

Conserved Structures of Cell Wall Protein Genes among Protein-Producing *Bacillus brevis* Strains

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Bacillus brevis HPD31 contains a surface (S)-layer protein, termed the HWP, which forms a hexagonal array in the cell wall. The 5' region of the HWP gene was isolated from a DNA library constructed in bacteriophage vector EMBL3 from a partial *Bam*HI digest of the chromosomal DNA. The 3' region contained in a 2.7-kilobase *Bgl*III fragment of the DNA was cloned into *Escherichia coli*, using pUC118 as a vector. On the basis of the chemically determined N-terminal amino acid sequence, the HWP gene was found to encode a polypeptide consisting of 1,087 amino acid residues with a signal peptide of 53 or 23 amino acid residues. The deduced amino acid composition was similar to the chemical amino acid compositions of other S-layer proteins in the predominance of acidic relative to basic amino acids and in the very low content of sulfur-containing amino acids. The deduced amino acid sequence showed high homology (78%) with that of the middle wall protein of *B. brevis* 47. Furthermore, the multiple 5' ends of the HWP gene transcripts detected on S1 nuclease analysis closely resembled those of the middle wall protein gene transcripts. This complex structure was also conserved (greater than 85%) in the regulatory regions of two other cell wall protein genes isolated from *B. brevis* HPD52 and HP033, suggesting that the synthesis of the cell wall proteins is intricately regulated through a similar mechanism in protein-producing *B. brevis*.

A number of gram-positive and gram-negative bacteria possess a regular surface layer, the so-called S layer, which is now defined as a two-dimensional crystalline array of proteinaceous subunits forming a surface layer on procaryotic cells (28, 29). The morphological properties of S layers have been extensively characterized for a wide range of microorganisms (25-27), whereas the genes for S-layer-forming proteins have been isolated from only a few microorganisms, such as *Bacillus brevis* 47 (35, 37, 39, 41), *Halobacterium halobium* (11), *Deinococcus radiodurans* (18, 19), *Caulobacter crescentus* (30), and *Aeromonas salmonicida* (4). Too little is known at present about the regulation of these genes to elucidate the mechanisms involved in the biosynthesis, transport, and assembly of S-layer proteins. Isolation and characterization of the genes encoding S-layer-forming proteins from diverse origins are essential.

Recently, we newly isolated many protein-producing *B. brevis* strains from soil from diverse origins (32) for comparison with *B. brevis* 47, the S layer of which has been extensively characterized from various aspects: morphology (40), chemical and immunological properties of the S-layer-forming proteins, and characterization of genes for those proteins (35, 37). Protein-producing *B. brevis* strains all have S layers showing hexagonal symmetry, with a lattice constant of approximately 18 nm (8).

The genes for two S-layer proteins, termed the outer wall protein and middle wall protein (MWP), of *B. brevis* 47 constitute a cotranscriptional unit (*cwp* operon) and are transcribed from several tandem promoters located upstream of the MWP gene. The MWP gene contains two translation initiation sites (41). The complex structure of the 5' region of the *cwp* operon has been suggested to play an

important role in differential regulation of the gene expression (1).

B. brevis HPD31 secretes a protein derived from a cell wall component, up to 30 g/liter under optimal growth conditions (32). *B. brevis* HPD31 contains one major protein, designated HWP, with an approximate molecular mass of 135 kilodaltons in its cell wall, the structure of which is different from that of *B. brevis* 47 (8). Immunological analysis indicated that HWP of *B. brevis* HPD31 is closely related to MWP of *B. brevis* 47 (8). To further characterize HWP and the regulation of its synthesis in *B. brevis* HPD31, the gene for HWP was cloned and sequenced. Both the HWP and MWP genes showed highly homologous sequences. Multiple promoters and dual translation initiation sites were also found in the 5' region of the HWP gene. To demonstrate further the existence of such a complex structure in other cell wall protein genes, we cloned and sequenced the genes from two other strains, *B. brevis* HPD52 and HP033. All sequences in the regulatory regions of the four cell wall protein genes were highly conserved. The synthesis of cell wall proteins appears to be regulated strictly among protein-producing *B. brevis* strains.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, plasmids, media, and transformation. The bacterial strains used were *B. brevis* 47 (16), HPD31, HPD52, and HP033 (32) and *Escherichia coli* Q359 (13) and JM103 (42). The surface properties of the *B. brevis* strains used are shown in Table 1. *B. brevis* was grown at 37°C in T2 medium (43). *E. coli* Q359 and JM103 were grown at 37°C in TB broth (1% tryptone, 0.5% NaCl, pH 7.4) and L broth (22), respectively. When required, ampicillin was added at a concentration of 50 µg/ml. *E. coli* Q359 was used for the growth of recombinant λEMBL3 phages; JM103 was used as the cloning host and for DNA manipulation and growth of M13 phages. Phage vector

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TABLE 1. Properties of surface structures of protein-producing *B. brevis* strains

Strain	No. of S layers	Molecular masses of S-layer proteins (kilodaltons)	Lattice constant (nm)	Reference
47	2	104, 115	18.3	35-37, 40
HPD31	1	135, 150 ^a	18.1	8
HPD52	1	75, 115	17.4	8
HP033	2	110, 150	18.4	8

^a Minor component.

EMBL3 (7) and pUC plasmids (44) were used as cloning vectors. Infection and transformation of *E. coli* were performed as described by Frishauf et al. (7) and Lederberg et al. (12), respectively.

DNA and RNA preparations. Chromosomal DNAs were prepared from *B. brevis* HPD31, HPD52, and HP033 as described by Saito and Miura (21) and stored at 4°C in TE buffer (39). Phage and plasmid DNAs were isolated as described by Maniatis et al. (13) and Birnboim (6), respectively. Total RNA was extracted from both *B. brevis* HPD31 and *B. brevis* 47 grown to the exponential phase of growth by the hot-phenol method of Aiba et al. (3) and then stored at -80°C in distilled water.

Construction of the gene library of *B. brevis* HPD31. The *B. brevis* HPD31 chromosomal DNA was partially digested with *Bam*HI, ligated to phage vector EMBL3 DNA, which had been cleaved completely with *Bam*HI and *Eco*RI, followed by treatment with bacterial alkaline phosphatase, and then packaged into the phage particles, using an in vitro packaging kit (Stratagene Inc.).

Cloning of the 5' regions of the *B. brevis* HPD52 and HP033 cell wall protein genes. *Hind*III and *Hpa*I digests of *B. brevis* HPD52 and HP033 chromosomal DNAs were subjected to

preparative agarose gel electrophoresis (24). The 650-base-pair (bp) and 1.2-kilobase-pair (kb) *Hind*III-*Hpa*I fragments of the respective digests were eluted electrophoretically from the corresponding gel slices. These fragments were ligated to pUC119, which had been cleaved completely with *Hinc*II and *Hind*III, followed by treatment with bacterial alkaline phosphatase. The ligated DNAs were used for transformation of *E. coli* JM103 to ampicillin resistance, and the transformants were screened by means of in situ colony hybridization, using the ³²P labeled 640-bp *Cl*aI-*Hpa*I fragment prepared from ϕ -SK10 as a probe (Fig. 1C, probe D).

DNA and RNA blot hybridization. In situ plaque and colony hybridizations were performed as described by Benton and Davis (5), using the ³²P-labeled 2.5-kb *Hpa*I fragment isolated from pCWP300 (35) and the 400-bp *Cl*aI-*Bam*HI fragment on ϕ -SK10 as hybridization probes (Fig. 1). Southern blot analysis of restriction fragments of the *B. brevis* HPD31 chromosomal DNA was performed as described by Southern (31). Northern (RNA) blot analysis of total cellular RNAs from *B. brevis* HPD31 and 47 was carried out as described previously (33). The apparent RNA sizes were determined by using 16S and 23S rRNAs prepared from *E. coli* as standards.

Purification and amino-terminal amino acid sequence analysis of HWP. *B. brevis* HPD31 was harvested in the mid-logarithmic phase of growth by centrifugation (7,000 × *g*, 15 min, 4°C) and washed once with 50 mM Tris hydrochloride buffer (pH 7.5). For a 1-liter culture, the washed cell pellet was suspended in 20 ml of the same buffer and then disrupted by sonication for six 30-s periods with intervals of cooling in ice in between. Crude cell envelopes were centrifuged (48,000 × *g*, 30 min, 4°C) after removal of unbroken cells by centrifugation, washed three times by suspension in the same buffer, and then treated with Triton X-100 to prepare Triton-insoluble envelopes according to the method of

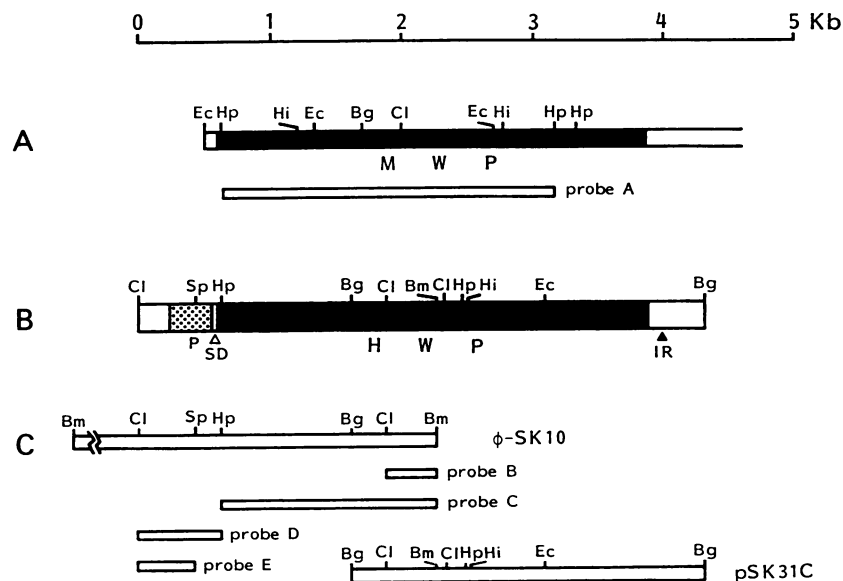


FIG. 1. Restriction map of the cell wall protein gene of *B. brevis* HPD31. (A) Restriction map of the MWP gene of *B. brevis* 47. Symbols: ■, coding region for MWP; □, probe A, the DNA fragment used as the hybridization probe to obtain ϕ -SK10. (B) Restriction map of the region around the HWP gene of *B. brevis* HPD31. Symbol and abbreviations: ■, coding region for HWP; P, promoter region; SD, Shine-Dalgarno sequence; IR, inverted-repeat structure. (C) DNA fragments cloned on ϕ -SK10 and pSK31C. Probes B, C, D, and E represent the DNA fragments used as hybridization probes. Probe B was used to obtain pSK31C; probe C was used for Northern blot analysis; probe D was used to obtain pSK52P and pYN033; probes D and E were used for S1 mapping analysis. Restriction sites: Bm, *Bam*HI; Cl, *Cl*aI; Sp, *Spe*I; Hp, *Hpa*I; Bg, *Bgl*II; Hi, *Hind*III; Ec, *Eco*RI.

<u>Clai</u> ATCGATTACCAAAAAACCGCGAAAGATTCGCGCGCTGAGAGCTATCGTTTGACCAATTTGCGCTTTGAAACGGTCTGGCAACGACCT	88
CGAAGTGATTACGCCGAGGAGGAATTATCCAACCGTGAGAATCAGTACCAAAAAGCGATCCTGCAGTACAATCTGGCTGTGGTAACTTT	178
GAAACTGCCCTCGGAAGCTAAGAAGAATTTGTAAGGTTTTGAATGGAAGTGGAAAAAGTTCAATGGTGACAGTCCGTCGGCGTACCT	268
ATAATACGAGTTGTGGCGGATGTCACCTGCTTACATATTACAAGTGAATACGACCCATGAAAATTTCTTTAACTTTTTTCTGAGGCGCC	358
GCAACTTTTGTCTCGACTGAGGCGTTAATAGGGTGTACACGAAAAACGGGGAATTGTGTAATAAAGATTCACGGATTCTAGCATTGTG	448
TTACACTAGTGATTGTTACATTTTACACAATAGCTGAATATACTAGAGATTTTAAACAAAAAGCGAAGCTGCTCGCAAAGGAGGT	538
GACACCGCTTGCAGGATTCGGGCTTAAAAAGAAAGATAGATCAACAAATATCCCAAGAACAATTTGTTTATACTAGAGGAGGAGAA	628
fMetGlnAspSerGlyPheLysLysLysAspArgSerThrAsnIleProGlnGluGlnPheValTyrThrArgGlyGlyGlu	
-53	
CACAAGGTTATGAAAAAGGTCGTTAACAGTGTATTGGCTAGTGCCTCGCCATCACAGTTGCTCCAATGGCTTTCGCAGCAGAGGACACA	718
HisLysValMetLysLysValValAsnSerValLeuAlaSerAlaLeuAlaIleThrValAlaProMetAlaPheAlaAlaGluAspThr	
-23	
ACTACAGTCTCTAAATGGATGCTGCAATGGAGAAAACCGTAAAACGCTCTGGAAGCTCTTGGCCTGGTAGCAGTTATGGCAACGGCGAT	808
ThrThrAlaProLysMetAspAlaAlaMetGluLysThrValLysArgLeuGluAlaLeuGlyLeuValAlaGlyTyrGlyAsnGlyAsp	
30	
TTCGGTGCAGACAAAACCATCACTCGTGCAGAGTTCGCTACTCTAATCGTTCGCGCTCGCGGTCTGGAGCAAGGTGGCAACTGGCACA	898
PheGlyAlaAspLysThrIleThrArgAlaGluPheAlaThrLeuIleValArgAlaArgGlyLeuGluGlnGlyAlaLysLeuAlaGln	
60	
TTCAACTACTTACACAGATGTTAGATCCACTGACTGGTTCGCTGGTTTTGTAACGTAGCTTCCGGTGAAGAAATCGTAAAAGGTTTC	988
PheAsnThrThrTyrThrAspValArgSerThrAspTrpPheAlaGlyPheValAsnValAlaSerGlyGluGluIleValLysGlyPhe	
90	
CCGACAAATCTTTTAAACCACAAAACCAAGTTACTTATGCTGAAGCAGTAACCATGATCGTTCGTGCTTTGGGTTATGAGCCATCCGTT	1078
ProAspLysSerPheLysProGlnAsnGlnValThrTyrAlaGluAlaValThrMetIleValArgAlaLeuGlyTyrGluProSerVal	
120	
GAAGGTGTATGGCCGAACAGCATGATCTCCAAAGTTCGCAACTGAACATTGCAAAAGGTATCAACAACCTAACATGCAGCAGTTCGCG	1168
ArgGlyValTrpProAsnSerMetIleSerLysGlySerGluLeuAsnIleAlaLysGlyIleAsnAsnProAsnMetGlnGlnPheAla	
150	
GCGACAATCTTCAAAATGCTGGACAACGCTCTTCGCGTTAAGCTGATGGAGCAAATCGAATACGGTACTGACATCCGTTTAAACGTA	1258
AlaThrIlePheLysMetLeuAspAsnAlaLeuArgValLysLeuMetGluGlnIleGluTyrGlyThrAspIleArgLeuAsnValThr	
180	
GACGAACTCTCTTGACTAAATATTTGAAAGTTACCGTACGTGATATGGACTGGGCTCACGAAAAGGTAACAATCTGATGAATTGCCA	1348
AspGluThrLeuLeuThrLysTyrLeuLysValThrValArgAspMetAspTrpAlaHisGluLysGlyAsnAsnSerAspGluLeuPro	
210	
CTTGTAAACAAACGTACCTGCTATTGGTCTGGGTAGTTTGAAGCAAATGAAGTTACTTTGAATGGAAAAGATGCTGATCTGGGTAGCAAC	1438
LeuValThrAsnValProAlaIleGlyLeuGlySerLeuLysAlaAsnGluValThrLeuAsnGlyLysAspAlaAspLeuGlySerAsn	
240	
ACTACTTATAAAGTAGCTGAAGGCATCAATCCTAACGCATTTGATGGTCAAAAAGTACAAGTGTGGATCAAAGATGACCGAGAAAATGTC	1528
ThrThrTyrLysValAlaGluGlyIleAsnProAsnAlaPheAspGlyGlnLysValGlnValTrpIleLysAspAspArgGluAsnVal	
270	
ATCGTTTGGATGGAAGGTTCCGAAGACGAAGATGTCGTTATGGACCGTGTGAGTCTCTGTACCTGAAAGGTAAAGCCTTACAGATGAT	1618
IleValTrpMetGluGlySerGluAspGluAspValValMetAspArgValSerAlaLeuTyrLeuLysGlyLysAlaPheThrAspAsp	
300	
ATTGTAAGATCTTAGCAAGTCTGATTTGGATGATGTAATAATCGAAATGGATGGTAGCGAGAAATCGTATCGCTTGACGGAAGATACG	1708
IleValLysAspLeuSerLysSerAspLeuAspAspValLysIleGluMetAspGlySerGluLysSerTyrArgLeuThrGluAspThr	
330	
AAAACTACTTATACTTCACTCGTTTCAACGATCCAGTAGATGCTCTGAGCAAAATTTACAAAGACAACGACACGTTTGGTGTAAAGTT	1798
LysIleThrTyrAsnPheThrArgPheAsnAspProValAspAlaLeuSerLysIleTyrLysAspAsnAspThrPheGlyValLysVal	
360	
GTTTTGAATGATAACAATGAAGTTGCATACCTCCACATCATTGACGATCAAACAATTGATAAAAAGCGTAAAAGGCGTTAAATACGGTTCC	1888
ValLeuAsnAspAsnAsnGluValAlaTyrLeuHisIleIleAspAspGlnThrIleAspLysSerValLysGlyValLysTyrGlySer	
390	
AAAGTTATTAGCAAAATCGATGCTGATAAGAAGAAAATCACCAACTTGGATAACTCCAATTTAGCGACTTGAAGATCAGGATGAAGGA	1978
LysValIleSerLysIleAspAlaAspLysLysLysIleThrAsnLeuAspAsnSerLysPheSerAspLeuGluAspGlnAspGluGly	
420	
AAAGACTTCTTGGTATTCCTTGATGGCCAACCGCTAAACTGGGTGATCTGAAAGAATCCGATGTTTACTCGGTATACTATGCTGATGGT	2068
LysAspPheLeuValPheLeuAspGlyGlnProAlaLysLeuGlyAspLeuLysGluSerAspValTyrSerValTyrTyrAlaAspGly	
450	
GATAAGATAAGTACTTGGTGTTTGCTAACCGTAACGTTGCAGAAGGAAAAGTAGAAAAAGTGGTAAGCCGTAACAAGACAGACATTCGT	2150
AspLysAspLysTyrLeuValPheAlaAsnArgAsnValAlaGluGlyLysValGluLysValValSerArgAsnLysThrAspIleArg	
480	
CTGACTGTTGGTGGTAAAACCTACAAGTATATCCAGATGCTTCTTACTCCGAAAACGCAATAAAGATGTTAAGAAGGTTAATCCGAC	2248
LeuThrValGlyGlyLysThrTyrLysValTyrProAspAlaSerTyrSerGluAsnAlaAsnLysAspValLysLysValAsnSerAsp	
510	

FIG. 2. Nucleotide and deduced amino acid sequences of the HWP gene of *B. brevis* HPD31. Numbers on the right indicate nucleotide positions; numbers below the sequence represent amino acid positions. The two Shine-Dalgarno sequences (SDa and SDb) are overlined. The amino-terminal amino acid sequence of HWP, confirmed by the Edman method, is underlined. The cleavage site of the signal sequence is marked (▼). Palindromic sequences (arrows below the nucleotide sequence) and cleavage sites for representative restriction enzymes are indicated.

BamHI

TTGGATCTGATTAGCAACCTTGATGGAGAAGAAGTAAACTTCTCTTGGATCCATCCGGTCGAGTTCGTACATCGAAACAAAAGACGGC 2338
 LeuAspLeuIleSerAsnLeuAspGlyGluGluValLysLeuLeuLeuAspProSerGlyArgValArgHisIleGluThrLysAspAla
 540

Clai

ATCGATGATCGTAAACCATTGGCAATCATCACTAAGGGTGCTACCTATAACTCCAGCAAAGATACTTATGACTTTACTGTTATGACCCAA 2428
 IleAspAspArgLysProLeuAlaIleIleThrLysGlyAlaThrTyrAsnSerSerLysAspThrTyrAspPheThrValMetThrGln
 570

HpaI

AAAGGTAACACACAAATCGTATCTTTGGATCAAAGGACATCTACGATAGATATGGGGTTAACTACGATAAATCCAATGATAAGCGTCA 2518
 LysGlyLysThrGlnIleValSerLeuAspGlnLysAspIleTyrAspArgTyrGlyValAsnTyrAspLysSerAsnAspLysArgGln
 600

HindIII

GCTTTCGAAAAGGATTTAGTTGAGCTTCTGCAACCTAAGGTAGTTAAAGAGGACTCAGTACTGATGCCAATCAAACCGTTCTTTTGGAA 2608
 AlaPheGluLysAspLeuValGluLeuLeuGlnProLysValValLysGluAspSerAlaThrAspAlaAsnGlnThrValLeuLeuGlu
 630

GTTAATTTTGATTCCAAAGCGGAAGTTGATAAAGTCAAAGTCTTGATAGCAAGCTGAAATACTCCGAGAAAAGCACTGGGATAAACTT 2698
 ValAsnPheAspSerLysGlyGluValAspLysValLysValLeuAspSerLysLeuLysTyrSerGluLysSerThrTrpAspLysLeu
 660

GCGGATGAAGATGACGACGCTCGTTGGTGATTATGAAGTAAACCGATAAGACTGCTGTCTTCAAAATGACTGGTGACCTTACTCCAGCTACT 2788
 AlaAspGluAspAspValValGlyAspTyrGluValThrAspLysThrAlaValPheLysMetThrGlyAspLeuThrProAlaThr
 690

GGAACATAACCGTGGTGAACGTGAAAACGCTGGTACTGCTAAATTTAAGGATGTTGCTAAGAAGAGCGACTCTGAAGGTTTGGTACTCAGTG 2878
 GlyThrLysArgGlyGluLeuLysAsnAlaGlyThrAlaLysPheLysAspValAlaLysLysSerAspLeuLysValTrpTyrSerVal
 720

GATGAAGACAAAAGGTGAAGTTCGAAGCGATCTTCGATAGATGCGAGTGGTTGGGTGGCGACCATCAGTTTGGTATGGTCAAACAATAT 2968
 AspGluAspLysGlyGluValGlnAlaIlePheValValAspGlySerGlyLeuGlyGlyAspHisGlnPheGlyMetValLysGlnTyr
 750

GGTACTGCTAGTAAACAGGACACTATCACTATAGTTACAAAAGATGGTGATTCTGTCACTGAAAAAGAGTACAATTTGGATGGCGACGCT 3058
 GlyThrAlaSerLysGlnAspThrIleThrIleValThrLysAspGlyAspSerValThrGluLysGluTyrLysLeuAspGlyAspAla
 780

EcoRI

GATGATTTGAAAGTTGATCAAGACATCAGACGTTGGAGATGTCATTTCTTTCACACTGAAATTTCTGATGGAGAAGTTATTGTTGACGATGTA 3148
 AspAspLeuLysValAspGlnAspIleArgArgGlyAspValIleSerPheThrLeuAsnSerAspGlyGluValIleValAspAspVal
 810

GTCGAGGTTGTAATAACAACCACATTGATAAACAAGTCTTAAATCAGCTACGCTCATGCCTGAAGACGAACGTCAAAAAGCAGGAATC 3238
 ValGluValValAsnAsnAsnHisIleAspAsnThrAlaSerLysSerAlaThrLeuMetProGluAspGluArgGlnLysAlaGlyIle
 840

GACAAATGGTTGTTGCTCGCGTTGACGAAGTTGATGGTAACACTATTTCTTGAACACTATGCTGACGGAAAGCACAAAAATATTACACA 3328
 AspLysLeuValValAlaArgValAspGluValAspGlyAsnThrIleSerLeuAsnTyrAlaAspGlyLysThrGlnLysTyrTyrThr
 870

AAAGCATCCACTGCGTTCATTGATGTGTATGACGGTCTTGAAAGGAATTGATGGAGTAGATGAAGGGCACTACATCGTAATGATCGATAGC 3418
 LysAlaSerThrAlaPheIleAspValTyrAspGlyLeuGluGlyIleAspGlyValAspGluGlyAspTyrIleValMetIleAspSer
 900

GCCGATATTGACGGAACCTCGTTGACTATGTACTGGTAGTTCTAGCGATGATGAGATCCGCACGCAACACATCTCCACTAAAGCAGTT 3508
 AlaAspIleAspGlyThrArgPheAspTyrValLeuValValSerSerAspAspGluIleArgThrGlnHisIleSerThrLysAlaVal
 930

ACGGACTTCTGAACAAGCCAACCAGACTATGTACCAAATCTGGCGATGGGGAAGAAGTAGTCACGGCACAAAAGTTAATACAGTTAAC 3598
 ThrAspPheLeuAsnLysProThrArgLeuCysThrLysSerTrpArgTrpGlyArgSerSerHisGlyThrLysValAsnThrValAsn
 960

GATGAAGCAGTTGTAGATGGTATTGTAACCTTCCAGCTGATGCATCTGTTAGAAACTTCAACATTGCATTTGATCAAGAAATTAACAGC 3688
 AspGluAlaValValAspGlyIleValThrLeuProAlaAspAlaSerValArgAsnPheAsnIleAlaPheAspGlnGluIleAsnSer
 990

AAAGATGCAACGGTAACTGTTACTAATGAAGATACGCTTGGTAACGTAACGGTATCTGAGGTTGCGACAGATGCAAAAAGTATTGAGCTTC 3778
 LysAspAlaThrValThrValThrAsnGluAspThrLeuGlyAsnValThrValSerGluValAlaThrAspAlaLysValLeuSerPhe
 1020

AAGACTGCTAAACTGGACACTACAAAACTTACATCATCACAGTTAAAGGCTTGAAGATAAAAAACGGTAAAGCAGTGAAGATGTAAC 3868
 LysThrAlaLysLeuAspThrThrLysThrTyrIleIleThrValLysGlyLeuLysAspLysAsnGlyLysAlaValLysAspValThr
 1050

CTTTACGTTGAATTTGTTGCTGGAGTGTGACTTGTGCTCTATAAACACTAATAGTGTGAAATGAGTGAAGAAGGAGAAGGATTAATAAC 3958
 LeuTyrValGluPheValAlaGlyVal*** → ← -

CTTCTCCCTTCTTTAACTGTTTTGGCGAATTGAAGGGTGAACCCCTTCCGTGGAACAACCATTAATCCACGTGACTAATCCTCCTGC 4048

ATGCGTTGTAAGCTAACTCTTACAGGGTGTGAAGCCAGGTGAAGACGTAACCCCTTAGTGGTCCGGGTCTCCGTA AAAAGCTAACTATGGTT 4138

AGGAAACGAATCTACGCTGAGGCGTGCAGGTTTGGTAATTAGCCCTTCAGCTGCTAATACCCACAGGCTGAGATGCTGGAGGGTT 4228
 ← →

CTAACGAATTAAGGTTGTTAACCTTAGTTCATCTTGATATGTATAGTAGGAACCTAAGGTTTACGAAAAAAGGGATAGCCAAAATGGAA 4318

BglII

CAAGTGAGATCT

FIG. 2—Continued.

Tsuboi et al. (34). The cell wall proteins were solubilized with 8 M urea, precipitated with 70% cold acetone, and then dissolved in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis buffer (10). The 135-kilodalton cell wall protein (HWP) was separated by electrophoresis on preparative SDS-polyacrylamide gels (7.5%) as described by Laemmli (10), extracted from the gels, and then dissolved in 50 mM Tris hydrochloride buffer (pH 7.5) containing 2%

SDS as described by Ohmizu et al. (17). The purity of HWP was greater than 95%, as judged on scanning of electropherograms obtained by using different amounts of protein. The NH₂-terminal amino acid sequence of HWP was determined with an ABI 477A-120A protein sequencer after extensive dialysis against a 0.01% SDS solution.

DNA sequencing and S1 mapping analysis. Nucleotide sequences were determined by the dideoxy-chain termina-

AED--TTTAPKMDAAMEKTVKRLEALGLVAGYGNDFGADKTIITRAEFATLIVRARGLEQGAKLAQFNNTTYDVRSTDFW	78
AAAATTTAPKMDADMEKTVKRLEALGLVAGYGNGEYGVDKTIITRAEFATLVVVRARGLEQGAKLAQFSNTTYDVKSTDFW	80
AGFVNVASGEEIVKGFDPKSFKPQNVTYAEAVTMIVRALGYEPSVRGVWPNMSMISKGSSELNIAKGINNPNMQQFAATIF	158
AGFVNVASGEEIVKGFDPKSFKPQNVTYAEAVTMIVRALGYEPSVKGWPNMSMISKASELNIARSITTPNNAATRGDIF	160
KMLDNALRVKLMQIEYGTDIRLNVTDDELLTKYLKVTVRMDWAHEKGNNSDELPLVTNPVPAIGLGLKANEVTLNGKD	238
KMLDNALRVDLMEQVEFGTDIRHEITKETLLTKYLKVTVRDMEWAQEAAGNDSDELPLVTNPVPAIGLGLKANEVTLNGKD	240
ADLGSNTTYKVAEGINPNAFDGGKQVQVWIKDDRENVIVWMEGSEDEDDVMDRVVSALYLKGAFTDDIVKDLKSDLDLDDVK	318
AGIG--NTTYKVADGINANDFDGQHVQVWYKDDKEDVIVWMEGSTDQEVIMDRVGEFTLKGKTFED--PKDLSNSDLADLK	317
IEMDGSEKSYRLTEDTKITYNFRFNDPVDALSKEYKND---TFGVKVLNDNNEVAYLHIIDDQTIKSVKGVKYG	394
LELDASEKSYRFNKNKVTYNTFRFNDTVLGLKEIKDNADGGFTFGAKVVLNDNNEIAYIHVIDDQSMNKEDEGVKYG	397
KVISKIDADKKKITNLDNSKFSLEDQDEGKDFLVFLDGGPAKGLDKESDVYSVYYADGDKDKYLVFANRNAEKGVEK	474
EVISKIDTDDKKKITNRDNDKFNLDLGGKEGKDFLVFLNGKPAKFSDLKEGMVYSVYYADGDEKLLVFATDTVVEGKVDK	477
VVSRNKTDIRLTVGGKTYKVPDASYSANANKDVKVNSD-LDLISNLDGEEVKLLDPSGRVRHIETKDAIDDRKPLAI	553
VVSRNNNDYRLTIGDKTYRVYEGATFSDDGKNDVQIDKDHWDLVSLDDETVKLYTDASGRVRHIETKDAIDDRKQKAI	557
ITKGATYNSKDYDFVTMTQKQKTIQVSLDQKDIYDRYGVNYDKSNDRQAFKDLVELLQPKVVKEDSATDANQTVLL	633
VTRSATFNTSKDTWDFRVLTVQKQKEIVVSLKAKNIYDFDGNFSRDNKNQDDLEDILV-----PSKDKDITLL	625
EVNFDKSGEVDKVKVLDLDS--KLKYEKSTWDLADEDDVVDYEVTDKTAVFKMTGDLTPATGTRKGLKNAKTAKFKDV	712
EVTLDADGKPGKVEFLKPKVVEQESGKAWDDLADDDMVGDYEVTDKTAVFNMGTGL--EESKRKELKNAKTAKFKDV	703
AKKSDLKVVYSVEEDKGEVQAIFFVVDGSLGGDHQFGMVKQYGTASKQDTITIVTKDGSVTEKEYKLDGDADDLKVDD	792
ADENDLSVIYTNV--DKDEVEAIFVVEGDGLTGDHAHYGQVIDFGRKGGKDPVIRVWEKDGKVVKEYKLDGDQDDLK--DED	781
IRRGDVISFTLNSDGEVIVDDVVEVVNNHIDNTASKSATLMPEDERQKAGIDKLVARVDEVDGNTISLNYADGKTQKY	872
IRRNDFIAFTVDSNDEVVDDVVDVVKNAKGMLA----EVTDEKGMKDANIDKMVVLVSDVRKDTITYKDADDNKKKA	857
YTKASTAFIDVYDGLGIDGVEGDYIVMIDSADIDGTRFDYVLLVSSDDEIRTQHISTKAVTDFLNKPTRLCTKSWRWG	952
SIKSATVYFDLYDDFGADGVNEGDYVVMIDSGDISGTYDYVLLVSDAKTVRDKKLEDDAEAFKQEPSEKPPDPTKWD	937
RSSHGTVKNTVNDVAVVDGIVTLPADASVRNFNIAFDQEIINSKDATVTVTNEEDTLGNVTVSEVATDAKVLVSKTAKLDTT	1037
ALPSKVEGKFTSAGPVKLYRATVELNSKVAEDV-----DAIEFYFNGKKEVPSLLNFKDGVITIGYNTEDRVTS	1007
KTYIITVKGLKDKNGKAVKDVTLVYVEFVAGV H W P	1063
SKIKVTNKNKGDSEATVTFVEA-----M W P	1030

FIG. 3. Alignment of the HWP and MWP amino acid sequences. The sequences are aligned to obtain maximum homology. Symbols: ., identical residues; ., similar but nonidentical residues; -, gaps in the sequences. Numbers on the right indicate amino acid positions.

tion method of Sanger et al. (23) after subcloning of appropriate restriction fragments into derivatives of M13 phages or pUC plasmids. S1 mapping analysis of RNA was performed as described previously (3). The 640-bp *ClaI-HpaI* fragment and 450-bp *ClaI-SpeI* fragment isolated from ϕ -SK10 (Fig. 1), which were labeled with ³²P at the 5' ends of their sense strands, were used as hybridization probes. The DNA (100 ng) was hybridized at 45°C for 3 h with 100 μ g of total *B. brevis* HPD31 RNA in the presence of 80% formamide. The hybridized molecules were treated with 50 U of S1 nuclease (Bethesda Research Laboratories, Inc.) in a 0.3-ml reaction mixture at 37°C for 30 min and then subjected to chain length determination on a sequence gel. The modification and cleavage reaction of Maxam and Gilbert (14) was performed on the hybridization probe, which was coelectrophoresed with the samples and used as a marker.

RESULTS AND DISCUSSION

Cloning of the cell wall protein (HWP) gene from *B. brevis* HPD31. An antibody to the MWP of *B. brevis* 47 cross-reacts with HWP purified from *B. brevis* HPD31 (8). This finding raised the possibility that the MWP gene of *B. brevis* 47 could be used as a DNA probe for cloning of the *B. brevis* HPD31 HWP gene.

A genomic DNA library, constructed in phage vector EMBL3 from a partial *Bam*HI digest of the *B. brevis* HPD31 chromosomal DNA, was screened with a 2.5-kb *HpaI* fragment coding for the structural portion of the *B. brevis* 47 MWP gene as a probe (Fig. 1A, probe A). Of approximately 1.8×10^5 bacteriophages screened, one positive clone, designated ϕ -SK10, was obtained and found to contain a 14-kb *Bam*HI insert. Southern blots of the restriction endonuclease digestion products were sequentially probed with

probe A, and a restriction map around the HWP gene was constructed (Fig. 1B). Comparison of the restriction map of the ϕ -SK10 insert with the genomic restriction map constructed simultaneously indicates that the insert on ϕ -SK10 contained approximately one half of the HWP gene on its lefthand side (Fig. 1C). Then, the 2.7-kb *Bgl*II fragment indicated in Fig. 1C was cloned as follows. The 2.7-kb *Bgl*II chromosomal DNA fragment was eluted electrophoretically from an agarose gel after electrophoresis, ligated to pUC118, which had been cleaved completely with *Bam*HI followed by treatment with bacterial alkaline phosphatase, and then used for transformation of *E. coli* JM103 to ampicillin resistance. The transformants were screened by means of in situ colony hybridization, with the 32 P-labeled 450-bp *Cla*I-*Bam*HI fragment used as a probe (Fig. 1C, probe B). A plasmid designated pSK31C, carried by one of the positive transformants was subsequently analyzed. The 2.7-kb insert on pSK31C was confirmed to be the exact counterpart of the *B. brevis* HPD31 chromosomal DNA by genomic Southern analysis (data not shown).

Nucleotide sequence and characterization of the HWP gene. The nucleotide sequence of the 4,330-bp fragment from the upstream *Cla*I site to the downstream *Bgl*II site (Fig. 1C) was determined by using the inserts on ϕ -SK10 and pSK31C. Analysis of the DNA sequence revealed that there is an open reading frame (ORF) in the fragment oriented from the *Cla*I site to the *Bgl*II site (Fig. 2). The ORF starts from the codon, TTG, at nucleotides 548 to 550 and terminates in a TGA nonsense codon at nucleotides 3896 to 3898. No significant ORF was found either upstream of the ORF or in the reverse strand.

The ORF, consisting of 3,261 bp, encodes a polypeptide of 1,087 amino acid residues with a molecular weight of 123,456. The 15-residue NH₂-terminal amino acid sequence of HWP determined chemically, Ala-Glu-Asp-Thr-Thr-Thr-Ala-Pro-Lys-Met-Asp-Ala-Ala-Met-Glu, was in complete agreement with that deduced from the DNA sequence starting at GCA (nucleotides 707 to 709; Ala) (Fig. 2). This confirmed that the ORF is the coding sequence for HWP.

Following the promoter sequence, the properties of which are described below, two potential ribosome-binding sites, designated SDA and SDB, were located tandemly in the same reading frame, at nucleotides 529 to 540 and 617 to 627. SDA and SDB have 12 and 9 bases complementary to the 3' ends of *B. brevis* and *B. subtilis* 16S RNA, respectively (9, 15). SDA is located 8 bases upstream of the TTG codon (nucleotides 548 to 550), and SDB is located 11 bases upstream of the ATG codon (nucleotides 638 to 640), which is in frame with the first initiation codon, TTG. It is not yet known whether one or both of the two potential translation initiation sites are utilized for the synthesis of HWP. The NH₂-terminal amino acid sequence of HWP indicates that it is synthesized in *B. brevis* HPD31 as a precursor protein with a signal peptide of 53 or 23 amino acid residues. These properties of translation initiation of the HWP gene highly resemble those of the MWP gene in *B. brevis* 47 (41). In the 3' noncoding region, two palindromic sequences were found 49 and 276 bp downstream of the termination codon of the HWP gene. In the first region, a very stable stem-and-loop structure of $\Delta G = -34.8$ kcal (ca. -146.4 kJ)/mol, as calculated by the method of Salser (22), can be formed, followed by a short T-rich sequence. Such a structure most likely functions as a transcription terminator because of its resemblance, in structural characteristics, to the *E. coli* Rho-independent transcription terminator (2, 20). The second palindromic sequence ($\Delta G = -22$ kcal [ca. -92.0 kJ)/mol) also shows some

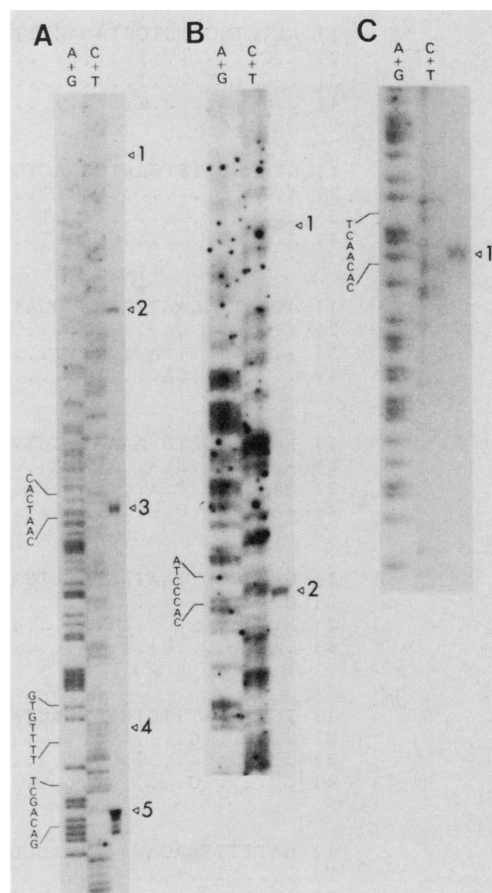


FIG. 4. S1 mapping analysis of the transcripts from the HWP gene. The total RNA fraction prepared from exponentially growing *B. brevis* HPD31 cells was analyzed as described in Materials and Methods. (A and B) The *Cla*I-*Hpa*I (probe D) fragment (Fig. 1) was labeled with 32 P at its 5' end and then used for hybridization at 45°C. The S1-treated DNA was subjected to electrophoresis on a 6% polyacrylamide gel containing 8 M urea. The 32 P-labeled probe DNA was simultaneously processed for nucleotide sequence determination (A+G and C+T). DNA sequences of the sense strands around the positions of S1-protected fragments (arrowheads 1 through 5) are shown. Gels were electrophoresed for 4 h (A) and 7 h (B). (C) The *Cla*I-*Spe*I (probe E) fragment was used as a hybridization probe. The analytical conditions were the same as for gels A and B.

features in common with well-known transcription termination signals (2, 20).

To determine how the HWP gene is transcribed in vivo, Northern blot analysis of the total cellular RNA prepared from *B. brevis* HPD31 was performed as described in Materials and Methods. When the 1.4-kb *Hpa*I-*Bam*HI fragment within the coding region of the HWP gene was used as a probe (Fig. 1C, probe C), one transcript of approximately 3.5 kb was detected (data not shown). The 3.5-kb transcript is long enough to cover the HWP gene and might be generated as a consequence of transcriptional arrest at the putative terminators described above.

The amino acid composition deduced from the nucleotide sequence of the HWP gene shares several features with S-layer proteins, such as those of *B. brevis* 47, *Bacillus sphaericus*, *Clostridium thermosaccharolyticum*, and *Clostridium thermohydrosulfuricum* (25, 26, 35, 37): a high content of hydrophobic amino acids (43%), a higher proportion

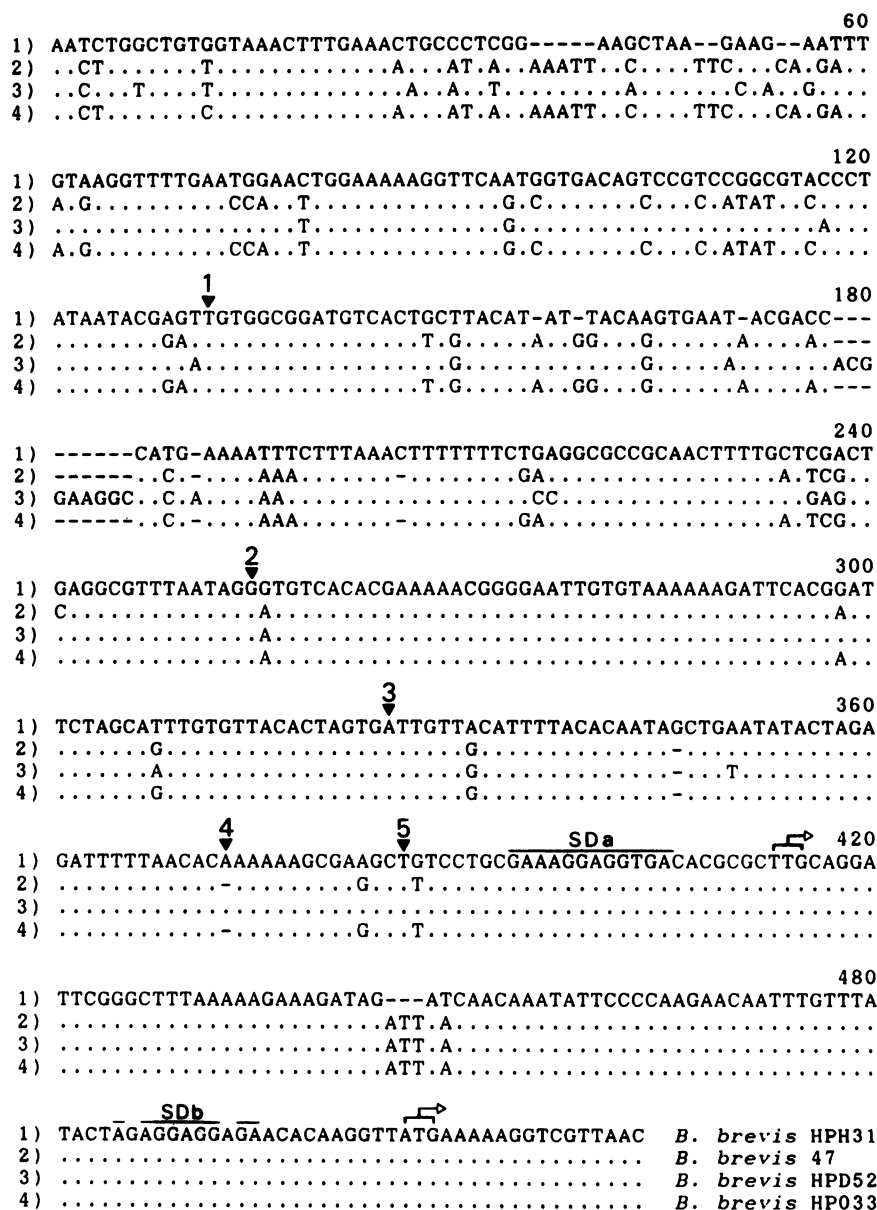


FIG. 5. Comparison of 5' region of the cell wall protein genes of *B. brevis* 4 strains. The 5'-region sequences of the cell wall protein genes of the following four strains are compared: 1, HPD31; 2, 47; 3, HPD52; 4, HP033. Symbols: ., identical residues; -, deletion of the corresponding residues. Two potential ribosome-binding sites are overlined. Arrowheads 1 through 5 indicate positions of the 5' ends of the HWP gene transcripts determined by S1 analysis.

of acidic amino acids (17%) than of basic amino acids (8%), and a very low content of Met, His, Tyr, and Cys.

The deduced amino acid sequence of HWP showed high homology (78%) with that of MWP (Fig. 3). In the NH₂-terminal region (amino acids 1 to 548), they show significantly higher homology (90%) than in the COOH-terminal region (65%; amino acids 549 to 1034). In the rather low-homology region of the COOH-terminal portion, however, several sequences ranging in size from 8 to 19 amino acid residues were exactly the same in the two proteins. It is tempting to suggest that these homologous regions play an important role in assembly of the regular surface array through interactions with the peptidoglycan layer.

Further comparison of the predicted amino acid sequence of HWP with those of other eubacterial S-layer proteins

revealed that HWP contains no sequence homology with either the outer wall protein of *B. brevis* 47 (37) or the hexagonally packed intermediate-layer protein of *D. radiodurans* (19), although the latter two proteins show a statistically significant homology in the COOH-terminal portions (19). Any of these sequences may not apply to the structure of S-layer proteins in general. More S-layer protein sequences, such as sequences responsible for the formation of paracrystalline structure and those participating in the interaction with other cell wall components, are required to shed light on the characteristic structures of S-layer proteins and to arrive at conclusions about evolutionary relationships among S-layer proteins.

Unique structure in the regulatory region of the cell wall protein genes among protein-producing *B. brevis* strains. The

5' ends of the transcripts from the HWP gene were determined by means of an S1 nuclease protection assay with total RNA prepared from *B. brevis* HPD31 and with 640-bp *ClaI-HpaI* and 450-bp *ClaI-SpeI* fragments on ϕ -SK10 (Fig. 1C, probes D and E) as hybridization probes. Five S1-protected fragments of different sizes were detected on a sequencing gel (Fig. 4) and mapped within 300 bp upstream of the HWP gene (Fig. 5). The HWP gene showed surprisingly high homology (>85%) with the regulatory region of the MWP gene. Moreover, the 5' ends of five HWP gene transcripts were nearly the same as those of the MWP gene transcripts (41). These complex structures in the regulatory regions of the cell wall protein genes appear to be unique among protein-producing *B. brevis* strains. This finding prompted us to characterize further the cell wall protein genes from two other protein-producing *B. brevis* strains, HPD52 and HP033. DNAs homologous to the 5' region of the HWP gene were isolated from these strains as described in Materials and Methods. The resultant plasmids, pSK52P and pYN033, contained the 1.2-kb *HindIII-HpaI* fragment derived from *B. brevis* HPD52 chromosomal DNA and the 650-bp fragment derived from *B. brevis* HP033 chromosomal DNA, respectively. The nucleotide sequences of these inserts were determined and compared with those of the HWP and MWP genes (Fig. 5). In all four genes, the nucleotide sequences of the regulatory regions were nearly identical. Only 13 bases were different in the region from nucleotides 374 to 640, the putative translation initiation site, ATG, whereas the upstream region was less conserved. A striking feature of these cell wall protein genes, from the standpoint of transcription and translation, is that all of them contain multiple transcription initiation sites and two possible ribosome-binding sites in the same reading frame at the same distances from the potential translation initiation codons, TTG and ATG.

The advantage of the complex structure in the regulatory region is not readily apparent, but the region should be important in regulating strictly and growth phase dependently the synthesis of the cell wall protein, as reported by Adachi et al. (1).

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