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Cloning of the Crystalline Cell Wall Protein Gene of *Bacillus licheniformis* NM 105

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A protein with a tetragonal pattern, defined as RS protein, was found on the wall surface of an alkaline phosphatase secretion-deficient mutant (NM 105) of *Bacillus licheniformis* 749/C. The protein was present on the wall surface of the exponential-growth-phase cells, but at the stationary growth phase it was overproduced and hypersecreted. This protein was precipitated to homogeneity from the culture fluid by 80% ammonium sulfate saturation and chilled acetone. The molecular mass of the protein was 98 kilodaltons, and it had a single subunit in a sodium dodecyl sulfate gel. Specific anti-RS antibody was generated in rabbits and used to immunolabel the RS protein on the cells at different growth phases. In early-exponential-growth-phase cells, the outside surface of the wall, the cytoplasm, and the inside surface of the cytoplasmic membrane were labeled. In stationary-growth-phase cells, the cytoplasm was poorly labeled, but the labeling on the outside surface of the wall was high. A *B. licheniformis* NM 105 gene library was made by using the lambda phage EMBL3. The RS protein expression from this gene library was detected by a modified autoradiographic procedure. One of the amplified RS protein-positive plaques (4213-1) containing recombinant DNA was chosen, and the restriction map of this DNA was prepared. The RS protein expressed in *Escherichia coli* NM 539 infected with 4213-1 recombinant phage had a lower molecular mass than the purified authentic RS protein. The 4.5-kilobase-pair (kbp) *SalI-EcoRI* fragment of the recombinant DNA was cloned in the shuttle plasmid pMK4 to construct pMK462, which was expressed in *B. subtilis* MI112 and produced the RS protein identical in molecular mass to the purified authentic RS protein. The RS protein expression was also demonstrated in cryosections of transformed *E. coli* and *B. subtilis* cells by immunoelectron microscopy. The 1.2-kbp *SalI-Hin*dIII and 1.8-kbp *Hin*dIII-*Hin*dIII recombinant DNA restriction enzyme fragments, respectively, from the right of the restriction map produced anti-RS antibody cross-reacting proteins. The expression of the 1.2-kbp *SalI-Hin*dIII DNA fragment cloned in pUC8 could be induced with isopropyl-β-D-thiogalactopyranoside. The 1.8-kbp DNA restriction fragment hybridized with both the chromosomal DNA of strain NM 105 and the recombinant phage 4213-1 DNA. The RS gene expression was finally demonstrated in transformed *E. coli* 539 cells by in situ hybridization of frozen thin sections with the 1.8-kbp *Hin*dIII biotin-dATP probe and immunolabeling these with anti-biotin immunoglobulin G and protein A-gold.

Bacillus licheniformis NM 105, an alkaline phosphatase secretion-deficient mutant of strain 749/C, has a thin electron-dense layer on the outside surface of the cell wall (14). Both the cellular location and the thin section appearance of this layer are reminiscent of the regular surface layer or crystalline layer found on many gram-positive, gram-negative, and archaebacterial cells. This layer, referred to as S layer, RS layer, paracrystalline surface layer, and cell wall protein, will be referred to in this paper as RS layer. In general, these layers are composed of monomolecular layers of proteins (20, 23) or glycoproteins (30, 31), and perhaps RS layers are primitive protein membranes (20). The lattice structure of RS layer, first demonstrated in electron micrographs of shadowed preparations of bacteria (11), varies among tetragonal, hexagonal, and oblique arrangements (20, 29; U. B. Sleytr and G. B. Friers, Proc. 9th Int. Congr. Electr. Microsc. Soc. 2:346–347, 1978). Theoretical models explain the association between individual subunits (3, 20, 23). The RS protein dissolves in detergents, urea, guanidine hydrochloride, EDTA, and a variety of other metal-chelating agents (1, 16, 20, 25). The RS protein subunits are suggested to be associated with each other and with the underlying cell wall by noncovalent bonds.

Although RS layers are present on the wall surfaces of a wide variety of bacteria, no definite information is available on their specific physiological functions. Data suggest that the uniform pore size of RS layers prevents diffusion of protein molecules and may enable RS layers to function as passive permeability barriers (18, 22, 24; Sleytr and Friers, Proc. 9th Int. Congr. Electr. Microsc. Soc. 1978) which prevent the actions of toxic and lytic proteins and the dilutions of extracellular digestive and protective proteins. Some RS layers with glycoprotein characteristics may aid bacterial adhesion to substrates (6, 19).

The RS layers in many species of the genus *Bacillus* show tetragonal lattice structures and 7- to 14-nm spacing between the subunits (16). Molecular masses of the RS proteins as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) vary between 150 and 200 kilodaltons (kDa). Although single layers are common, double RS protein layers are known and the genes of these proteins from *B. brevis* 47 have been cloned and sequenced (27–29). The typical secretory character of the RS proteins is indicated by the presence of signal sequences in the nucleotide sequences of these proteins (28). The RS proteins appear to be assembled on the outside surface of the

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membrane, and they never show a repeating structure inside the cells.

This paper demonstrates that the RS protein with a tetragonal repeating structure was present on the wall surface of *B. licheniformis* NM 105. A large quantity of this protein was synthesized and secreted in the stationary growth phase. The RS protein was purified, and rabbit antibody was prepared against this protein. The gene coding for the RS protein was cloned from a *B. licheniformis* NM 105 gene library and was expressed in *Escherichia coli* NM 539 and JM 109 and *B. subtilis* MI112(pMK462). The expression of the RS gene was tested by the Western blot (immunoblot) technique and was confirmed by immunoelectron microscopy and in situ hybridization at the electron microscopic level.

MATERIALS AND METHODS

B. licheniformis NM 105 was grown in phosphate-free Casitone-glucose medium (12, 13). *E. coli* NM 539, JM 109, and JM 83 were grown in LB medium (10 g of Bacto-tryptone, 5 g of Bacto-yeast extract, and 10 g of sodium chloride per liter; the pH was adjusted to 7.5) or 2× TY medium (16 g of Bacto-tryptone, 10 g of yeast extract, and 5 g of sodium chloride per liter). *B. subtilis* MI112 (r^- m^- Rec^-) was grown in LB medium. The bacteriophage EMBL3 was grown in *E. coli* NM 539.

Purification of RS protein. *B. licheniformis* NM 105 was grown with constant shaking at 37°C for 72 h. The culture supernatant, containing both intact sheets and dissociated subunits of the RS protein, was separated from the cells by centrifugation at 10,000 × *g* for 10 min and concentrated by overnight dialysis at 4°C through Spectrapor semipermeable membrane tubing (Spectrum Medical Industries Inc., Los Angeles, Calif.) with a 14,000-molecular-weight cutoff against polyethylene glycol (20,000 molecular weight). The concentrated protein was precipitated with 80% ammonium sulfate saturation, dissolved in 10 mM Tris hydrochloride buffer (pH 7.5), and reprecipitated with 70% chilled acetone. The precipitate was dissolved in 20% SDS for SDS-PAGE, and the 94-kDa protein was extracted by electroelution in Bio-Rad Laboratories equipment.

Preparation of polyclonal antibody. A female New Zealand rabbit was used for the production of polyclonal antibody. Blood collection was always from the ear veins of the rabbit. Preimmune serum was collected before antibody production for determining the specificity and cross-reactivity of the anti-RS antibody. The purified RS protein (1 mg/ml) mixed with an equal volume of Freund adjuvant was injected into both thighs on a weekly basis for 5 weeks, and the antibody titer was determined by an enzyme-linked immunosorbent assay technique. Two weeks after injection 5, about 50 ml of blood was collected and the serum from this blood was frozen at −20°C. The immunoglobulin G (IgG) was purified from this serum by $(NH_4)_2SO_4$ precipitation followed by Blue gel (Bio-Rad) chromatography.

Electron microscopy. (i) Ruthenium red treatment. The cells of the strain NM 105 were harvested after 8 to 18 h of growth in inorganic phosphate-deficient medium by centrifugation at 10,000 × *g* and washed in 0.1 M cacodylate buffer, pH 6.9. The cells were fixed and stained in a mixture of ruthenium red and glutaraldehyde (1% each) for 1 h followed by a mixture of ruthenium red and osmium tetroxide (1% each) in the above buffer for 2 h at room temperature (10). The cells were then washed in the same buffer and treated for dehydration and embedding (7). Ultrathin sections were prepared by using a Saphratome knife and examined without any staining.

(ii) Immunoelectron microscopy. Details of the immunoelectron microscopy method have been described earlier (8, 9, 21, 26). In brief, the following protocol was followed. Washed cells were fixed in a glutaraldehyde (0.5%) and tannic acid (0.6%) mixture or a glutaraldehyde (1%) and paraformaldehyde (1%) mixture for 30 min at room temperature and cryoprotected by several washes in 30% glycerol or 2.3 M sucrose dissolved in 0.1 M phosphate buffer, pH 7.5, containing 0.15 M NaCl. Drops of thick cell suspensions, placed on Cryonova specimen pins, were rapidly frozen in liquid nitrogen-cooled propane and preserved in liquid nitrogen. The pins containing frozen specimens were mounted on the specimen-holding arm of a Cryonova cryoultramicrotome (LKB), and 600- to 800-nm-thick sections were prepared by using a diamond knife or a freshly broken glass knife. The dry sections, accumulated on the knife edge, were removed by 2.3 M drops of freezing sucrose and then set on electron microscope grids. These were placed on liquefied gelatin to remove sucrose and immunostained by floating the electron microscope grids containing the sections on the following drops of solutions in sequence (8, 21): (i) 0.02 M glycine (three times for 10 min each); (ii) normal rabbit serum (20 min; 1:1,000 dilution); (iii) 1% bovine serum albumin (BSA) (four times for 2 min each); (iv) anti-RS IgG (1 μg/ml; 20 min); (v) 1% BSA (four times for 2 min each); (vi) protein A-gold (PAG) (1:100 dilution, 5 nm; Jansen Life Sciences, Piscataway, N.J.) (20 min); (vii) phosphate-buffered saline (four times for 5 min each); (viii) deionized water (four times for 1 min each); (ix) uranyl acetate oxalate (5 min in the dark); (x) deionized water (30 s); (xi) uranyl acetate with polyvinyl alcohol (10 min in the dark). After these treatments, the grids were drained and air dried for 1 h before being examined in the electron microscope. The stained sections were examined by a transmission electron microscope using 100 kV of acceleration voltage, a 20-μm objective aperture, and a 100-μm condenser aperture.

Isolation of chromosomal, plasmid, and lambda DNA. The chromosomal DNA of *B. licheniformis* NM 105 was purified by a phenol-chloroform method, and plasmid DNA was purified by an alkaline lysis method (15). Lambda DNA extracted from purified lambda phages by proteinase and SDS treatment was purified by the phenol-chloroform method.

Construction of *B. licheniformis* NM 105 genomic library. The chromosomal DNA fragments (9 to 22 kilobase pairs [kbp]) of *B. licheniformis* NM 105 produced by partial digestion with *Sau*3A was ligated with the EMBL3 *Eco*RI-*Bam*HI-digested DNA by overnight T4 ligase treatment (15). The recombined DNA was subjected to electrophoresis and packaged in vitro by using Packagene (Promega Biotec). A genetic selection was done, and the titer was determined by growing the phage containing recombinant DNA in *E. coli* NM 539 (*supF hsdRCP2 cox3*). The library contained 15,000 to 20,000 of the initial plaques. Probability calculation based on some theoretical parameters indicated that 2,065 independent plaques should be screened to clone one RS gene at 99% probability (5).

Screening by autoradiography. The RS-positive plaques were transferred on nitrocellulose filters for screening by using a modified transfer procedure. The usual technique for this transfer is to touch the surface of the plaques with a nitrocellulose filter. The modification used was agar medium on a perforated petri dish for the growth of the plaques or bacterial colonies; upon completion of growth, the petri dish

was overlaid with a nitrocellulose filter and a thick layer of tissue paper. This petri dish was partially immersed in buffer contained in a larger petri dish and incubated for several hours at room temperature. The proteins from the plaques or colonies were transferred to the nitrocellulose filter while the buffer was soaked up by the tissue paper. These filters were treated with chloroform vapor, lysozyme, 3% BSA, and 10 μg of the anti-RS IgG per ml and then preabsorbed with the total protein of *E. coli* NM 539, 1% BSA, and protein A labeled with 5×10^6 cpm of ^{125}I. The RS-positive plaques or colonies appeared as dark spots after autoradiography on X-ray film.

The DNA fragment, which was presumed to contain the RS gene, was subcloned in *E. coli* JM 109 containing the plasmids pUC8 or pMK4. The recombinant phage DNA (4213-1) coding for the RS protein and the plasmid DNA were digested with suitable restriction enzymes. Different-sized DNA bands were isolated from agarose gels by extraction with phenol-chloroform followed by precipitation with ethyl alcohol. These DNA fragments were dried in a vacuum, dissolved in ligase buffer, and incubated with restricted vectors overnight at 10°C with T4 ligase. *E. coli* JM 109 transformed with this recombined DNA was grown for 90 min at 37°C in 1 ml of 2× TY medium and finally plated on 2× TY agar plates in the presence of 40 μg of ampicillin per ml, isopropyl-β-D-thiogalactopyranoside (IPTG), and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (14). White colonies grown on these plates were picked and again grown in the presence of 40 μg of ampicillin per ml.

B. subtilis MI112 was transformed with pMK4 DNA containing the RS gene by the method of Niandet and Ehrlich (17). The colonies were grown in LB medium, and both the cells and the culture medium were screened for the RS protein by Western blotting (4). The culture filtrates were mixed with 0.1 mM phenylmethylsulfonyl fluoride (PMSF) to prevent proteolysis and precipitated with 10% trichloroacetic acid. The precipitates were subjected to centrifugation and dissolved in 20% SDS for SDS-PAGE.

Western blot. The proteins dissolved in SDS were subjected to electrophoresis by SDS-PAGE, and the protein bands from the gels were transferred to nitrocellulose filters by using a Transblot apparatus (Bio-Rad). This filter was treated sequentially with 3% BSA, 10 μg of anti-RS IgG per ml preabsorbed with *E. coli* JM 539 cell lysate at 4°C overnight, and 5×10^6 cpm of ^{125}I-labeled protein A (Amersham Corp.) for 1 h. Autoradiography was done by exposing the labeled filter to Kodak X-ray film overnight or for several days.

Analysis of DNA restriction fragments. All restriction enzymes were obtained from commercial suppliers (Bethesda Research Laboratories, New England BioLabs, or Promega Biotec). Restriction fragments were analyzed by agarose gel electrophoresis as described by Maniatis et al. (15). Lambda phage DNA digested by *Hin*dIII-*Eco*RI was used as molecular weight markers.

DNA hybridization procedure. DNA and colony hybridizations of the DNA restriction fragments were performed by using nitrocellulose filters (Schleicher & Schuell, Inc.) and suitable ^{32}P-labeled DNA fragments (15).

In situ DNA hybridization using immunoelectron microscopy. The method of Binder et al. was used for this experiment (2). The pUC9 plasmid DNA containing a 1.8-kbp *Hin*dIII fragment of the RS gene was isolated. This was digested with *Hin*dIII, and the fragments were separated by soft agarose gel electrophoresis for 4 h at 40 V. The 1.8-kbp band was recovered by a Geneclean Kit (Bio 101 Inc.). Biotin-11-dATP (Sigma Chemical Co.) was incorporated into the 1.8-kbp fragment by nick translation and precipitated with 2 volumes of ethanol, vacuum dried, and preserved at −20°C for future use. In order to verify the successful hybridization, a [^{32}P]dCTP-labeled *Hin*dIII DNA fragment was used instead of the biotin-labeled DNA fragment, and the hybridized DNA was examined by autoradiography.

The immunoelectron microscopy procedure was used to immunolabel the hybridized DNA. Fixed frozen thin sections of the transformed and untransformed cells of *E. coli* were subjected to hybridization with biotinylated DNA probe. The biotin-labeled DNA was detected by anti-biotin rabbit IgG treatment followed by labeling with protein A-conjugated colloidal gold.

RESULTS

Demonstration of RS in *B. licheniformis* NM 105. The RS layer uniformly covers the entire envelope surface. The cells from 8-h cultures, when gently homogenized in a Bead Beater (Biospec Products, Bartlesville, Okla.), released large sheets of RS protein attached to the wall surface. The tetragonal arrangement of these RS layer fragments was visible after negative staining with ammonium molybdate (Fig. 1a). The diagonal distances between the individual subunits of the tetragons were 11.6 nm. The organization of the subunits was clearly seen in the high-magnification micrographs (Fig. 1b), which show that the spacings form uniform-size pores. The symmetric extensions of the subunits can also be seen in this micrograph (Fig. 1b [small circle]).

Sheets of the RS protein showing tetragonal structures comparable to the wall-attached material shown in Fig. 1a and b were released into the growth medium (Fig. 1c). The tetragonal assembly of the RS protein was unstable in the growth medium. In the absence of divalent cation and protease inhibitors, there was almost total disorganization at 4°C or higher temperatures. An example of a partially disorganized RS layer is seen in Fig. 1c. It appears from this micrograph that the subunits of the tetragonal assembly were arranged in a linear order but were not associated with each other. Although the RS layer could be demonstrated on the wall surface of exponential-phase cells, at the stationary growth phase it was overproduced. Substantial amounts of this overproduced RS protein were released from the cells, and both the fragments of wall-attached sheets of the RS protein showing tetragonal assembly and the dissociated subunits constituting the tetragonal array of the protein accumulated in the culture medium. Cells fixed and stained in ruthenium red showed layers of material associated with the wall surface. Frequently the wall-associated electron-dense layers were seen to be partially peeled from the wall surface of the stationary-phase cells (Fig. 2a, arrows). Partially wall-attached single or multiple electron-dense layers were seen (Fig. 2b, arrows) in glutaraldehyde- and tannic acid-fixed cells. In addition, a large amount of amorphous material was bound diffusely to the wall surface (Fig. 2b, arrowheads). In exponential-phase cells, a single layer (5 to 10 nm thick) was firmly attached to the wall surface with an intervening space (20 nm or less), and release of this layer was not seen. The wall surfaces of neither the exponential-phase nor the stationary-phase cells of strain 749/C showed any electron-dense layer (Fig. 2c).

The cell culture supernatant of strain NM 105 (grown in phosphate-limiting medium) obtained by centrifugation of the culture at 10,000 × *g* was concentrated, and the charac-

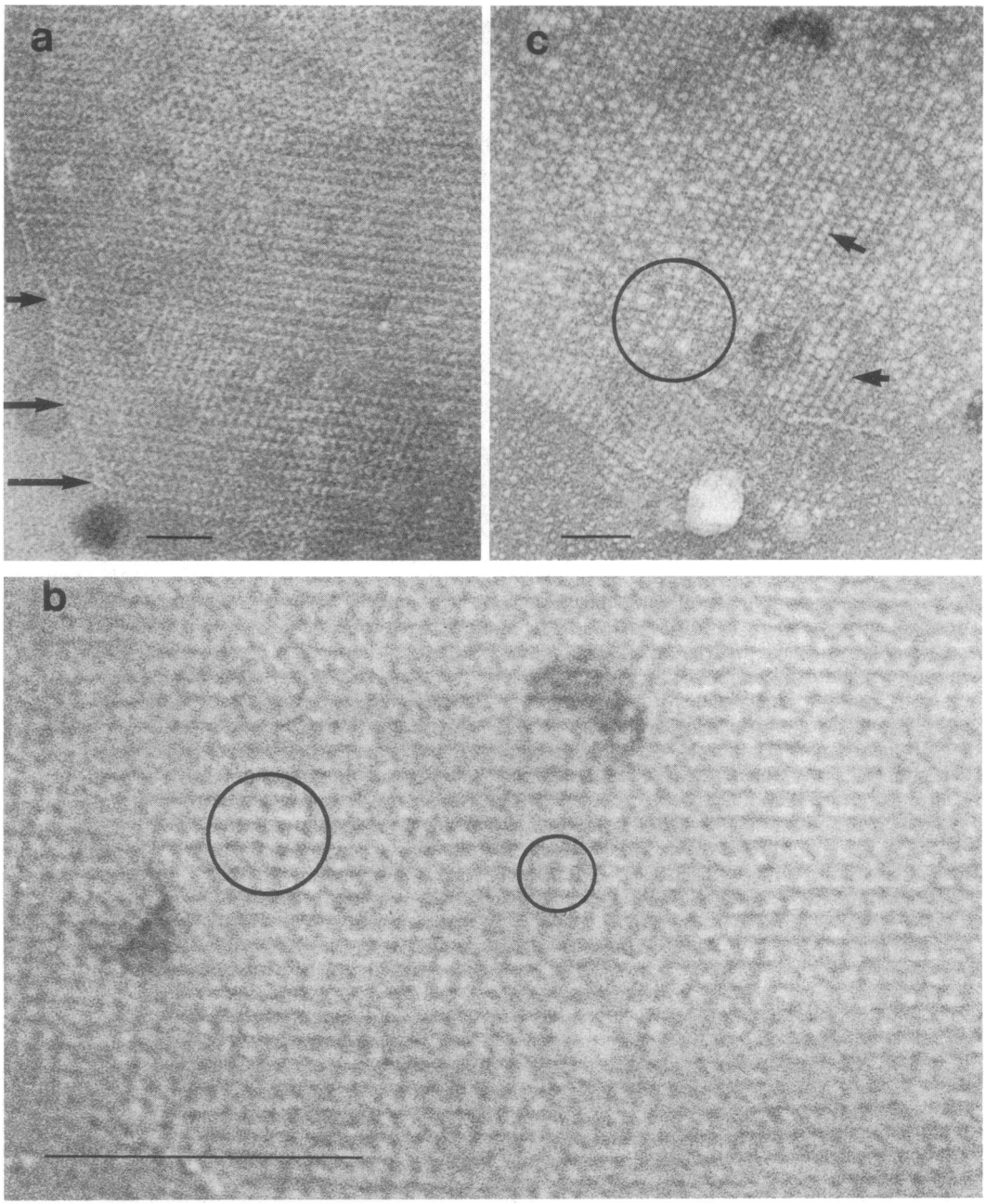


FIG. 1. Structure of the RS layer of *B. licheniformis* NM 105. (a) Segment of the RS layer released from the envelope, showing tetragonal array of the subunits. Arrows indicate the curvature at which the RS layer was separated from the envelope. (b) High magnification of a fragment of the released RS layer. The organization of the subunits of the tetragonal array is shown. Diagonal distances between the subunits were 11.6 nm (circles). (c) Partially dissociated array of the subunits (arrows). Many of these subunits appear to have aggregated, causing an increase of their apparent sizes (circle). Bars in each panel, 0.1 μm.

teristics of the proteins in this supernatant were determined by SDS-PAGE. The result presented in Fig. 3a shows that, although a significant number of protein bands are present, a band comparable to the 94-kDa standard is the heaviest. While comparable concentrations of proteins (32 to 38 μg, besides lane 1) from the culture supernatants were used for electrophoresis, there were significant increases in the densities of these bands as the age of the cultures increased. The relative densities of these protein bands (lanes 4 to 7) remained constant in cultures older than 3 days. The net protein concentrations (determined by the Lowry method) of the culture supernatants (results not shown) increased until day 3 and then remained constant. The protein in the heaviest band, corresponding to the 94-kDa standard, was tentatively identified as RS protein. In the culture supernatants of the wild-type strain (749/C), only a trace amount of this protein was found (Fig. 3b).

Purification of the RS protein. The RS protein was purified from 3-day-old cultures because of the presence of high amounts of this secreted protein. Preliminary attempts at gel filtration and ion-exchange chromatography were unsuccessful because of the tendency of the protein to aggregate, so an

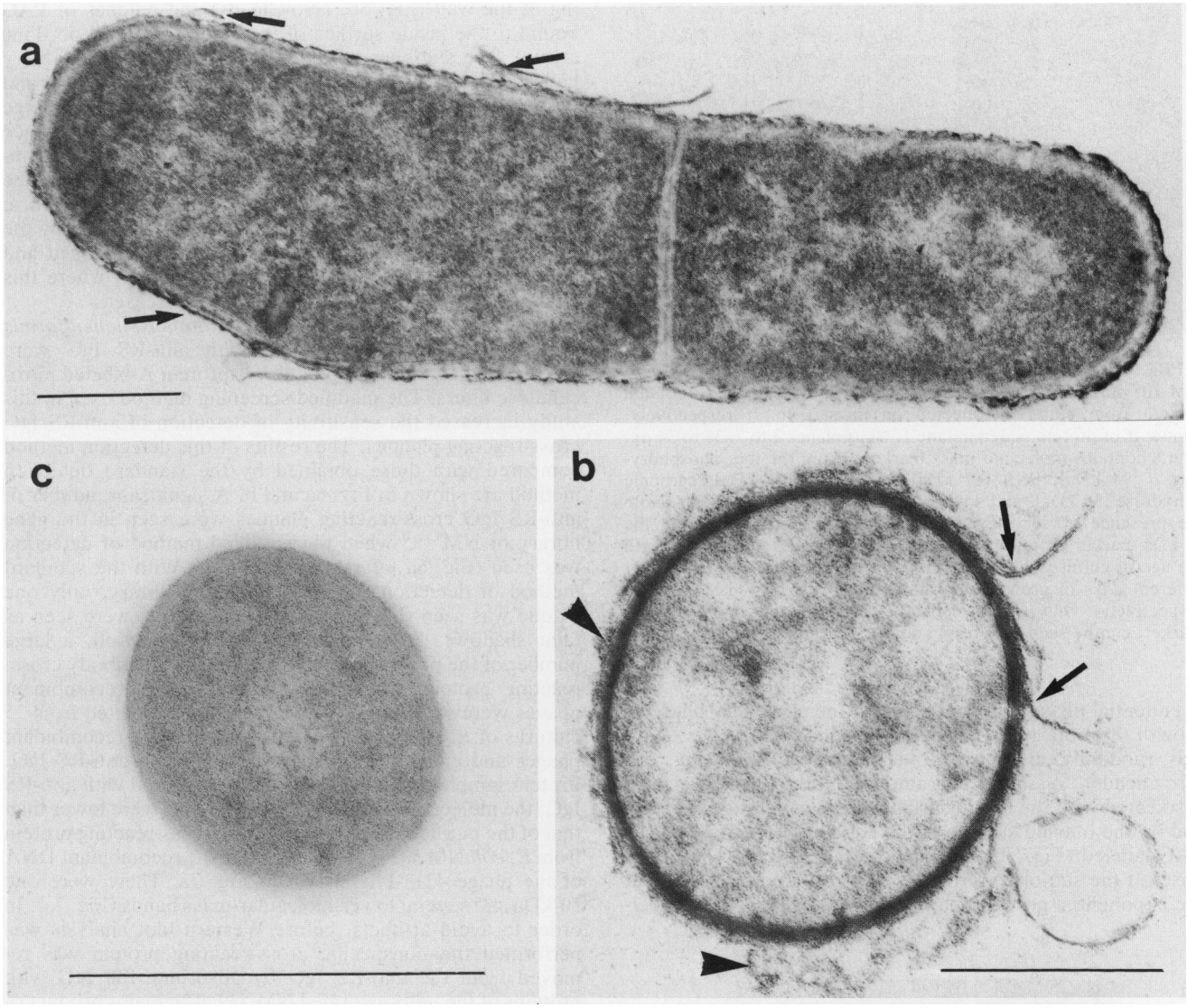


FIG. 2. Detection of RS in thin sections of *B. licheniformis* NM 105. (a) Section of *B. licheniformis* NM 105 fixed and stained with glutaraldehyde, ruthenium red, and osmium tetroxide. The presence of an electron-dense layer on the surface of the wall (arrows) is shown. (b) Section fixed in glutaraldehyde and tannic acid followed by osmium tetroxide shows partially released electron-dense layers (arrows) and amorphous material on the wall surface (arrowheads). (c) Section of *B. licheniformis* 749/C, fixed and stained as described for panel a, shows no electron-dense material on the wall surface. Bars in each panel, 0.5 μm.

electroelution method was used for the purification. The protein eluted from the SDS gels was concentrated and examined by SDS-PAGE. The results presented in Fig. 4a show a single band of the electroeluted protein. Careful examination of the band obtained from 40 μg of protein (Fig. 4a, lane 2) shows trace amounts of material which was eliminated by a second cycle of electroelution. Comparison of the purified RS protein band with the heaviest bands in the SDS gels of acetone precipitate, ammonium sulfate precipitate, and concentrated medium (results not shown) indicated that the purified protein corresponded to the tentatively identified RS protein. The R_f value calculated from the calibration curve of the standard proteins suggested that the molecular mass of the purified RS protein was 98 kDa.

This purified protein was used for antibody preparation in rabbits. The antiserum was used for IgG purification by CM Affi-Gel Blue (Bio-Rad) chromatography. The antibody was used to check the purity of protein and possible heterogeneity of the antibody by immunoelectrophoresis. The results presented in Fig. 4b show distinct single arcs in the immunoelectrophoresis gel, suggesting homogeneity of the antibody and purity of the RS protein.

The result of a Western blot analysis is shown in Fig. 4c. This autoradiogram shows 20 μg of the purified RS protein (lane 1) and 1 μg of the crude protein extracted from *B. licheniformis* NM 105. Both these proteins have matching single bands, confirming the homogeneity of the purified material. The presence of anti-RS IgG-reacting protein in the crude cell extract without any other cross-reacting protein suggested that the IgG was specific for a single protein identified as the RS protein.

Immunoelectron microscopic localization of RS protein. Frozen thin sections of the cells of *B. licheniformis* NM 105, grown in phosphate-limiting CH/S medium, were treated with anti-RS antibody and then labeled with 5-nm PAG. In Fig. 5a and b, PAG-labeled sections of the cells from

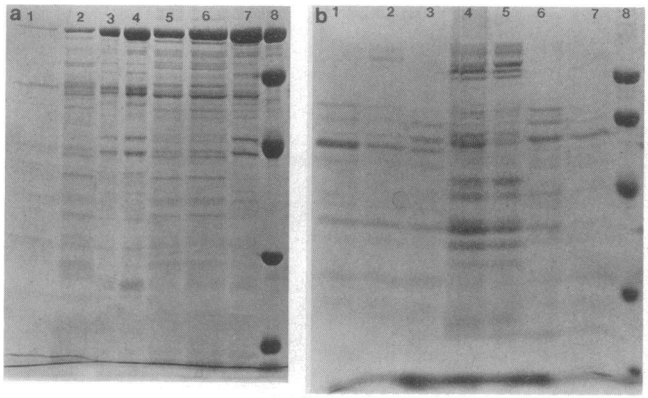


FIG. 3. (a) SDS-PAGE of protein secreted from *B. licheniformis* NM 105 culture at different times of growth. Lanes 1 to 7 contain protein from cultures collected on days 1 to 7, respectively. Amounts of protein added in lane 1, 10 μg; lanes 2 to 7, 31 to 34 μg; lane 8 contains molecular mass markers (from the top: phosphorylase *b* [94 kDa], BSA [67 kDa], ovalbumin [43 kDa], carbonic anhydrase [30 kDa], and soybean trypsin inhibitor [20 kDa]). Note the presence of very heavy bands, approximately matching the 94-kDa marker protein band, showing differences in densities with the age of culture. (b) Preparations from *B. licheniformis* 749/C at different days of growth (lanes 1 to 7 show 1- to 7-day cultures, respectively). Only traces of bands, corresponding to the 94-kDa marker, can be seen in lanes 4 and 5.

exponential phase (4 h) and onset of the stationary phase of growth (8 h) are seen. In the exponential-phase cells, PAG was randomly distributed over the cytoplasm (Fig. 5a, arrowheads). A significant amount of the PAG was also associated with the inside surface of the membrane (Fig. 5a) and on the outside surface of the envelope (Fig. 5a, arrow). PAG-labeled (Fig. 5b) sections, obtained from cells at the onset of the stationary growth phase, differed from those at the exponential growth phase in showing higher PAG labeling of the wall (Fig. 5b, arrowheads) and patches of PAG bound to the inside surface of the membrane (Fig. 5b, long arrow). The stationary-phase cells (Fig. 5c) showed a very high density of PAG on the outside surface of the wall and very little PAG within the cytoplasm. Frequently, large patches of PAG particles were observed at the inside surface of the membrane (Fig. 5c, arrows). The immunocytochemical results showed that the RS protein was synthesized in the exponential growth phase and secreted to the wall to form the RS layer. A substantial amount was also retained by the cytoplasm. This protein, however, was overproduced and hypersecreted to the wall and growth medium, where this continued to accumulate as culture age increased.

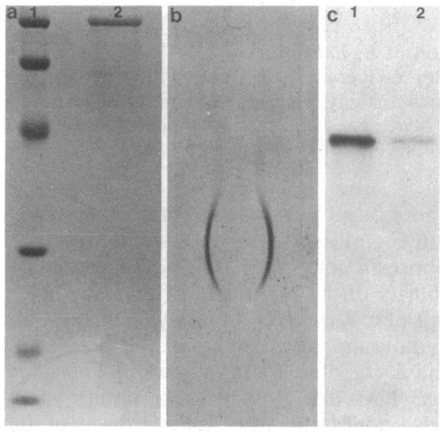


FIG. 4. (a) SDS-PAGE of RS protein purified by electroelution. Lanes: 1, molecular mass markers as described for Fig. 3; 2, purified RS protein (40 μg) shows only a single band. The faint shadow of material in lane 2 was removed by a second run of electroelution. (b) Immunoelectrophoresis of the purified RS protein; the wells contained purified RS protein (5 μg), and the trough contained anti-RS antiserum (undiluted). The single arc shows monospecificity of the antibody and purity of the protein. (c) Western blot analysis of the purified RS protein. Lane 1, 20 μg of purified protein; lane 2, 1 μg of crude cell extract from *B. licheniformis* NM 105.

Cloning of the RS protein. Plaques from a *B. licheniformis* NM 105 gene library reacting with anti-RS IgG were screened by autoradiography of ^{125}I-protein A-labeled nitrocellulose filters. The modified screening method used in this study increased the sensitivity of detection of anti-RS IgG cross-reacting plaques. The results of this detection method compared with those obtained by the standard detection method are shown in Fig. 6a and b. A significant number of anti-RS IgG cross-reacting plaques were seen in the gene library of NM 105 when the modified method of detection was used (Fig. 6a [after amplification]). With the standard method of detection and the same gene library, only one plaque was seen (Fig. 6b), but many plaques were seen as faint shadows. After selection and amplification, a large number of the plaques produced the anti-RS antibody cross-reacting protein. Many RS protein-positive recombinant phages were screened. Total proteins were isolated from 25 cultures of *E. coli* NM 539 infected with these recombinant phages and examined by Western blot using anti-RS IgG. Protein samples from all cultures cross-reacted with anti-RS IgG; the molecular masses of these proteins were lower than that of the purified RS protein. RS IgG cross-reacting protein from *E. coli* NM 539 transformed with the recombinant DNA of the phage 4213-1 is shown in Fig. 7a. There were one 50-kDa and several lower-molecular-mass bands (Fig. 7a). In order to avoid artifacts, before Western blot analysis was performed the nonspecific cross-reacting protein was removed from the anti-RS IgG by adsorbing the IgG with proteins of the untransformed NM 539. The major product of the expression of the RS gene in *E. coli*, therefore, was substantially smaller than the authentic RS protein, which was 98 kDa (Fig. 7b, lane 3).

DNA from the positive recombinant phage 4213-1 (Fig. 6c) was isolated and purified. The restriction sites of this DNA were mapped and are shown in Fig. 8. A 12.6-kbp DNA fragment was inserted in EMBL3 DNA at *Sal*I sites. Different restriction fragments of this DNA were subcloned in *E. coli* JM 109 by using the pUC8 vector for identifying the specific fragments producing proteins cross-reacting with anti-RS IgG. The first 1.2-kbp *Sal*I-*Hin*dIII fragment and the second 1.8-kbp *Hin*dIII-*Hin*dIII fragment of the inserted DNA (Fig. 8) produced proteins cross-reacting with anti-RS IgG. Both DNA fragments, when subcloned in pUC8, could be induced by IPTG, producing higher amounts of anti-RS IgG cross-reacting proteins compared with in the uninduced culture.

A hybridization experiment was done by using the 1.8-kbp *Hin*dIII fragment as a probe. The results (Fig. 9) showed that the recombinant 4213-1 DNA and the *B. licheniformis* NM 105 DNA hybridized with this probe, but the DNA of lambda EMBL3 and *B. subtilis* did not.

Although the DNA fragment in transformed *E. coli* NM 539 coded for the anti-RS IgG cross-reacting protein, it had

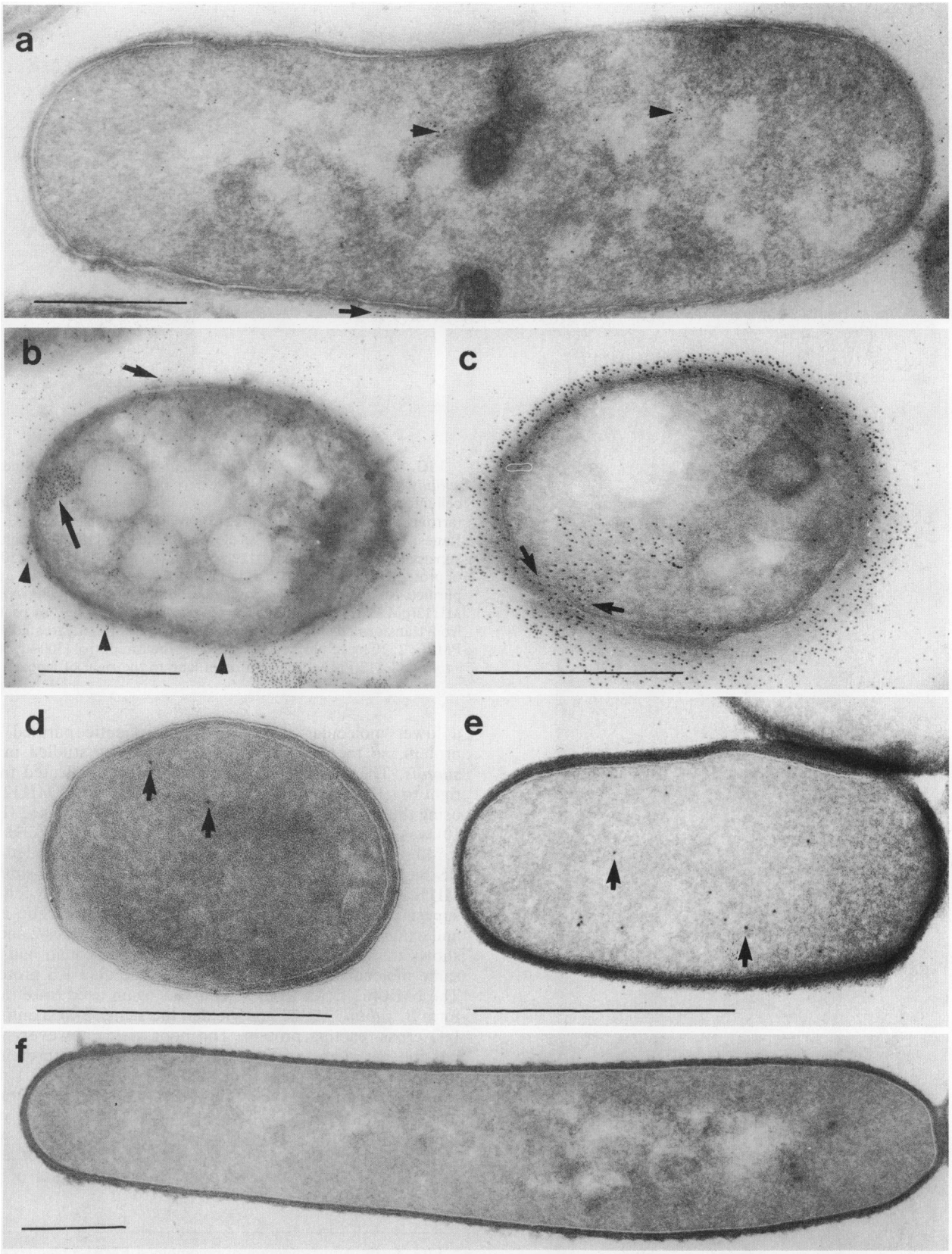
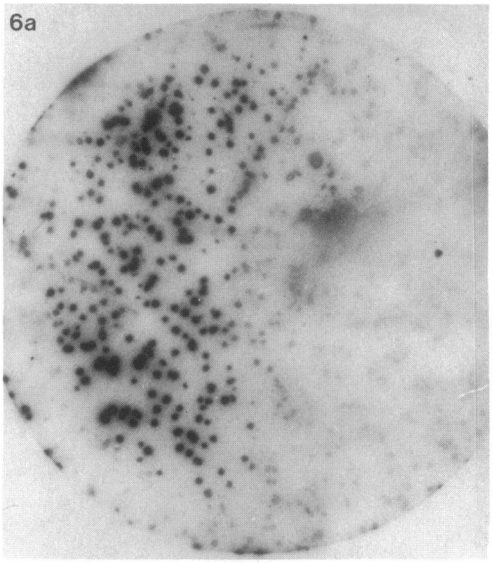
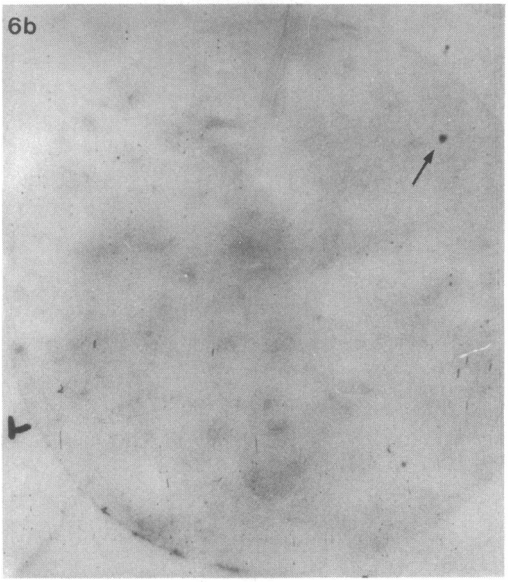


FIG. 5. Immunoelectron microscopic demonstration of RS protein in cells labeled with 5-nm gold particles. (a) Four-hour *B. licheniformis* NM 105 cell shows labeling of the wall (arrow) and random labeling of the cytoplasm (arrowheads). (b) Eight-hour *B. licheniformis* NM 105 cell shows labeling of the cytoplasm, increase of wall labeling (arrowheads), and membrane-bound cluster of label (long arrow). (c) Twenty-four-hour *B. licheniformis* NM 105 cell shows dense labeling of the wall. Random labeling of the cytoplasm is almost absent; a large membrane-bound cluster of PAG can be seen (arrows). (d) Section of transformed *E. coli* JM 109 shows sparse labeling of the cytoplasm (arrows). (e) Section of *B. subtilis* MI112 transformed with pMK462 shows significant cytoplasmic labeling (arrows). (f) Section of untransformed control of *B. subtilis* shows no reaction with PAG. Bars in all panels, 0.5 μm.





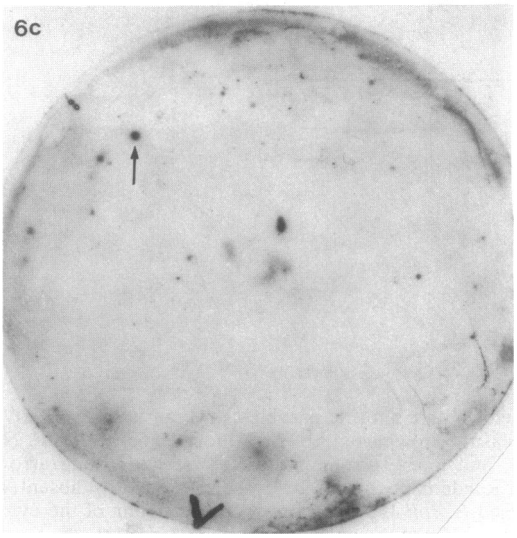


FIG. 6. (a) Autoradiogram of recombined EMBL3 plaques on a nitrocellulose filter; proteins transferred by modified method. A large number of plaques expressing anti-RS IgG cross-reacting protein are shown. (b) Plaques from the same phage culture transferred onto a nitrocellulose filter by the conventional method. Only one anti-RS IgG cross-reacting protein-positive plaque (arrow) is shown. (c) The plaque shown by the arrow (4213-1) was used for subcloning the RS gene.

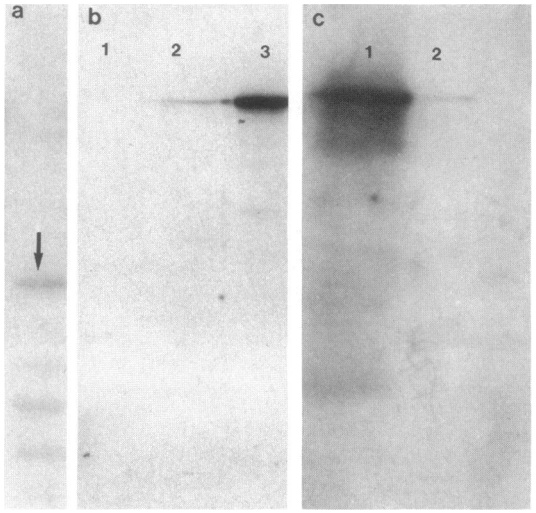


FIG. 7. Expression of RS protein in *E. coli* NM 539 and *B. subtilis* MI112 as determined by Western blots of proteins. (a) *E. coli* cell infected with 4213-1 phage shows a band at the 50-kDa position (arrow) and several other bands cross-reacting with anti-RS IgG. (b) Lanes: 1, vector control [50 μg of protein extracted from the culture supernatant of *B. subtilis* MI112(pMK4)] (no band shown); 2, 50 μg of protein from *B. subtilis* MI112(pMK462) (single band); 3, 10 μg of purified RS protein; correspondence with transformed *B. subtilis* MI112(pMK462) protein is shown. (c) Lanes: 1, 294 μg of protein from transformed culture of *B. subtilis* MI112(pMK462) treated with PMSF; 2, 10 ng of purified RS protein. Vector control [300 μg of *B. subtilis* MI112(PMK4) protein] is in lane to the right of lane 2.

a lower molecular mass than the authentic purified RS protein, so the expression of RS gene was studied in *B. subtilis*. The 4.5-kbp *Sal*I-*Eco*RI fragment (indicated from right to left in Fig. 8) was subcloned in *B. subtilis* MI112 by using the shuttle plasmid pMK4. The plasmid pMK462 (Fig. 10) was constructed from the shuttle plasmid pMK4 and the 4.5-kbp *Sal*I-*Eco*RI DNA fragment. This plasmid coded for RS-IgG-reacting protein in the transformed *B. subtilis* MI112. The protein was recovered from the cell culture supernatant by precipitation with 10% trichloroacetic acid and examined by Western blotting. Figure 7b, lanes 2 and 3, shows that this anti-RS IgG cross-reacting protein had the same molecular mass (98 kDa) as the purified RS protein. The pMK462 DNA from *E. coli* was again used to retransform *B. subtilis* MI112 and yielded the same 98-kDa anti-RS IgG cross-reacting protein. The poor yield, however, was possibly caused by protease degradation of the RS protein in the culture. In order to prevent this degradation, 0.1 mM PMSF was added to the culture of *B. subtilis* MI112 transformed with pMK462. The total anti-RS cross-reacting protein was precipitated from the cell culture supernatant with 10% trichloroacetic acid and treated for Western blot analysis. The results showed that the molecular mass of the

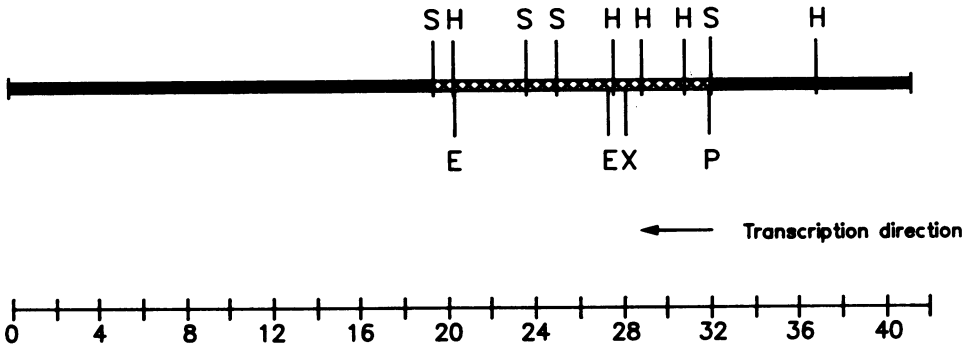


FIG. 8. Restriction map of the cloned 12.-6kbp DNA fragment of *B. licheniformis* NM 105 in 4213-1 phage; from right to left, first fragment is the 1.2-kbp *Sal*I-*Hin*dIII fragment, and the second fragment is the 1.8-kbp *Hin*dIII-*Hin*dIII fragment. When cloned in pUC8, the expression of these fragments was induced with IPTG to produce more anti-RS IgG cross-reacting protein (transcription direction shown in the figure is hypothetical). Restriction sites: P, *Pst*I; S, *Sal*I; H, *Hin*dIII; E, *Eco*RI; X, *Xho*I. Symbols: ▩, cloned NM 105 DNA (12.6 kilobases); ■, EMBL3 DNA.

protein recovered from the culture was comparable to that of the purified RS protein (Fig. 7c, lanes 1 and 2). Lane 1 of Fig. 7c, which was overloaded with six times more protein from the culture than Fig. 7b, lane 2, showed some minor cross-reacting bands. Without any PMSF treatment, there was substantial degradation. Although the RS protein was expressed, in the transformed *B. subtilis* MI112 the tetragonal structure could be detected neither on the wall surface nor in the cell culture supernatant.

The RS protein was not a functional protein, so the expression of the cloned DNA could not be confirmed by determining any enzymatic or other functional activity of the expressed protein. To confirm the expression of the RS gene, immunoelectron microscopic demonstration appeared to be suitable because the technique is specific for the detection of a protein molecule inside the cell and because application of this method clearly demonstrated the protein on the wall surface of *B. licheniformis* NM 105 (Fig. 5a through c). Frozen thin sections of *E. coli* JM 109 and *B. subtilis* MI112 transformed with pMK462 were labeled with PAG as described in Materials and Methods. Both the cells of *E. coli* (Fig. 5d, arrows) and *B. subtilis* MI112 (Fig. 5e, arrows) showed sparse labeling of the cytoplasm. The cells of *E. coli* (Fig. 5d) showed some labeling of the cell envelope, but no such labeling of the envelope could be detected in *B. subtilis* MI112(pMK462). This lack of envelope labeling was in contrast to that found in *B. licheniformis* NM 105 (Fig. 5a through c). Untransformed *B. subtilis* MI112 showed no labeling (Fig. 5d).

The RS gene expression was finally examined by in situ hybridization at the electron microscopic level. Frozen thin sections of *E. coli* transformed with plasmid pUC9 containing the RS gene were hybridized with the 1.8-kbp *Hin*dIII DNA fragment with biotin-11-dATP and immunolabeled with anti-biotin IgG treatment and PAG labeling. The result presented in Fig. 11 shows that the transformed cells reacted highly with the 1.8-kbp *Hin*dIII biotin probe because large numbers of gold particles are present in the section. Both the untransformed control and transformed control sections (without the addition of the probe) showed poor labeling with PAG. The result indicated that the growing transformed cells of *E. coli* had significant copies of pUC9 plasmid which contained RS gene and which hybridized with biotinylated *Hin*dIII DNA probe.

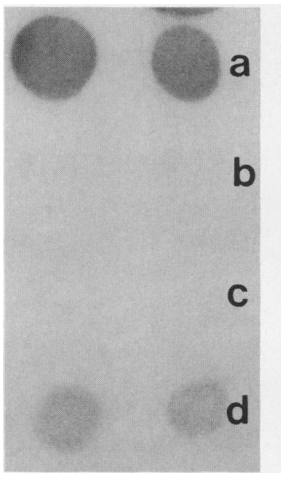


FIG. 9. Results of the hybridization experiment with the 1.8-kbp *Hin*dIII fragment as the probe. (a) Recombinant 4213-1 DNA. (b) *B. subtilis* DNA (no hybridization). (c) EMBL3 DNA (no hybridization). (d) *B. licheniformis* NM 105 DNA.

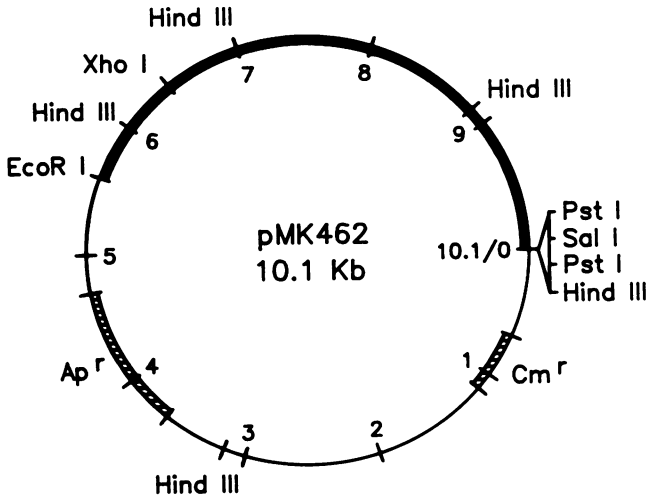
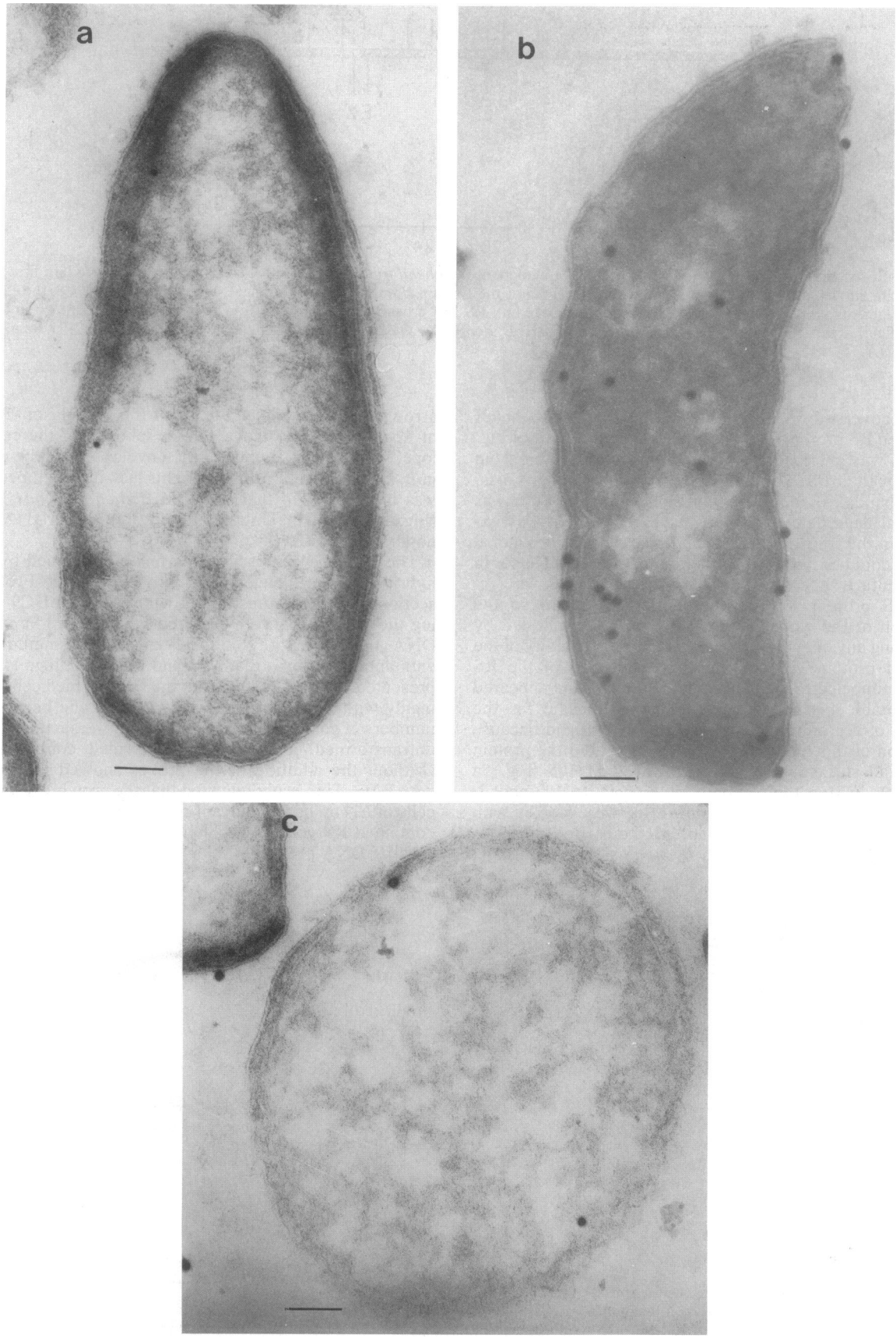


FIG. 10. Map of pMK462. Symbols: ——, vector pMK4 DNA; ■, inserted NM105 DNA; ▨, Ap^r or Cm^r.


a
b
c

DISCUSSION

RS proteins are present throughout the bacterial kingdom, particularly in natural habitats (20, 23). In laboratory cultures, however, many bacteria do not have this highly ordered protein layer on their wall surface. The present study describes the presence of this layer in a *B. licheniformis* NM 105 mutant exhibiting an alkaline phosphatase secretion deficiency phenotype. The presence of the RS protein, its organization, its cellular localization, and purification and cloning of the gene coding for the RS protein have all been explored. Information which correlates alkaline phosphatase secretion with RS protein synthesis or secretion is not available. Although the RS gene has been cloned from some gram-positive and gram-negative organisms, this study was intriguing because RS protein was overproduced and hypersecreted only in the stationary growth phase.

Electron microscopic examination revealed that large amounts of the assembled RS layer were shed from the wall surface and accumulated in the growth medium. This process continued for a considerable period while the cells were holding in the stationary growth phase. A comparison of the distribution of immunolabel (i.e., PAG) in cells at different growth phases showed that there was substantial accumulation of the RS protein in the cytoplasm during the exponential growth phase. Random distribution of the RS protein in cytoplasm was significantly reduced at the onset of the stationary growth phase and could not be detected as the age of the culture increased. Significant amounts of membrane-bound RS protein were present during all phases of growth. The pattern of membrane labeling of stationary-growth-phase cells differed from that of the exponential-growth-phase cells in showing large clusters of PAG associated with the membrane. It was possible that hypersecretion of overproduced protein required specific membrane-binding sites. The wall labeling with PAG was low in the exponential-growth-phase cells and very high in the stationary-growth-phase cells. It appeared from correlating the RS protein secretion and PAG labeling data that appreciable amounts of the RS protein were synthesized, but were poorly secreted, during the exponential growth phase. It was possible that posttranslational processing was required for both the secretion and assembly of the RS protein into tetragonal arrays; hence, when this activity was low, the RS protein accumulated in the cytoplasm. A large accumulation of this protein may have been prevented by intracellular proteolytic activity.

Purification of the RS protein indicated that the properties of this protein are comparable with those of RS proteins of other organisms (20). Attempts to purify this protein by a gel filtration procedure were unsuccessful due to the tendency of the protein to form tetragonal repeating structures and amorphous aggregates of the dissociated subunits. The molecular mass of the RS protein (98 kDa) was lower than those of the RS proteins of many bacilli. Immunoelectrophoresis results suggested that the polyclonal antibody prepared against the purified RS protein was monospecific. Single bands of the purified RS protein and of the crude extracts in Western blots indicated both monospecificity of the anti-RS antibody and purity of the RS protein. Since there was no functional assay for the RS protein, interaction with RS antibody was used to identify this protein for cloning work.

B. brevis 47 RS protein (known as the cell wall protein) gene has been cloned, and its nucleotide sequence has been determined (28, 29). The technique used was ligation of *Hin*dIII fragments into pBR322 DNA and then transformation of *E. coli*. In the present investigation, a gene library was constructed for the identification of the DNA fragment containing the RS gene. It was calculated that there was a 99% probability (5) for selecting one RS gene in 2,065 plaques of the recombinant phages. The strategy followed here may be helpful for cloning proteins comparable to the RS protein without any functional assay.

The standard procedure for nitrocellulose filter autoradiography is frequently found to be inefficient for the transfer of proteins from the plaques of recombinant phages, making detection of low-expression genes very difficult. The modification of the procedure for protein transfer to nitrocellulose filters described in this paper substantially increased the sensitivity of the method. Slow diffusion of the buffer through the agar transferred significant amounts of protein for detection by autoradiography. The results showed that without this modification, plaques reacting with anti-RS IgG could be rarely detected, but when the modification was used multiple plaques were observed. Although the recombinant phages were effectively screened for the production of the RS protein in *E. coli* JM 539, the molecular mass of this protein produced in the transformed cells was lower (75 kDa) than that of the authentic RS protein (98 kDa). Western blots showed that in transformed *E. coli* cell extract, several protein bands cross-reacting with the anti-RS IgG were present in addition to the 75-kDa band. It was previously reported by Tsukagoshi et al. (29) that MWP and CWP cell wall protein genes of *B. brevis* strain 47, when expressed in *E. coli*, produced proteins lower in molecular mass than the authentic ones. Production of the lower-molecular-mass proteins in *E. coli* may be explained by rapid intracellular degradation of the RS protein by cytoplasmic proteases, particularly when the protein existed in an unassembled condition. It was also possible that the RS protein expressed in *E. coli* had a different conformation than the authentic RS protein and was susceptible to protease degradation. It was hypothesized that the protease degradation was a regulatory response preventing the crystallization of the RS protein within the cytoplasm.

The authentic RS protein purified from NM 105 and that expressed in *B. subtilis* MI112(pMK462) had the same molecular weight. The latter protein, however, did not form tetragonal arrays on the wall surface. Subtle alterations of conditions (e.g., addition of protease inhibitor or divalent cation) did not help in the formation of tetragonal structure. The presence of the RS protein was detected by immunoelectron microscopy in the transformed cells of *E. coli* and *B. subtilis*. Immunolabeled materials were found primarily in the cytoplasm and in the growth medium, but none was wall associated. It is postulated from the results presented here that processing RS protein after its synthesis is essential for

FIG. 11. Result of in situ hybridization of frozen thin sections of *B. licheniformis* NM 105 with biotinylated 1.8-kbp *Hin*dIII DNA fragment and immunolabeling of the sections with PAG. The sections were treated with anti-biotin rabbit IgG followed by 15-nm PAG. (a) Hybridized and immunolabeled section of an untransformed cell of *E. coli* JM 109. (b) Section of a transformed and hybridized cell; many gold particles, indicating a significant number of copies of plasmid containing RS gene, can be seen. (c) Section of a transformed cell without hybridization shows very few gold particles. Bar in each panel, 0.1 μm.

the formation of the tetragonal array. Both the *E. coli* and *B. subtilis* cells were deficient in this processing activity. Further study is in progress to attempt to explain this phenomenon. Poor recovery of RS protein from the cell culture supernatant in the absence of the protease inhibitor PMSF suggested that the protein was unstable because of high sensitivity to protease digestion.

The entire 4.5-kbp fragment subcloned in *B. subtilis* expressed the authentic RS protein. The 1.2-kbp *Sal*I-*Hin*dIII and 1.8-kbp *Hin*dIII-*Hin*dIII fragments subcloned in *E. coli* also produced the anti-RS IgG cross-reacting protein. The expression of these subcloned DNA fragments could be induced in *E. coli* with the β-galactosidase inducer IPTG. Finally, hybridization with the 1.8-kbp *Hin*dIII fragment showed that the above-mentioned 4.5-kbp DNA fragment originated from the DNA of *B. licheniformis* NM 105. In situ hybridization showed that the RS gene was highly expressed from the plasmid pUC9. Substantial immunolabeling of frozen thin sections of transformed *E. coli* indicated that high copy numbers of plasmid were present in the cells. Other gene factors may have been absent, preventing tetragonal structure formation. It was concluded from these observations that the 4.5-kbp *Sal*I-*Eco*RI fragment contained the RS gene. On the basis of this conclusion, work is in progress to sequence the 1.2- and 1.8-kbp fragments of the cloned DNA.

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