Identification of the \textit{Methanococcus voltae} S-Layer Structural Gene

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We have established that the gene which we had previously identified as encoding the \textit{Methanococcus voltae} P-type ATPase is, in fact, the structural gene for the \textit{M. voltae} S-layer protein. This conclusion is based on a comparison of the N-terminal sequence of S-layer protein prepared by two independent methods with that derived from the nucleotide sequence of the cloned gene. This conclusion was further supported by immunocytochemical localization of the antigen directed against the antibodies used in the cloning experiments.

High-resolution electron microscopy of thin-sectioned, freeze-etched, freeze-dried, and shadowed or negatively stained prokaryotes has shown that the outermost cell envelope in many species of bacteria and archaea consists of a crystalline cell surface layer, designated the S-layer. In most archaea the S-layer is the only cell envelope component outside the cytoplasmic membrane (1, 12). Among members of the methanogenic archaean order \textit{Methanococcales}, the S-layer is composed of a regular hexagonal array of protein subunits that completely covers the cell surface (4, 5). Among the methanogens, primary sequence information is available only for the related species \textit{Methanothermus fervidus} and \textit{Methanothermus sociabilis} (2).

**Structural analysis.** We have reported the nucleotide sequence of a gene that we believed was the structural gene for a membrane-associated \textit{Methanococcus voltae} P-type ATPase (GenBank accession number M59200) (3). We now believe that the gene that we cloned encodes the \textit{M. voltae} S-layer protein.

Patel and coworkers developed a procedure for the conversion of \textit{M. voltae} cells into protoplasts that led to the release of S-layer protein into the protoplasting buffer medium (8). We grew \textit{M. voltae} FS (DSM 1537) in a medium containing vitamin and mineral supplements under a gas atmosphere of 20$\%$ H$_2$ and 80$\%$ CO$_2$ at 37$^\circ$C (13). Cells were taken from the logarithmic growth phase of the culture, and the protoplasts were prepared according to the method described by Patel and coworkers (8) with the modification that 2$\%$ NaCl replaced 0.34 M NaCl in the original cell suspension. After removal of the protoplasts by centrifugation, we found that the supernatant was highly enriched in S-layer protein, which we resolved on a one-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel (Fig. 1, lane 2). This preparation had no ATPase activity.

The S-layer protein had an apparent molecular mass of 74 kDa, which is similar to that previously assigned to the \textit{M. voltae} S-layer protein (7). In a Western blot (immunoblot), the protein reacted with a purified antibody preparation that we had used in our gene cloning (3). This protein was next electroblotted from an SDS-PAGE gel to a polyvinylidene difluoride membrane. After transfer, the protein was excised from the membrane and applied to a pulsed-liquid-phase sequencer (Applied Biosystems) for amino acid sequencing. We found that the N-terminal sequence (determined for 28 residues) of this protein matched perfectly in positions 13 to 40 the amino acid sequence derived from the gene sequence that we previously reported. We have previously commented on the finding that this gene contains a leader sequence (3). Amino acid analysis showed that the protein had an amino acid composition that was very similar to that of the protein that we had sequenced as well as to that reported for the \textit{M. voltae} S-layer protein (4) (data not shown).

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**FIG. 1.** SDS-PAGE. Electrophoresis with 10$\%$ polyacrylamide was carried out as described by Laemmli (6). Lane 1, Sigma SDS-6H molecular mass markers: myosin (205 kDa), $\beta$-galactosidase (116 kDa), phosphorylase b (97.4 kDa), bovine albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa). Lane 2, protein (22 $\mu$g) present in the protoplasting wash medium. Lane 3, same standards as depicted in lane 1. Lane 4, S-layer protein (20 $\mu$g) prepared by the method of Nusser and König (7). In lanes 1 and 2, protein was visualized by Coomassie brilliant blue R-250 staining. In lanes 3 and 4, protein was visualized by silver staining. The arrowhead indicates the position of the S-layer protein.
We next purified the S-layer protein by using a scheme that is based on the differential solubility of the S-layer protein in Triton X-100 chloroform-methanol-water and guanidinium chloride (7). Our preparation contained a major protein component that ran as a 74-kDa protein in one-dimensional SDS-PAGE (Fig. 1, lane 4). In a Western blot, this protein reacted with the purified antibody that we had used in our cloning (3). The N-terminal amino acid sequence (determined for 17 residues) of this protein matched positions 13 to 29 derived from the sequence of the cloned gene.

**Immunocytochemical localization.** We next used immunocytochemical techniques to localize the antigen against which the antibody was raised. Cells were taken from the logarithmic growth phase of the culture, fixed with a solution containing 0.3% paraformaldehyde and 0.2% glutaraldehyde at room temperature under anaerobic conditions, dehydrated at 0 to −35°C with methanol (10 to 100% solutions), and embedded in Lowicryl KM4 resin at −40°C (10). Ultrathin sections obtained from the polymerized samples were mounted on Formvar-coated nickel grids, and the samples were etched with H2O2. Immunocytochemical localization in the bacterial cells was performed by application of the protein A-gold technique (9, 11). The grids were rinsed with distilled water prior to incubation (overnight at 4°C) with antibodies. The grids were next rinsed with phosphate-buffered saline (pH 7.0) containing 0.01% Tween 20 and incubated with protein A-coated colloidal gold (about 5 nm in diameter) for 30 min. After a second rinsing step, the samples were stained with 4% uranyl acetate for 3 min. Electron micrographs were taken with a Philips EM 301 instrument (Philips, Eindhoven, The Netherlands).

The labeling pattern obtained by application of the postembedding procedure to ultrathin sections of cells of *M. voltae* with antibodies used in the cloning experiments is depicted in Fig. 2A and B. The colloidal gold was predominantly found in the region of the cell envelope. The cells depicted in Fig. 2C were incubated with nonspecific antibodies as a negative control. While this technique does not provide the level of resolution that can distinguish between localization to the cell surface and to the underlying membrane structure, the results support our conclusion that the antibody that was used for gene cloning was directed against the S-layer protein.

The simplest explanation for our combined results is that the protein preparation that we used to generate the antibodies used in our cloning experiments (3) consisted primarily of S-layer protein. This conclusion is consistent with our finding that the N-terminal sequence of the protein preparation used to prepare antibodies matched the gene sequences of the two independently isolated S-layer preparations sequenced in this study (Fig. 1) (3).

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**REFERENCES**


FIG. 2. Immunocytochemical localization of the S-layer protein from *M. voltae* at low (A) and high (B) magnifications. Antibody (3) was diluted 1/100 before use. (C) Control (nonspecific antibodies and protein A-colloidal gold). The colloidal gold (black dots) is predominantly found at the periphery of the cells. Bars, 0.2 μm.