

## Characterization of the *cro-ori* Region of the *Streptococcus thermophilus* Virulent Bacteriophage DT1

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The virulent *cos*-type *Streptococcus thermophilus* phage DT1 was previously isolated from a mozzarella whey sample, and its complete genomic sequence is available. The putative *ori* of phage DT1 is characterized by three inverted and two direct repeats located in a noncoding region between *orf36* and *orf37*. As the replication ability of the putative *ori* and flanking genes could not be established, its ability to confer phage resistance was tested. When *ori* is cloned on a high-copy-number plasmid, it provides protection to *S. thermophilus* strains against phage infection during milk fermentation. This protection is phage specific and strain dependent. Then, a detailed transcriptional map was established for the region located between the *cro*-like gene (*orf29*) and the *ori*. The results of the Northern blots indicated that the transcription of this region started 5 min after the onset of phage infection. Comparative analysis of the expression of the *cro-ori* region in the three *S. thermophilus* *cos*-type phages DT1, Sfi19 (virulent), and Sfi21 (temperate) reveals significant differences in the number and size of transcripts. The promoter upstream of *orf29* was further investigated by primer extension analysis, and its activity was confirmed by a chloramphenicol acetyltransferase assay, which showed that the phage promoter is more efficient than the constitutive bacterial promoter of the *S. thermophilus* operon encoding the general proteins of the phosphoenolpyruvate:sugar phosphotransferase system. However, the phage promoter is less efficient than the *pts* promoter in *Lactococcus lactis* and in *Escherichia coli*.

Thermophilic lactic acid bacteria, such as *Streptococcus thermophilus*, are widely used as starter cultures in many yogurt and cheese-manufacturing processes. Bacteriophage infection of these lactic acid bacteria is the main factor impairing lactic acid fermentations, which ultimately decreases the efficiency of the production process and the value of the final product.

*S. thermophilus* phages are currently classified into two general groups based on their mode of DNA packaging and their major structural proteins (27). The first group includes phages with cohesive genome extremities (*cos* type), and the second group contains phages with a DNA packaging scheme that proceeds via a headful mechanism (*pac* type) (27). To date, the complete nucleotide sequence of six *S. thermophilus* phage genomes is available through public databases: four *cos*-type phages, Sfi21 (temperate) (6), DT1 (virulent) (52), Sfi19 (virulent) (33), and 7201 (virulent) (49), and two *pac*-type phages, O1205 (temperate) (48) and Sfi11 (virulent) (31). Comparative analysis indicated a close genetic relationship between temper-

ate and virulent *S. thermophilus* phages. It was even proposed that virulent *S. thermophilus* phages were derived from temperate phages by a combination of rearrangements and deletion events within the lysogeny module (4, 33). Despite these genetic modifications in the lysogeny module, a gene coding for a *cro*-like repressor is still found among all the virulent *S. thermophilus* phages analyzed so far (32), and it could play a role in the replication of these phages.

Our knowledge of phage DNA replication and gene expression is still limited for *S. thermophilus* phages. The availability of such information will likely point towards novel means of controlling phage infections. For instance, phage resistance mechanisms have been engineered with phage genetic elements, such as the origin of replication (*ori*) and antisense RNA genes (for a review, see reference 10). Phage *oris* are mainly characterized by a noncoding region containing several inverted and direct repeats (20, 36). An antiphage system named PER (phage-encoded resistance) was previously associated with the presence of the bacteriophage *ori* provided on a plasmid (20). It is presumed that phage replication factors are titrated by the plasmid harboring the phage *ori*. This effect was first described in *Lactococcus lactis* (20) and later in *S. thermophilus* with the *ori* from phages Sfi19, Sfi21, O1205, 7201, and  $\kappa$ 3 (15, 49, 50).

The gene organization in the vicinity of *ori* is relatively conserved among *S. thermophilus* phages. This region is essentially composed of genes expressed early after the onset of infection, such as those encoding the helicase and primase. Interestingly,

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TABLE 1. Bacterial strains, phages, and plasmids

| Strain, phage, or plasmid | Relevant characteristics <sup>a</sup>   | Reference or source              |
|---------------------------|---|----------------------------------|
| <i>E. coli</i>            |   |                                  |
| DH5 $\alpha$              | Cloning host strain   | Invitrogen                       |
| SMQ-538                   | DH5 $\alpha$ (pFB1); Em <sup>r</sup> Cm <sup>r</sup>                            | This work                        |
| SMQ-636                   | DH5 $\alpha$ (pFB4); Ap <sup>r</sup> Cm <sup>r</sup>                            | This work                        |
| SMQ-822                   | DH5 $\alpha$ (pBV5030); Em <sup>r</sup>   | This work                        |
| SMQ-823                   | DH5 $\alpha$ (pGA1); Em <sup>r</sup> Cm <sup>r</sup>                            | This work                        |
| <i>S. thermophilus</i>    |   |                                  |
| ATCC 19258                | Source of <i>pts</i> promoter   | American Type Culture Collection |
| SMQ-119                   | Host for phages Q1 and Q3   | 39                               |
| SMQ-301                   | Host for phage DT1  | 52                               |
| SMQ-495                   | Host for phages DT1 and MD2   | 14                               |
| SMQ-533                   | SMQ-301(pFB3); Cm <sup>r</sup>  | This work                        |
| SMQ-535                   | SMQ-495(pFB3); Cm <sup>r</sup>  | This work                        |
| SMQ-536                   | SMQ-495(pNZ123); Cm <sup>r</sup>  | This work                        |
| SMQ-629                   | SMQ-301(pFB1); Em <sup>r</sup> Cm <sup>r</sup>                                  | This work                        |
| SMQ-630                   | SMQ-301(pBV5030); Em <sup>r</sup>   | This work                        |
| SMQ-633                   | SMQ-301(pNZ123); Cm <sup>r</sup>  | This work                        |
| SMQ-634                   | SMQ-119(pNZ123); Cm <sup>r</sup>  | This work                        |
| SMQ-635                   | SMQ-119(pFB3); Cm <sup>r</sup>  | This work                        |
| SMQ-817                   | SMQ-301(pGA1); Em <sup>r</sup> Cm <sup>r</sup>                                  | This work                        |
| <i>L. lactis</i>          |   |                                  |
| MG1363                    | Plasmid-free strain   | 16                               |
| SMQ-537                   | MG1363(pFB1); Em <sup>r</sup> Cm <sup>r</sup>                                   | This work                        |
| SMQ-819                   | MG1363(pBV5030); Em <sup>r</sup>  | This work                        |
| SMQ-820                   | MG1363(pGA1); Em <sup>r</sup> Cm <sup>r</sup>                                   | This work                        |
| Bacteriophages            |   |                                  |
| DT1                       | <i>cos</i> type   | 52                               |
| MD2                       | <i>cos</i> type   | 14                               |
| Q1                        | <i>pac</i> type   | 39                               |
| Q3                        | <i>pac</i> type   | 39                               |
| Plasmids                  |   |                                  |
| pBV5030                   | Promoter probe vector; Em <sup>r</sup>  | 2                                |
| pFB1                      | 268-bp amplified fragment of P1 promoter cloned into pBV5030                    | This work                        |
| pFB3                      | 750-bp EcoRI restriction fragment from DT1 cloned into pNZ123; PER <sup>+</sup> | This work                        |
| pFB4                      | 5-kb HindIII restriction fragment from DT1 cloned into pTRK333                  | This work                        |
| pGA1                      | 273-bp amplified fragment of <i>pts</i> promoter cloned into pBV5030            | This work                        |
| pNZ123                    | Cloning vector; Cm <sup>r</sup>   | 13                               |
| pTRK333                   | Origin-screening vector; Ap <sup>r</sup> Tet <sup>r</sup> Cm <sup>r</sup>       | 38                               |

<sup>a</sup> Ap<sup>r</sup>, ampicillin resistance; Cm<sup>r</sup>, chloramphenicol resistance; Em<sup>r</sup>, erythromycin resistance; PER<sup>+</sup>, phage-encoded resistance; Tet<sup>r</sup>, tetracycline resistance.

antiphage systems based on the expression of an antisense RNA, complementary to the helicase (50) as well as the primase (51) mRNAs of a *cos*-type *S. thermophilus* bacteriophage, were recently designed. The antisense RNA strategy is effective against *cos*-type and *pac*-type bacteriophages that have homologous genes. The gene silencing technology is even more effective if combined with the PER system. It is noteworthy that these phage-based antiviral strategies were developed without prior analysis of the phage genome expression. Only recently, the complete transcriptional maps of the *S. thermophilus cos*-type phages Sfi19 (54) and Sfi21 (55, 56) were reported. Transcriptional maps of some lactococcal phages are also available (30, 34, 40). Usually, three classes of transcripts are observed: early, middle, and late. Most of them are not preceded by a consensus promoter sequence (55, 56).

The *cos*-type phage DT1 was previously isolated from a failed milk fermentation, and its complete genomic sequence was the first available for a virulent *S. thermophilus* phage (52). In this study, we report the genetic analysis of the *cro-ori* region of phage DT1.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and bacteriophages.** The bacteria, phages, and plasmids used in this study are listed in Table 1. *Escherichia coli* strains were grown aerobically at 37°C in Luria-Bertani (LB) medium. *S. thermophilus* strains were grown at 42°C without agitation in M17 broth (Quélab) supplemented with 0.5% (wt/vol) lactose (LM17). *L. lactis* strains were grown at 30°C without agitation in M17 broth supplemented with 0.5% (wt/vol) glucose (GM17). When needed, antibiotics were added as follows: 20  $\mu$ g of chloramphenicol per ml or 250  $\mu$ g of erythromycin per ml for *E. coli*, and 5  $\mu$ g of chloramphenicol per ml or 5  $\mu$ g of erythromycin per ml for *L. lactis* and *S. thermophilus*. The culture media used in phage experiments were supplemented with 10 mM CaCl<sub>2</sub>.

Phages were propagated according to Jarvis (22) and purified on a discontinuous-step CsCl gradient followed by a one-step continuous gradient with a CsCl solution density of 1.4 g/ml instead of 1.5 g/ml. For plaque enumeration, 100  $\mu$ l of phage suspension and 500  $\mu$ l of a *S. thermophilus* culture (optical density at 600 nm [OD<sub>600</sub>] of 1.0) were added to 3 ml of LM17 top agar and poured on LM17 plates supplemented with 0.1% (vol/vol) milk and 0.25% (wt/vol) glycine to increase plaque size. Growth curves, burst size, and the efficiency at which center of infection formed (ECOI) were performed as reported earlier (37). The efficiency of plaque formation (EOP) was calculated as described previously (42).

**DNA techniques.** Routine DNA manipulations were performed according to Sambrook and Russell (41). The Maxi Lambda DNA purification kit (Qiagen) was used for phage DNA purification as specified previously (25). To determine

TABLE 2. Probes and primers used in the transcriptional analysis

| Probe | Region                       | Primers            | Sequence (5' to 3')                            | Probe length (bp) |
|-------|------------------------------|--------------------|--|-------------------|
| A     | <i>orf29</i>                 | DT1-29F<br>DT1-29R | GTTAGTTAGGAAGGAGGAAC<br>TCAAAAGCCATACAAATCAGAC | 226               |
| B     | <i>orf30</i> to <i>orf32</i> | DT95<br>DT83       | CCAAACGCACAAACTGGTTT<br>TACACTCTCCACGCAGTC     | 1,426             |
| C     | <i>orf30</i>                 | DT1-30F<br>DT1-30R | ACTAGTCACCTAGTGCGGT<br>GTTGGTACGGATTGAAACA     | 222               |
| C1    | <i>orf31</i>                 | DT1-31F<br>DT1-31R | TTTTAGCACCAGAAGTTTTTG<br>TCACTCATCGCTGCTAAAC   | 259               |
| C2    | End of <i>orf32</i>          | DT84<br>DT1-32R    | GACTGCGTGGGAAGATGTA<br>GGTCTTCAATCTGCAAGCTG    | 227               |
| C3    | <i>orf33</i>                 | DT76<br>DT61       | CAGACGACATTGTTCTCGGAA<br>TTACTAGCAACCTCTTGCC   | 771               |
| D     | <i>orf33</i> to <i>orf36</i> | DT62<br>DT20       | CGGCAAGAGGTTGCTAGTAA<br>GCTCGGTTTACTTGGTGAGA   | 1,484             |
| E     | <i>orf36</i>                 | DT19<br>DT12       | TCACCAAGTAAACCGAGC<br>CTACTGCTGCTTCTTCGCC      | 1,440             |
| F     | <i>orf37</i>                 | DT1-37F<br>DT1-37R | TTCTGAAGCAGCAACAGTTA<br>TGCTTGTTGGAATAAGCAG    | 294               |
| G     | <i>orf38</i>                 | DT1-38F<br>DT1-38R | TCACTAATTCAAATCAAATTCG<br>CAACTATCCTCATAGCATCT | 291               |

the ability of the phage DT1 *ori* to drive plasmid replication, DNA restriction fragments obtained from digestion of the phage genome with XbaI or HindIII were cloned into the vector pTRK333 (38), which can replicate in *E. coli* but not in *S. thermophilus*. This vector also contains a chloramphenicol acetyltransferase gene functional in *S. thermophilus*. Plasmid DNA transformation from the clones obtained in *E. coli* was then attempted in *S. thermophilus* SMQ-301. Ligation mixtures were also electroporated directly into *S. thermophilus*. Growth of *S. thermophilus* transformants on chloramphenicol-containing media can only occur in the presence of a functional replicon.

To verify the PER effect of phage DT1 *ori* on various *S. thermophilus* phages, a 750-bp EcoRI fragment (coordinates 29805 to 30555) containing the *ori* was cloned into the high-copy vector pNZ123 (13). The resulting plasmid, pFB3, was then transformed into *S. thermophilus* with the usual procedures (7, 21).

The P1 promoter region of phage DT1 was amplified with the primers ORIPRO (5'-GAATTCGTCGACCCGGTGCAGCAAAAACCGACTTGAGTGG A-3') and FB2 (5'-CTGCAGCTGCAGTCAATTTGTTGTTCTCCTCCT-3') containing restriction sites for EcoRI and PstI. The amplicon was then cloned into the promoter-screening vector pBV5030 (2) to generate pFB1. The promoter region of the *pts* operon of *S. thermophilus* ATCC 19258 was amplified with the primers PTSP1 (5'-GAATTCGAATTCATATGATGTTTTGTCAC AAAAATG-3') and PTSP2 (5'-CTGCAGCTGCAGGCCATAATGGAGTCTC CTTCT-3'). The amplicon was cloned into pBV5030 to generate pGA1. Clones were confirmed by DNA sequencing performed by the DNA sequencing service of the Université Laval with an ABI Prism 3100 apparatus. Sequence analysis was performed with the Wisconsin Package software (version 10.3) of the Genetics Computer Group (12). The sequences used in this study were from accession numbers AF085222 (complete genome of phage DT1), AF115102 (complete genome of phage Sfi19), and NC\_000872 (complete genome of phage Sfi21).

**Replication of DT1 in the presence of its *ori* in *trans*.** The experiment was conducted with *S. thermophilus* SMQ-301(pNZ123) and *S. thermophilus* SMQ-301(pFB3). Phage DT1 replication in infected cells was monitored according to Hill et al. (19, 20) with the following modifications: LM17 broth was inoculated at 1% (vol/vol) with an overnight culture and incubated at 42°C until an OD<sub>600</sub> of 1.0 was reached. Two samples of 1 ml each were then taken. Total DNA of the first sample was extracted, while the second sample was incubated at 42°C and DNA extraction was carried out at the end of the experiment. CaCl<sub>2</sub> (10 mM) was then added to the culture before infection with phage DT1 at a multiplicity of infection (MOI) of 1. One-milliliter samples were taken immediately after DT1 inoculation and every 5 min thereafter for 30 min.

To extract the total DNA from the samples, cells were immediately centrifuged and washed with 1 ml of a cold washing solution containing 50 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), and 100 mM NaCl. Cell pellets were resuspended in a lysis solution containing 20 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 150 mM NaCl, and 30 mg/ml lysozyme and left on ice for 30 min. Fifty microliters of 10% (wt/vol) sodium dodecyl sulfate and 20 µl of

proteinase K (20 mg/ml) were added, and the mixture was incubated at 65°C for 20 min. Phenol-chloroform extractions were performed, and the DNA was precipitated with 0.1 volume of 3 M potassium acetate (pH 4.8) and 1 volume of isopropanol. The DNA pellet was resuspended in 20 µl of Tris-HCl (pH 8.0) and digested with XbaI. The resulting fragments were separated by electrophoresis on a 0.8% (wt/vol) agarose gel.

The antiviral efficacy of pFB3 was also evaluated by milk fermentation assays. Both phage-sensitive and -resistant strains were inoculated at 5% (vol/vol) from an overnight culture (LM17) in 10 ml of pasteurized milk (Natre). Dilutions of a given concentration of phages were added, and an aliquot was immediately taken for pH reading at time zero. The tubes were incubated at 42°C in a water bath for 4 h. Finally, a second pH reading was taken, and the ΔpH was obtained by subtracting both values.

**Northern blots.** A 100-ml LM17 broth was inoculated with 1% (vol/vol) of an overnight culture of *S. thermophilus* SMQ-301 and incubated at 42°C until the OD<sub>600</sub> reached 0.5. CaCl<sub>2</sub> was added to the culture at a final concentration of 10 mM. The cells were then challenged with the virulent phage DT1 at an MOI of 10. Samples of 1.5 ml were taken at 0, 2, 5, 7, 12, 17, and 22 min after the beginning of infection. The transcription was stopped by adding rifampicin at 150 µg per ml. The cells were collected by centrifugation and kept at -80°C until RNA extraction. Total RNA was extracted with the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. Then, the RNA samples were treated with 1 µl of RNase-free DNase I (10 U/µl) in the presence of 2 µl of RNase inhibitor (40 U/µl) for 30 min at 37°C. Five micrograms of each RNA sample and ladder (Invitrogen) were separated by electrophoresis on a 1% (wt/vol) agarose-formaldehyde gel and transferred to a nylon membrane as described by Sambrook and Russell (41).

RNA was stained with methylene blue after transfer from the agarose-formaldehyde gels to nylon membranes as described elsewhere (18, 41). Immobilized RNA was hybridized with DNA probes labeled with digoxigenin-dUTP with the PCR digoxigenin labeling mix (Roche) as specified by the manufacturer. The various probes were obtained following PCR amplification with phage DT1 genomic DNA, and the primers presented in Table 2. Hybridization and detection conditions were carried out according to the manufacturer's protocol (Roche). Transcripts were detected with the chemiluminescent substrate CDP-STAR (Roche) and Biomax films (Kodak).

**Primer extension.** The primer Cat86Rev (5'-ATCACCAGCTATATGAGC ATCGGG-3') was 5' end labeled with T4 polynucleotide kinase and [γ-<sup>32</sup>P] dATP. Primer extension was performed with 20 µg of total RNA, 0.036 µM of the labeled primer, 2X PCR buffer (20 mM Tris-HCl [pH 8.3], 3.0 mM MgCl<sub>2</sub>, 100 mM KCl), and 5.6 mM dithiothreitol. The deoxynucleoside triphosphates were added to a final concentration of 5 mM. The reaction mixture was incubated at 80°C for 10 min and at 55°C for 50 min. The extension was performed with 200 U of Expand reverse transcriptase (Roche) for 1 h at 42°C. DNase-free RNase was added at a final concentration of 20 µg per ml, and the mixture was

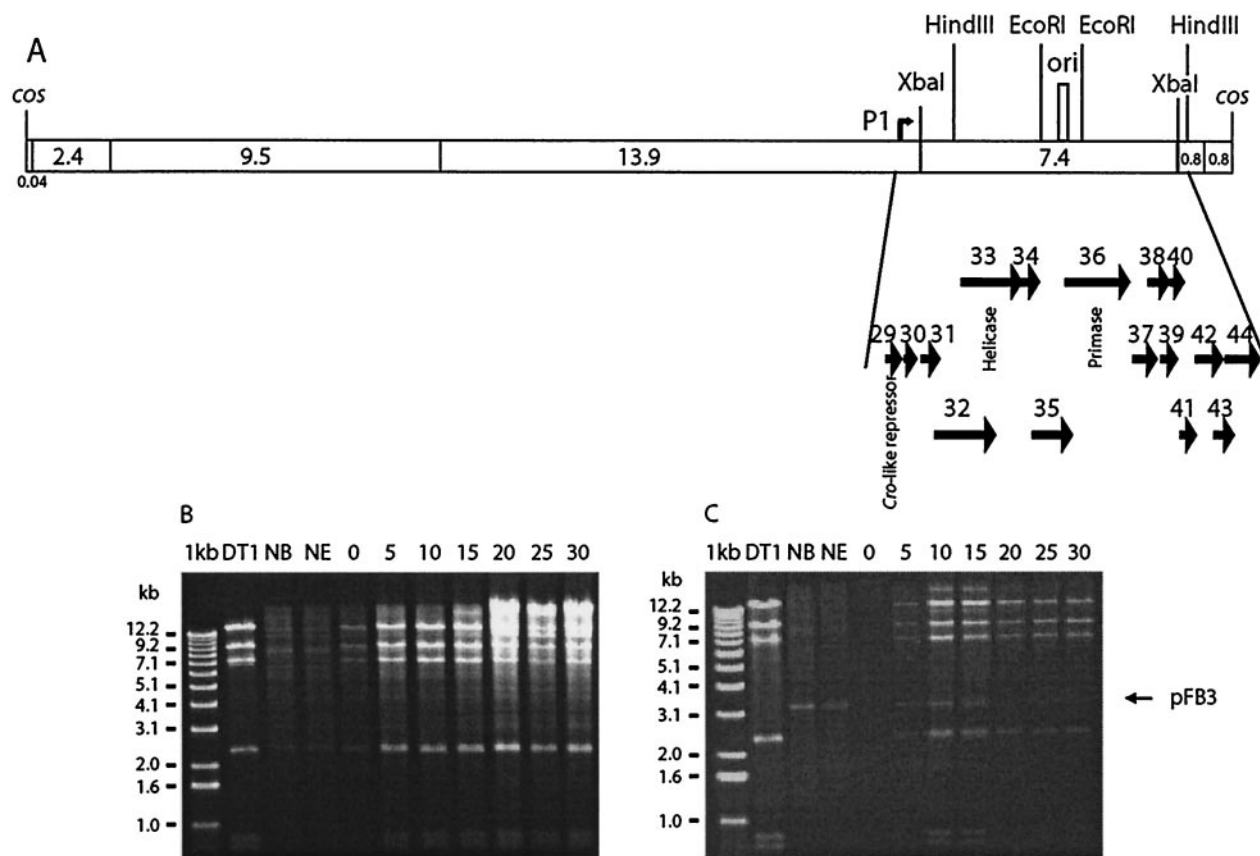


FIG. 1. Schematic illustration of the phage DT1 genome analyzed in this study and its replication in *S. thermophilus* SMQ-301 infected cells. (A) XbaI restriction map of the phage DT1 genome. The bent arrow marks the position of promoter P1. (B) Agarose gel electrophoresis of DNA from SMQ-301(pNZ123). (C) Agarose gel electrophoresis of DNA from SMQ-301(pFB3). NB, uninfected cells before addition of DT1; NE, uninfected cells at the end of the experiment. Lane numbers correspond to the time (in minutes) after the beginning of the infection. Plasmid pFB3 is indicated by an arrow.

incubated at 37°C for 15 min. The extended product was ethanol precipitated and dissolved in 5  $\mu$ l of stop solution (T7 sequencing kit, Amersham Biosciences). The product was separated on a 6% (wt/vol) polyacrylamide gel containing 7 M urea. The DNA sequence of the relevant portion was sequenced with the same primer and pFB1 as the template. The sequence reaction was performed with the T7 sequencing kit.

**Chloramphenicol acetyltransferase assays.** *E. coli* cells were grown overnight at 37°C with agitation in 20 ml of YT medium (41). Cells were harvested by centrifugation, washed, and resuspended in 2 ml of Tris-HCl, 1 mM (pH 7.6). Cells were disrupted by sonication, and the cell debris was removed by centrifugation. The supernatants containing the total protein extracts were used for the assays. For *S. thermophilus* and *L. lactis*, cells from a 500-ml culture ( $OD_{600}$  of 0.4) were harvested by centrifugation and washed with sodium phosphate buffer (8 mM  $NaH_2PO_4$ , 42 mM  $Na_2HPO_4$  [pH 7.5]) containing 5 mM  $\beta$ -mercaptoethanol. Cell extracts were prepared by grinding with alumina (53) and kept at -80°C until used. Chloramphenicol acetyltransferase assays were performed at 37°C for 10 and 60 min as reported elsewhere (28), except that 250 ng of protein from the total cell extracts was used for each assay.

## RESULTS

**Analysis of a putative phage *ori*.** It has been suggested that the *ori* of phage DT1 was located between *orf36* and *orf37* (52). A noncoding region containing direct and indirect repeats, characteristics of phage *oris*, was found between these two open reading frames. Cloning of different restriction fragments containing this putative *ori* into the *ori*-probe vector pTRK333

were undertaken in *E. coli* and *S. thermophilus* in order to find a DNA fragment from the DT1 genome capable of sustaining plasmid replication. Clones containing selected DNA fragments of various length covering the *ori* region could not be obtained or were unstable in *E. coli*. Only one stable clone (pFB4) was obtained in *E. coli* and it contained a 5-kb HindIII fragment covering *orf36* to *orf44* (Fig. 1A).

Despite several attempts, *S. thermophilus* could not be transformed with pFB4. As the replication ability of the putative *ori* and flanking genes could not be established in *S. thermophilus*, its ability to confer phage resistance (PER effect) was tested. A 750-bp EcoRI fragment containing the *ori* (coordinates 29805 to 30555) (Fig. 1A) was cloned into the high-copy vector pNZ123, generating plasmid pFB3, and then transformed directly into *S. thermophilus* SMQ-301. The strains *S. thermophilus* SMQ-301(pNZ123) and SMQ-301(pFB3) were then infected with DT1, and the phage DNA replication was monitored over time. Large amounts of phage DNA were observed in the infected *S. thermophilus* SMQ-301(pNZ123) strain after 5 min (Fig. 1B). However, only small amounts of phage DNA were detected in the cells harboring the *ori* plasmid, and the amount decreased 10 min after infection (Fig. 1C). The copy number of pFB3 increased slightly after phage infection.



TABLE 3. Effect of the *ori*-containing plasmid pFB3 on various *S. thermophilus* phages<sup>a</sup>

| Strain  | Phage | Type       | EOP   | ECOI (%) | Burst size |           |
|---------|-------|------------|---|----------|------------|-----------|
|         |       |            |   |          | pNZ123     | pFB3      |
| SMQ-119 | Q1    | <i>pac</i> | 0.5 ± 0.03                                  | ND       | ND         | ND        |
|         | Q3    | <i>pac</i> | 0.8 ± 0.10                                  | ND       | ND         | ND        |
| SMQ-301 | DT1   | <i>cos</i> | 3 × 10 <sup>-5</sup> ± 2 × 10 <sup>-5</sup> | 3 ± 2    | 142 ± 47   | 1.3 ± 0.2 |
| SMQ-495 | DT1   | <i>cos</i> | 9 × 10 <sup>-2</sup> ± 2 × 10 <sup>-2</sup> | 57 ± 1   | 143 ± 23   | 31 ± 10   |
|         | MD2   | <i>cos</i> | 0.1 ± 0.01                                  | ND       | ND         | ND        |

<sup>a</sup> Results are the means of two independent experiments. ND, not determined.

**Effect of pFB3 on infectivity of various *S. thermophilus* phages.** Following the demonstration that the *ori* provided a PER effect, the EOP, ECOI, and burst size were measured with different phage-host systems. Although pFB3 conferred on *S. thermophilus* SMQ-301 a phage resistance phenotype (EOP 10<sup>-5</sup>, ECOI 3%) against the virulent *cos*-type phage DT1, its efficiency was somewhat limited (EOP 10<sup>-2</sup>, ECOI 57%) in strain *S. thermophilus* SMQ-495 (Table 3). The plasmid pFB3 provided only slight resistance (EOP 10<sup>-1</sup>) against the virulent phage MD2, which is also a member of the *cos* group, and had no significant effect against the virulent *pac*-type phages Q1 and Q3.

To determine the efficiency of the antiviral system in dairy fermentation, the strains were grown in milk with various phage concentrations. In the absence of phage, the milk assay showed a small decrease in the acidification ( $\Delta\text{pH} = 0.2$ ) with strains carrying the pFB3 plasmid (Table 4), indicating that plasmid maintenance had a slight negative effect on cellular metabolic activity. For *S. thermophilus* SMQ-301, in the presence of pFB3 the decreased pH was the same with or without phages even at a high phage concentration (10<sup>7</sup> PFU/ml). Interestingly, even *S. thermophilus* SMQ-495 showed a marked increase in phage resistance, as 10<sup>6</sup> phages were needed to reduce milk acidification, compared to 10<sup>2</sup> phages for the sensitive strain. Overall, the milk fermentation assay confirmed the efficiency of pFB3 in protecting *S. thermophilus* strains against phage DT1 infection.

**Transcriptional analysis of *cro-ori* region.** Comparative genome analyses previously showed the presence of a gene coding for a Cro-like repressor in virulent and temperate *S. thermophilus* phages as well as significant nucleotide differences in this region. In order to investigate the putative involvement of this regulator in the replication of *S. thermophilus* phages, a detailed transcriptional map was established for the region

located between the *cro*-like gene (*orf29*) and the *ori* of DT1. Northern blot experiments were performed to study the transcription of the eight DT1 genes located between *orf29* and the *ori* (Fig. 2). Ten probes were used to analyze this region and to identify the genes associated with the transcripts (Table 2).

Eight transcripts of various sizes were detected in this region with these probes (Fig. 3) and they were all detected 5 min after the beginning of the phage infection (Fig. 2). The amounts of some transcripts decreased over time, but the signals were detected up to 22 min, the last time point examined. Interestingly, at least two mRNAs covered each open reading frame, thereby securing an efficient transcription of this genomic area. Two large transcripts (5.3 and 6.1 kb) covered seven open reading frames (*orf32* to *orf38*) and the *ori*, while several small transcripts spanned three open reading frames (*orf29* to *orf31*). The 6.1-kb mRNA was more abundant throughout the period tested, while the 5.3-kb mRNA was the least abundant. Two other probes covering *orf28* and *orf39* were also used to confirm the start and the end of some transcripts (data not shown). Putative promoters were identified by in silico sequence analysis upstream of *orf29* (TTGACA-N<sub>17</sub>-TATAAT), *orf30* (TTGACA-N<sub>17</sub>-TATATA), and *orf32* (TTGATG-N<sub>17</sub>-GATAAT).

**Comparison of the *cro-ori* region of phages DT1, Sfi19, and Sfi21.** To compare the transcriptional map of DT1 with the *S. thermophilus* phages Sfi19 (virulent) and Sfi21 (temperate), the *cro-ori* region of DT1 was aligned with the homologous DNA region of Sfi19 (*orf69-ori*) and Sfi21 (*orf75-ori*) (Fig. 3). The sequence analysis revealed that the *cro*-like repressor gene (*orf29*) of DT1 did not have homology with the *cro*-like repressors of Sfi19 (*orf69*) and Sfi21 (*orf75*). Nevertheless, a promoter was identified upstream of these genes. In phage Sfi21, the promoter upstream of *orf75* generated a single 2.1-kb transcript covering four open reading frames, while a smaller tran-

TABLE 4. Efficiency of *S. thermophilus* PER system in milk fermentation

| Phage DT1 titer<br>(PFU/ml) | $\Delta\text{pH}^a$ |                |                  |                |
|-----------------------------|---------------------|----------------|------------------|----------------|
|                             | SMQ-301(pNZ123)     | SMQ-301Z(pFB3) | SMQ-495Z(pNZ123) | SMQ-495Z(pFB3) |
| 0                           | 2.09 ± 0.04         | 1.83 ± 0.11    | 1.94 ± 0.06      | 1.75 ± 0.13    |
| 2 × 10 <sup>1</sup>         | 2.04 ± 0.13         | 1.82 ± 0.09    | 1.71 ± 0.11      | 1.76 ± 0.02    |
| 2 × 10 <sup>2</sup>         | 1.98 ± 0.07         | 1.85 ± 0.11    | 1.18 ± 0.19      | 1.83 ± 0.12    |
| 2 × 10 <sup>3</sup>         | 1.39 ± 0.36         | 1.83 ± 0.13    | 0.77 ± 0.14      | 1.81 ± 0.14    |
| 2 × 10 <sup>4</sup>         | 0.77 ± 0.13         | 1.83 ± 0.10    | 0.37 ± 0.11      | 1.79 ± 0.07    |
| 2 × 10 <sup>5</sup>         | 0.34 ± 0.10         | 1.78 ± 0.12    | 0.23 ± 0.07      | 1.77 ± 0.06    |
| 2 × 10 <sup>6</sup>         | 0.11 ± 0.04         | 1.91 ± 0.01    | 0.16 ± 0.11      | 1.35 ± 0.35    |
| 2 × 10 <sup>7</sup>         | 0.03 ± 0.03         | 1.83 ± 0.13    | 0.08 ± 0.05      | 0.50 ± 0.26    |

<sup>a</sup> The  $\Delta\text{pH}$  was the difference in pH at time zero and after 4 h of incubation at 42°C. Results are the mean of three independent experiments.

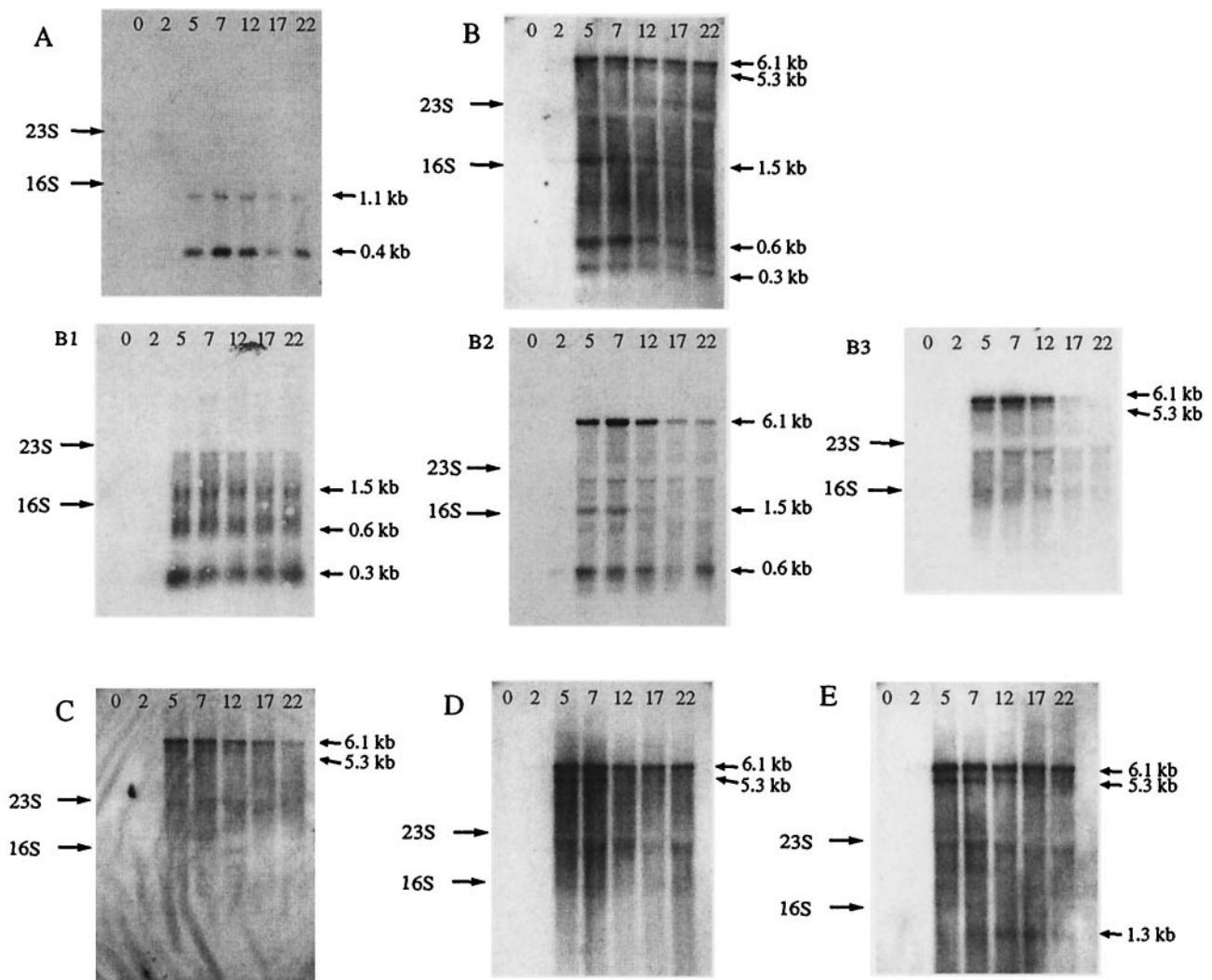


FIG. 2. Northern blots of total RNA isolated from *S. thermophilus* SMQ-301 at the indicated time (in minutes) after infection with phage DT1. The letters A to G on the left side represent the probes used (see Table 2 and Fig. 3 for details). The estimated sizes of the observed transcripts are indicated on the right.

script was observed for DT1 (0.4 kb) and Sfi19 (0.6 kb) as it covered only the *cro*-like gene. Contrary to phages Sfi19 and Sfi21, several small transcripts were detected in the region spanning *orf29* and *orf31* of phage DT1 (Fig. 3). *orf30* and *orf31* are unique to phage DT1, as they had no counterpart in the genomes of phages Sfi19 and Sfi21.

Downstream of *orf31* of DT1, a region of approximately 5 kb is almost identical between the three phages and covers *orf32* up to the *ori* in DT1 and from *orf233* to the *ori* in Sfi19 and Sfi21. Two large transcripts (5.3 and 6.1 kb) spanned this region in DT1, while two smaller transcripts (3.6 and 5.8 kb) were found in phages Sfi19 and Sfi21. For the three phages, a shorter mRNA (1.3 or 1.5 kb) corresponding to the transcription of the putative primase gene was also observed. The *ori* of phage DT1, characterized by the presence of many secondary structures, may serve as terminator of the 1.3-kb transcript covering the putative primase gene. However, it appears to be leaky, as the two larger transcripts bypassed the *ori*. On the

contrary, the *ori* appears to terminate two transcripts (1.5 and 5.8 kb) in phages Sfi19 and Sfi21.

**Characterization of P1 promoter.** Since a gene coding for a *cro*-like repressor is present in all *S. thermophilus* phage genomes analyzed thus far, we further analyzed the putative promoter (P1) identified upstream of *orf29*. As indicated above, P1 possesses the  $-35$  and  $-10$  consensus sequence (TTGACA- $N_{17}$ -TATAAT) (17). This region was amplified from the DT1 genome and the amplicon was cloned upstream of a promoterless chloramphenicol acetyltransferase (*cat*) gene into the pBV5030 vector, and the resulting plasmid was designated pFB1. A primer extension experiment with a primer specific to the *cat* gene was performed with total RNA isolated from *S. thermophilus* SMQ-301(pFB1). Primer extension analysis located the transcriptional start point 6 nucleotides downstream from the last nucleotide of the inferred  $-10$  box (Fig. 4).

The relative strength of the P1 promoter was then compared

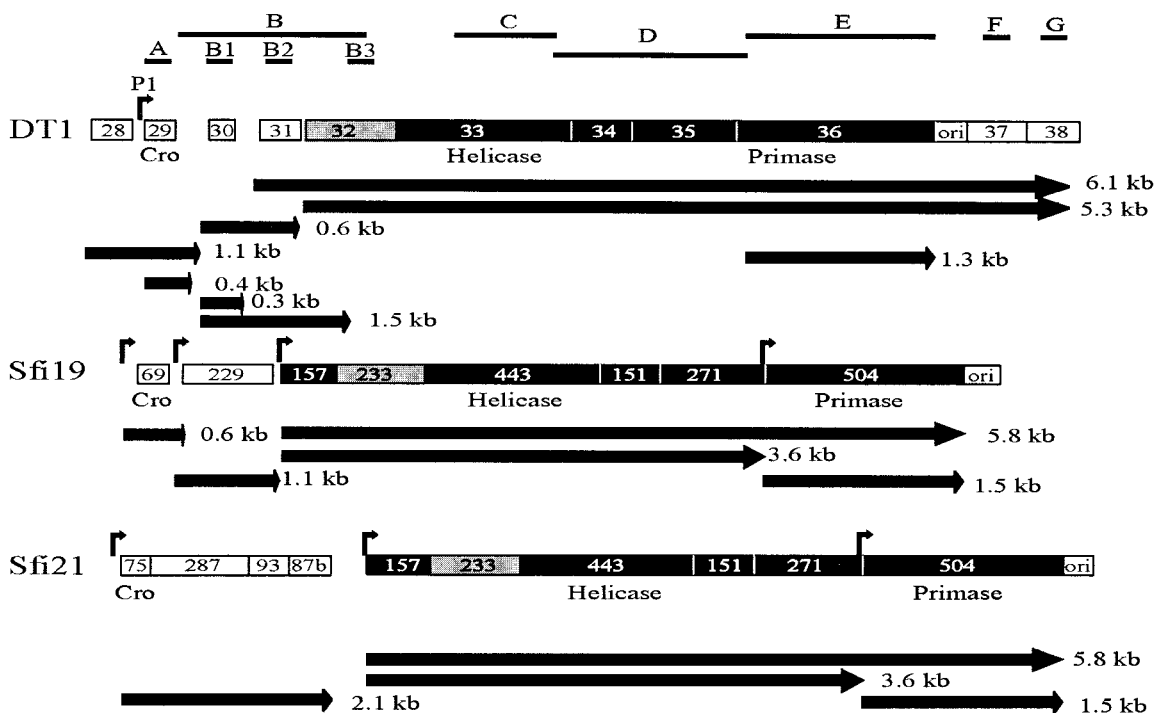


FIG. 3. Comparison of the transcriptional maps of the *cro-ori* regions of *S. thermophilus* phages DT1, Sfi19, and Sfi21. The DNA probes used in the Northern blot experiments are indicated by the letters A to G and black lines above the transcripts. The arrows indicate the direction of transcription. The estimated sizes of the observed transcripts are also indicated. Bent arrows designate promoter sequences. Deduced proteins showing between 70% and 80% amino acid identity are in grey, while those sharing more than 90% amino acid identity are in black.

to the strong bacterial promoter (TTGATA-N<sub>17</sub>-TATAAT) of the operon coding for the general proteins of the phosphoenolpyruvate:sugar phosphotransferase system *pts* of *S. thermophilus* ATCC 19258 (9). The promoter of the *pts* operon was amplified from *S. thermophilus* ATCC 19258 and cloned in pBV5030, and the resulting plasmid (pGA1) was introduced by electroporation into *E. coli* DH5- $\alpha$ , *L. lactis* MG1363, and *S. thermophilus* SMQ-301. The activity of the P1 and PTS promoters was measured by determining the CAT activity in these

three strains (Table 5). While no CAT activity was detected in cells carrying only the promoter-probe vector pBV5030, it was detected in strains bearing pFB1 and pGA1, confirming the functionality of these two promoters. The CAT activity was approximately twofold higher in *S. thermophilus* when the *cat* gene was under the control of P1 compared with the promoter of the *pts* operon. Conversely, in *E. coli* and *L. lactis*, control of *cat* expression by the *pts* promoter resulted in higher levels of CAT activity than with promoter P1.

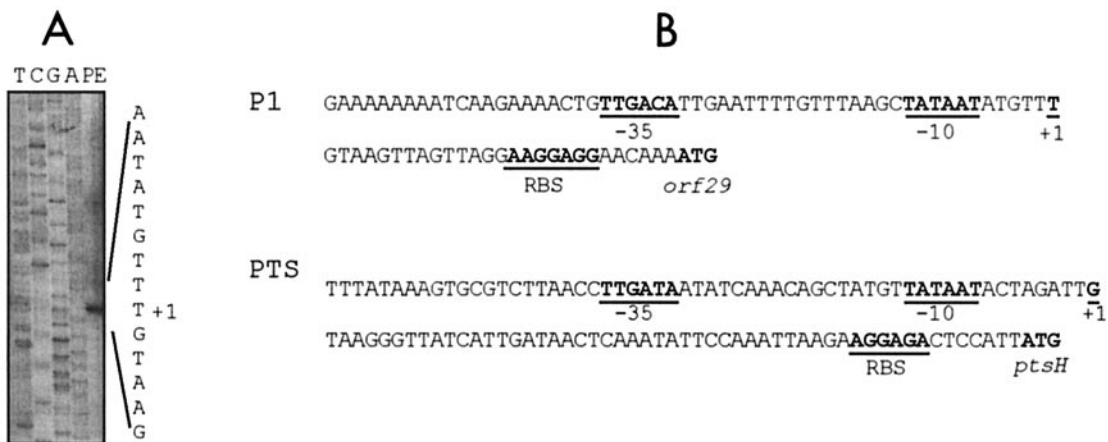


FIG. 4. (A) Mapping of the transcription start site from the P1 promoter by primer extension (PE). Nucleotide position +1 is indicated. The nucleotide sequence of pFB1 was determined with the same primer (lanes T, C, G, and A). (B) Partial nucleotide sequence of the P1 and PTS promoter regions. Putative -35 and -10 boxes and the putative ribosome binding sites (RBS) are indicated.

TABLE 5. Chloramphenicol acetylation in various strains<sup>a</sup>

| Bacteria                       | Acetylated chloramphenicol (%/min) |               |                     |
|--------------------------------|------------------------------------|---------------|---------------------|
|                                | pBV5030                            | pFB1 (P1)     | pGA1 ( <i>pts</i> ) |
| <i>E. coli</i> DH5 $\alpha$    | 0.0 $\pm$ 0.0                      | 1.0 $\pm$ 0.3 | 1.5 $\pm$ 0.2       |
| <i>L. lactis</i> MG1363        | 0.0 $\pm$ 0.0                      | 0.3 $\pm$ 0.1 | 2.5 $\pm$ 0.3       |
| <i>S. thermophilus</i> SMQ-301 | 0.0 $\pm$ 0.0                      | 1.5 $\pm$ 0.4 | 0.8 $\pm$ 0.0       |

<sup>a</sup> The activity is expressed as the percentage of chloramphenicol acetylated per minute at 37°C by 250 ng of protein from the total cell extracts. The means of three independent experiments are presented.

## DISCUSSION

**Analysis of the phage *ori*.** Despite several attempts, we were unable to obtain a stable plasmid containing DNA from phage DT1 that could replicate autonomously in *S. thermophilus*. This may be explained by the presence of early genes in the vicinity of the *ori*, as early-transcribed phage genes are often toxic to the bacterial cell (29). Other groups were also unsuccessful in cloning a functional *ori* from an *S. thermophilus* phage (15, 49). The fact that similar results were obtained in this study is not surprising, as the *ori* region appears to be very well conserved among *S. thermophilus* phages. Since no self-replicating plasmids were obtained, the PER phenotype usually conferred by phage *ori* was verified to assess the functionality of the putative DT1 *ori* identified by in silico analysis. A strong inhibition of phage DT1 DNA replication was observed in cells carrying the *ori* cloned on a high-copy plasmid. This is presumably the result of the titration of phage replication factors by the *ori*. The copy number of the *ori*-containing plasmid increased but only slightly, which is likely due to the fact that the *ori* was already cloned on a high-copy plasmid.

The assays conducted with other phage-host systems showed that the resistance conferred by plasmid pFB3 was phage specific, which is consistent with observations made by Foley et al. (15). As noticed by Stanley et al. (49), the resistance was also strain dependent in *S. thermophilus*. Indeed, the presence of pFB3 conferred stronger protection against phage DT1 in *S. thermophilus* SMQ-301 than in SMQ-495. Plasmid-strain interactions probably account for this difference, as the burst size of DT1 was similar in the wild-type strains SMQ-301 and SMQ-495. These data demonstrate that the *ori* of phage DT1 confers a PER phenotype during milk fermentation but the protection is limited to certain phages and thus is likely to be used only for specific applications.

**Transcriptional analysis.** The transcriptional map of the *cro-ori* region of DT1 was compared with that of Sfi19 (54) and Sfi21 (56). In contrast to the virulent phages DT1 and Sfi19, which contain only a *cro*-like repressor gene, the temperate phage Sfi21 has a complete and functional lysogeny module. These three phages have a consensus promoter upstream of the *cro*-like repressor gene. For the virulent phages DT1 and Sfi19, the promoter enabled the transcription of a short mRNA that covered only the *cro*-like gene. For Sfi21, the corresponding promoter generated a 2.1-kb mRNA covering part of the lysogeny module (*orf75* to *orf87b*). Additional transcriptional start points are necessary for the transcription of the conserved downstream genes of these three phages.

Interestingly, the *oris* of phages Sfi19 and Sfi21 appear to act as stronger terminators than the *ori* of phage DT1. A transcript

covering only the putative primase gene was also detected for the three phages. This small transcript suggests that the primase has to be produced at higher levels for efficient phage genome replication. It was shown recently that antisense RNA targeting an *S. thermophilus* phage primase was found to be a more effective phage defense strategy than antisense RNA targeting helicase (51), strengthening the observation that the primase is essential for phage replication and is required in substantial amounts.

It was previously hypothesized that the ancestor of DT1 (likely a temperate phage) may have lost part of a lysogenic module to become a lytic variant (52). Bruttin et al. (4–6) showed the emergence of lytic phages after several passages of the temperate phage Sfi21 on an indicator strain. These phage mutants had two types of deletions. The first deletion eliminated the genes involved in immunity control, and the second deletion resulted in the loss of genes coding for the integrase, immunity, and part of the *cI* repressor. Similar genetic rearrangements may have occurred in the ancestor of phage DT1. As a consequence, in the lytic phage DT1, the transcription profile was altered, resulting in several short transcripts. Expression analysis of the *cro-ori* region of these three phages clearly showed that a given transcriptional map should not be assumed for all phages of the same group. Additional analysis should be performed with other phages to have a comprehensive view of phage gene expression. Such analyses should lead to identification of a suitable target for the development of effective phage-resistant *S. thermophilus* strains based on antisense RNA (50, 51).

**Characterization of the P1 promoter.** To our knowledge, this is the first study on the characterization of an *S. thermophilus* phage promoter. By contrast, several promoters have been studied in various *S. thermophilus* strains (43–47) and other lactic acid bacteria phages (1, 3, 11, 24, 35, 36, 58, 59). The P1 promoter possesses the bacterial consensus promoter sequence, while the promoter of the *pts* operon differs from the consensus by one nucleotide (TTGATA-N<sub>17</sub>-TATAAT). The transcriptional start point of the P1 promoter is located 6 nucleotides downstream from the last nucleotide of the –10 region, while that of the *pts* promoter is initiated at 9 nucleotides downstream from the last nucleotide of the –10 box (9). The P1 promoter is more active than the promoter of the *pts* operon in *S. thermophilus*, while the opposite is found in *E. coli* and *L. lactis*. The divergence of activity for these two promoters suggests that the phage P1 promoter has an optimal sequence for the RNA polymerase of *S. thermophilus* to ensure efficient transcription of the early genes, while the *pts* promoter is subjected to regulatory expression mechanisms according to cell requirements. Similarly, the streptococcal phage promoter may not possess the optimal sequence for its recognition by the RNA polymerases from *E. coli* and *L. lactis*.

The analysis of several promoters of *Bacillus* spp. and other gram-positive bacteria indicated that several conserved regions are necessary for the recognition of the promoter by the RNA polymerase (8, 57). The most significant region (named –16) consists of a TRTG motif (57). The P1 promoter has internal and external sequences similar to those identified in *Bacillus subtilis* promoters. The P1 promoter has a partial –16 box (TAAG) as well as A<sub>n</sub> stretches from positions –54 to –51 and from –44 to –38. It also possesses an A downstream from the



–10 box and a CTG located just upstream of the –35 box. Several T's are also present between the –35 and –10 consensus sequences. The *pts* promoter also has some sequences present among promoters of *B. subtilis* and other gram-positive bacteria, such as the –16 box (TATG) and  $A_n$  stretches (Fig. 4). With synthetic promoters introduced in *L. lactis*, Jensen and Hammer (23) also demonstrated that the sequences internal and external to the –35 and –10 boxes are also critical for promoter strength. The dinucleotide TG is often located one nucleotide upstream of the –10 box. A 4-bp consensus sequence (ATTC) located just upstream from the –35 sequence is also frequently found among lactococcal promoters. These differences, and possibly others, may be responsible for promoter strength in the three bacteria analyzed in this study.

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