Molecular Cloning of a Major Cell Wall Protein Gene from Protein-Producing *Bacillus brevis* 47 and Its Expression in *Escherichia coli* and *Bacillus subtilis*

NORIHIRO TSUKAGOSHI,* RYO TABATA, TOMOAKI TAKEMURA, HIDEO YAMAGATA, AND SHIGEZO UDADA

Department of Food Science and Technology, Faculty of Agriculture, Nagoya University, Nagoya 464, Japan

Received 31 January 1984/Accepted 14 March 1984

*Bacillus brevis* 47 contains two major cell wall proteins. Each protein forms a hexagonal array in the cell wall. A 4.8-kilobase *HindIII* fragment of *B. brevis* 47 DNA cloned into *Escherichia coli* with pBR322 as a vector directed the synthesis of polypeptides cross-reactive with antibody to the middle wall protein. A 700-base-pair *BamHI-HpaI* fragment was shown to be the essential region for the synthesis of immunoreactive polypeptides. Furthermore, this fragment appeared to contain the promoter activity. The 3.5-kilobase *BamHI* fragment covering the essential region as well as its downstream sequence was subcloned into the corresponding restriction site of pUB110 by using *Bacillus subtilis* as the cloning host. Both *E. coli* and *B. subtilis* carrying the cloned DNA synthesized several immunoreactive polypeptides which were mainly found in the cytoplasm. *B. subtilis* secreted polypeptides cross-reactive with antibody to the middle wall protein. These extracellular polypeptides were degraded upon prolonged culture.

The cell wall protein gene of *B. brevis* 47 was expressed in both *E. coli* and *B. subtilis*. *B. subtilis* carrying the cloned DNA fragment secreted into the medium polypeptides cross-reactive with antibody to the cell wall protein.

**MATERIALS AND METHODS**

**Bacterial strains, media, and transformation.** *B. brevis* 47 was used for the isolation of the cell wall protein genes. *E. coli* HB101 (3) was used as the cloning host. *B. subtilis* RM141 (restrictionless, modificationless, *recE*, and a generous gift from T. Uozumi, University of Tokyo) and *B. subtilis* BD630 were used to subclone the cell wall protein gene and determine insertional activation of the CAT (chloramphenicol acetyltransferase) gene on pGR71 (a generous gift from R. H. Doi, University of California, Davis), respectively. T$_2$ medium (32) was used to grow *B. brevis* 47 and transformants of *B. subtilis* RM141. Difco antibiotic medium 3 was used to grow *B. subtilis* strains RM141 and BD630. L broth (30) was used to grow *E. coli*. All strains were grown at 37°C with either reciprocal or rotary shaking. Transformation of *E. coli* HB101 and *B. subtilis* strains RM141 and BD630 was done by the methods of Lederberg and Cohen (11) and Chang and Cohen (4), respectively. Transformants of *E. coli* HB101 were selected on L broth plates supplemented with 50 μg of ampicillin per ml. Protoplasts of *B. subtilis* were regenerated on DM3 plates supplemented with 300 μg of kanamycin per ml, and transformants were selected by replication on Difco antibiotic medium 3 plates supplemented with 10 μg of kanamycin per ml.

**Extraction of DNA.** *B. brevis* 47 grown in T$_2$ medium was harvested at the end of the exponential growth phase. Chromosomal DNA was prepared as described by Saito and Miura (20) and stored at 4°C in TE buffer (10 mM Tris-hydrochloride [pH 7.5], 1 mM EDTA). Plasmid pBR322, pGR71, and pUB110 DNAs were prepared as described by Tanaka and Weisblum (27) from the cleared lysates of *E. coli* KY2562(pBR322) (21), *E. coli* HB101(pGR71) (7), and *B. subtilis* NIG1121(pUB110) (19), respectively. Rapid small-scale plasmid extraction from *E. coli* was carried out as described by Birnboim and Doly (2). Since this method did...
not give satisfactory results for *B. subtilis* RM141, the procedure was modified as follows. *B. subtilis* RM141 carrying a plasmid isolated to the middle logarithmic phase in Difco antibiotic medium 3 supplemented with 10 µg of kanamycin per ml was first converted to spheroplasts in SMMP solution containing 10 µg of kanamycin and 2 mg of lysozyme per ml, followed by incubation at 37°C for 1 h as described by Chang and Cohen (4). Then, plasmid DNA was extracted from the spheroplasts (2), except that lysozyme was removed from solution 1.

Cloning procedures. pBR322 DNA was cleaved with restriction endonuclease HindIII and then treated with bacterial alkaline phosphatase (Millipore Corp., Freehold, N.J.). Chromosomal DNA isolated from *B. brevis* 47 was partially digested with HindIII. The HindIII-digested DNA (0.5 µg of pBR322 and 1.6 µg of chromosomal DNA) were annealed in 100 µl of 66 mM Tris-hydrochloride buffer (pH 7.5) containing 10 mM MgCl₂ and 10 mM dithiothreitol in an ice bath for 7 to 8 h and then ligated with 2 U of T4 DNA ligase per ml at 12.5°C for 18 h after the addition of neutralized ATP to a final concentration of 0.4 mM. The ligated DNA was used for the transformation of *E. coli*.

When pUB110 was used to subclone a DNA fragment coding for the cell wall protein, pUB110 was cleaved with *Bam*H1I, followed by treatment with bacterial alkaline phosphatase and ligation with the DNA fragment generated from pBR322 derivatives by digestion with *Bam*H1I as described above. The ligated DNA was used for the transformation of *B. subtilis* RM141.

Biochemical procedures. CNBr-activated paper was prepared as described by Clarke et al. (5). Rabbit antiserum to the two major cell wall proteins, 130,000- and 150,000-dalton proteins, were prepared as in earlier studies (17, 29). The IgG fraction was prepared from each antiserum by precipitation with ammonium sulfate as described by Livington (12) and kept at -20°C in 25 mM potassium phosphate buffer (pH 7.5) containing 100 mM NaCl. Protein A of *Staphylococcus aureus* (Pharmacia Fine Chemicals) was iodinated by using lactoperoxidase and 125I to a specific activity of 5 x 10⁶ to 10 x 10⁶ cpm/µg.

Analysis of restriction fragments. All restriction enzymes used were obtained from commercial suppliers and were used under the conditions recommended by the suppliers. Restriction fragments were analyzed by agarose gel (1%) and polyacrylamide gel electrophoresis as described by Sharp et al. (23) and Manniatis et al. (14). DNA cleaved with HindIII was used as a molecular weight reference.

Radioimmune screening of bacterial colonies and detection of polypeptide antigens. Colonies carrying *B. brevis* cell wall protein genes were detected by an in situ colony immunosay by using antibodies to cell wall proteins and 125I-labeled protein A as described by Kemp and Cowman (9). Colonies containing material capable of reacting with the cell wall protein antibodies were detected by radioautography. Protein samples were electrophoresed on 10% sodium dodecyl sulfate-polyacrylamide slab gels along with RNA polymerase B of *Bacillus steatorrhophilus* (Seikagaku Kogyo Ltd.) as a molecular weight marker as described by Laemmli (10) and transferred electrophoretically (60 V, 20 h, 4°C) to nitrocellulose as described by Towbin et al. (28). To visualize proteins, the nitrocellulose sheets were stained briefly with amido black (0.1% in 45% methanol–10% acetic acid) and destained with 90% methanol–2% acetic acid (22).

The nitrocellulose sheets stained with amido black were incubated at room temperature for 24 h with 3% bovine serum albumin dissolved in 10 mM Tris-hydrochloride buffer (pH 7.4) containing 0.9% NaCl and 0.01% NaN₃, followed by incubation with antibody to the 150,000-dalton protein (230 µg of protein per ml) and 125I-labeled protein A (10⁶ cpm/ml) as described by Towbin et al. (28). The same antibody solution was used repeatedly in this experiment. The reaction of transferred peptides with antibody and 125I-labeled protein A was detected by radioautography.

Localization of the cloned gene products in *E. coli* and *B. subtilis*. *E. coli* grown in 250 ml of LB broth containing 50 µg of ampicillin per ml was subjected to osmotic shock (16) or converted to spheroplasts (1) to obtain periplasmic protein. Periplasmic protein was that found in the supernatant after cold-water treatment or that released into the sucrose solution when spheroplasts were formed.

Cytoplasmic materials and cell envelope fractions were also prepared by disrupting cells by sonication, followed by high-speed centrifugation.

Other analytical procedures. Protein was determined as described by Lowry et al. (13), with bovine serum albumin as a standard. When extracellular protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the protein was first precipitated with 5% trichloroacetic acid, solubilized with 1% sodium dodecyl sulfate, and heated at 100°C for 5 min in the presence of 1% β-mercaptoethanol.

RESULTS

Construction and identification of clones carrying cell wall protein genes. The *B. brevis* 47 DNA was cut with HindIII, and the fragments were ligated to the pBR322 DNA cleaved with HindIII as described above. The ligated molecules were used to transform *E. coli* HB101 to ampicillin resistance. Transformants carrying *B. brevis* DNA inserts were screened for the production of cell wall protein antigens by using antibodies and 125I-labeled protein A. A mixture of antibody to the outer wall protein (130,000-dalton protein) and that to the middle wall protein (150,000-dalton protein) was used for the initial screening, since we aimed to clone both major cell wall protein genes. Three clones, designated NT100, NT200, and NT300, were found to react with the mixture of antibodies in the initial screening of ca. 4,000 colonies. After purification of these clones, the specificity of the reaction with antibody either to the 130,000-dalton protein or to the 150,000-dalton protein was determined. Strain NT100 produced polypeptides cross-reactive only with the antibody to the 150,000-dalton protein, whereas the other two strains, NT200 and NT300, produced polypeptides cross-reactive with both the antibody to the 130,000-dalton protein and that to the 150,000-dalton protein. Strains NT200 and NT300 were found to carry the same recombinant plasmid DNA as described below.

Analysis of recombinant plasmid DNA. Three plasmids, pNT100, pNT200, and pNT300, were isolated from strains NT100, NT200, and NT300, respectively. On digestion with HindIII, pNT100 was found to contain three HindIII inserts (3, 1.5, and 0.3 kilobases [kb]), whereas pNT200 and pNT300 contained two HindIII inserts (1.8 and 0.17 kb). pNT200 and pNT300, on further restriction analysis, were shown to have an identical insert. Restriction maps of pNT100 and pNT200 are shown in Fig. 1. pNT200 directed the synthesis of polypeptides cross-reactive with both the antibody to the 130,000-dalton protein and that to the 150,000-dalton protein, although the size of the insert was considerably smaller than that of pNT100. pNT200 was not characterized further.

Reduction of the size of the insert. pNT100 was cut with
HindIII, and the fragments were religated. Transformants (Ap⁺Tc³) were selected and evaluated by the colony immunoassay to determine which contained polypeptides cross-reactive with the antibody to the 150,000-dalton protein. All colonies positive to the colony immunoassay contained at least the 3-kb HindIII insert with the same orientation as pNT100. One of the strains, carrying only the 3-kb HindIII insert, was designated as NT110.

We further reduced the size of the insert to identify the essential region on pNT100 for the synthesis of polypeptides cross-reactive with the antibody to the 150,000-dalton protein. After much empirical experimentation, the 1.3-kb HpaI-Hpal region appeared to be essential for the synthesis of polypeptides cross-reactive with the antibody to the 150,000-dalton protein. This region was divided into three portions: (i) a 400-base-pair (bp) Hpal-BamHI fragment, (ii) a 700-bp BamHI-Hpal fragment, and (iii) a 200-bp Hpal-Hpal fragment (Fig. 1 and 2). We then determined which portions were essential for the synthesis of immunoreactive polypeptides. pNT100 was cut with Hpal, and the fragments were religated. Transformants were randomly selected and colonies positive to the colony immunoassay were analyzed for plasmid DNA (Fig. 2). On pNT22, fragments i, ii, and iii were integrated in the opposite orientation to those on pNT100, suggesting that the essential region might be localized in these regions. The absence of fragment iii on pNT21 did not affect the synthesis of polypeptides cross-reactive with the antibody to the 150,000-dalton protein, indicating that fragment iii is not essential for the synthesis of polypeptides. Therefore, fragment i or ii or both should contain the N-terminal portion of the cloned DNA. Then, pNT100 was cut doubly with BamHI and Hpal, and the fragments were religated. One of transformants positive to the colony immunoassay carried pNT132, which contained fragment ii in addition to two iii fragments. The orientation of these two iii fragments on pNT132 was not determined. These analyses indicate that fragment ii should be most essential for the synthesis of the immunoreactive polypeptides. Although we tried to further reduce the size of the insert, clones positive to the colony immunoassay were not obtained, possibly because the size of the polypeptide synthesized was not large enough for an immunoreaction.

Subcloning into pUB110 of the 3.5-kb BamHI fragment of pNT100. The 3.5-kb BamHI fragment on pNT100 was ligated with pUB110 cleaved with BamHI and used to transform B. subtilis RM141 to kanamycin resistance. Ten clones of ca. 1,000 kanamycin-resistant colonies were found to react with the antibody to the 150,000-dalton protein. Plasmids isolated from these clones were observed to have undergone spontaneous deletions of inserts to various extents. All plasmids were maintained stably in B. subtilis RM141. For two of these plasmids, the insert was readily released by digestion with BamHI. For other plasmids, the insert was not released by BamHI digestion. Restriction maps of three representative plasmids are shown in Fig. 3. pTT5 contained the longest insert (1.7 kb), with two BamHI sites intact, whereas other plasmids contained 0.95- to 1.65-kb inserts. All plasmids, however, contained fragment ii of pNT100 and directed the synthesis of polypeptides cross-reactive with the antibody to the 150,000-dalton protein.

Detection of promoter activity on pNT100. An expression vector, pGR71 (7), was used to demonstrate which region of the cloned DNA fragment on pNT100 contained the promoter activity for the 150,000-dalton protein. Insertional activation of the Tn9-derived chloramphenicol resistance in B. subtilis was examined by using various DNA fragments isolated from pNT110. The 680-bp Hinfl fragment shown in Fig. 1, one of the DNA fragments examined, insertional activated the CAT gene in B. subtilis. The Hinfl fragment was inserted into the HindIII site of pGR71 as follows. The
HindII fragment was electroeluted from a polyacrylamide gel after HindII-digested pNT110 DNA was electrophoresed on a 5% polyacrylamide gel. Both termini of the fragment were filled in with the Klenow fragment of E. coli DNA polymerase I and then ligated with a phosphorylated HindIII adaptor (CAAGCTTGT), followed by digestion with HindIII and isolation of the fragment on a small Sephadex G150 column. This HindIII-adapted restriction fragment was inserted into the HindIII site of pG7R1.

Transformants were first selected for the presence of plasmid on kanamycin plates and then replica plated onto Difco antibiotic medium 3 plates containing 5 μg of chloramphenicol per ml. When analyzed with restriction enzymes, one plasmid isolated from a Cm' colony was found which had the HindII fragment inserted immediately before the CAT gene in the same orientation that on pNT100. This plasmid conferred chloramphenicol resistance. The CAT gene was also expressed when the plasmid was introduced into E. coli. This indicates that the HindII fragment contained a promoter activity, and the direction of transcription on pNT100 shown in Fig. 1 should be clockwise.

Products of the cloned gene. Products of E. coli and B. subtilis carrying the cell wall protein gene were examined by the Western blot procedure as described above (Fig. 4). E. coli(pNT100) produced six polypeptides cross-reactive with the antibody to the 150,000-dalton protein, although the antibody cross-reacted nonspecifically with some cellular polypeptides of E. coli(pBR322) (Fig. 4A). The largest molecular weight of immunoreactive polypeptides synthesized in E. coli(pNT100) was ca. 130,000, and major products were polypeptides with approximate molecular weights of 85,000, 68,000, and 32,000. E. coli(pNT110) mainly produced a 32,000-molecular-weight polypeptide. B. subtilis(pTT5), which carried a much shorter insert, also produced several polypeptides cross-reactive with the antibody (Fig. 4B). The antibody, however, did not cross-react with any cellular polypeptides of B. subtilis(pUB110). The largest molecular weight of immunoreactive polypeptides produced in B. subtilis(pTT5) was ca. 100,000, and major products were polypeptides with approximate molecular weights of 100,000, 65,000, and 60,000.

Comparison of the intensity of polypeptide bands shown in Fig. 4B indicates that the cloned gene appears to be expressed more efficiently in B. subtilis than in E. coli.

Localization of immunoreactive polypeptides synthesized in E. coli and B. subtilis. Immunoreactive polypeptides synthesized in E. coli(pNT100) were found both in the periplasm and the cytoplasm of the cells (Fig. 5A). The composition of polypeptides in the periplasmic fraction (Fig. 5A, lanes 5 and 8) prepared by two different methods was essentially the same as that of total cellular polypeptides (Fig. 5A, lanes 7 and 10), indicating that some of the products in E. coli were secreted into the periplasmic space. The cell envelope fraction (Fig. 5A, lane 2) prepared by sonication contained one major cross-reacting polypeptide with an approximate molecular weight of 110,000. This polypeptide, however, should be due to contamination by either periplasmic or cytoplasmic materials or both, since neither the outer nor inner membrane fraction enriched by centrifugation on dis-
continuous sucrose gradients contained immunoreactive polypeptides (data not shown).

*B. subtilis* pTT5 secreted polypeptides cross-reactive with the antibody to the 150,000-dalton protein (Fig. 5B, lanes 1 and 5), although most polypeptides were observed both in the cytoplasm and cell envelope fraction (Fig. 5B, lanes 3, 4, 7, and 8). Polypeptides secreted at the middle logarithmic phase of growth (Fig. 5B, lane 1, and Fig. 5C, lane 1) consisted mainly of a polypeptide with a molecular weight of 65,000. The amount of the major extracellular polypeptide increased up to the end of the logarithmic phase of growth (Fig. 5C, lane 2) judging from the intensity of the band. At the early stationary phase of growth (Fig. 5C, lane 3) polypeptides began to be degraded, and new polypeptides with lower molecular weights were detected. After 24 h of growth (Fig. 5C, lane 4), almost all polypeptides were degraded into small peptides.

**DISCUSSION**

Isolation and chemical characterization of regular surface arrays have been undertaken in only a few bacterial strains, whereas morphological studies of the regular surface arrays have been performed for a wide range of gram-positive and gram-negative organisms. The regular structure consists mainly of protein, which appears to contain all the information needed to form the regular arrays (25). The amino acid sequences of proteins forming the regular arrays, however, are not yet known for any organism.

*B. brevis* 47 contains two major cell wall proteins. Each protein forms hexagonal arrays in the cell wall (29, 31). Since no biological activities were known for either protein, immunological procedures were used to clone genes coding for the two proteins as described above. Although no clones were isolated that produced polypeptides cross-reactive only with the antibody to the outer wall protein (130,000-dalton protein), a clone (NT100) was obtained that produced polypeptides cross-reactive only with the antibody to the middle wall protein (150,000-dalton protein). pNT100 contained a 4.8-kb insert at the HindIII site of pBR322 (Fig. 1). The minimum size of the insert for immunological detection was the ca. 700-bp fragment (ii fragment) on pNT100 (Fig. 1 and 2). Fragment ii was found to be the essential region for the synthesis of immunoreactive polypeptides. When the 3.5-kb BamHI fragment on pNT100 was subcloned into the BamHI site of pUB110 by using *B. subtilis* as the cloning host, all clones positive to the colony immunooassay contained plasmids which had undergone large deletions (Fig. 3). All plasmids isolated from positive clones, however, contained fragment ii and were maintained stably. This also indicates that fragment ii is essential for the synthesis of immunoreactive polypeptides. The HindII fragment shown in Fig. 1, which covered about two-thirds of fragment ii, insertionally activated the promoter-deficient CAT gene on pGR71 in *B. subtilis*. Taken together with the results of immunological assays, this suggests that fragment ii contains the promoter region for the 150,000-dalton protein and that the direction of transcription should be clockwise. The distance between fragment ii and the end of the HindIII insert was 3.1 kb, indicating that pNT100 contains about two-thirds of the entire gene for the 150,000-dalton protein.

pTT5, one of the plasmids that directed the synthesis of immunoreactive polypeptides in *B. subtilis*, was used to transform *B. brevis* 47 to neomycin resistance by the Tris-polyethylene glycol procedure (26). The plasmids isolated from transformants of *B. brevis* 47 were identical with pTT5 when analyzed with restriction enzymes. Plasmids prepared from *B. subtilis* seem to be introduced intact into *B. brevis* 47. We are now in the process of constructing a secretion vector in *B. brevis* 47 with the DNA fragment on pNT100.

Products of the cloned gene in *E. coli* and *B. subtilis* were different from each other and consisted of several polypeptides (Fig. 4). For now we assume that most of these polypeptides are degradation products, although this remains to be proved. Immunoreactive polypeptides were found mainly in the cytoplasm of both *E. coli* and *B. subtilis*. *E. coli* secreted into the periplasm polypeptides with the same composition as the cellular polypeptides. *B. subtilis* secreted into the medium polypeptides whose composition differed significantly from that of the cellular polypeptides (Fig. 5B). Upon prolonged incubation, the extracellular polypeptides were degraded into small polypeptides, whereas the composition of the cellular polypeptides remained almost constant (Fig. 5B and C). This is also the case with *B. subtilis* carrying the Semliki Forest virus E1 protein gene (18). Even though the full-sized E1 protein is found in the cell, very little full-size E1 protein can be detected extracellularly.

Although we have not isolated clones which produce polypeptides cross-reactive only with the antibody to the 130,000-dalton protein, clones carrying pNT200 with the 170-bp HindIII fragment deleted were negative to the colony immunooassay when the antibody to the 130,000-dalton protein was used. This suggests that the 170-bp HindIII fragment on pNT200 might be specific for the 130,000-dalton protein. We are now in the process of cloning the entire gene.
coding specifically for the 130,000-dalton protein by using the 170-bp HindIII fragment as a probe. We are also in the process of determining the nucleotide sequence of fragment II as well as its downstream sequence. The amino acid sequence deduced from the nucleotide sequence may facilitate understanding of the properties of subunits forming crystalline structures on the cell surface.

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LITERATURE CITED