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Short communication

Differential expression of *cro*, the lysogenic cycle repressor determinant of bacteriophage A2, in *Lactobacillus casei* and *Escherichia coli*

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ABSTRACT

Expression of bacteriophage A2-encoded *cro* in *Escherichia coli* gives rise to two co-linear polypeptides, Cro and Cro*, which were proposed to form a regulatory tandem to modulate the frequency with which the phage would choose between the lytic and the lysogenic cycles. In this communication, it is reported that Cro is the canonical product of the gene *cro* while Cro* results from a −1 ribosome frameshift during translation and is twelve amino acids shorter than Cro. However, frameshifting was not observed during phage development in *Lactobacillus casei*. Furthermore, wild type phages and *cro*-frameshifting negative mutants present the same phenotype, thus corroborating that only the canonical form of Cro is needed to produce a viable phage progeny.

Most food fermentations are driven by the so called lactic acid bacteria, a group of anaerobic, Gram positive bacteria that obtain their energy through fermentation of sugars to lactic and other organic acids (Roissart de and Luquet, 1994).

Bacteriophages constitute the major biological problem of industrial food fermentations (Garneau and Moineau, 2011). This is because the stockbreeding products used as raw materials become altered upon sterilization, thus precluding heat-killing of phages that contaminate the fermentable mass (Madera et al., 2004). As a consequence, many phages that infect lactococci and lactobacilli have been isolated and characterized (Garneau and Moineau, 2011; Brussöw and Suarez, 2006; Deveau et al., 2006). One of the best known is A2, a temperate phage of *Lactobacillus casei*, isolated from a failed cheese fermentation (Herrero et al., 1994). A2 has a 60 nm isodiametric capsid, and a 290 nm × 12 nm non-contractile tail (family *Siphoviridae*) that presents a basal plate and a 28 nm long spike (García et al., 2003). Its dsDNA genome (Gene Bank accession number AJ251789) presents 3′-protruding cohesive ends (García et al., 1997) and 61 *orf*s, clustered into functional categories (García et al., 2003) (Fig. 1A). The temperate nature of A2, its cohesive ends and the production of a single polypeptide during the lytic cycle that is processed to give the scaffolding and the major capsid proteins, allows its placement in the Sfi-21 group of phages (Brussöw and Desiere, 2001). The center of the A2 genome is occupied by the genetic switch that governs whether the phage, upon infection, will follow a lysogenic or a lytic cycle (Fig. 1B). The switch is composed of two non-overlapping, divergent promoters: P_L that supports transcription of the lytic repressor determinant *cI* and P_R that governs expression of the lysogenic cycle repressor (*cro*) and the replication genes (Brussöw and Suarez, 2006; García et al., 1999; Ladero et al., 1999). Interspersed between them three operator sequences have been identified (O_1–O_3) to which CI and Cro bind although with opposite affinities. Binding of Cro is maximal to O_3, which overlaps the −35 region of P_L, and results in repression of *cI* transcription and entry of the phage into the lytic cycle (Ladero et al., 1999). Further accumulation of Cro provokes its binding to O_1 and O_2 as well, blockage of P_R, bending of the intervening DNA and attenuation of *cro* expression, which may coincide with the entry of A2 into its late stage of development (Ladero et al., 2002).

Purification of the expected translation product of *cro* from over-expressing *E. coli* cells produced two polypeptides with an identical

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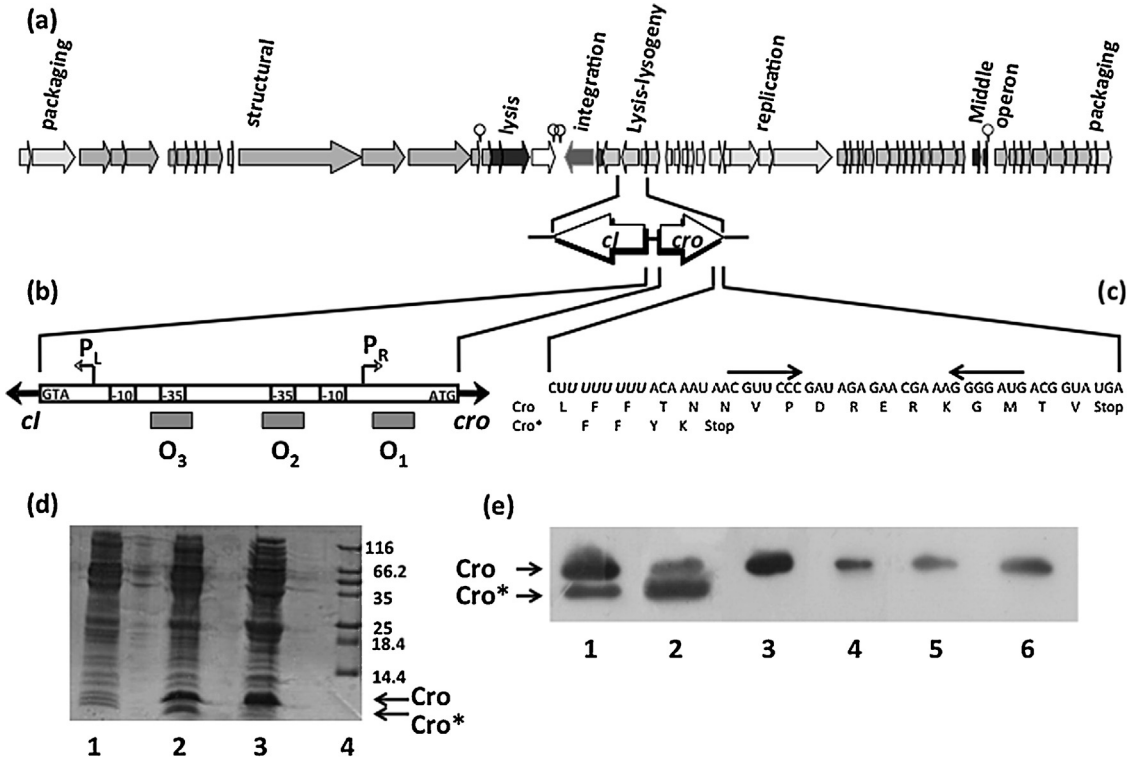

Fig. 1. (A) Diagram of the phage A2 genome with functional gene clusters indicated. (B) The *cI-cro* intergenic region. The relative positions of the three operators with respect to the -35 and −10 hexamers of P_L and P_R, the transcription start sites and the first triplets of *cI* and *cro* are shown. (C) Sequence of the 3′-end of *cro* mRNA, with the slippery heptanucleotide (bold, italics) and the stem-loop located in its vicinity (converging arrows) indicated. (D) SDS-PAGE of *E. coli* BL21(DE3)pLysS cell extracts containing pET11a with different versions of *cro* inserted under the control of its T7 promoter; lane 1: non-induced culture; lane 2: induced culture expressing the wild type *cro*; lane 3: induced culture harboring a mutant *cro* with a transition T to C in the fourth position of the slippery sequence; lane 4: size standards in kDa. (E) Immunodetection of Cro and Cro*; lanes 1 and 2: cultures of *E. coli* BL21(DE3)pLysS expressing the wild type *cro* in the presence or absence of rifampicin respectively at 4 h postinduction; lane 3: *E. coli* BL21(DE3)pLysS carrying the non-frameshifting *cro* mutant; lane 4: *L. casei* infected with phage A2; lanes 5 and 6: mitomycin C induced *L. casei* lysogens harboring the wild type and mutant prophages respectively.

NH_2-end, but of different sizes. The largest, of 9180 Da as determined by MALDI/TOF, fitted with the mass of the canonical product of the gene and consequently was called Cro. The shorter form presented a size that might correspond to a polypeptide resulting from proteolysis between the residues Asn70-Val71 or Asn69-Asn70 and was named Cro*. Both proteins were monomers in solution at nanomolar concentrations and form dimers and even tetramers at micromolar concentrations, reason why the dimerization motif was not predicted to be allocated toward its N-terminal end. Cro and Cro* were able to bind the operator region, but the latter did so with about 5-fold lower affinity than the canonical protein (Ladero et al., 1999). These data suggested that the two polypeptides might form a regulatory tandem that would modulate the frequency with which the phages of a population of infected cells would choose between the lytic and lysogenic cycles (Ladero et al., 1999). In this communication, we address two questions: first, what is the mechanism for production of Cro and Cro* in *E. coli* and second, whether both proteins are being synthesized in *L. casei* and, if so, whether both are needed to give rise to a viable phage progeny upon infection with A2 or after induction of its lysogenic cultures.

Cro and Cro* were purified from IPTG-induced *E. coli* BL21(DE3)pLysS (Studier and Moffatt, 1986) cultures as described in Ladero et al. (1999) and subjected to trypsin digestion and MALDI-TOF-TOF analysis of the resulting peptides in the Proteomic facilities of the National Center for Biotechnology (Madrid, Spain). Both proteins generated identical fragments with one exception; in the case of Cro, it corresponded to amino acids 57–74 while in that of Cro* it was identical to the former up to residue 67, presenting two extra amino acids. The coding sequences of these two would correspond to those in positions 68–69 of *cro* but in the −1 reading frame, the third being a stop codon that would explain the shorter size of Cro* with respect to Cro (Fig. 1C). Both the slippery heptanucleotide and the 3′-neighboring stem-loop necessary for −1 sliding of ribosomes (Baranov et al., 2002) are present in the appropriate positions of the *cro* mRNA (Fig. 1C), suggesting that this is precisely the mechanism of Cro* generation. To confirm this assumption, a mutation was introduced by overlapping PCR (Adey et al., 1996) into the slippery sequence T TTT TTT to give T TTC TTT. This would still encode F–F in frame 0, but would change to F–L in the −1 frame, thus making it impossible for the ribosomes backwards displacement due to the lack of complementarity between the codon-anticodon sequences located at the A and P sites. As expected, the mutant gene was unable to give rise to Cro* (Fig. 1D, compare lanes 2 and 3) thus confirming the functionality of the frameshift mechanism. This result contradicts the previously published assumption on the generation of Cro* through proteolysis of Cro (Ladero et al., 1999) and explains the ambiguity obtained when its molecular mass was determined by MALDI/TOF. Furthermore, this would be the third gene of phage A2 that experiments a frameshift during its translation, the other two being the major head and tail protein determinants; each of them gives rise to two proteins that become included in the virion and are indispensable for the generation of infectious particles (García et al., 2004; Rodríguez et al., 2005).

To determine whether *cro* produced the two polypeptides in *L. casei* as well, exponential cultures of *L. casei* ATCC 393, growing in MRS (Difco) supplemented with 10 mM of each $CaCl_2$ and $MgSO_4$, were infected with A2 at a multiplicity of infection of 1 and