Cloning and nucleotide sequence of the major capsid proteins of Lactobacillus bacteriophage Φgle

Cloning and nucleotide sequence of the major capsid proteins of *Lactobacillus* bacteriophage ϕgle

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Abstract

Bacteriophage ϕgle was induced from a lysogenic *Lactobacillus* strain Gle. ϕgle genome is double-stranded DNA of approximately 42.5 kilo-base (kb) pairs. SDS poly-acrylamide gel electrophoresis demonstrated that the phage particles contain 4 major structural (capsid) proteins, gpB, gpG, gpO, and gpP, whose molecular weights (MW) are estimated to be 64, 43, 29 and 26 kilodaltons (kDa), respectively. More than 16 minor proteins ranging from 113 to 9.6 kDa were also detected. The genes for the major capsid proteins were cloned and each DNA sequence was determined. N-terminal amino acid alignments determined by protein sequencing completely coincided with those deduced from the nucleotide sequences.

Keywords: Temperate phage; *Lactobacillus*; Capsid protein; Phage structural gene; Protein sequence; DNA sequence

1. Introduction

Lysogeny among an abundance of lactic acid bacteria has been recognized widely (Davidson et al., 1990). On the analogy of *Escherichia coli* lambda system (Campbell, 1994), temperate phages of lactic acid bacteria, such as *Lactobacillus* and *Lactococcus* species, have been regarded as valuable genetic tools of gene transfer (transduction) and cloning in these industrially and medically important genera (Lillehaug and Birkeland, 1993; Raya et al., 1992).

In contrast to coliphages, molecular details of the temperate phages of lactic acid bacteria are still unclear: host-range, gene-organization, replication, classification, evolution, and so on (Relano et al. 1987; Davidson et al., 1990; Raya et al., 1992).

In the genus *Lactobacillus*, three temperate phages, ϕadh from *gasseri* ADH (Raya et al., 1989, 1992), ϕFSW from *casei* (Shimizu-Kadota and Tsuchida, 1984) and PL-1 from *casei ATCC 27092* (Nakashima et al., 1994) have been so far characterized extensively. These results have demonstrated that they are significantly different in the structures of phage particle and genome, and there exist hard barriers to industrial applications of *Lactobacillus* temperate phages.

More recently, we have identified a bacteriophage, designated ϕgle, which was induced from a lysogenic *Lactobacillus* strain Gle in a fermented plant in Japan. Phage ϕgle considerably differs from other *Lactobacillus* phages, ϕadh, ϕFSW, and PL-1 in genome size and morphology.

In this study, we electrophoretically analyzed ϕgle capsid proteins, and sequenced N-terminal aa alignments of the 4 major proteins, gpB, gpG, gpO and gpP, and determined DNA sequences of the ϕgle genome encoding these 4 proteins.

These results afford an important clue to further studies of molecular biology of *Lactobacillus* phages.

2. Materials and methods

2.1. Bacteria, phage, and plasmids

The lysogenic *Lactobacillus* strain Gle was originally isolated in our laboratory in a fermented plant (unpub-

lished result). The Gle cells were grown in GYP (Okada et al., 1986) or M17 (Terzaghi and Sandine, 1975) broth at 30 °C. Development of bacteriophage ϕgle was induced from Gle by mitomycin C (MMC), and the phage particles were then purified as follows. When the A_{660} of the culture (GYP broth) had reached 0.25, MMC was added to a final concentration of 5.0 μg/ml. After incubation for further 20 min at 30 °C, the cells were harvested by centrifugation, suspended in fresh medium, and incubated at 30 °C. After lysis, the cell lysate was centrifuged at 5000 × *g* for 15 min. Liberated phages in the supernatant were purified by the method of Yamamoto et al. (1970): phage particles were concentrated with 10% polyethylene glycol 6000 and 0.5 M NaCl, and then purified by CsCl step and equilibrium gradient centrifugations. Phages thus banded were collected and dialyzed against phage buffer containing 20 mM Tris·HCl (pH 7.5), 0.1 M NaCl, and 0.001 M $MgSO_4$. Cells of *E. coli* XL1-blue were grown in LB or 2YT broth, and the *E. coli* vector plasmids (pUC18, pUC19, pUC118 and pUC119) were prepared by the method of Sambrook et al. (1989).

2.2. *Electron microscopy*

Phages were applied on carbon-coated grids (200 mesh; Oken Shoji Co., Ltd., Tokyo) and negatively stained with 1% uranyl acetate. Micrographs were taken with a JEOL 100SX electron microscope at 80 kV.

2.3. *Analysis of phage proteins*

For detection of phage proteins, the purified phage particles were subjected to electrophoresis on 12% SDS polyacrylamide slab gels (SDS-PAGE) together with authentic marker proteins as described by Kodaira et al. (1984). The electrophoresed gel was stained by Coomassie brilliant blue (CBB) R-250, and then subjected to silver staining. The bands stained with CBB were scanned using an imaging densitometer (BIO-RAD model GS-700). Two-dimensional (isoelectric focusing-SDS polyacrylamide slab gel) electrophoresis was performed as described previously by Tomoda et al. (1984). The first isoelectric gel contained ampholine covering pH from 3.5 to 10. The foregoing 12% polyacrylamide containing 0.1% SDS was used as the second-dimensional gel. For amino-acid sequencing, the purified ϕgle particles were subjected to 12% SDS-PAGE and then electroblotted to a PVDF membrane (Perkin Elmer Applied Biosystems division). After transfer, the PVDF membrane was stained with CBB R-250. The protein bands were excised from the CBB-stained PVDF membrane, and then subjected to N-terminal aa sequence analysis using an Applied Biosystems Procise or 476A sequencer.

2.4. *Analysis of phage DNA*

Southern blot hybridization was performed according to a procedure described by Hosono et al. (1992). DNA was electrophoresed on 1% agarose gel, followed by transfer to a positively charged nylon membrane (Boehringer Mannheim). After transfer, the membrane was baked at 121 °C for 15 min. Probes were labeled with digoxigenin-11-dUTP, and hybridizations were performed according to the supplier (Boehringer Mannheim). ^{32}P-Labeled probes were prepared by nick translation (Kodaira et al., 1994a). Cloning of ϕgle DNA was carried out essentially as described by Kodaira et al. (1992). Hybrid DNAs between restriction DNA fragment of ϕgle and *E. coli* vector plasmid were introduced into *E. coli* XL1-blue by Ca^{2+}-dependent transformation or electroporation (Taketo, 1988). For genome analysis, several series of restriction library from ϕgle DNA in *E. coli* plasmids (pUC series) were constructed using restriction enzymes. For DNA sequencing, various deletion clones were constructed from the libraries by *Bal*31 nuclease digestion (Kodaira et al., 1994b). The DNA sequence was determined by the chain termination method (Sanger et al., 1977). All other procedures were performed as described previously by Kodaira et al. (1992).

2.5. *Enzymes and biochemicals*

Restriction enzymes, phage T4 DNA ligase, and alkaline phosphatase (calf intestine) were purchased from Takara Shuzo (Kyoto) and Nippon Gene (Toyama). Buffers for each enzymes were as recommended by the manufacturers.

[α-^{32}P]dCTP was from NEN. All other materials were described previously by Kodaira et al. (1992).

3. Results and discussion

3.1. *Structure of ϕgle phage particle*

Structure of the ϕgle phage particle was analyzed by electron microscopy. As presented in Fig. 1, ϕgle has (i) an isometric and hexagonal head (63 nm in diameter), (ii) a remarkably long noncontractile and flexible tail (260 nm in length and 10 nm in width) with regularly spaced transversal striations, and (iii) a complicated stacked-baseplate (from 45 to 24 nm in diameter) ending in several short fibers (12 nm long) (see Fig. 4B and C).

Phage ϕgle thus exhibited a morphotype that has not been previously reported for *Lactobacillus* and *Lactococcus* phages.

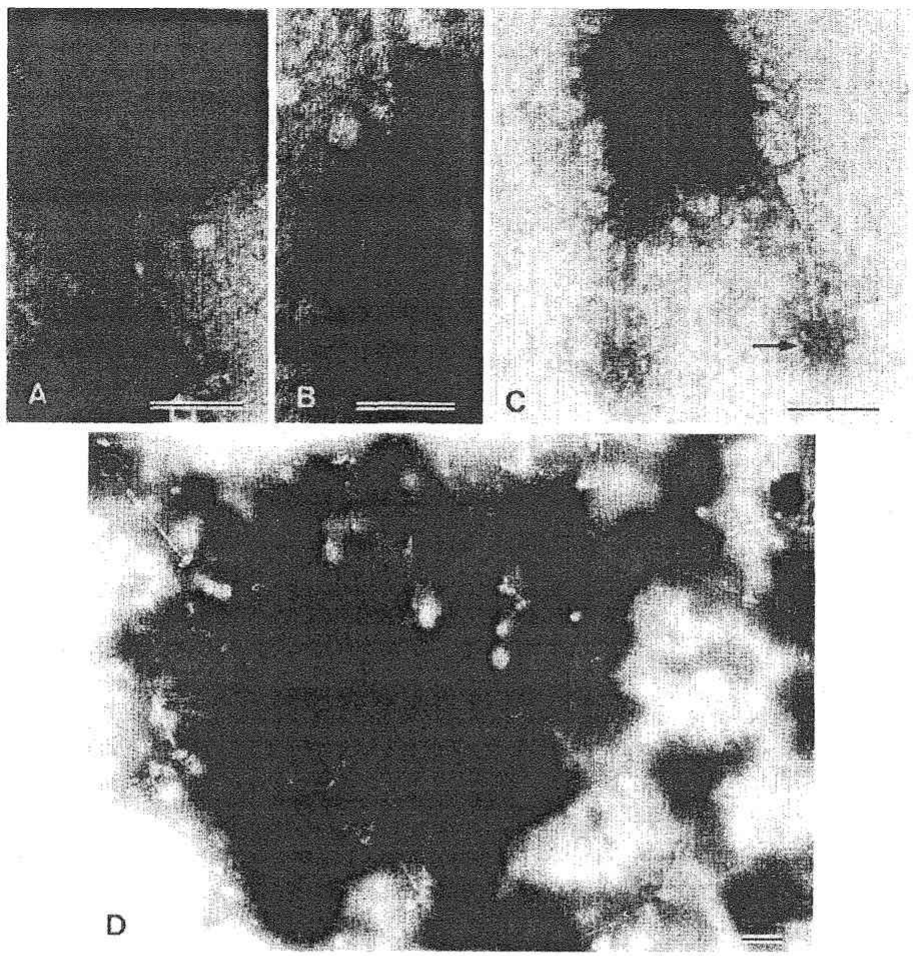


Fig. 1. Electron micrograph of ϕgle virion particles. Phages were negatively stained with uranyl acetate. (**A**) ϕgle filtered (0.45 μm) from cell lysates; (**B**) same as (**A**), magnified two stacked base plates and short fibers; (**C**) same as (**A**), magnified two stacked base plates from the bottom; (**D**) ϕgle purified by CsCl centrifugation. Bars: 100 nm (A, C, D); 50 nm (B).

3.2. Analysis of ϕgle capsid proteins

Purified ϕgle particles were disrupted by heating in the denaturing buffer containing SDS and mercaptoethanol (see Section Materials and methods), and subjected to 12% SDS-PAGE together with authentic marker proteins. Staining the electrophoresed gel with CBB R-250 revealed that ϕgle particles are composed of 4 major capsid proteins, referred to as gpB, gpG, gpO and gpP, whose molecular weights are estimated to be 64, 43, 29 and 26 kDa, respectively (Fig. 2A, lane 2); the relative abundance estimated by densitometric analysis is 1.0 (gpB), 4.0 (gpG), 0.5 (gpO) and 2.6 (gpP). In addition to these 4 major proteins, at least 16 minor proteins ranging from 113 to 9.6 kDa were detected after silver staining (Fig. 2A, lane 3).

Upon two-dimensional electrophoresis (see Section Materials and methods), each of the 4 proteins, gpB, gpG, gpO and gpP, gave a single spot on a second silver-stained gel (Fig. 2B). The 3 proteins gpB, gpG and gpP were significantly acidic, whose isoelectric points (p*I*) were estimated to be approximately 5.0, whereas the gpO protein was neutral (p*I*, approximately 6.5).

These results suggested that ϕgle differs from lactic acid phages reported so far (see above) in capsid protein and genome structure (see below).

We determined N-terminal aa sequences of the 4 major proteins, gpB, gpG, gpO and gpP, by a method of protein-blotting to a PVDF sequencing membrane (see Section Materials and methods). Their aa alignments are presented in Table 1: the first methionine was absent in the 3 proteins gpG, gpO and gpP.

3.3. Cloning and DNA sequence of the ϕgle genes coding for the major proteins

Fig. 3 shows a physical map of the ϕgle genome DNA composed of approximately 42.5 kb. The ϕgle DNA is circularly permuted; whether ϕgle belongs to *pac* or *cos* type is now under investigation.

To map structural genes for the 4 major capsid proteins gpB, gpG, gpO and gpP, mixed oligonucleotide probes were prepared based on their N-terminal aa alignments (see Table 1): for example, 5′-AA(A/G)TT-(T/C) AA (A/G) ATGAA (T/C) TA (T/C) AA (A/G) AA-(T/C)-3′ for gpP. By Southern blot experiment (data not shown), each of the probes for gpB, gpG, gpO and gpP hybridized to a single unique restriction fragment: 5′ regions of gene B (for gpB), gene G (gpG), gene O (gpO) and gene P (gpP) are located in 2.5 kb of an *Eco*RI fragment, 4.1 kb of a *SalI*/*Eco*RI fragment, 1.8 kb of an *Eco*RI fragment and 4.8 kb of a *Sal*I fragment, respectively (see Fig. 3).

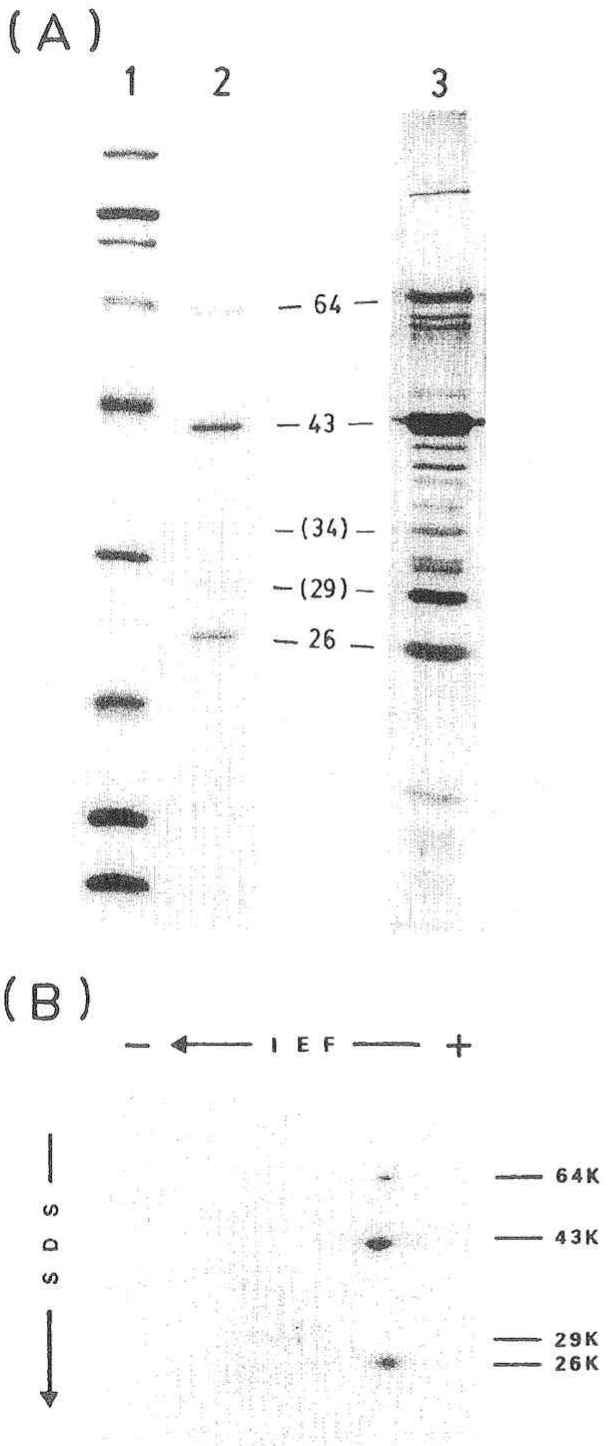


Fig. 2. Proteins of ϕgle phage particles. (**A**) Phage particles disrupted by SDS and mercaptoethanol were analyzed on a SDS polyacrylamide slab gel. Phage proteins were stained with Coomassie brilliant blue R-250 (lane 2) and then subjected to silver staining (lane 3). Lane 1, authentic marker proteins: myosin (200 kDa), β-galactosidase (116.3 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbmin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa). (**B**) Phage particles denatured as in (**A**) were subjected to two-dimensional electrophoresis: first, on isoelectric focusing gel (IEF); second, on SDS gel. After electrophoresis, phage proteins were visualized by staining with Coomassie brilliant blue R-250.

Based on the Southern blot experiments, we sequenced 3 regions of ϕgle genome (see Fig. 3): (1) 1.5 kb from *Hin*dIII to *Eco*RV (containing gene G), (2) 1.0 kb from *Hinc*II to *Eco*O109I (gene P), and (3) 3.6 kb from *Eco*RV to *Xba*I (genes B and O).

The sequencing analysis revealed the expected 4 genes, G, P, B and O, in addition to 6 truncated open reading frames (ORF): RorfU1, RorfU2, RorfU3, RorfU4, RorfU5 and RorfU6. Fig. 4 shows the DNA sequence of these genes and ORFs, together with their aa alignment. They are encoded on one strand, referred to as R-strand, whereas, no ORF of significant length could be found in the complementary strand, referred to as L-strand. The 4 genes (G, P, B and O) and 3 ORFs (RorfU2, RorfU4 and RorfU6) are preceded by a potential ribosome binding sequence (see Table 2), in good agreement with those of several *Lactobacillus* (5′-AGGAGG-3′; Pouwel and Leer, 1993) and *Lactococcus* (5′-AGA-AAGGAGGT-3′; Ludwig et al., 1985; Schouler et al., 1994) genes.

In the 4 major capsid proteins, gpG, gpP, gpB and gpO, N-terminal aa alignments predicted by the DNA sequence were identical to those determined by the protein sequence (Table 1). These results confirmed that the genes B, G, O and P are structural genes for gpB, gpG, gpO and gpP, respectively, and suggested that like the major phage protein gpE of coliphage lambda (Georgopoulos et al., 1983), there is no cleavage of a signal peptide from each ϕgle capsid protein, prior to or during phage capsid formation.

In the 3 proteins gpG, gpO and gpP, the N-terminal methionine was deleted probably by posttranslational processing (Table 1); the result agrees with the rule that the N-terminal methionine is generally processed when the second aa residue is alanine (Ben-Bassat and Bauer, 1987).

TABLE 1
Properties of the four major structural proteins of φgle phage particle

Protein	Molecular weight[a]	N-terminal amino acid sequence[b]
gpB	64.0	*MQTLTYVIGQDRRTLVKDIINFKIDF*.....
gpG	43.0	*AETHLSDLIVPEVFGNYVVNQIIKTNRFVQSGILT*.....
gpO	29.0	*ANIVDKTNQIGRKTTELAANMIPA*.....
gpP	26.0	*AKFKMNYKNVFEIDTTGSQDPQDTxKAWFVPLAAxI*....

[a] Molecular weights of the proteins were estimated from SDS-PAGE (Fig. 1A).
[b] N-terminal amino acid sequence was determined by a method of protein-blotting to a PVDF sequencing membrane (see Section Materials and methods).
x, undetermined residue.

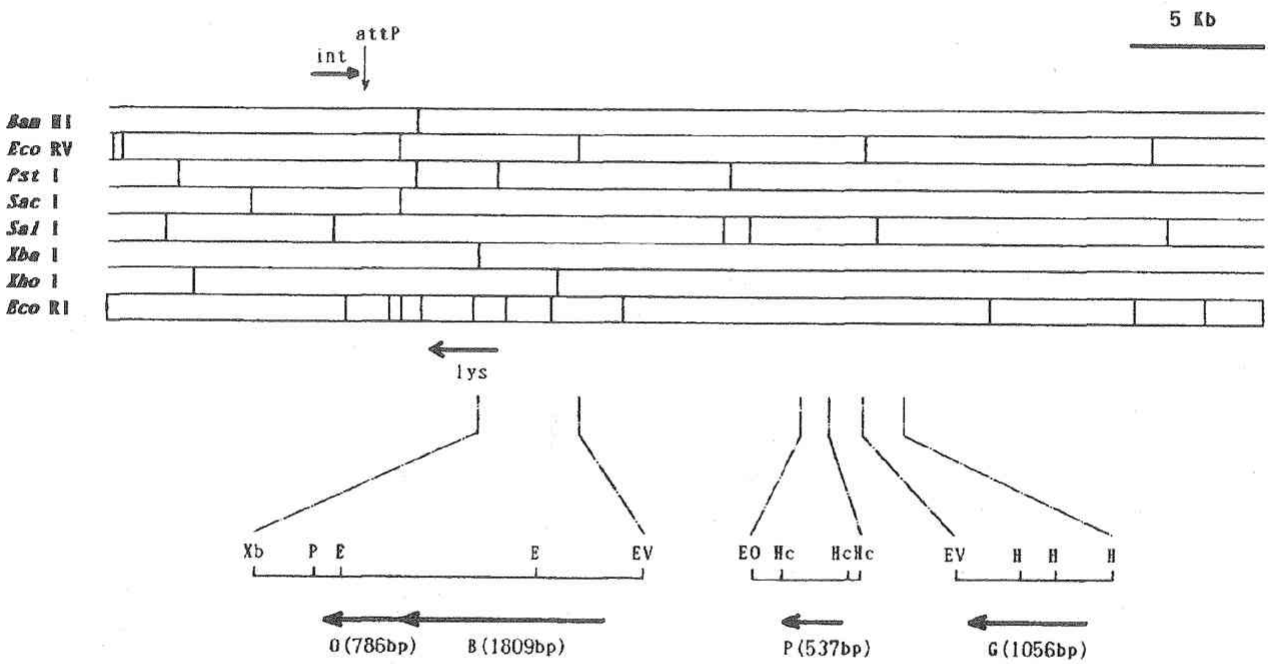


Fig. 3. Physical map of φgle genome. Restriction physical map is linearized at one site of *Eco*RI. Locations of the 1.5 kb from *Hin*dIII to *Eco*RV, the 1.0 kb from *Hin*cII to *Eco*O109I, and the 3.6 kb from *Eco*RV to *Xba*I are enlarged, and the genes G, P, B and O with their sizes and locations are indicated. E, *Eco*RI; EO, *Eco*O109I; EV, *Eco*RV, H, *Hin*dIII; Hc, *Hin*cII; P, *Pst*I; Xb, *Xba*I. *Int* encodes the phage integrase, *attP* is the site of recombination with the host chromosome, and *Lys* encodes the phage lysin (unpublished result).

The 3 proteins gpB, gpG and gpP contain an high molar percentage of acidic aa residues, and their p*I* points were calculated as 4.75, 4.85 and 4.70, respectively, which were in good agreement with those estimated from two-dimensional SDS-PAGE (Fig. 2B).

As shown Fig. 4A, gpG is encoded from nucleotide (nt) position 263 to 1318, and composed of 350 aa residues; its MW was calculated to be 37.6 kDa, which is slightly smaller than that (43.0 kDa) predicted from SDS-PAGE (Fig. 1A). In *Lactococcus* phage F4-1, Kim and Batt (1991) reported that proteins with low p*I* points migrate slower on SDS-PAGE.

Immediately downstream of the putative ribosome binding site (5′-AAAAAGGAGG-3′) for gpG, an inverted repeat of 5 bases (5′-GCCAT-3′) was detected (see Fig. 4A); it can form a stem-loop secondary structure, which may play a role in transcriptional regulation. Two truncated ORFs RorfU1 and RorfU2 were located upstream and downstream of gene G, respectively. RorfU1 is separated from gene G by 16 bases, and the stop codon (TAA) of RorfU1 overlaps the ribosome binding site of gene G. Between gene G and RorfU2, an intergenic space consisting of 23 bases exists, which contains the ribosome binding site of RorfU2 (5′-GGAAGT-3′), but no possible secondary structure.

Gene P is located approximately 1.6 kb downstream of gene G (Fig. 3), and is positioned from nt 190 to 726 (Fig. 3B). Like gene G, gene P encodes the acidic protein of 177 aa residues, whose calculated mass is 18.8 kDa, and is smaller than that (26.0 kDa) estimated from SDS-PAGE (Fig. 2A). Directly downstream of gene P, a stem-loop structure was detected, whose stem is composed of

6 bases (5′-GAGGGCT-3′) (Fig. 4B); it may play a role in transcriptional regulation.

RorfU3 was located upstream of gene P with an intergenic space of 32 bases, which contains the gene P ribosome binding sequence (5′-AGGAAGGAAGT-3′), but no secondary structure. RorfU4 was located downstream of gene P by 49 bases containing the RorfU4 ribosome binding site (5′-AGGAGG-3′).

As presented in Fig. 4C, genes B (from nt 354 to 2162) and O (from nt 2177 to 2962) are in close proximity; they are separated by 14 bases, in a configuration in which the stop codon (TAG) of gene B overlaps the ribosome binding site of gene O (5′-GGAGGT-3′). The acidic protein gpB is composed of 602 aa residues, which have the N-terminal methionine (Table 1). Its calculated mass of 70.0 kDa is slightly larger than that (64.0 kDa) predicted from SDS-PAGE (Fig. 1A). Contrary to gpG, gpP and gpB, gene O product of 260 aa residues is neutral, and its mass is calculated to be 28.6 kDa, which matched that (29.0 kDa) from SDS-PAGE (Fig. 2A).

RorfU5 was located upstream of gene B by 23 bases containing the ribosome-binding site of gene B (5′-AAAAAGGAGAGT-3′). An intergenic space between gene O and RorfU6 is 278 bases long, and rich in several possible regulatory sequences: a large inverted repeat and two promoter like sequences (see Fig. 4C). The stem-loop secondary structure located from nt 3056 to 3084 is ending in a run of 5 T residues, and is able to function as a rho-independent transcription terminator.

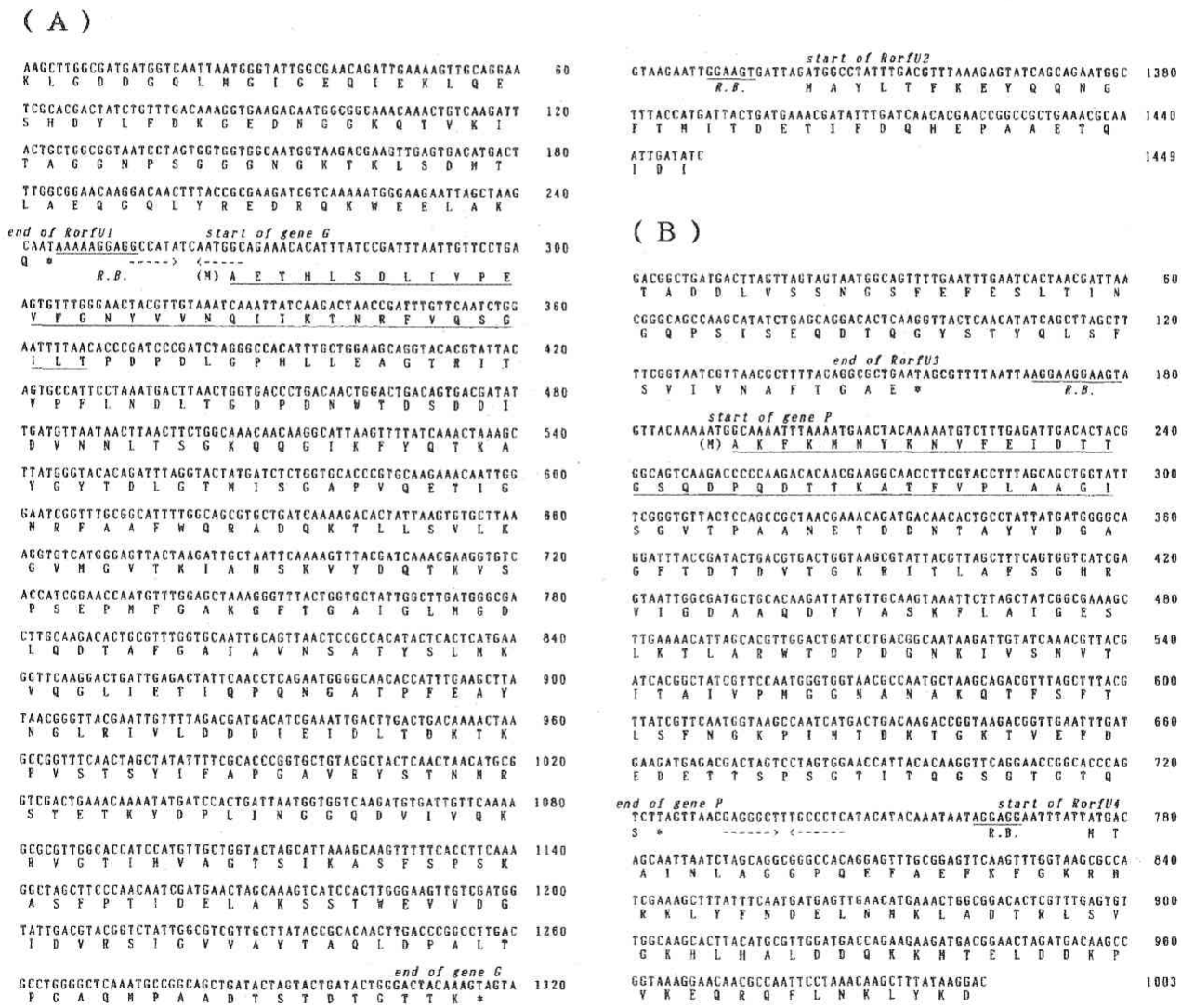
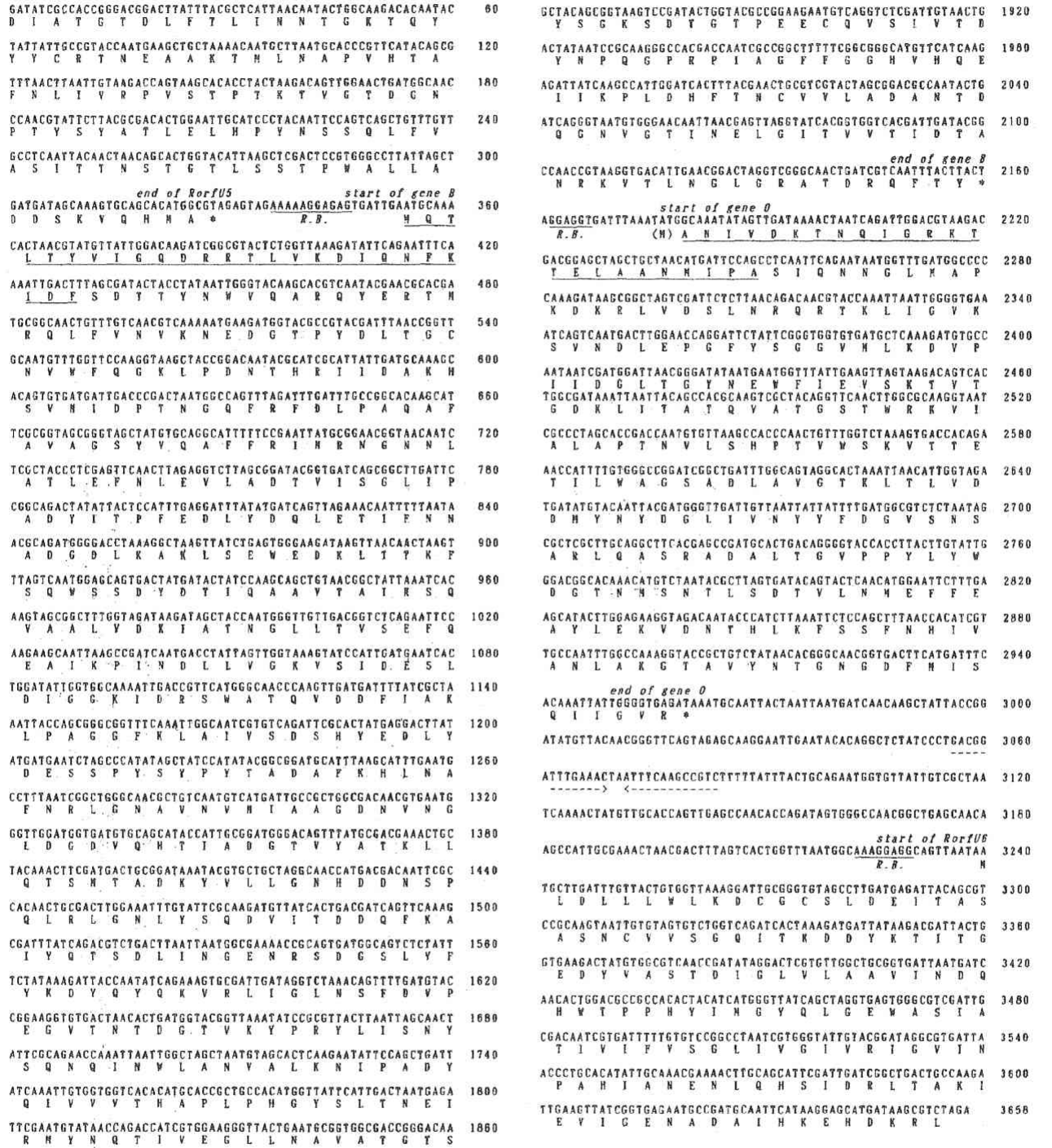


Fig. 4. DNA sequences and predicted amino acid alignments of genes G (A), P (B), B (C) and O (C). Amino acids underlined are those confirmed by N-terminal sequencing as shown in Table 1; the N-terminal methionines of gene products, G, P and O, were absent. Asterisks indicate stop codons. Dotted arrows show inverted repeats. R.B., putative ribosome binding site.

(C)



Sequences of the two promoter sequences are similar to those of *E. coli*: for −35 region, 5′-TTGAAA-3′ from nt 3063 to 3068 and 5′-TTGCAC-3′ from nt 3132 to 3137, and for −10 region, 5′-TTTTAT-3′ from nt 3086 to 3091, and 5′-GATAGT-3′ from nt 3155 to 3160.

4. Conclusions

In this study, we characterized the capsid proteins of bacteriophage ϕgle induced from *Lactobacillus* strain Gle, and determined structural genes encoding the 4

TABLE 2
Genes encoding the major capsid proteins of φgle particle

Gene	Ribosome binding sequence[a]	Gene product[b]			
		Protein	AA	MW ($\times 10^{-3}$)	p*I*
B	AaAAAGGAGag---tgattgaATG	gpB	602	70.0	4.75
G	AaAAAGGAGGc---catatcaATG	gpG	350	37.5	4.85
O	tactAGGAGGT-gatttaaatATG	gpO	260	28.5	6.35
P	AGgAAGGAaGTagttacaaaaATG	gpP	177	18.7	4.70
16s rRNA	AGAAAGGAGGT				

[a] The sequence of *Lactococcus* 16s rRNA is from Ludwig et al. (1985). Capital letters indicate the nucleotides identical to those of 16s rRNA. Dashes represent gaps inserted to align nucleotides for maximal homology. ATG, starting codon.
[b] AA, number of amino acid residue (for gpG, gpO, and gpP, N-terminal methionine is omitted); MW, molecular weight calculated from DNA sequence; p*I*, isoelectric point predicted from DNA sequence.

major capsid proteins gpB, gpG, gpO and gpP. Several properties of genes G, P, B and O are summarized in Table 2.

Information on phage capsid proteins of lactic acid bacteriophage is still scarce (Relano et al. 1987), and a few sequences are available at present: (i) in *Lactobacillus* temperate phage mv4, as well as virulent phage LL-H (Vasala et al., 1993), a major capsid protein g34, a capsid protein g20, and three minor capsid proteins (ORF-4, ORF-5, and ORF-6): (ii) in *Lactococcus* temperate phage Tuc2009 (Arendt et al., 1994), two minor capsid proteins (mp1 and mp2): (iii) in *Lactococcus* phage F4-1 (Chung et al., 1991), a major capsid protein MCP, and three minor capsid proteins (p35, p11 and p9.5). The 4 capsid proteins of φgle identified in this study have no significant homology with those of mv4, LL-H, Tuc2009 or F4-1, in addition to coliphage lambda (Sanger et al., 1982).

The two φgle proteins, gpG and gpP, resemble tail sheath and tube proteins of P2 (Temple et al., 1991) and T4 (Arisaka et al., 1988) in size and p*I*. The tail sheath proteins of F_I (P2) and gp18 (T4) are estimated to be acidic with a p*I* point of 4.85 and 4.79, respectively, and the tail tube proteins of F_{II} (P2) and gp19 (T4) are also acidic with a p*I* point of 4.85 and 4.51, respectively. However, they are clearly different in amino acid sequences, and have no homology in the secondary structures estimated by a method of Chou and Fasman (1978).

Our recent sequencing results on φgle DNA have shown that (i) the DNA region from gene G to gene O of approximately 14 kb contains many ORFs which are considerably compact, and probably encode the 16 minor phage structural proteins, in addition to the four major proteins (this study), suggesting that this region forms an operon for phage head/tail proteins (unpublished data), and (ii) following gene O, there are a few ORFs, one of which is RorfU6 (see Fig. 4C); these ORFs are located in an approximately 2.5 kb region, and probably form another operon (referred to as Lysis operon), which is involved in disruption of the bacterial cell envelope (unpublished data). The possible regulatory structures of stem-loop and promoter sequences found between gene G and RorfU6 (see above) might function as a terminator of the head/tail operon, and promotor(s) for the lysis operon, respectively.

Unfortunately, φgle forms no plaques on the indicator strains used in this study, as previously reported in many other temperate phages of lactic acid bacteria (Teuber and Lembke, 1983). We are now attempting to isolate a φgle-cured strain supporting plaque formation with φgle, although there remains a possibility that φgle is a defective phage.

Further studies on genome structure, gene functions and molecular taxonomy of phage φgle are in progress.

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