Clear Plaque Mutants of Lactococcal Phage TP901-1

Kot, Witold; Kilstrup, Mogens; Vogensen, Finn K.; Hammer, Karin

Published in:
P l o S One

Link to article, DOI:
10.1371/journal.pone.0155233

Publication date:
2016

Document Version
Publisher's PDF, also known as Version of record

Link back to DTU Orbit

Citation (APA):

General rights
Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.
Clear Plaque Mutants of Lactococcal Phage TP901-1

Witold Kot1,*, Mogens Kilstrup2, Finn K. Vogensen3, Karin Hammer2*

1 Department of Environmental Science, Aarhus University, Frederiksborgvej 399, DK-4000, Roskilde, Denmark, 2 Department of Systems Biology, The Technical University of Denmark, Matematiktorvet Building 301, DK-2800, Lyngby, Denmark, 3 Department of Food Science, University of Copenhagen, Rolighedsvej 26, DK-1958, Frederiksberg C, Denmark

* kha@bio.dtu.dk (KH); wk@envs.au.dk (WK)

Abstract

We report a method for obtaining turbid plaques of the lactococcal bacteriophage TP901-1 and its derivative TP901-BC1034. We have further used the method to isolate clear plaque mutants of this phage. Analysis of 8 such mutants that were unable to lysogenize the host included whole genome resequencing. Four of the mutants had different mutations in structural genes with no relation to the genetic switch. However all 8 mutants had a mutation in the \( cI \) repressor gene region. Three of these were located in the promoter and Shine-Dalgarno sequences and five in the N-terminal part of the encoded CI protein involved in the DNA binding. The conclusion is that \( cI \) is the only gene involved in clear plaque formation i.e. the CI protein is the determining factor for the lysogenic pathway and its maintenance in the lactococcal phage TP901-1.

Introduction

The birth of phage genetics was based on the visual inspection of the morphology of plaques. Isolations of clear plaques mutants have been the classic route for obtaining mutations in the major regulators responsible for immunity. The model for temperate phages, bacteriophage lambda, forms turbid plaques on its \( Escherichia coli \) host, and this phenotype was used for isolation of clear plaque mutants (CI) more than fifty years ago[1]. In accordance with the nomenclature in lambda, phage genes in other phages coding for repressors of the lytic cycle are usually named \( cI \). The isolation and analysis of such clear plaque mutants has also been used to identify additional genes involved in the maintenance of the lysogenic state or in the decision process leading to lysogenisation. In lambda, mutations in three different genes \( cI \), \( cII \) and \( cIII \)[1] were found to result in clear plaques. The CI protein is necessary for establishment and maintenance of lysogenic state, the activator CII is necessary for efficient establishment of lysogeny, and CIII is needed for increasing the stability of the otherwise unstable CII protein [2]. The lactococcal temperate phage TP901-1 has an interesting and simple switch mechanism consisting of a repressor CI and a small protein, Mor, which apparently is counteracting CI repression of the lytic promoter \( P_L \)[3]. CI and Mor are expressed from two divergent promoters. When a 1kb DNA fragment containing the entire switch region is inserted into a plasmid,
transformation of *Lactococcus lactis* will lead to two alternative stable phenotypes with the lytic promoter being either open or closed, thus mimicking the choice after phage infection.

In the study of the genetic switch of TP901-1 we wanted to isolate and analyze clear plaque mutants to investigate if genes other than CI could be involved in the decision process. This procedure had never been used before because the wild type TP901-1 forms clear plaques on its host *Lactococcus lactis* subsp. *cremoris* 3107 using standard plating procedures. We expected that the reason for the lack of turbidity was caused by the inability of the immune lysogens to grow in the plaques due to the low pH produced by the lactic acid bacterial lawn. Hence a reduction in the acidity of the plates during growth of the host layer might allow for further growth of the lysogens allowing formation of a turbid plaque. Adding additional MOPS buffer to the plates and reducing the glucose concentration resulted in slightly turbid plaques of the parent phage and allowed the selection of clear plaques. Isolation and characterization of the clear plaque mutants resulted in mutations only in the *ci* gene and thus supports the model for the TP901-1 phage that the CI repressor is the determining factor in lysogeny and no other gene product is necessary for its synthesis in TP901-1. The CI protein, transcribed from the PR promoter in TP901-1, has been purified and shown to form a hexamer in solution. The DNA binding domain is located in the N-terminal part of the 180 amino acid long CI monomer, while the C-terminal end is responsible for the oligomerization [4]. The three dimensional structure of the N-terminus of CI has been determined [5]. Mor (Modulator of Repression) is a small protein of 72 amino acids, it is transcribed from P_L (the lytic promoter in TP901-1), which is located in the opposite direction of PR. Using transcriptional fusions it has been shown that a surplus of Mor counteracts repression by CI at P_L, but also increases repression by CI at P_R [3]. CI-Mor protein-protein interactions have been proposed to be responsible for this behaviour [5,6].

**Methods**

**Growth medium**

M17 with 1% glucose (GM17) was used for growth of the lactococci [7], except when clear plaques were isolated, in that case M17/7 was used. M17/7 corresponds to M17 supplemented with 200mM MOPS instead of beta-glycerophosphate and adjusted to pH 7.2 with NaOH, glucose was added to 0.5%[8]. SAL is a defined SA medium[9] where the Sodium Acetate has been substituted with 2μg/ml lipoic acid. *E. coli* strains were grown in LB medium [9] with selection for erythomycin at 150 μg/ml.

**Bacterial stains, plasmids and phages**

The bacterial strains and plasmids used are found in Table 1. Lactococcal bacteriophage TP901-1[13] and TP901-BC1034[14] were used. The TP901-BC1034 is a TP901-1 derivative, which encodes erythromycin resistance and was used to aid measurement of lysogenization.

**Mutagenesis and isolation of clear plaque mutants**

The TP901-BC1034 phage stock was diluted in 0.9% NaCl and mutagenized by UV light resulting in a 50 fold reduction in titer. The mutagenized stock was wrapped in foil and kept in the dark until use the next day.

The host *L. lactis* subsp. *cremoris* 3107 was grown exponentially until OD_{600} of 1.0 in M17/7, then it was diluted in 0.9% NaCl and mutagenized by UV for 12 seconds, this affected viability with a factor 2 or less. The UV treatment was performed in order to induce the SOS system in the host and hence increase the mutagenic effect of the damaged phage DNA. The bacteria
were used immediately for plaquing using the mutagenized phage stock. The mixture was incubated for 10 min at 30°C and then plated in M17 top agar with 0.5% agar and 5 mM CaCl$_2$ on M17/7 plates containing 1.1% agar and 5 mM CaCl$_2$; they were incubated anaerobically for approx. 24h at 30°C, aerobic incubation gave slightly turbid plaques.

**Lysate**

Single plaque lysate was made using a fresh plaque to which 0.1 ml O/N culture of *L. lactis* 3107 and 5 mM CaCl$_2$ was added. After 5–10 min at room temp, 10 ml GM17 with 5 mM CaCl$_2$ was added and the mixture was incubated at 30°C until lysis occurred. Lysates were filtered using Q-Max Syringe filter with a pore size of 0.45 μm.

**DNA preparation**

Phage DNA from clear plaque mutants and from the wild type phage TP901-BC1034 that was used for mutagenesis was isolated from CsCl purified phage preparations by phenol-chloroform extraction as described previously for phage lambda by Sambrook and Russell[15].

**Sequencing and sequence analysis**

DNA sequencing libraries were prepared using the Nextera XT DNA kit (Illumina, San Diego, USA) according to the manufacturer’s protocol. Individually tagged libraries were sequenced as a part of a flowcell as 2 × 250 bases paired-end reads using the Illumina MiSeq platform (Illumina, San Diego, USA). Reads were trimmed, analyzed and assembled using CLC Genomic Workbench 7.5 (CLCbio, Aarhus, Denmark). Briefly, reads were trimmed (0.05 quality limit) and mapped to reference (length fraction = 0.5, similarity fraction = 0.8, reference accession- AF304433). Afterwards reads mapping were searched for mutations using the probabilistic variant detection tool followed by filtering variants against control reads derived from the TP901-BC1034 phage.

**Lysogenization**

*Lactococcus lactis* subsp. *cremoris* 3107 was grown at 30°C in GM17+ 0.5% glucose. When the growth was exponential at OD$_{600}$ 0.8–1.0, CaCl$_2$ was added to 10 mM and the culture was

<table>
<thead>
<tr>
<th>Plasmid or strain</th>
<th>Description</th>
<th>Reference or Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pLB86</td>
<td>Promoter probe vector containing attP (TP901-1) and used for integration in <em>Lactococcus lactis</em></td>
<td>Brandstedt and Hammer [10]</td>
</tr>
<tr>
<td>pMK1228</td>
<td>pLB86 containing cl P$_R$ O$_R$ O$_L$ P$_L$</td>
<td>This work</td>
</tr>
<tr>
<td>pMK1223</td>
<td>pLB86 containing cl with mutation C3, P$_R$ O$_R$ O$_L$ P$_L$</td>
<td>This work</td>
</tr>
<tr>
<td>pMK1224</td>
<td>pLB86 containing cl with mutation C8, P$_R$ O$_R$ O$_L$ P$_L$</td>
<td>This work</td>
</tr>
<tr>
<td>pMK1231</td>
<td>pLB86 containing cl with mutation C18, P$_R$ O$_R$ O$_L$ P$_L$</td>
<td>This work</td>
</tr>
<tr>
<td>LB504</td>
<td><em>Lactococcus lactis</em> subsp. <em>cremoris</em> MG1363 / pLB95 containing the int (TP901-1) used for integration of pLB86 derivatives</td>
<td>Brandstedt and Hammer [10]</td>
</tr>
<tr>
<td>MK755</td>
<td>LB 504 attB::pMK1228</td>
<td>This work</td>
</tr>
<tr>
<td>MK750</td>
<td>LB 504 attB::pMK1223</td>
<td>This work</td>
</tr>
<tr>
<td>MK751</td>
<td>LB 504 attB::pMK1224</td>
<td>This work</td>
</tr>
<tr>
<td>MK763</td>
<td>LB 504 attB::pMK1231</td>
<td>This work</td>
</tr>
<tr>
<td>Lactococcus lactis subsp. cremoris 3107</td>
<td>Host for TP901-1 and TP901-BC1034</td>
<td>Braun et al. [11]</td>
</tr>
<tr>
<td>Escherichia coli MC1061</td>
<td>Used as host for plasmids during cloning</td>
<td>Casadaban and Cohen [12]</td>
</tr>
</tbody>
</table>

doi:10.1371/journal.pone.0155233.t001
diluted ten fold in prewarmed medium. TP901-BC1034 phages carrying erythromycin resistance were added at a multiplicity of 0.2 and incubated for 35 min. Topagar containing 20 mM sodium citrate was added and the mixture plated on GM17 plates containing 10 mM citrate and 2 μg erythromycin selecting for erm resistant transductants. Using these conditions the wild type phage TP901-BC1034 gave 2 transductants per 10^3 infected bacteria.

**Analysis of CI repression of the P_L promoter**

Promoter fusions were created in the integration plasmid pLB86 [10] by PCR amplification of the region from base pair 2627 to 3351 in TP901-BC1034 containing cl, P_R, O_R, O_L and P_L from wild type and mutant phages. Purified phage chromosomal DNA and primers MK638 (5'-AAAAAA GCTTCTCGAGAAAGCTCTCTAAG-3') and MK660 (5'-AAAATGCAGTTTCTCCTTCTCTTTCT TT CAGTTCACGTTTCAT-3') were used, resulting in DNA fragments containing the cl gene expressed from its own P_R promoter, and the P_L promoter directed towards a PstI restriction site. Insertion of the PCR fragments into the pLB86 transcriptional fusion plasmid by use of the XhoI and PstI restriction sites followed by transformation of E. coli MC1061 resulted in plasmids pMK1228, pMK1223, pMK1224, and pMK1231 for the wild type, C3, C8, and C18 mutant phages, respectively. In all plasmids, the P_L promoter was inserted in front of the promoterless lacLM reporter. Following plasmid preparation and transformation of L. lactis strain LB504, the strains MK755 (LB504 attB::pMK12528 (wt)), MK750 (LB504 attB::pMK1223 (C3)), MK751 (LB504 attB::pMK1224 (C8)), and MK763 (LB504 attB::pMK1231 (C18)) were created, in which the fusion plasmids had integrated into the attB site on the chromosome.

Fusion strains were cultured in triplicate (inoculated from individual bacterial colonies) at 37°C in SAL medium containing 1% glucose and erythromycin at 2 μg/ml. Dilutions were prepared to ensure exponential growth for at least 8 generation, and each culture was harvested after approximately 8, 11, and 14 generations. The resulting nine samples for each strain were harvested and assayed for β-galactosidase activity as described earlier[6] except that the specific activity was based upon OD450 instead of OD600.

**Results**

**Reduced acidification of plates results in turbid plaques of TP901-BC1034**

The standard plating procedure for obtaining TP901-1 plaques results in clear plaques, which has prevented the selection of CI mutants based upon plaque turbidity. Since a large amount of lactic acid is produced during growth of L. lactis, we tried to prolong the growth of the bacterial lawn by counteracting the lowering of the pH through a combination of higher buffer capacity and lower sugar availability in the medium. Addition of 200 mM MOPS pH 7.2 and lowering of the glucose concentration from 1% to 0.5% and anaerobic incubation had the required effect and we obtained large turbid plaques upon infection of L. lactis strain 3107 with the wild type bacteriophage TP901-1 as well as the TP901-BC1034 labelled with an Erm^R^ cassette. No clear plaques were identified by screening more than 1000 plaques from a normal phage stock.

**Mutagenization and screening for clear plaques with lowered lysogenization efficiency**

After UV mutagenesis of a TP901-BC1034 phage stock with reduction of the phage titer to 2%, clear plaques were found at a frequency of 0.1%. The plaques were purified to single plaques 3 times for obtaining a stable clear plaque phenotype. Lysates prepared from 8 such mutants
(C3, C6, C8, C9, C18, C23, C25, and C26) were used for lysogenization tests and phage genome sequencing.

Lysogenization was analysed by measuring the efficiency of transducing the Erm$^R$ resistance cassette, harboured in TP901-BC1034, to the host *L. lactis* 3107. It was shown that all mutant phages were unable to lysogenize (less than $10^{-6}$ to $10^{-8}$ transductants per infected bacterium).

**Genome sequencing and identification of clear plaque mutations**

DNA was extracted from the phage lysates and the genome sequences were determined. The genomic sequence of the parent TP901-BC1034 phage used in this experiment exhibited changes in comparison to the GenBank reference of TP901-1 (AF304433). One of them was the insertion of an Erm$^R$ cassette in ORF56TP901-1, as reported earlier for TP901-BC1034[14]. The second rearrangement region was more complex and was localized in the region responsible for morphogenesis of the phage, precisely in region ranging from the *orf31* coding for large terminase subunit (*terL*) to *orf36* coding for major head protein (*mhp*). We reasoned that the second rearrangement was likely to be caused by homologous recombination with a prophage during the Erm$^R$ labelling process or while maintaining the TP901-BC1034. Sequencing and analysis of the host genome, *L. lactis* subsp. *cremoris* 3107, indeed revealed that it has a prophage sequence that could recombine with TP901-BC1034 (data not shown). In order to see if the recombination between 3107 and TP901-1 had taken place we designed a PCR test, which verified that TP901-BC1034 has a fragment of a prophage from 3107. The rearrangement was regarded as irrelevant for the clear plaque morphology as it was present in both the parent phage TP901-BC1034 (turbid plaques) and all the clear plaque mutants. *S1 Fig* and *S1 Table* show the sequence differences between the mutants and the parent phage TP901-BC1034 sequence together with the region of recombination between TP901-1 and a 3107 prophage. Half of the mutant phages were single mutants (C3, C8, C18, and C26) while the other half had additional mutations in the late structural genes (*S1 Table*).

Due to the late expression of these genes the mutations were not likely to influence the regulatory switch determining the lysogenization of the phage. As the phage mutants were able to form fine plaques it was known that the mutations did not affect the viability of the phages either. C9 had amino acid changes in the C-terminal end of the integrase gene (Leu-Gly to Leu-Ser change) in addition to a mutation in the end of the *nps* structural gene coding for collar-whisker structure. Deletion of A in *nps* resulted in frame shift mutation from 433 amino acid. Collar whisker structure was shown before not to be an essential part of a TP901-1 virion structure[16]. A mutation in *int* might affect lysogenization of C9, however it has a secondary frameshift mutation located in the *cl* gene. This mutation is located just besides the position of a single frameshift mutation in C26 with a similar phenotype, therefore it is more likely that the mutation in *cl* is responsible for the observed phenotype of C9. C23 had 3 mutations in the *tal* gene encoding the tail-associated lysis protein[17] (positions in TP901-1 27069–27089), which resulted in Gln-Lys, Ser-Tyr and Gly-Asp substitutions.

No attempts were made to separate the mutations in the double and triple mutants because all eight mutant phages had mutations in the *cl* region and these are highly likely to result in clear plaque phenotypes. In the lower part of *Fig 1*, it can be seen that mutant C3 had acquired an early nonsense mutation in the *cl* gene, and that the C9, C25 and C26 mutants had early frame shift mutations. None of these mutations would be expected to result in functional CI proteins since the changes occurred either in the DNA binding motif (helix α2 and helix α3 in *Fig 1*) or just after.

The stop codon in C3 mutant is located only two amino acids after helix α3, the DNA recognition helix. The C23 mutant had a mutation that changed the -35 region of the P$_R$ promoter.
from TTGAAC to TTGAGC. Since a G in position 5 of the -35 element is the least common nucleotide for RpoD promoters [18], the mutation is likely to lower the promoter activity.
C6 mutant changed the sequence immediately upstream of the -10 element in the P_B promoter from TGGGCTATAAT to TGCACTATAAT. Promoters with extended -10 regions (TaTGg-TATAAT) are known to be much stronger than their non-extended counterparts [19], so the C6 mutation is consistent with a weaker P_B promoter. Along the same lines, the C18 mutant had acquired a mutation that changed its SD sequence AAAAGAGG to AAAAAAGG, fitting the consensus of the ribosomal binding site to a lesser degree. The C6, C18, and C23 mutants are therefore likely to express lower levels of CI compared to the wild type phage therefore being unable to lysogenize. No mutations were found in the binding sites for CI, OR, OL and OD.

The most unexpected mutant is C8 that has acquired a conservative mutation, changing the amino acid 57 from arginine to a lysine. It appeared strange that this mutation should render the CI protein unable to repress, as the altered amino acid is distant from the DNA binding region. However, the region where the C8 mutation is located has previously been proposed to be involved in binding to the small regulatory Mor protein, which has been suggested to function as an antirepressor for CI repression at the P_L promoter [3]. The proposed Mor binding region in CI is based upon homology to other CI-Mor partners and the non-repressible phenotype in the presence of Mor of two 5 amino acid insertion mutations in CI [5,6]. It might be possible that a CI mutation could result in a clear plaque phenotype if it resulted in increased binding between CI and Mor without disrupting CI repression in the absence of Mor.

The mutation in C8 does not inactivate P_L repression in the absence of the Mor protein

Because the C8 mutant was a clear plaque mutant, we knew that CI was not able to repress in the presence of Mor. To pursue the possibility that the C8 mutation was involved in Mor binding we had to show that the C8 mutation did not prevent CI repression of P_L in the absence of Mor. From the wild type phage, and the C3, C8, and C18 mutants we amplified the cl gene with its own promoter (P_B) and the adjacent P_L promoter, but without the mor gene. After insertion into plasmid pLB86, with P_L upstream from the promoterless lacLM gene, and integration of the fusion plasmids into the attP locus on the chromosome of L. lactis LB504, the fragments were assayed for P_L promoter activity. The cloned piece of DNA contains OR and OL, but not OD (Table 1). According to Pedersen and Hammer [20], P_L is repressed almost the same on the plasmid containing OR and OL, but without OD, as in the presence of all three sites. Assuming the P_L activity seen with the C3 mutant (with a stop codon early in the cl gene) represents fully unrepressed P_L, the wild type fusion gives ~1000-fold repression (Table 2). Although the C8 mutation was slightly de-repressed (6-fold compared to the wildtype), it still retained 160-fold repression indicating that the mutant repressor is still functional in the absence of Mor. Interestingly the SD mutation in C18 retained 260-fold repression of P_L in the absence of Mor, despite the fact that the mutation result in a clear plaque phenotype. This may

<table>
<thead>
<tr>
<th>Strain</th>
<th>cl mutation</th>
<th>β-galactosidase activity (+/- SD)*</th>
<th>Repression fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>MK755</td>
<td>None</td>
<td>0.3 (+/- 0.1)</td>
<td>1000</td>
</tr>
<tr>
<td>MK750</td>
<td>Nonsense (C3)</td>
<td>310 (+/- 70)</td>
<td>1</td>
</tr>
<tr>
<td>MK751</td>
<td>R57K (C8)</td>
<td>2.0 (+/- 0.5)</td>
<td>160</td>
</tr>
<tr>
<td>MK763</td>
<td>SD (C18)</td>
<td>1.2 (+/- 0.4)</td>
<td>260</td>
</tr>
</tbody>
</table>

Table 2. Analysis of CI repression in P_L-lacLM fusions. The cl region from wt TP901-1-BC1034 and mutants C3, C8, C18 were inserted in plasmid pLB86 and integrated into the attB locus of L. lactis subsp. cremoris LB504. Three biological replicates, each determined after exponential growth for approximately 10, 13 and 16 generations. Activity is given as 1000 x A_{420}/(min x OD_{450}).

doi:10.1371/journal.pone.0155233.t002
be explained by the presence of a wild type functional CI repressor combined with the autoregulation of the Pr promoter by CI. The reduced translation efficiency caused by the C18 mutation is outbalanced by increased expression from the Pr promoter. However in the phage it results in a clear plaque. This shows that the expression levels of CI and Mor in the wild type phage have to be finely tuned to produce the normal lysogenization frequency.

Discussion

Temperate bacteriophages have been found to have a significant impact on bacterial evolution [21]. The study of the genetic switches are therefore important for understanding of the transfer mechanisms. Such a study usually implies isolation of clear plaque mutants in the phage.

A clear plaque mutant in a temperate lactococcal phage has to our knowledge so far only been isolated in φLC3[22]. The mutant had lost its ability to lysogenize and later DNA sequencing[23] showed that it had acquired a stop codon in amino acid 197 in the repressor protein (total size 286 amino acids).

In order to get turbid plaques of the wild type TP901-1 on its host strain, we first had to design a modified growth medium. Afterwards we then succeeded in isolating clear plaque mutants of TP901-BC1034 after UV mutagenesis. We analysed 8 mutants further as reported in results.

They were all defective in lysogeny and they all had mutations in the cl gene. The phages were fully genome sequenced and four of them had additional mutations (S1 Table). Of these only the int mutation in C9 might affect lysogenization, all other mutations in C6, 23 and 25 were in late structural genes with no relevance for lysogenization.

All 8 cl mutations map in the first third of the cl gene region, three of them in the promoter and the Shine-Dalgarno sequence, respectively. Three of the remaining 5 mutations within the structural gene give rise to frame shift mutations before or in the DNA binding motive (C26, C9 and C25). One, C3, gives rise to a stop codon just after the DNA binding motive and the last mutation C8 is a conservative amino acid exchange from Arg to Lys in amino acid 57, located 9 amino acids after the DNA binding motive.

The localization of the mutations might indicate that the last part of CI is not necessary for DNA binding. This is in accordance with previous results, that 5 amino acid insertions in the CI protein from amino acid 78 and further downstream do not abolish repression of the lytic P1 promoter[6]. The purified CI protein has been shown to be a hexamer in solution. However when the last 43 amino acids in the C-terminal end are removed, it forms mainly a dimer, but the truncated CI protein is still able to repress the P1 promoter on a plasmid containing the switch region with the Ol and Or sites [4]. Our results presented here may also indicate that multimerization to a hexameric form is not needed for lysogen formation but probably results in more stable lysogens.

No mutations were found in the three operator regions. The Or site, located between the Pr and the P1 promoter, is a poor binding site for CI, while the Ol site located in the start of the transcript from P1 is a strong binding site, this is also the case for the Od site located at the end of the Mor transcript. When only Ol is present in plasmid P1 is also repressed, however a Od mutation alone does not abolish P1 repression if both Or and Od is present [20]. This is probably the reason why none of the phage mutants obtained had mutations in Ol, since it would have required an additional mutation in Od or Or.

The finding that only mutations in cl are responsible for the clear plaque phenotype strongly indicates that only CI is required for the choice of lysogenization and its maintenance in TP901-1.

It was an unexpected result that the conservative exchange of Arg to Lys in amino acid 57 should result in defective lysogenization. Since the mutation is localised in a region which has
been proposed to interact with the Mor protein [5,6], we hypothesized that the mutant CI protein might bind with higher affinity to the Mor protein, which should then be able to counteract repression by CI of the lytic promoter P_L. Therefore this mutant in the cl gene was cloned without mor and it was shown that is was able to repress the P_L promoter almost as well as the wild type cl gene. This proves that the CI protein carrying the C8 mutation is functional. However, as the phenotype of the SD mutation in C18 can be explained by auto regulation of P_R we cannot exclude that auto regulation of P_R also is involved in the phenotype of C8. Further studies are thus needed to disclose whether C8 has an increased affinity for Mor. In conclusion our study shows that CI is the only protein involved in the lysogenization decision and the maintenance of the lysogenic state.

Supporting Information

S1 Fig. Physical map of clear plaque mutants. A physical map of ORF’s in the TP901-1 genome is shown below a size indicator. Localization of the mutations in TP 901-BC1034 is indicated by a red bar. The dark blue boxes indicate regions of similarity between TP901-1 and the prophage in 3101, the red box indicate the region that was recombined into TP 901-BC1034 and was verified by PCR. ERM indicates the position where the erythromycin resistance gene was inserted in TP 901-BC1034.

(TIF)

S1 Table. List of additional mutations outside the lysogeny module of TP901-BC1034 in four of the clear plaque mutants. FS- Frame shift.

(DOCX)

Acknowledgments

This work was supported by the Danish Council for Independent Research grants 4093-00198 (PHAGEMID), 0602-02271B (MetaPhageLAB) and DFF-4002-00107. We thank Lars H. Hansen from the Department of Environmental Science, Aarhus University for providing sequencing facilities. We thank Bashir Aideh from Department of Food Science, University of Copenhagen for his technical support. Marzanna Pulka-Amin and Lise Sørensen from Department of Systems Biology, The Technical University of Denmark, are also thanked for their technical support.

Author Contributions

Conceived and designed the experiments: WK MK FKV KH. Performed the experiments: WK MK KH. Analyzed the data: WK MK FKV KH. Contributed reagents/materials/analysis tools: WK MK FKV KH. Wrote the paper: WK MK FKV KH.

References

5. Frandsen KH, Rasmussen KK, Jensen MR, Hammer K, Pedersen M, Poulsen J-CN, et al. Binding of the N-terminal domain of the lactococcal bacteriophage TP901-1 CI repressor to its target DNA: a crys-
tallography, small angle scattering, and nuclear magnetic resonance study. Biochem. 2013; 52: 6892–
6904.

6. Pedersen M, Ligowska M, Hammer K. Characterization of the CI repressor protein encoded by the tem-
PMID: 20118255

7. Terzaghi BE, Sandine WE. Improved medium for lactic streptococci and their bacteriophages. Appl
Microbiol. 1975; 29: 807–813. PMID:16350018

8. Rallu F, Gruss A, Ehrlich SD, Maguin E. Acid- and multistress-resistant mutants of Lactococcus lactis:
2958.2000.01711.x PMID: 10672175

9. Jensen PR, Hammer K. Minimal requirements for exponential growth of Lactococcus lactis
Appl Envi-
ron Microbiol. 1993; 59: 4363–4366. PMID:16349136

10. Brøndsted L, Hammer K. Use of the integration elements encoded by the temperate lactococcal bacte-
riophage TP901-1 to obtain chromosomal single-copy transcriptional fusions in Lactococcus lactis.

Lactococcus lactis by electron microscopy, DNA-DNA hybridization, and protein profiles. J Gen Microbiol.

12. Casadaban MJ, Cohen SN. Analysis of gene control signals by DNA fusion and cloning in Escherichia

13. Brøndsted L, Østergaard S, Pedersen M, Hammer K, Vogensen FK. Analysis of the complete DNA
sequence of the temperate bacteriophage TP901-1: evolution, structure, and organization of lactococcal

resistance mutations in the temperate lactococcal bacteriophage TP901-1 and their use in studies of


16. Vegge CS, Neve H, Brøndsted L, Heller KJ, Vogensen FK. Analysis of the collar-whisker structure of
temperate lactococcal bacteriophage TP901-1. Appl Environ Microbiol. 2006; 72: 6815–6818. PMID:
17021234

17. Vegge CS, Brøndsted L, Neve H, Mc Grath S, van Sinderen D, Vogensen FK. Structural characterization
and assembly of the distal tail structure of the temperate lactococcal bacteriophage TP901-1. J

18. Shimada T, Yamazaki Y, Tanaka K, Ishihama A. The whole set of constitutive promoters recognized by
0090447

of Escherichia coli sigma 70 is inessential for initiation of transcription at an “extended minus 10” pro-
mo

20. Pedersen M, Hammer K. The Role of MOR and the CI operator sites on the genetic switch of the tem-

21. Nanda AM, Thormann K, Frunzke J. Impact of spontaneous prophage induction on the fitness of bacte-
14

22. Lillehaug D, Lindqvist B, Birkeland NK. Characterization of qLC3, a Lactococcus lactis subsp. cremoris
temperature bacteriophage with cohesive single-stranded DNA ends. Appl Environ Microbiol. 1991; 57:
3206–3211. PMID: 1840480

23. Blatny JM, Risoen PA, Lillehaug D, Lunde M, and Nes IF. Analysis of a regulator involved in the genetic
switch between lysis and lysogeny of the temperate Lactococcus lactis phage qLC3. Mol Genet Geno-