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Identification of the Repressor-Encoding Gene of the *Lactobacillus* Bacteriophage A2

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The repressor gene of the *Lactobacillus* phage A2 has the following properties: it (i) encodes a 224-residue polypeptide with DNA binding and RecA cleavage motifs, (ii) is expressed in lysogenic cultures, and (iii) confers superinfection immunity on the host. Adjacent, but divergently transcribed, lies another open reading frame whose product resembles the λ Cro protein. In the 161-bp intergenic segment, putative promoters and operators have been detected.

Bacteriophages are recognized to be the main source of disruption in industrial food fermentations (5). The temperate phage A2 infects strains of *Lactobacillus casei* and *Lactobacillus paracasei* of industrial relevance. The virions present isometric heads and noncontractile tails. The phage genome is a 44.02-kb double-stranded DNA molecule with 3′-protruding cohesive ends (6, 7). A2 can be recovered from lysogens through mitomycin C induction, suggesting that the phage repressor becomes inactivated by proteolytic cleavage during the mitomycin-induced SOS response as it occurs with bacteriophage λ (17). The λ repressor binds to promoters P_L and P_R, which results in repression of the genes that lead to the lytic development. One consequence of this regulation is that lysogens are immune to superinfection by the same or related viruses. In addition, *cI* is autogenously regulated through differential binding to three adjacent operator sites.

Characterization of A2 clear plaque deletion mutants. The gene that encodes the viral repressor was localized through the selection of deletion mutants unable to lysogenize *L. casei* ATCC 393. To get them, phage suspensions were treated with 10 mM sodium pyrophosphate, pH 7.4, at 37°C for 30 min, which resulted in survival of 7×10^{-4} phage. Appropriate dilutions were plated onto MCM (4), and the surviving phage was collected, suspended in SM buffer, and subjected to new rounds of treatment until a plateau was reached at around 10% survival. Phage from isolated plaques, obtained after each round, was repurified, and their DNA restriction patterns were compared with that of the wild type. The deletions ranged from 0.5 to 3.5 kb and mapped in three *Eco*RI fragments that defined two regions of the genome, comprising up to 7.9 kb dispensable for lytic development (Fig. 1). The phage whose deletion was located in the center of the physical map showed a clear plaque phenotype and was unable to lysogenize its hosts. In contrast, lysogenization was easily obtained with the mutants lacking segments in the right arm of the genome.

Structural characterization of the repressor region of bacteriophage A2. Since the deletions located at the center of the A2 physical map resulted in impairment of lysogenization, we started the analysis of this region by cloning and sequencing it. In this sequence, four open reading frames (*orfA* to *orfD*), which read in opposite directions, were found (Fig. 1). The products of *orfB* and *orfC* were hypothesized to be the functional homologs of the λ proteins CI and Cro, respectively. This was based on their sizes (224 and 81 amino acids for ORFB and ORFC, respectively, which correspond to 25,277- and 9,180-Da polypeptides), their transcription in opposite directions, with an intergenic region of 161 nucleotides (Fig. 2), and the similarities shown by ORFB (pI, 4.56) to phage repressors and also to regulatory proteins involved in SOS induction (Fig. 3). The NH_2-terminal end of ORFB presents a helix-turn-helix motif, which is possibly involved in binding of a specific DNA target, while its carboxy-terminal part shows a domain for protease RecA recognition. It includes conserved Ser and Lys residues and the Ala-Gly motif (marked with asterisks in Fig. 3), in front of which cleavage has been reported to occur in the λ repressor, as the first step towards lytic development of the prophage (18).

As stated above, ORFC is hypothesized to be the functional homolog of Cro, a competitor of CI for binding at the operators of the promoters that regulate the lytic-lysogenic pathways. Nevertheless, ORFC does not show any significant similarity to Cro at the amino acid sequence level, although it does with a putative DNA binding transcription repressor of a *Pseudomonas aeruginosa* bacteriophage (9), and with a *cro* topological homolog of φSfi21, which infects *Streptococcus thermophilus* (3). Additionally, it shares some of the essential amino acids present in Cro homologs and has a basic isoelectric point (pI, 10.80).

In the intergenic region lying between *orfB* and *orfC*, two putative divergent promoters were identified. Both presented the dinucleotide TG positioned 1 base upstream of the −10 hexamer, a feature that has been shown to enhance both promoter strength and utilization (20). These are followed by po-

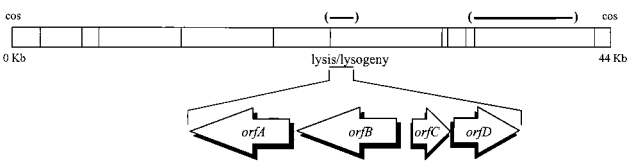

FIG. 1. *Eco*RI restriction map of A2 DNA with indication of dispensable regions for lytic development (thick lines in parentheses). Below the map is shown the organization of the region that controls the phage cycles. The arrows indicate the relative sizes and directions of transcription of the indicated genes.

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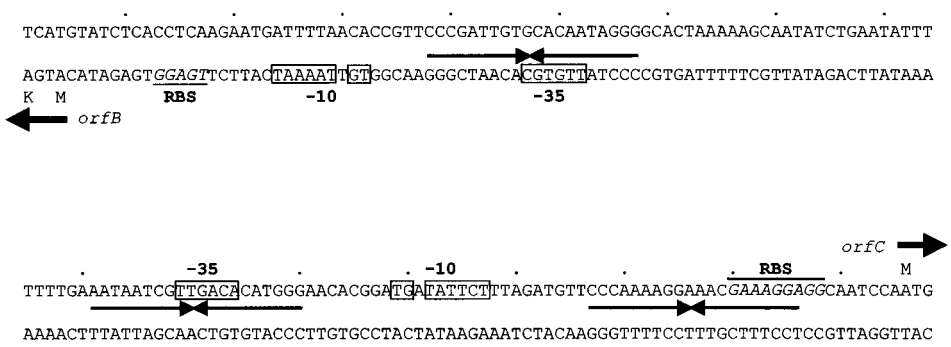

FIG. 2. Nucleotide sequence of the region between *cI* and the putative *cro*. Potential ribosomal binding sites (RBS), consensus promoter sequences (−35 and extended −10 boxes), the starts of translation, and the inverted repeats found are indicated.

tential ribosomal binding sites, complementary to the *Lactobacillus delbrueckii* 3′ end of the 16S rRNA (10). In addition, three imperfect palindromic sequences that might act as operators in regulation of the phage developmental cycles were observed (Fig. 2).

The stop triplet of *orfC* overlaps with the start of *orfD* in the sequence ATGA, which suggests that both have a coupled translation. The predicted ORFD polypeptide is 160 amino acids long, with a mass of 17,844 Da and a pI of 5.39. It shows some homology to putative proteins encoded by open reading frames located in similar genomic positions of several bacteriophages, such as BK5-T, ϕSfi21, and r1t, that infect other lactic acid bacteria (2, 3, 11). In turn, the corresponding gene of the temperate *S. thermophilus* phage ϕSfi21 has some homology with the gene *ant* of P1, which encodes an antirepressor (15).

The start codon of *orfA* is located 58 nucleotides downstream of *orfB*. In this region an inverted repeat with a ΔG of −17 kcal/mol, followed by a stretch of T's, was found; it may act as a rho-independent transcription terminator (see below). *orfA* encodes a 225-amino-acid polypeptide with a mass of 24,447 Da and a pI of 4.4, which is preceded by a canonical ribosome binding site. Comparison of the sequence deduced from *orfA* with those present in databases did not reveal similarities to relevant proteins.

Expression of *orfB* confers immunity to A2 superinfection. If *orfB* coded for the repressor, it should confer superinfection immunity against A2 when cloned into *L. casei* cells. To test this possibility, a 0.8-kb DNA segment containing *orfB* was amplified by using a primer that included its putative promoter sequence and a converging one located just after the stop codon, into which *Eco*RI restriction sites were introduced. The amplified DNA segment was purified, *Eco*RI cleaved, and ligated to pEM40 digested with the same enzyme to generate pEM40::*orfB*. This plasmid is a pUC18 derivative that contains an erythromycin resistance gene for selection in gram-positive bacteria. It does not replicate in *L. casei* but carries the integrase gene and the *attP* sequence of A2, which allows its insertion into a tRNA gene of several lactobacilli (1). Challenge of several independently obtained pEM40::*orfB* transformants with bacteriophage A2 resulted in complete immunity to superinfection (no plaques were produced by a phage suspension with a titer on the untransformed host of 10^{10} PFU/ml). This is consistent with the suggested function of *orfB* as the gene that encodes the A2 repressor (*cI*).

***cI*-specific transcripts produced during the lytic and lysogenic cycles of A2.** As a final test of *cI* identity, its transcription pattern was investigated. A Northern blot of total RNA from *L. casei* ATCC 393 (without infection and at several times postinfection) and from one A2 lysogen derivative was probed with a PCR-generated ^{32}P-labelled DNA fragment that exactly spans *cI*. Two transcripts, of 0.8 and 1.4 kb, were observed in the lysogen (Fig. 4A). The size of the first fits with the distance between the putative *cI* promoter and the rho-independent terminator identified 3′ of that gene. The 1.4-kb transcript most probably corresponds to *cI* plus *orfA*. In productively infected cultures of *L. casei* ATCC 393 (Fig. 4B), the same pattern of *cI*-specific RNAs was found at early times postinfection (from 15 to 25 min) and fading afterwards (the eclipse period of the phage under the propagation conditions used lasts about 120 min).

Several lines of evidence seem to indicate that the central

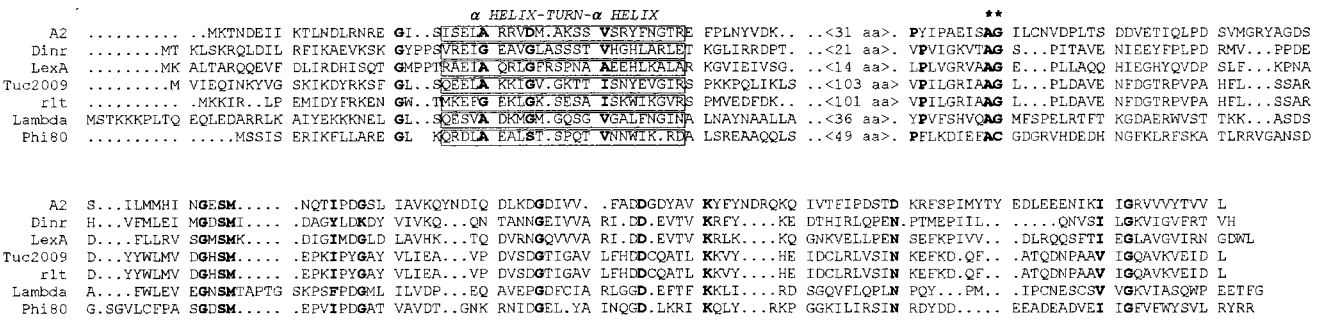

FIG. 3. Amino acid sequence alignment of ORFB with the CI proteins of phages λ (16), ϕ80 (12), and r1t (11); the putative repressor of Tuc2009 (19); and the SOS response-related proteins LexA (8) and DinR (14). The DNA binding motifs of the amino termini are boxed. Conserved amino acids are shown in boldface, and the RecA cleavage point is marked with asterisks.

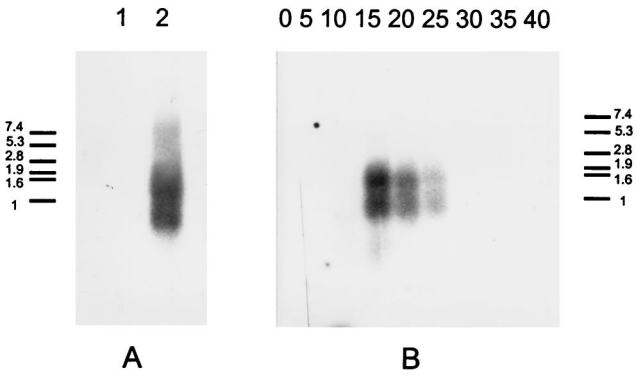

FIG. 4. Northern blot analysis of the *cI* transcripts. (A) Equal amounts of total RNA from uninfected *L. casei* (lane 1) and from an A2 lysogen (lane 2) were used. (B) Total RNA from *L. casei* at various times postinfection (in minutes) with bacteriophage A2. The numbers beside the size standards are in kilobases.

region of the A2 genome is involved in the genetic switch that directs phage development into the lytic or lysogenic cycles. First is its resemblance to the homologous region of bacteriophage λ (13), with two genes reading in opposite directions, separated by a short intergenic region in which three putative operator sequences could be discerned. In addition, the role of ORFB as the λ CI homolog is suggested by its sequence, in which the relevant DNA binding and RecA protease recognition motifs are present; by its transcription pattern, both in lysogens and at early times postinfection; and, surely most important, through the superinfection immunity phenotype conferred on *L. casei* upon insertion of *cI* into its genome. The overall resemblance of the genetic switch regions of phage A2 and λ is a proof of the suitability of this regulation system, which is present in phages not only of gram-negative but also of gram-positive bacteria. However, the distance between the putative *cro* and *cI* genes of phage A2 (161 bp) is longer than it is in λ (100 bp), possibly indicating that the DNA-protein and protein-protein interactions that lead to growth cycle regulation may differ between the phages.

Furthermore, lactobacilli are used in many food fermentations and also as probiotics in health promotion. The confirmation that phage repressors, even in single copy but stably integrated into the host's genome, protect the host from phage infection may suggest ways to avoid one of the main causes of industrial fermentation failure; in fact, we have determined that protection by CI against A2 infection is extended to *L. casei* growing under fermented milk production conditions (unpublished data). Of course, further refinements would be necessary before it can be used for industrial purposes, mainly to replace the *Escherichia coli* plasmid-derived sequences of the vector with generally-regarded-as-safe bacterial sequences. Our efforts will be devoted to demonstrating, we hope, the general utility of stable repressor expression in protection against phage attack on valuable bacterial strains.

Nucleotide sequence accession number. The sequence of the repressor region of bacteriophage A2 has been submitted to EMBL under accession no. Y12813.

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