BIOLOGIJA. 2009. Vol. 55. No. 1–2. P. 14–17
DOI: 10.2478/v10054-009-0003-6

Substitution *in vivo* of the holin inhibitor RI of bacteriophage T4 by the homologous protein of T-even phage RB69

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Lysis inhibition is the ability to delay lysis of a bacterial cell primarily infected by a T-even phage for several hours in response to secondary T-even phage adsorption. This phenomenon is a unique attribute of the T-even family of phages. Recently, it has been established that the interaction between the T holin and the holin inhibitor RI plays the major role in the mechanism of lysis inhibition of phage T4. Bacteriophage RB69 is a member of this family, yet the relation of RB69 to lysis inhibition is undefined. In the present study, we constructed a T4 mutant which had its lysis inhibition gene *rI* replaced by a homologous gene of the phage RB69. The lysis profile of the recombinant phage showed that the lysis of infected cells was delayed for several hours. This result indicates that protein RI of RB69 can effectively substitute the holin inhibitor RI of phage T4 in lysis inhibition.

Key words: bacteriophage RB69, lysis inhibition, holin inhibitor RI

INTRODUCTION

The lysis of a bacterial cell infected by bacteriophage T4 or its close T-even relative normally occurs at 20–30 min postinfection at 37 °C [1]. The process of cell lysis is determined by the action of two phage T4 proteins: an endolysin encoded by gene *e* [2] and a holin encoded by gene *t* [3]. At the end of the infection cycle, the T holin permeabilizes the cytoplasmic membrane and permits the passage of endolysin to the periplasm where it degrades the peptidoglycan layer [4]. However, the lysis is inhibited for several hours if the external phage adsorbs an already infected cell [5]. It was determined that the periplasmic protein RI encoded by T4 gene *rI* senses the adsorption of the external phage and inhibits the T holin [6].

The ability to control the length of the infection cycle in response to the availability of bacterial hosts in the environment makes it seem likely that lysis inhibition is widely conserved among the T4 related phages [1]. Nevertheless, until recently the lysis inhibition phenomenon has mostly been examined in the bacteriophage T4 and its close relatives T2 and T6 [5–7]. A few studies present some indirect results concerning the lysis inhibition in the T-even bacteriophage RB69, a more diverged relative of T4 [1, 8]. As distinct from other T-even phages, RB69 does not sport traditional large-plaque, *r*-type mutants [8]. It was reported that the RB69-infected *E. coli* K-12 strain B cells undergo lysis inhibition in a liquid culture at standard cell densities according to the observation of a several-hour delayed lysis [1, 8]. Yet, later Abedon et al. [9] reported that their own RB69 did not display lysis inhibition when grown in *E. coli* K-12 strain CR63 cells. Taken together, these ambivalent results do not give a definite answer regarding RB69 relation to lysis inhibition. Moreover, they raise the question about the extent to which the lysis inhibition mechanism is conserved among the T-even phages.

Our recent studies of lysis inhibition in phages outside the T-even group have shown that the pseudo T-even phage RB49 does not display lysis inhibition [10]. Regardless of the obvious rapid lysis phenotype, RB49 still owns the gene *rI* that encodes highly diverged homologue of the basic component of the T4 lysis inhibition mechanism [11]. The T-even phage RB69 occupies an intermediate position between T-even and pseudo T-even phages and appears to be a chimera composed of segments derived from both T-even and pseudo T-even genomes [12]. Its RI protein sequence encoded by gene *rI* differs from the homologous protein T4 by 47% – the level that would be expected for a pseudo T-even phage (Fig. 1) (see http://phage.bioc.tulane.edu).

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T4   MALKATALFAMLGLSFVLSPSI-EANVDPHFDKFMESGIRHVYMLFENKSVESSEQF 56
     MAL+A A+ AMLG     +P +  A VDP+FD FMESGI++VY LFE ++VE+SE+F
RB69 MALRAIAIVAMLGFFAATTPIVGTAYVDPYFDNFMESGIKNVYTLFEIQNVENSEKF 57

T4   YSFMRTTYKNDPCSSDFECIERGAEMAQSYARIMNIKLE 95
     Y +M   YKN PC   FEC E+G + A+ +A  M IKLE
RB69 YKYMAKHYKNSPCDDAFECHEQGIKTARKFAEFMKIKLE 96
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Fig. 1. Alignment of predicted amino acid sequence of RB69 RI (GenBank accession No. AAP76011) with the sequence of T4 RI (GenBank accession No. AAB26962). The sequences were aligned using BLAST 2.0 program. A plus sign (**+**) indicates conserved substitutions. A dash indicates a space inserted in the sequence to preserve the alignment

The aim of this work was to evaluate the potential of the RI protein of RB69 to act as a holin inhibitor. We report the construction of the T4 mutant that has its lysis inhibition gene rI replaced by a homologous gene of the phage RB69. Lysis profiles of the mutant phage demonstrate that RI of RB69 can substitute for RI of phage T4 in lysis inhibition.

MATERIALS AND METHODS

Bacterial and bacteriophage strains. *Escherichia coli* strain B^E (*sup*^{0}) was kindly supplied by Dr. Lindsay Black. *E. coli* JM101 (Amersham Biosciences) was used for the transformation and preparation of plasmid DNA.

Bacteriophage T4D wild-type was kindly supplied by Dr. William Wood. Phages RB69 and T4 *rI* mutant *r*48 were kindly provided by Dr. Karin Carlson. The recombinant phage $T4rI_{RB69}$ was constructed in this study.

Plasmid constructions. The plasmid pTZrI.1/69rI/rI.-1 for insertion of the RB69 gene *rI* into the phage T4 genome was constructed as follows. Initially, plasmids carrying T4 DNA fragments for homologous recombination were constructed. The 486 bp DNA fragment spanning the distal part of *tk* and *rI.1* was amplified from the T4 genome using a direct primer, 5'-TGGGCTAAGGACTGAATTCGCTGGA, carrying the EcoRI restriction site, and a reverse primer, 5'-CCTGTATTTACTTTGTGCCGATGCACG. The obtained DNA fragment, after treatment with T4 DNA polymerase and digestion with EcoRI, was inserted into the EcoRI and SmaI sites of pTZ19R, resulting in pTZrI.1. The 485 bp DNA fragment containing the distal part of *rI* and *rI.-1* was amplified from the T4 genome using the direct primer, 5'-CGACCTATAAAAAGGATCCGTGCTCTTCTG, carrying a BamHI restriction site and a reverse primer, 5'-GGGAAAGAAGATACTTACTCTGGT. The obtained PCR product, after treatment with T4 DNA polymerase and digestion with BamHI, was inserted into the BamHI-HincII sites of pTZ19R, generating the plasmid pTZrI.-1. The resulting plasmid, after digestion with BamHI and treatment with T4 DNA polymerase, was religated to eliminate the BamHI site. Then, the SmaI-PstI fragment was cut from pTZrI.-1 and cloned into the HincII-PstI sites of pTZrI.1, resulting in pTZrI.1/rI.-1. The 340 kb DNA fragment carrying the gene *rI* with its Shine–Dalgarno sequence was amplified from the phage RB69 genome, using a direct primer, 5'-TACTGGCCACAATTGACAGGAGG, containing a MunI restriction site, and a reverse primer, 5'-GGCTAGTAATCTAGATAGATGTAGG, containing an XbaI restriction site. The obtained DNA fragment, after treatment with T4 DNA polymerase and digestion with XbaI, was inserted into the SmaI and XbaI sites of pTZ19R. The resulting plasmid, pTZrb69rI, was digested with MunI and HindIII to excise the gene *rI* containing fragment which was cloned into the EcoRI-HindIII restriction sites of the plasmid vector pET21(+). The obtained plasmid, pETrb69rI, was digested with BamHI and XbaI, and the excised DNA fragment was cloned into the same sites of pTZrI.1/rI.-1.

All the clones constructed using PCR fragments were sequenced to ascertain the absence of intrusive mutations. Standard procedures for the isolation and manipulation of plasmid DNA and for the construction and identification of recombinant plasmids were used throughout. DNA restriction endonucleases, T4 DNA ligase and polymerase, DNA polymerase I (Klenow fragment), *Taq* DNA polymerase, *Pfu* DNA polymerase and the CycleReader™ DNA sequencing kit were obtained from Fermentas AB.

Substitution of T4 gene *rI* by the plasmid–phage recombination. *E. coli* B^E cells harbouring the recombinant plasmid pTZrI.1/69rI/rI.-1 were grown at 37 °C to a density of 2×10^8 cells/ml in Luria–Bretani (LB) broth containing 40 μg/ml ampicillin. Cells were infected at a MOI of 0.1 with the T4D. The infected culture was aerated for two hours at 37 °C and then lysed with chloroform. T4 mutants carrying the gene *rI* of RB69 were selected by plating phage progeny on B^E cells and picking out the plaques of *r* phenotype.

Lysis inhibition assays. *E. coli* strain B^E cells were grown overnight in standard LB broth; 5 ml LB were inoculated with 0.05 ml of an overnight culture, and the diluted cells were grown at 37 °C with rigorous aeration (200 rpm) to an $A_{600} = 0.8$. Based on an assumed cell titer of 2×0^8 cells/ml at $A_{600} = 0.8$, phages were added to the flasks to give a multiplicity of infection (MOI) of 10. After 10 min, phage at MOIs of 10 was added to the culture to achieve secondary

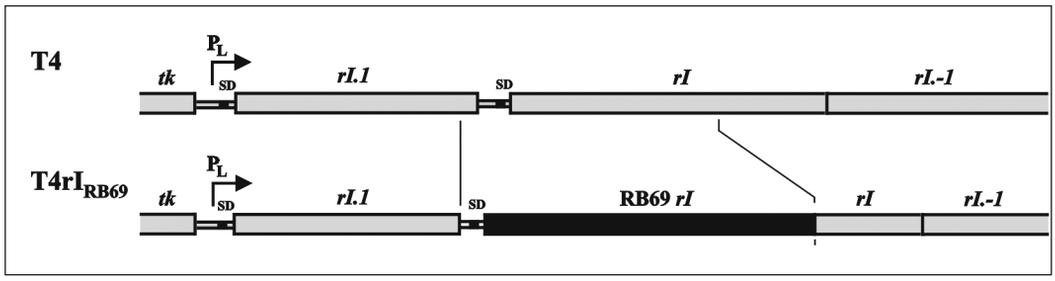

Fig. 2. Schematic representation of the gene *rI* region of the phage T4 and recombinant phage T4rI$_{RB69}$. T4 sequences are shaded in grey. The name of a gene is indicated above the corresponding box. The arrow represents the late promoter (P_L). The positions of the Shine–Dalgarno sequences (SD) are indicated as well. The scheme was designed according to the complete genomic sequence of T4 (AF158101)

infection. Starting at 0 min (time of initial infection), absorbance at 600 nm was measured at appointed times, using a spectrophotometer (Eppendorf Biophotometer). All values were normalized to the value at 0 min.

RESULTS AND DISCUSSION

A genomic sequence analysis revealed significant alterations in the primary structure of the major lysis inhibition protein RI of the T-even phage RB69. This finding prompted us to examine whether RI encoded by this phage was functionally compatible with the lysis inhibition system of T4. We decided to carry out the complementation assay by inserting the gene *rI* of RB69 into the genome of the T4 phage. The plasmid pTZrI.1/69rI/rI.-1, containing gene *rI* of RB69, flanked by the appropriate T4 DNA for the homologous recombination, was constructed to replace the 5' part of T4 gene *rI* with the full length gene *rI* of RB69 in the T4 genome (see Materials and Methods).

Then we used a plasmid–phage homologous recombination, expecting to screen recombinant phages by sharp-edged, larger *r*-type plaques. T4 mutants deficient in lysis inhibition form this type of plaques instead of the small, rough-edged plaques generated by the wild-type phage [5]. We sequenced the gene *rI* region of the several phages that formed *r*-type plaques and successfully isolated the recombinant phage T4rI$_{RB69}$ (Fig. 2). In contrast to the observed morphology of *r*-type plaques, the lysis profile of T4rI$_{RB69}$-infected cells clearly demonstrated the presence of lysis inhibition: after a slight drop at 25 min after infection, the turbidity of the culture constantly rose for up to several hours (Fig. 3). On the other hand, T4rI$_{RB69}$ was unable to maintain lysis inhibition for so long as the wild-type phage did under the same experimental conditions. We supposed that the *r*-type plaque morphology that allowed us to isolate the recombinant phage was determined by the notably shorter period of lysis inhibition.

Our results evidently indicate that the level of sequence homology retained by the RI of T-even phage RB69 enables it to be functionally compatible with the other components of the lysis inhibition mechanism of T4, particularly with the holin T. Herewith, a comparison of the RI sequences that have substantially diverged between T4 and RB69 phages (Fig. 1) provides information about the motifs that are important for the function and thus conserved. Since the RI of RB69 is able to substitute the holin inhibitor of T4, it seems reasonable to expect that in the regulation of infection cycle of RB69, it plays the role of holin inhibitor as well. In this respect, our results are consistent with the previously reported observations of lysis inhibition in the T-even phage RB69 infected cells.

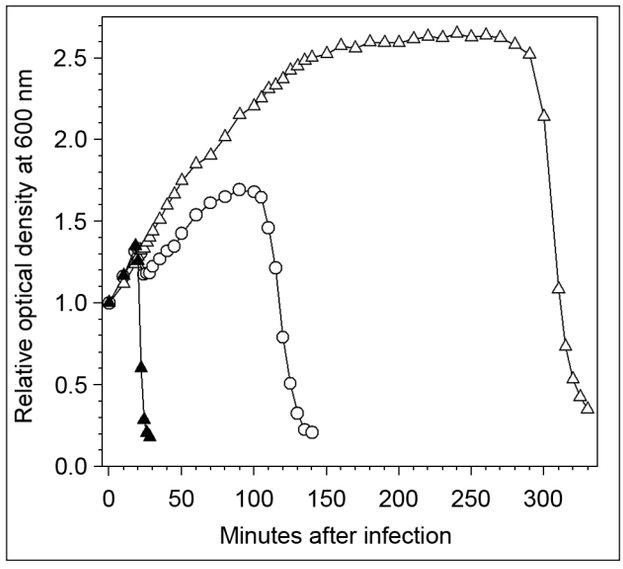

Fig. 3. Lysis profile of the recombinant phage T4rI$_{RB69}$ (○), compared with the lysis profiles of T4D (△) and T4D *r*48 (▲). *E. coli* B^E cells were infected with phages at a MOI of 10. Superinfection at a MOI of 10 was carried out 10 min later with the same phage used for initial infection

ACKNOWLEDGEMENTS

This work was supported in part by the Lithuanian State Science and Studies Foundation.

Received 2 February 2009
Accepted 10 March 2009

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T4 BAKTERIOFAGO RI HOLINO INHIBITORIAUS PAKEITIMAS *IN VIVO* HOMOLOGINIU T LYGINIO RB69 FAGO BALTYMU

Santrauka

Lizės inhibicija – tai T4 giminingų fagų sugebėjimas sustabdyti infekuotų ląstelių lizę kelioms valandoms ir reaguoti į išorėje esančių giminingų fagų adsorbciją prie ląstelės. Neseniai buvo nustatyta, kad T4 fago lizės inhibicijos mechanizmo pagrindas yra T holino ir RI holino inhibitoriaus tarpusavio sąveika. Tai unikali T lyginių bakteriofagų šeimos savybė. Šiai grupei priklauso ir RB69 bakteriofagas, tačiau neaišku, ar šiame fage veikia lizės inhibicijos sistema. Šio darbo metu mes sukonstravome rekombinantinį T4 fagą, kurio genome *rI* genas buvo pakeistas homologišku RB69 genu. Lizės profilio tyrimas atskleidė, kad rekombinantiniu fagu infekuotų ląstelių lizė buvo sustabdyta kelioms valandoms. Gautas rezultatas rodo, kad sustabdant ląstelių lizę, RB69 fago koduojamas RI holino inhibitorius gali efektyviai pavaduoti T4 fago koduojamą RI.