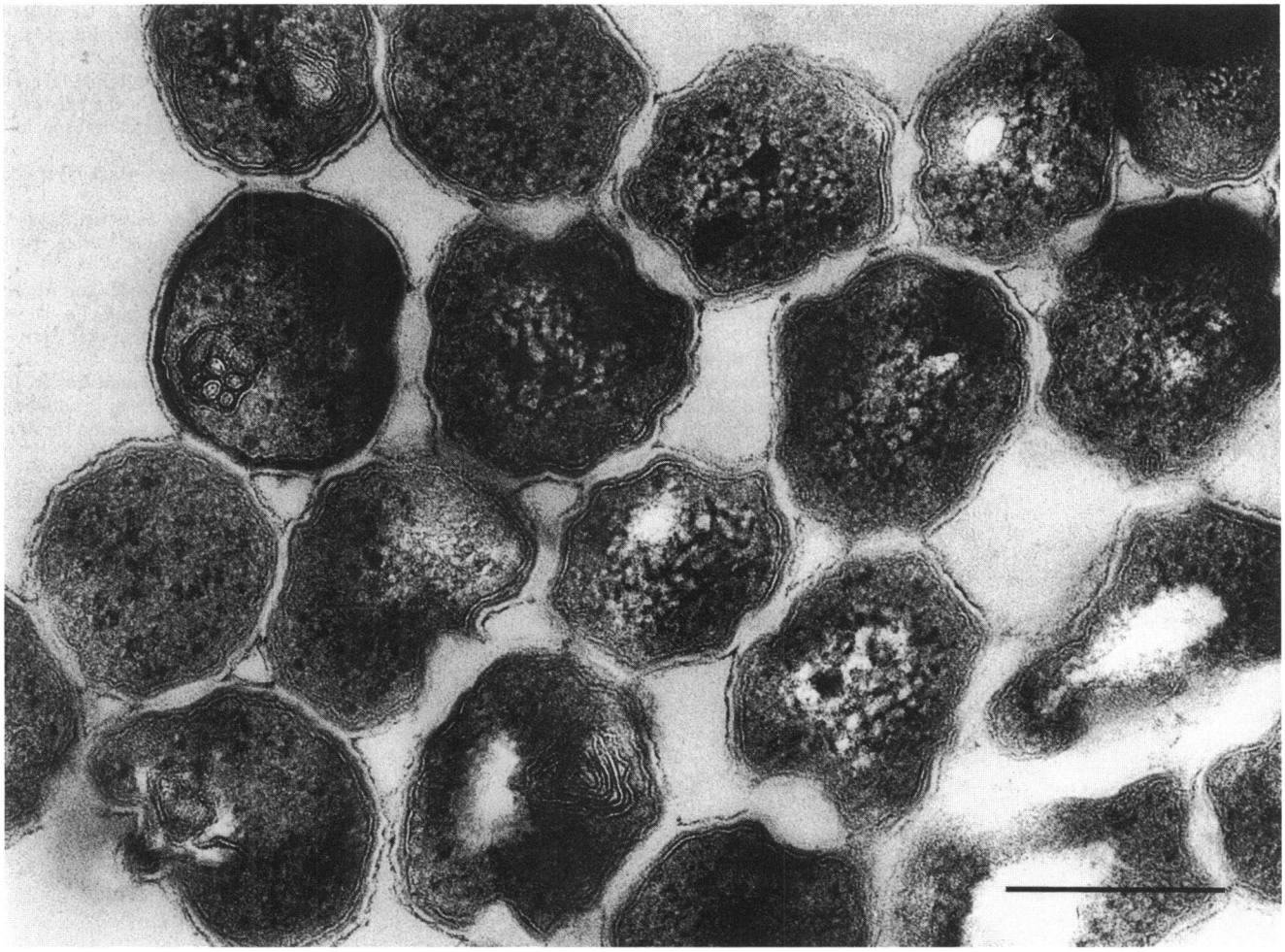

FIG. 1. Thin-sectioned cells of *Mycobacterium bovis* Russian strain, with the electron-dense outermost layer of the cell wall. Bar, 0.2 μm.

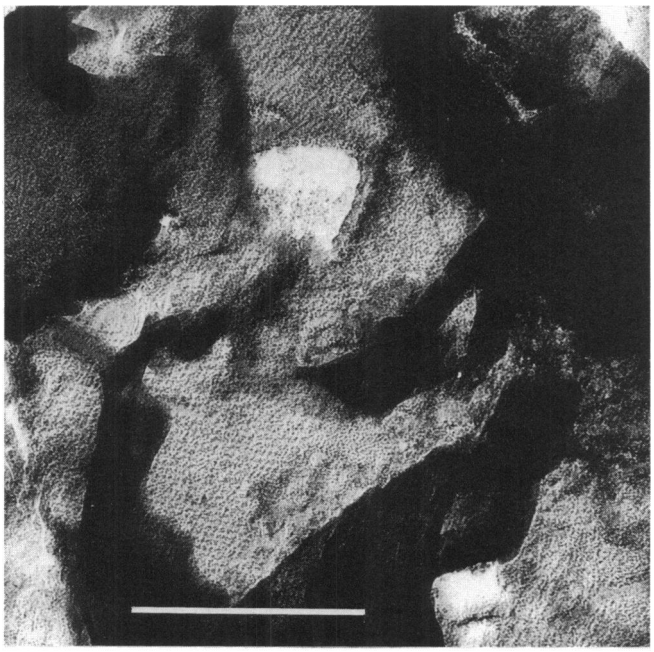

FIG. 2. Freeze-etched cells (Russian strain) with periodic layers. Bar, 0.2 μm.

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LITERATURE CITED

1. **Abou-Zeid, C., I. Smith, J. Grange, J. Steele, and G. Rook.** 1986. Subdivision of daughter strains of bacille Calmette-Guérin (BCG) according to secreted protein patterns. J. Gen. Microbiol. **132**:3047–3053.
2. **Carter, P. B., R. J. Zahorchak, and R. R. Brubaker.** 1980. Plaque virulence antigens from *Yersinia enterocolitica*. Infect. Immun. **28**:638–640.
3. **David, H. L., N. Rastogi, S. Clavel-Sérés, F. Clément, and M.-F. Thorel.** 1987. Structure of the cell envelope of *Mycobacterium avium*. Zentralbl. Bakteriol. Hyg. A **264**:49–66.
4. **Fischetti, V. A., E. C. Gotschlich, G. Siviglia, and J. B. Zabriskie.** 1976. Streptococcal M protein extracted by nonionic detergent. I. Properties of the antiphagocytic and type specific molecules. J. Exp. Med. **144**:32–53.
5. **Gillis, T. P., R. A. Miller, D. B. Yong, S. R. Khanolkar, and T. M. Buchanan.** 1985. Immunochemical characterization of a protein associated with *Mycobacterium leprae* cell wall. Infect. Immun. **49**:371–377.
6. **Husson, R. N., and R. A. Yong.** 1987. Genes for the major protein antigens of *Mycobacterium tuberculosis*: the etiologic agents of tuberculosis and leprosy share an immunodominant antigen. Proc. Natl. Acad. Sci. USA **84**:1679–1683.

7. **Ishiguro, E. E., W. W. Kay, T. Ainsworth, J. T. Buckley, and T. J. Trust.** 1981. Loss of virulence during culture of *Aeromonas salmonicida* at high temperature. J. Bacteriol. **148**:333–340.
8. **Kerosuo, E.** 1988. Ultrastructure of the cell envelope of *Bacteroides forsythus* strain ATCC 43037. Oral Microbiol. Immunol. **3**:134–137.
9. **Lamb, F. I., A. E. Kingston, I. Estrada-G, and M. J. Golston.** 1988. Heterologous expression of the 65-kilodalton antigen of *Mycobacterium leprae* and murine T-cell responses to the gene product. Infect. Immun. **56**:1237–1241.
10. **Lounatmaa, K.** 1985. Electron microscopic methods for the study of bacterial surface structures, p. 243–261. *In* T. K. Korhonen, E. A. Dawes, and P. H. Mäkelä (ed.), Enterobacterial surface antigens: methods for molecular characterization. Elsevier Science Publishers, Amsterdam.
11. **Melancon-Kaplan, J., S. W. Hunter, M. McNeil, C. Stewart, R. L. Modlin, T. H. Rea, J. Convit, P. Salgame, V. Mehra, B. R. Bloom, and P. J. Brennan.** 1988. Immunological signifience of *Mycobacterium leprae* cell walls. Proc. Natl. Acad. Sci. USA **85**:1917–1921.
12. **Radford, A. J., B. J. Duffield, and P. Plackett.** 1988. Cloning of a species-specific antigen of *Mycobacterium bovis*. Infect. Immun. **56**:921–925.
13. **Rastogi, N., C. Frehel, and H. L. David.** 1986. Triple-layered structure of mycobacterial cell wall: evidence for the existence of a polysaccharide-rich outer layer in 18 mycobacterial species. Curr. Microbiol. **13**:237–242.
14. **Sleytr, U. B., and P. Messner.** 1983. Crystalline surface layers on bacteria. Annu. Rev. Microbiol. **37**:311–339.
15. **Sleytr, U. B., P. Messner, D. Pum, and M. Sára (ed.).** 1988. Crystalline bacterial cell surface layers. Springer-Verlag, Berlin.
16. **Tanner, H. C. R., M. A. Listgarten, J. L. Ebersole, and M. N. Strzempko.** 1986. *Bacteroides forsythus* sp. nov., a slow-growing, fusiform *Bacteroides* sp. from the human oral cavity. Int. J. Syst. Bacteriol. **36**:213–221.
17. **Wood, P. R., J. Pipper, A. J. Radford, P. G. Bundesen, D. B. Rylatt, L. E. Cottis, M. John, and P. Plackett.** 1988. Production and characterization of monoclonal antibodies specific for *Mycobacterium bovis*. J. Gen. Microbiol. **134**:2599–2604.