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# In Vitro Reconstitution of a Hexagonal Array with a Surface Layer Protein Synthesized by *Bacillus subtilis* Harboring the Surface Layer Protein Gene from *Bacillus brevis* 47

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***Bacillus brevis* 47 contains two surface layer proteins, termed the outer wall protein and the middle wall protein (MWP), which form a hexagonal array in the cell wall. Introduction of the MWP structural gene into *Bacillus subtilis* by using a low-copy-number plasmid led to the synthesis of an immunoreactive polypeptide with a molecular mass almost the same as that of the MWP synthesized by *B. brevis* 47. Biochemical analysis indicated that most of the MWP synthesized by *B. subtilis* was localized in the cytoplasmic fraction. This was further confirmed by using immunogold electron microscopy. The amino-terminal amino acid sequence of the MWP purified from the cytoplasm of *B. subtilis* indicated that the MWP was a precursor with a signal peptide of 23 amino acid residues to the amino terminus of the mature protein. The precursor of the MWP possessed the ability to reassemble in vitro on the *B. brevis* 47 peptidoglycan layer, resulting in the formation of almost the same hexagonal arrays as with the mature MWP purified from *B. brevis* 47, judging from images averaged at a resolution of about 2.5 nm. Furthermore, a center-to-center distance of the hexagonal lattice on the envelope reconstituted by using the precursor MWP was calibrated as 18.3 nm, which was almost identical to the value of 17.8 nm obtained with the mature protein.**

Crystalline surface layers, the so-called S layers, have been observed as the outermost component of bacterial cell envelopes (17). S layers are an almost universal feature of archaebacterial cell envelopes, and they are found in gram-positive and gram-negative eubacteria from nearly all phylogenetic branches (3, 16). S layers are made of protein or glycoprotein protomers arranged on the cell surface with hexagonal (p6), square (p4), or oblique (p2) symmetry (16). A number of S-layer proteins have been not only characterized by biochemical studies (16) but also subjected to three-dimensional reconstruction (2). Furthermore, the entire primary amino acid sequences for a few S-layer proteins have recently been deduced from the cloned genes (8, 11, 21, 22). Although various functions of the S-layer have been proposed on the basis of both the location and the mesh-like structure (3, 4, 16), the S layer remains enigmatic because of the lack of complementary studies on the biochemistry, morphology, and function of a given S-layer protein.

*Bacillus brevis* 47 has a morphologically unique cell wall structure consisting of two protein layers and a thin peptidoglycan layer (24). The two protein layers, the outermost cell wall layer and the middle wall (MW) layer between the outer wall and peptidoglycan layers, are crystalline surface layers composed of single proteins, the outer wall protein and the MW protein (MWP), respectively (20). The structural genes coding for both proteins have been cloned, and the mature outer wall protein and MWP were deduced to consist of 980 and 1,030 amino acid residues with molecular masses of 104 and 115 kilodaltons, respectively (21, 22). Analysis of transcripts from *B. brevis* 47 revealed that these genes constitute a cotranscriptional unit and that they are transcribed from multiple and tandemly arranged promoters located upstream of the MWP gene (*cwp* [cell wall protein gene] operon) (22, 25). Furthermore, a recent study on the three-dimensional structure of the MW layer indicated that the MW layer protomer contains distinct domain structures (A. Tsuboi, H. Engelhardt, U. Santarius, N. Tsukagoshi, S. Udaka, and W. Baumeister, J. Ultrastruct. Mol. Struct. Res., in press).

In order to characterize the specific portion(s) of the MWP responsible for the formation of regular arrays in the cell wall, we first examined whether the MWP synthesized by *Bacillus subtilis* carrying the MWP gene possesses the ability to reassemble into a hexagonal array on the *B. brevis* 47 peptidoglycan layer.

## MATERIALS AND METHODS

**Bacterial strains and media.** *B. brevis* 47 (23) and 47-57 (a uracil-auxotrophic and outer-wall-defective mutant [20]) were grown in $T_2$ medium (23) (10 g of polypeptone, 5 g of meat extract, 2 g of yeast extract, 10 g of glucose per liter; pH 7.0) and $T_2$ medium supplemented with 0.01% uracil, respectively. *B. subtilis* RM141 (22) carrying plasmid pMWP1 (21) was grown in antibiotic medium 3 (Difco Laboratories, Detroit, Mich.) containing erythromycin at a concentration of 10 μg/ml. All cultivations were performed at 37°C with either reciprocal or rotary shaking.

**Analysis of MWP produced in *B. subtilis*.** Extracellular protein was precipitated with 5% trichloroacetic acid after cells were removed by centrifugation (10,000 × *g* for 10 min at 4°C), washed carefully with 50 mM Tris hydrochloride buffer (pH 7.5) without disturbing the pellets, and suspended in the same buffer. The cells were suspended in 50 mM Tris hydrochloride buffer (pH 7.5), sonicated on ice, and separated by centrifugation (10,000 × *g* for 10 min at 4°C) into membrane and cytoplasmic fractions. After the determina-

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tion of protein concentrations in the three fractions by the method of Lowry et al. (9), these fractions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (7), followed by immunoblot analysis using antiserum to the MWP, as described previously (19, 22). Quantitative analysis of the MWP in each fraction was performed with a densitometer (CS-9000; Shimazu, Kyoto, Japan), using the MWP purified from *B. brevis* as a standard.

**Immunoelectron microscopy.** Thin sectioning was performed by using epoxy resin as described previously (24), except for the following modifications. In the prefixation, the final concentration of glutaraldehyde was decreased from 1.6 to 1%, whereas that of formaldehyde was increased from 1.68 to 4%. In addition, the postfixation with osmium tetroxide was eliminated. Thin sections, cut with glass knives and picked up with 400-mesh copper grids, were immunostained with both the antiserum against the MWP (20) and protein A-gold colloidal particles (diameter, 15 nm; E-Y Laboratories) by the method of McCurdy and Pratt (10). The sections were counterstained with uranyl acetate and lead citrate (18). The specimens were examined under an electron microscope (JEM-100CX; Nihon-denshi, Tokyo, Japan) operating at 80 kV.

**Purification of MWP from *B. subtilis*.** The MWP synthesized intracellularly by *B. subtilis* harboring pMWP1 was purified for in vitro reconstitution as follows. *B. subtilis*(pMWP1) at the mid-log phase of growth ($A_{660}$ = 0.3) was harvested by centrifugation (3,000 × *g* for 5 min at 4°C), washed once with 50 mM Tris hydrochloride buffer (pH 7.5), suspended in the same buffer containing 10 mM $MgCl_2$ (TM buffer), and then sonicated on ice. After the removal of the membrane fraction (10,000 × *g* for 10 min at 4°C), the particulate fraction was obtained by centrifugation (48,000 × *g* for 60 min at 4°C) and suspended in TM buffer containing 2% Triton X-100, followed by incubation for 30 min at 4°C with slow shaking. Triton X-100-insoluble fraction was recovered by centrifugation (48,000 × *g* for 60 min at 4°C), solubilized in the sample buffer of 8 M urea-PAGE, and then separated by electrophoresis at 4°C as described previously (20). To the MWP fraction recovered from the gels by electroelution at 4°C, ammonium sulfate was added to 80% saturation. The precipitate was solubilized in 50 mM Tris hydrochloride buffer (pH 7.5) containing 8 M urea, dialyzed against the same buffer, and stored at 4°C.

Purification of the MWP from *B. brevis* 47-57 was performed as described previously (21).

**In vitro reconstitution and image processing.** Peptidoglycan sacculi were prepared from *B. brevis* 47-57, as described by Schwarz et al. (15), and stored in water containing 0.02% $NaN_3$ at 4°C.

The purified MWP (50 μg) was mixed with the peptidoglycan sacculi (25 μg [dry weight]) in the presence of 6 M urea and dialyzed against TM buffer at 4°C. The reconstituted MW layer was recovered by centrifugation, suspended in TM buffer, and negatively stained with 2% uranyl acetate on carbon-coated copper grids.

Electron micrographs were taken with a Philips EM420 microscope (operating at 100 kV) at a primary magnification of 36,000×. Micrographs for further analysis were selected by optical diffractometry, and then suitable areas were digitized by means of a flat-bed microdensitometer (Joice Loebl) in pixel arrays (512 by 512) at an interval of 20 μm, corresponding to a sampling of 0.57 nm at the specimen level. Correlation averaging (13) was carried out by using the SEMPER image-processing system (14). When images of double layers, rotated with respect to each other, were to be processed, a (quasi-optical) Fourier filtration was performed first in order to separate the two sets of reflections originating from the superimposed lattices. Small areas of the two filtered images were extracted and applied as references to the original image for correlation averaging, as described previously (5, 6).

**Amino-terminal amino acid sequence analysis.** The MWP synthesized by *B. subtilis*(pMWP1) was purified as described above, except that SDS-PAGE (7) was used instead of 8 M urea-PAGE (20). The protein separated by SDS-PAGE was recovered from the gels by electroelution, precipitated with 70% cold acetone, and solubilized in a 0.1% SDS solution. After extensive dialysis against the same solution, the $NH_2$-terminal amino acid sequence of the protein was determined with a gas-phase sequence analyzer (model 470A; Applied Biosystems).

## RESULTS AND DISCUSSION

**Production and cellular location of MWP in *B. subtilis* carrying the MWP gene.** *B. subtilis* carrying pMWP1 (Fig. 1A), which contains the entire coding sequence of the MWP gene, synthesized an immunoreactive polypeptide with a molecular mass almost the same as that of authentic MWP, as judged by SDS-PAGE (Fig. 2). To increase further the MWP production in *B. subtilis*, we attempted to construct a new plasmid containing the whole promoters of the *cwp* operon, since pMWP1 possessed only two promoters (P4 and P5; Fig. 1B) among five tandemly arranged ones (Fig. 1B) (1, 25). No transformants carrying such a plasmid, however, were obtained in the several screenings.

The cellular localization of the MWP in *B. subtilis* was examined by fractionating the cultures at the mid-log, late-log, and early-stationary phases into extracellular and cellular portions and analyzing the amount of MWP per 100-ml culture in these fractions by immunoblot (Fig. 2). As the total cellular protein increased concomitantly with the growth, the cellular MWP increased from 120 to 210 μg/100-ml culture (0.8 to 1% of the total cellular protein). In the extracellular fraction, the amount of MWP was invariable at all growth phases and quite small, 10 μg/100-ml culture (approximately 5% of that in the cellular fraction). These results revealed that the gene product was localized mainly intracellularly. Furthermore, only 9% of the MWP found in the cellular fraction was recovered in the membrane fraction (data not shown).

Minor bands below that of the MWP might be due to either degradation by proteases or some carry-over (Fig. 2).

To demonstrate visually the cellular location of the MWP in *B. subtilis*, immunoelectron microscopy of the mid-log-phase cells of *B. subtilis*(pMWP1) was performed by the gold-labeling procedure (10), using the antiserum to the MWP (Fig. 3). In thin sections of the mid-log-phase cells of *B. brevis* 47 with immunogold labeling, a number of gold particles were absorbed on the cell surface, mainly the MW layer consisting of the MWP (Fig. 3A), while few gold particles were detected in the section of the MWP-nonproducing *B. subtilis* (data not shown). In *B. subtilis* carrying the MWP gene, gold particles were detected mainly in the cytoplasm instead of associated with the membrane (Fig. 3B), confirming further the results obtained from the biochemical analysis.

When immunoelectron microscopy was also carried out for the *B. brevis* and *B. subtilis* cells by using the nonimmune rabbit serum, no gold particles were observed in either section (data not shown).

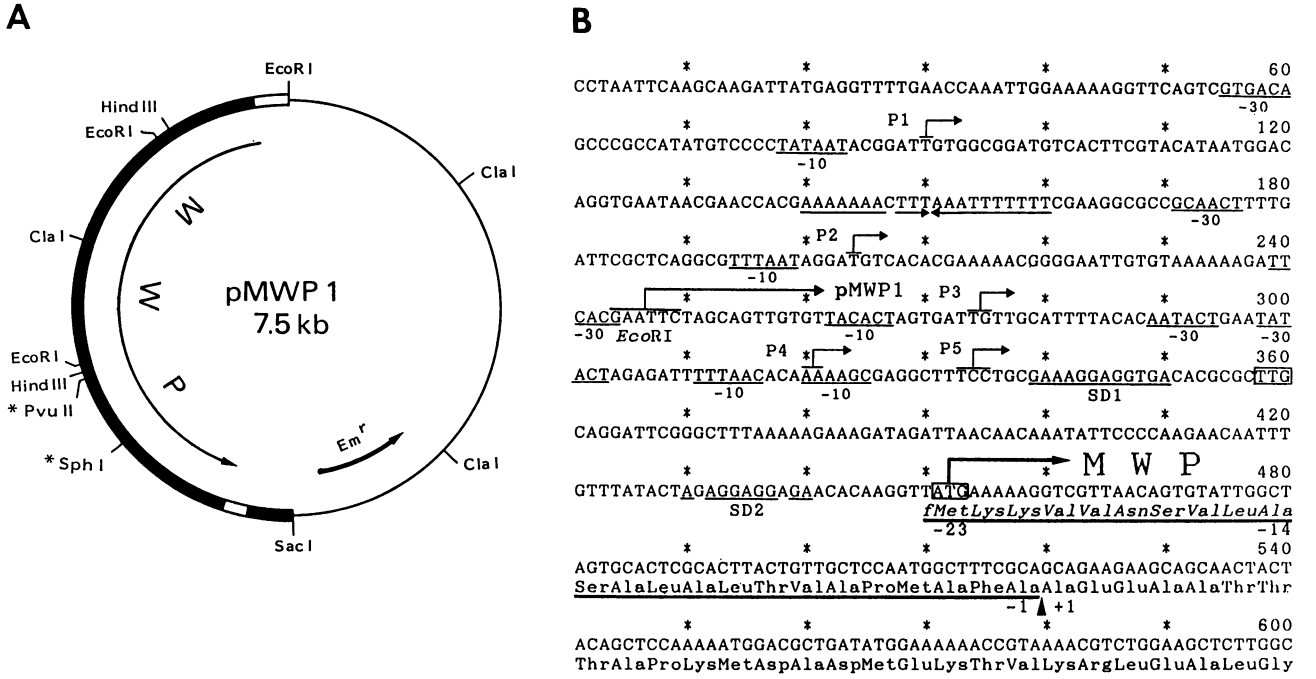


FIG. 1. Restriction map of pMWP1 and nucleotide sequence of the 5′ region of the *cwp* operon. (A) Plasmid pMWP1 contains the entire coding sequence of the MWP gene and two promoters (P4 and P5) among five tandemly arranged ones upstream of the MWP gene (B). The arrow indicates the direction of transcription. $Em^r$, Erythromycin resistance gene; *, sites used to construct the mutant MWP genes with the carboxy-terminal region deleted, as described in the text. (B) Horizontal arrows P1 through P5 above the DNA sequence denote the positions of the 5′ ends of the transcripts determined in previous studies (1, 25). Possible −35 and −10 regions are underlined on the basis of the transcriptional start sites. Two potential ribosome-binding sites (SD1 and SD2) are underlined, and two possible initiation codons are boxed. The amino acid sequence deduced from the DNA sequence is numbered from −23 on the basis of the cleavage site (vertical arrowhead) of the signal sequence (underlined). The chemically determined amino-terminal amino acid sequence of the MWP synthesized by *B. subtilis* is indicated in italics (*Met* through *Ala*).

**Characterization of the MWP from *B. subtilis*.** The MWP was purified from the cytoplasm of *B. subtilis*(pMWP1) to homogeneity, as described in Materials and Methods. The amino-terminal amino acid sequence determined chemically (up to 10 amino acid residues) was in complete agreement with the amino-terminal segment of the signal peptide deduced from the nucleotide sequence (from amino acids −23 to −14 [relative to the cleavage site]) (Fig. 1). This revealed that the MWP synthesized intracellularly by *B. subtilis* was a precursor with a signal peptide of 23 amino acid residues to the amino terminus of the mature protein. We term the precursor protein pre-MWP to discriminate it from the MWP purified from *B. brevis*.

Trials to purify the MWP produced extracellularly by *B. subtilis*, however, failed because of the extremely small amount of the protein in the medium fraction.

**In vitro reconstitution of a hexagonal array.** The MWP purified from *B. brevis* 47-57 by urea-PAGE was mixed with the *B. brevis* peptidoglycan sacculi in the presence of 6 M urea and dialyzed against $Mg^{2+}$-containing buffer. The negatively stained image indicates a hexagonal array reconstituted on the envelope (Fig. 4A). The calibration of the hexagonal lattice of the reconstituted layer revealed a center-to-center distance of 17.8 ± 0.4 nm, which is almost identical to the value (18.3 nm) for the native MW layer from *B. brevis* 47-57 (Tsuboi et al., in press). A mixture of the pre-MWP purified from *B. subtilis* and the *B. brevis* peptidoglycan also led to the formation of a hexagonal lattice array on the envelope (Fig. 4B). The center-to-center distance of the hexagonal lattice was calibrated as 18.3 ± 0.4

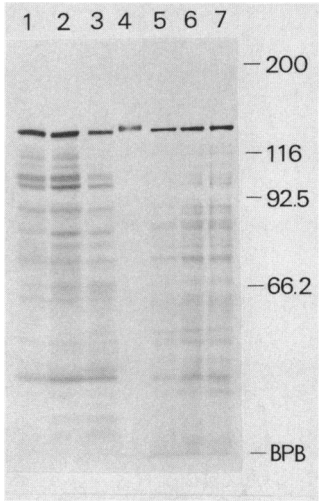


FIG. 2. Immunoblot analysis of the extracellular and cellular polypeptides synthesized at various growth phases of *B. subtilis* carrying pMWP1. Cultures of *B. subtilis*(pMWP1) at the mid-log ($A_{660}$ = 0.32; lanes 1 and 5), late-log ($A_{660}$ = 0.57; lanes 2 and 6), and early-stationary ($A_{660}$ = 0.63; lanes 3 and 7) phases were divided into the extracellular and cellular fractions. A 50-ng sample of the MWP purified from *B. brevis* (lane 4), 50 μg of the extracellular protein (lanes 1, 2, and 3), and 5 μg of the cellular protein (lanes 5, 6, and 7) were subjected to SDS-PAGE on a 7.5% gel and then analyzed by the immunoblot procedure. The following molecular mass standards (in kilodaltons) are shown at the right: myosin (200), β-galactosidase (116), phosphorylase *b* (92.5), and bovine serum albumin (66.2). BPB, Bromophenol blue.

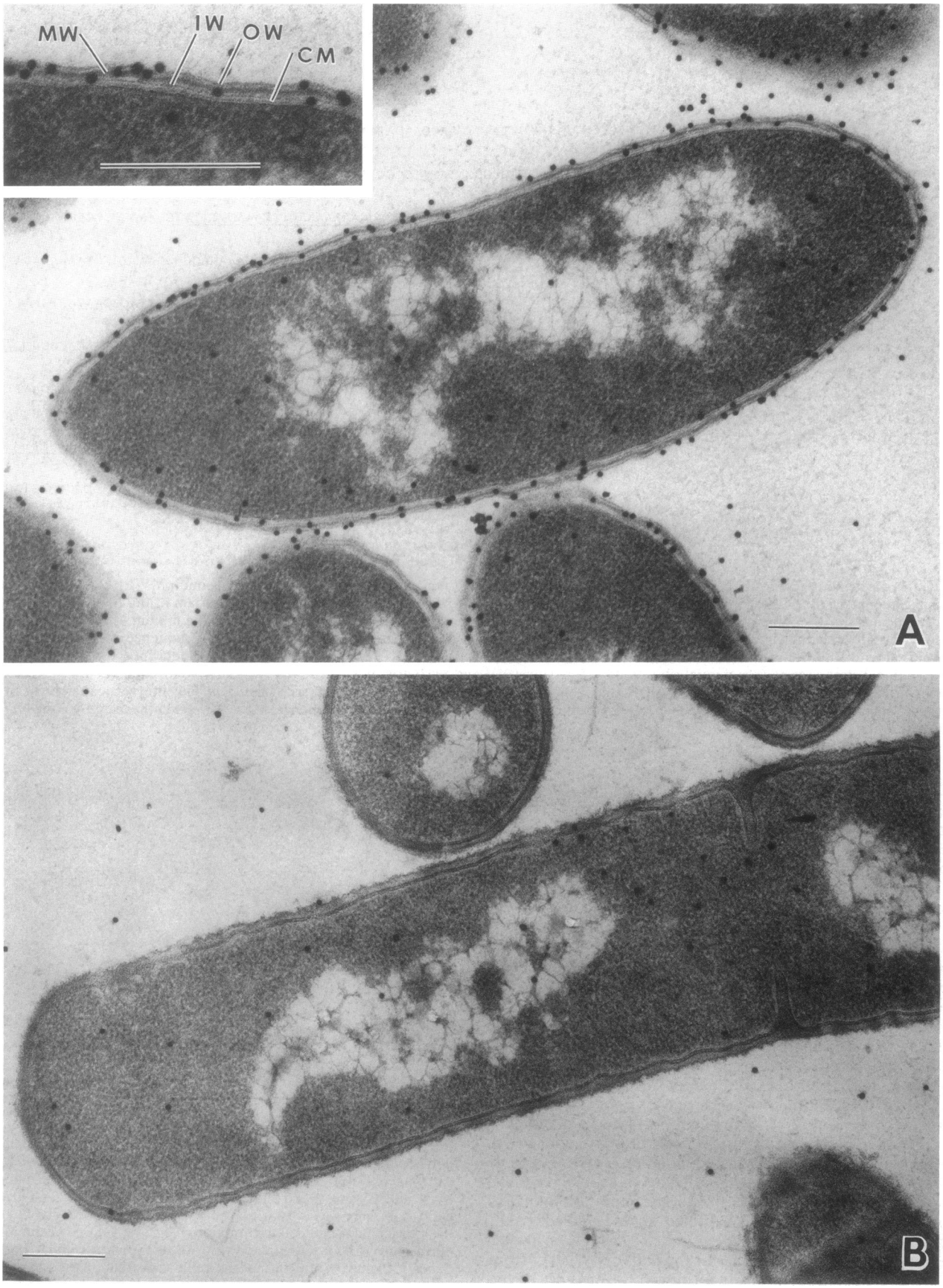


FIG. 3. Immunogold labeling of the MWP in the mid-log-phase cells of *B. brevis* 47 and *B. subtilis*(pMWP1) after thin sectioning. Thin sections of the *B. brevis* cells (A) and *B. subtilis* carrying pMWP1 (B) were incubated with antiserum to MWP, followed by labeling with protein A-gold colloidal particles (diameter, 15 nm), as described in the text. Bars, 300 nm. OW, outer wall; IW, inner wall; CM, cytoplasmic membrane.

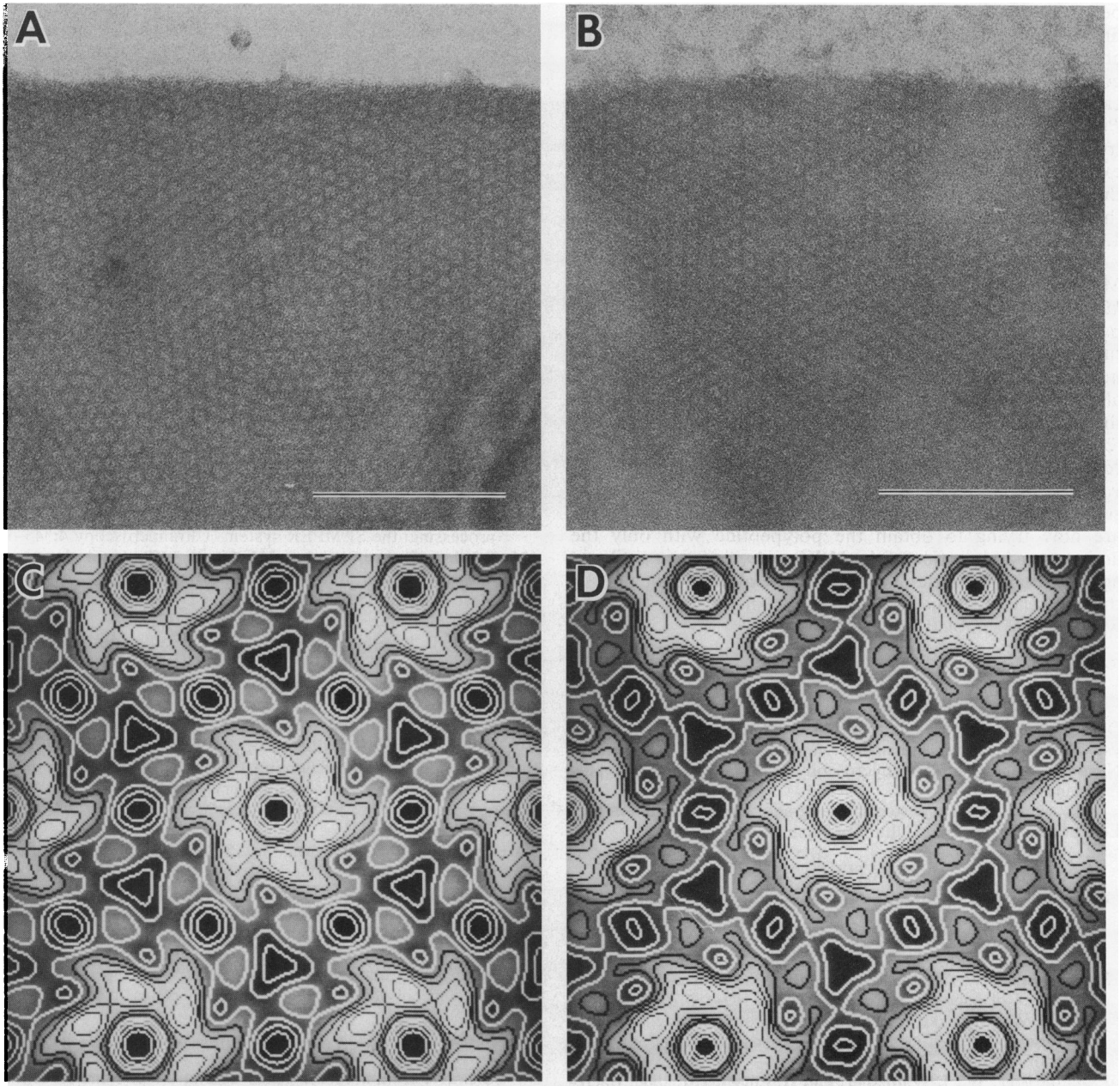


FIG. 4. Negatively stained images and the averaged structures of the reconstituted MW layers. Electron micrographs of the negatively stained preparations indicate that both MWPs purified from *B. brevis* (A) and *B. subtilis*(pMWP1) (B) form hexagonal arrays on the *B. brevis* peptidoglycan layer. Bars represent 200 nm. Contoured images (C and D) of the reconstituted MW layer were obtained from sixfold-symmetrized averages of the micrographs A and B, respectively. The dimensions of each image are 36 by 36 nm.

nm, in complete agreement with the value for the native MW layer.

Furthermore, the fine structures of both reconstituted layers after correlation averaging (13) reveal that there are no significant differences detectable between these images (Fig. 4C and D). The resolution, according to the radial correlation function criterion (13) is about 2.5 nm. Most of the molecular mass of each reconstituted S layer is contained in a sixfold centrosymmetric core (Fig. 4C and D), which is in good agreement with the structure of the native MW layer (Tsuboi et al., in press). This core resembles a toothed wheel subdivided into six centers of mass and appears to be penetrated by a central pore in projection.

The ratio (wt/wt) of protein to peptidoglycan in the reconstitution mixtures was 2 in both cases. There was an almost complete reassembly of hexagonal arrays on peptidoglycan at a ratio of more than 2, whereas only partial reassembly of hexagonal arrays was observed at a ratio of less than 1. Removal of $Mg^{2+}$ from the reconstitution mixture resulted in no formation of a hexagonal array (data not shown), suggesting that divalent cations such as $Mg^{2+}$ are essential for the reassembly into a hexagonal array on the peptidoglycan (16).

The charge distribution in the deduced amino acid sequence of the MWP indicated that there are a number of highly negatively charged regions in the protein (21). These regions appear to play a crucial role in the interaction

between the MWP and the peptidoglycan layer through salt linkage. To characterize the MWP which has negatively charged portion(s) involved in such an interaction, we have constructed a number of mutant MWP genes with the carboxy-terminal region deleted (for example, by using a *Pvu*II or *Sph*I site on pMWP1 [Fig. 1A]), purified the truncated MWPs from the cytoplasm of *B. subtilis*, and examined them for the ability to form a hexagonal array on the *B. brevis* peptidoglycan. The mutant MWP truncated in the carboxy-terminal portion by only 20% (with *Sph*I; Fig. 1A) failed to reassemble onto the peptidoglycan.

Comparative study with other S layers suggested that the MW layer of *B. brevis* resembles the S layer of *Acetogenium kivui* (2, 12) in various aspects: they have very similar lattice parameters and well-conserved three-dimensional structures, especially in the massive core domain. Furthermore, nucleotide sequence analysis revealed that a sequence of almost 200 amino acid residues in the $NH_2$-terminal region of the two polypeptides exhibits highly significant homology (21) (J. Peters et al., submitted for publication). These results lead to the possibility that the core region interacting with the peptidoglycan may correspond to the homologous sequence in the amino-terminal region of both proteins. We are now trying to obtain the polypeptide with only the amino-terminal portion of the MWP and prepare an antibody against the polypeptide in order to elucidate the location of the conservative and possibly functionally critical region within the assembled S layer.

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