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Abstract

Sensitivity of phage bIL66 to the AbiD1 *Lactococcus lactis* abortive infection mechanism was previously shown to be determined by the phage middle-time-expressed operon composed of four *orf*s. Using spontaneous bIL66 mutants resistant to AbiD1, we established that this sensitivity is determined by the *orf*1 encoded protein. Overproduction of Orf1 in trans in AbiD1^{+} cells was shown to increase AbiD1 efficiency on both wild-type phage bIL66 and mutants resistant to AbiD1. Such an increase was not observed following overproduction of mutant Orf1. We propose that wild-type, but not a mutant Orf1, activates AbiD1 expression or activity.

Keywords: Bacteriophage; Abortive infection; *Lactococcus lactis*

1. Introduction

Bacteriophage abortive infection (Abi) mechanisms are characterised by the interruption of an intracellular step of phage multiplication and death of the infected cell. As a consequence, further propagation of phages is prevented and bacterial population survives (for a review, see [1]).

Abis are widespread in lactococci, which could be due to the strong selective pressure determined by the presence of phages in the dairy environment [2,3]. Abi putative gene products share no homology with known proteins and the molecular basis of their action remains mostly unknown [2]. The AbiD1 mechanism active against small isometric- and prolate-headed lactococcal phages is one of the best characterised [4,5,6]. The sensitivity of the small isometric-headed phage bIL66 to AbiD1 is determined by the middle-time-expressed operon (M-operon), composed of four *orf*s [5]. By virtue of the complementation study, we suggested that *orf*3 would code for an essential phage product, the amount of which is decreased by AbiD1 [5]. Functional analysis of the M-operon established that Orf3 is an endonuclease (M-nuclease) homologous to *Escherichia coli* RuvC resolvase and specific for branched DNA structures [6]. By analogy with known structure-specific *E. coli* phage nucleases [7,8], Orf3 could participate in phage DNA maturation prior to packaging. bIL66 is the only phage known to give spontaneous AbiD1^{R} mutants [4,5]. Mutations conferring resistance to AbiD1 were localised within the *orf*1 gene [5].

Results presented here establish that phage sensitivity to AbiD1 requires the N-terminal part of Orf1. Deletion of the corresponding DNA region renders the phage resistant to AbiD1. We show that wild-type Orf1 expressed in trans increases the efficiency of AbiD1 and prevents the appearance of AbiD1^{R} mutant phages. We propose that phage Orf1 activates AbiD1.

2. Materials and methods

2.1. Bacterial strains, plasmids, phages and media

L. lactis ssp. *lactis* IL1403 [9] and derivatives were grown at 30°C in M17 medium supplemented with 0.5% glucose [10]. *E. coli* TG1 was grown at 37°C in Luria–Bertani medium [12]. Antibiotics, when needed, were added at the following concentrations: ampicillin (Ap), 100 μg ml^{-1}; erythromycin (Em), 200 μg ml^{-1} for *E. coli* and 5 μg ml^{-1} for *L. lactis*. Plasmid pIL105, which confers

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the AbiD1 phenotype, was from the wild-type *L. lactis* strain IL964 [11]. *L. lactis* plasmid vector pIL253 was used [13]. Phage bIL66 from our laboratory collection was enumerated as described in [4]. Isolation of mutant and recombinant AbiD1^R phages was described before [5].

2.2. DNA manipulations and sequence analysis

The procedures for DNA manipulation and transformation of *E. coli* were as described in [12]. Electrotransformation of *L. lactis* was carried out as described in [13]. Polymerase chain reaction (PCR) amplification was performed using the DNA Thermal cycler 9600 (Perkin-Elmer) and Ampli*Taq* DNA polymerase amplification kit (Boehringer, Mannheim, Germany). Nucleotide sequencing was performed on PCR products by the deoxy chain-termination method with big dye terminator cycle-sequencing ready reaction (Applied Biosystems) and Ampli*Taq* DNA polymerase (Perkin-Elmer) on a 377 DNA sequencer (Applied Biosystems).

2.3. Plasmid construction

Construction of plasmids pIL1940, pIL1975 and pIL1977 was described earlier [6]. All plasmids constructed in *E. coli* were made replicative in *L. lactis* by the insertion of *L. lactis* plasmid pIL253 at the *Bam*HI site. The resulting plasmids were designated pIL1923, pIL1989 and pIL1978, respectively.

M-nuclease gene was inactivated on pIL1978 plasmid by *Eco*RV–*Apa*I deletion (pIL1979). The *orf*1 gene from the M-operon was deleted by digestion of pIL1975 with *Hin*cII, followed successively by treatment with Bal31 nuclease and T4 DNA polymerase before religation. This construct was made replicative in *L. lactis* by the insertion of pIL253 at the *Bam*HI site (pIL1976). Deletion of the DNA region corresponding to the N-terminal part of Orf1 was introduced on plasmid pIL1940 using PCR amplification with following oligonucleotides complementary to the bIL66 sequence (accession number L35175): 5′-GA**AGATCT**GCTTTCCTCTCTTAACTTG-3′ (positions 474–459) and 5′-GA**AGATCT**GTCAATGGTTGACTTTAACG-3′ (positions 515–534). The *Bgl*II restriction site is shown in bold. The plasmid was made replicative in *L. lactis* by the insertion of pIL253 at the *Bam*HI site (pIL1982).

The phage M-operon region coding Orf1 was cloned under the control of the phage operon promoter (pIL2002). For this, the DNA fragment was amplified using phage bIL66 DNA and oligonucleotides 1 and 2. The sequences of oligonucleotides were as follows: no. 1: 5′-CG**GAATTC**ATGCCTACAAAGTCAATCATAG-3′ (positions 377–397); no. 2: 5′-**GGGGTACC**CATTTCTTTATTCTCCTTTAAAAATT-3′ (positions 611–687). *Kpn*I and *Eco*RI restriction sites are shown in bold. The PCR fragment was cloned in pBluescript plasmid with the following insertion of pIL253 at the *Bam*HI site (pIL2002). Plasmid pIL2001 carrying the *orf*1 gene from the bIL66.M1 phage was constructed as plasmid pIL2002, but DNA from the bIL66.M1 phage was used for PCR amplification. Plasmid pIL2022 expressing the N-terminal part of the Orf1 was constructed by PCR amplification of the corresponding DNA region using oligonucleotides complementary to the bIL66 sequence: 5′-G**GGGTACC**CTGCCTACAAAGTCAATCATAG-3′ (positions 378–397) and 5′-CG**GAATTC**GTCAACCATTGACAATGTT-3′ (positions 526–509). *Kpn*I and *Hin*cII restriction sites are shown in bold. The PCR fragment was cloned into pBluescript plasmid and made replicative in *L. lactis* by insertion of pIL253 plasmid at the *Bam*HI site.

3. Results and discussion

3.1. Analysis of AbiD1 resistant mutants of phage bIL66

Four bIL66 mutants were previously characterised [5]. Seven new independent bIL66 mutants were isolated and analysed using marker rescue experiments and following sequencing as described previously [5]. Results are presented in Fig. 1 together with those obtained earlier. A point mutation affecting *orf*1 was identified in each of the 11 mutants. Six different point mutations were revealed: four of them introduced a stop codon (M2, M3, M5 and M6) and the two others (M1 and M4) an amino acid change. These results suggest that the resistance of bIL66 mutant phages to AbiD1 could be due to the absence of the Orf1 protein or to its inactivation by mutation. To check this hypothesis, we attempted to construct phage bIL66 derivatives deleted for *orf*1.

3.2. A dual role for Orf1 from the bIL66 M-operon

To obtain bIL66 derivatives deleted for *orf*1, deletions were introduced on a plasmid-cloned phage M-operon and transferred to the phage genome by using its aptitude to recombine with a resident plasmid carrying a homologous segment [5].

bIL66	MTEEQLLFKQETLS**M**VDFNEFLLNAVECGLINLDTALIFKGE	
bIL66.M1	----------**M**----------D------	1[a]
bIL66.M2	--*----------**M**----------	3
bIL66.M3	---*----------**M**----------	3
bIL66.M4	-----P---------**M**----------	1
bIL66.M5	--------*-----**M**----------	2
bIL66.M6	----------*M----------	1

Fig. 1. Comparison of Orf1 from phage bIL66 to those of 11 spontaneous independent mutants resistant to AbiD1. Mutations M1, M3, M4 and M5 were described earlier [5]. Identical amino acids are indicated by –. M15 is indicated in bold. Stop codons are indicated by an asterisk (*); *a*: number of independent mutant phages isolated.

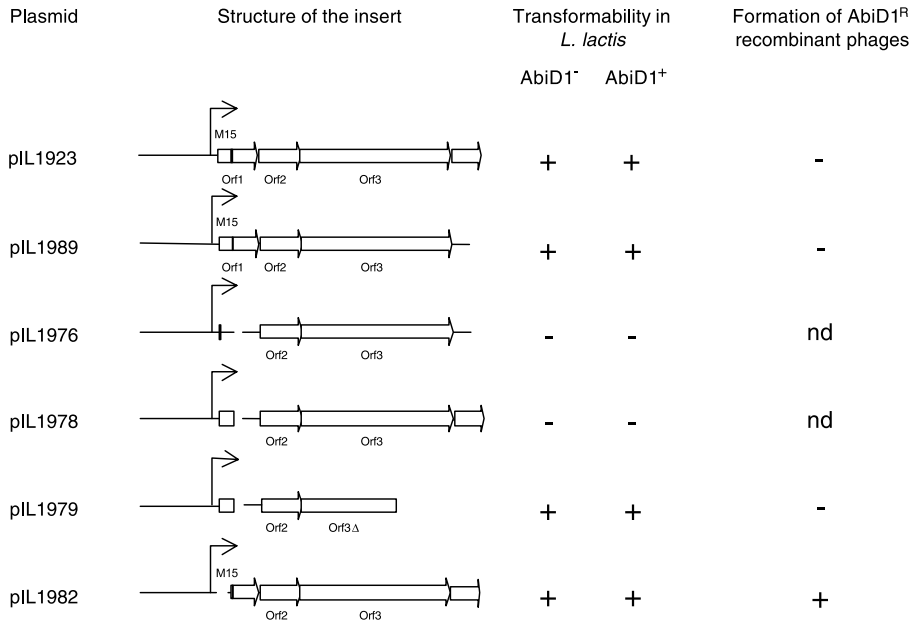

Fig. 2. Transformation of IL1403 AbiD1$^-$ and IL1403 (pIL105) AbiD1$^+$ by plasmids carrying the phage bIL66 M-operon. Deletions in *orf*1 were from positions 471–578 (pIL1976), 525–593 (pIL1978, pIL1979) and 476–513 (pIL1982) according to sequence L35175. Deletion in *orf*3 was from position 1047 to the end of the cloned segment (pIL1979). Formation of AbiD1^R phage following recombination between an infecting phage bIL66 and the cloned M-operon was measured in the IL1403 (pIL105) AbiD1$^+$ strain. nd: not determined.

Plasmids carrying a phage M-operon with whole or truncated *orf*1 gene were transformed in *L. lactis* AbiD1$^-$ cells IL1403 or *L. lactis* AbiD1$^+$ cells IL1403 (pIL105) (Fig. 2). Plasmids expressing either intact Orf1 (pIL1923 or pIL1989) or its C-terminal part (pIL1982), were successfully introduced in both strains. Repeated attempts to transform plasmids without *orf*1 (pIL1976) or expressing Orf1 deleted for the C-terminal part (pIL1978) failed.

Since the M-nuclease is highly toxic for *L. lactis* [6], these results suggest that Orf1, or at least its C-terminal part, is essential for viability of cells expressing the M-operon. Therefore, the M-nuclease gene was inactivated by deletion on pIL1978 plasmid (see Section 2). As expected, the resulting construct (pIL1979) was successfully transferred in IL1403 and IL1403 (pIL105) (Fig. 2).

Marker rescue experiments were then performed using pIL1982 and pIL1979 (Fig. 2). IL1403 (pIL105, pIL1982) infected with phage bIL66 formed clear plaques at a frequency of 10^{-3}. Phages isolated from 10 independent plaques, analysed by sequencing of the M-operon, all had the same deletion within Orf1 as plasmid pIL1982 (data not shown), indicating that they resulted from recombination between the infecting phage and the cloned M-operon.

Therefore, deletion of the N-terminal part of Orf1 confers phage resistance to AbiD1. In contrast, recombinant AbiD1^R phages were not isolated following infection of IL1403 (pIL105, pIL1979) with phage bIL66, indicating that the C-terminal part of Orf1 is essential for phage viability and/or does not confer resistance to AbiD1. Sequence analysis of AbiD1^R phages favours the first hypothesis, all non-sense mutations in *orf*1 being localised upstream of the methionine 15 (M15) codon (Fig. 1). We conjectured that M15 could serve as a second translational start in the case of a stop codon in the first third of the gene, as was described for many leaderless RNAs [14–16]. Moreover, the presence of M15 is a characteristic feature of the phage bIL66 *orf*1 gene, likely responsible for bIL66's capacity to form viable AbiD1^R mutants. Sequence analysis of the corresponding DNA region from other small isometric-headed phages (bIL170, sk1 and 712, accession numbers AF009630, NC001835 and AF087814, respectively) show that M15 (bIL66) is replaced by lysine (bIL170, 712) or glutamate (sk1). All these phages were shown not to form AbiD1^R mutants ([5], our unpublished results).

These results suggest that Orf1 plays a dual role. The N-terminal part renders the phage sensitive to AbiD1. The C-terminal part, essential for viability of both bacteria and phage, is most probably involved in regulation of the M-nuclease.

3.3. Expression of Orf1 increases AbiD1 efficiency

We have previously shown that expression in trans of the M-nuclease abolishes the AbiD1 phenotype and suggested that AbiD1 could decrease the amount of active M-nuclease below the level required for phage development [5]. This, together with the role of Orf1 in both phage sensitivity to AbiD1 and regulation of the M-nuclease, suggests that AbiD1 and Orf1 interact directly or indirectly to modulate the M-nuclease activity and thus phage development. To further elucidate Orf1/AbiD1 interaction, we studied the incidence of overexpression of

Table 1
Phage development in IL1403 and IL1403 AbiD1$^+$ cells expressing the Orf1 protein

Strain	Phage bIL66	
Protein in trans[a]	Relevant genotype[b]	Phage titre (plaques ml^{-1})
IL1403		
–	w$^+$	10^9
Orf1	w$^+$	10^9
Orf1.M1	w$^+$	10^9
–	M	10^9
Orf1	M	10^9
Orf1.M1	M	10^9
IL1403 AbiD1$^+$		
–	w$^+$	10^5t+10^4c
Orf1	w$^+$	<1
Orf1.M1	w$^+$	10^5t+10^4c
–	M	7×10^8
Orf1	M	<1
Orf1.M1	M	7×10^8

[a]bIL66 or bIL66.M1 Orf1 was expressed from phage M-operon promoter (plasmids pIL2002 and pIL2001, respectively).
[b]Relevant genotype of infecting phages (w$^+$ – phage bIL66, M – mutant phages bIL66.M1–M6). *t*: small turbid plaques, *c*: clear plaques formed by spontaneous AbiD1R mutants. Presented values are indicative.

Orf1 on phage development in the presence or absence of AbiD1.

Constructs with *orf*1 from phage bIL66 and mutant *orf*1 from phage bIL66.M1 cloned under the control of their own promoter (pIL2002 and pIL2001, respectively) were transformed in Il1403 AbiD1$^-$ and IL403 (pIL105) AbiD1$^+$ cells. In IL1403 expression of wild-type or mutant Orf1 had no effect on plaque-forming efficiency and plaque morphology of phage bIL66 and mutant AbiD1^R phages bIL66.M1–M6 (Table 1). These results indicate that plasmid-expressed Orf1 does not interfere with the development of infecting phages.

In contrast, in IL1403 (pIL105) AbiD1$^+$ cells expression of Orf1 prevented the growth of both bIL66 and bIL66.M phages (Table 1). Moreover, spontaneous AbiD1^R bIL66 mutants were not detected. No such effect was observed in the presence of the mutant *orf*1. Expression of the N-terminal part of Orf1 (pIL2022) also had no incidence on phage multiplication (data not shown). Therefore, expression of the wild-type Orf1 in trans drastically increases AbiD1 efficiency.

We have previously shown that the AbiD1 protein is toxic for the cell and suggested that its expression or activity could be induced during the phage infection [4]. In this work, we established that AbiD1 efficiency is increased by expression in trans of the wild-type phage Orf1. When Orf1 is overproduced, AbiD1 prevents the growth of AbiD1^R phages. This observation, joined to the incapacity of Orf1.M1 to increase AbiD1 efficiency, suggests that mutant phages are unable to activate AbiD1, but are still sensitive to it.

Two *E. coli* Abi mechanisms, Lit and Prr, were shown to be activated by small phage T4 encoded proteins Lit and Prr, respectively [17,18]. It was proposed that activating phage polypeptides interact directly with their protein target and change protein conformation [19–21]. In consequence, activated Abi mechanisms react with highly conserved cellular components and provoke cell death, thereby preventing propagation of phages [1]. We supposed that phage Orf1 protein could play a similar role in the development of *L. lactis* AbiD1.

This is the first description of a phage gene responsible for stimulation of Abi in *L. lactis*. Experiments will be carried out to determine the molecular mechanism by which Orf1 phage protein activates AbiD1 as well as its function in the regulation of phage M-operon activity.

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