A role for accessory genes *rI.-1* and *rI.1* in the regulation of lysis inhibition by bacteriophage T4

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Abstract Lysis inhibition (LIN) is a known feature of the T-even family of bacteriophages. Despite its historical role in the development of modern molecular genetics, many aspects of this phenomenon remain mostly unexplained. The key element of LIN is an interaction between two phage-encoded proteins, the T holin and the RI antiholin. This interaction is stabilized by RIII. In this report, we demonstrate the results of genetic experiments which suggest a synergistic action of two accessory proteins of bacteriophage T4, RI.-1, and RI.1 with RIII in the regulation of LIN.

Keywords Lysis inhibition · Accessory genes · Bacteriophage T4

Introduction

Lysis inhibition (LIN), as a response of bacteriophage T4-infected cells to superinfection by another T-even phage is a phenomenon known for more than 60 years. Up to date, the major players have been identified and characterized, but despite its crucial role in elucidating fundamental biological processes, the mechanism of this phenomenon remains mostly unexplained [1–3].

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During intracellular development of a T-even phage, superinfection by another T-even phage at least 3 min after the primary infection leads to prolongation of viral development inside the cell [1, 4, 5]. When repeated, superinfections may lead to very long latent periods, lasting for a few hours, increasing the number of progeny virions up to one order of magnitude [5]. In profiles of lysis of bacterial cultures, caused by T4 phage, this prolonged development is suddenly disrupted and rapid, almost synchronous, lysis occurs. This stage is called LIN collapse, and it may be caused by a damage of bacterial cell walls by superinfecting phages. When the damages accumulate to the critical point, the cell lyses and releases phage progeny. The locally increased concentration of phages causes a damage of neighboring cells, which also release phages. This kind of a chain reaction leads to quick lysis of the infected bacterial culture [6].

The most important factors in LIN are phage-encoded proteins: holin T and antiholin RI. RI is a small (97 aa, 11.1 kDa) periplasmic, hybrophobic protein with $pI = 4.0$ [1]. The *C*-terminal domain plays a key role in RI activity [7]. This protein is extremely unstable with a half-life of ~ 2 min [3]. According to a report by Tran et al. [3], RI protein is localized in the periplasmic side of cell membrane by its *N*-terminus, which was identified as a signal anchor release domain. When the *C*-terminal domain is released, it can interact with holin T and block its opening.

A signal triggering the LIN response is still not identified. It is assumed that small proteins and/or phage DNA may be a source of such a signal. This conclusion was drawn from experiments performed by Rutberg and Rutberg [5] which shows that phage ghost cannot trigger LIN. Thus, simple adsorption and penetration of the cell wall is not enough to provoke this phenomenon. However, the antiholin–holin system of T4 is also able to react to a signal

Table 1 Plasmids

Plasmids	Description	Source/Reference
pCattTrE18	Ori pMB1, Cm^R, P$_{tet,}$ MCS inside *lacZ*′	[12]
pCattTrE18 rI	As pCattTrE18, but bearing the *rI* gene. It was constructed by PCR amplification of the *rI* gene (with primers rIEcostart and rIPstend), digestion of the product with *Eco*RI-*Pst*I, and its insertion into MCS of pCattTrE18.	This study
pCattTrE18 rI.1	As pCattTrE18, but bearing the *rI.1* gene. It was constructed by PCR amplification of the *rI.1* gene (with primers rI.1Ecostart and rI.1Pstend), digestion of the product with *Eco*RI-*Pst*I, and its insertion into MCS of pCattTrE18.	This study
pCattTrE18 rI.-1	As pCattTrE18, but bearing the *rI.-1* gene. It was constructed by PCR amplification of the *rI.-1* gene (with primers rI.-1Ecostart and rI.-1Pstend), digestion of the product with *Eco*RI-*Pst*I, and its insertion into MCS of pCattTrE18.	This study
pCattTrE18 rIII	As pCattTrE18, but bearing the *rIII* gene. It was constructed by PCR amplification of the *rIII* gene (with primers rIIIPststart and rIIIHindend), digestion of the product with *Pst*I-*Hind*III, and its insertion into MCS of pCattTrE18.	This study
pCattTrE18 OP	As pCattTrE18, but bearing the *rI* operone. It was constructed by PCR amplification of the *rI* operone (with primers rI.1Ecostrat and rI.-1Pstend), digestion of the product with *Eco*RI-*Pst*I, and its insertion into MCS of pCattTrE18.	This study
pCattTrE18 OP rIII	As pCattTrE18 OP but bearing, in addition, *rIII*. The *rIII* gene was cut from pCattTrE18 rIII by *Pst*I-*Hind*III digestion and inserted into MCS of pCattTrE18 OP	This study
pCattTrE18 rI rIII	As pCattTrE18 rI but bearing, in addition, *rIII*. PCR product of gene *rIII* (rIIIPststart-rIIIHindend) was cut with *Pst*I-*Hind*III and inserted into MCS of pCattTrE18 rI	This study
pCattTrE18 rI rI.1	As pCattTrE18, but bearing the *rI* and *rI.1* genes. It was constructed by PCR amplification of the *rI* and *rI.1* genes (with primers rI.1Ecostart and rIPstend), digestion of the product with *Eco*RI-*Pst*I, and its insertion into MCS of pCattTrE18.	This study
pCattTrE18 rI rI-1	As pCattTrE18, but bearing the *rI.-1* and *rI* genes. It was constructed by PCR amplification of the *rI.-1* and *rI* genes (with primers rIEcostart and rI.-1Pstend), digestion of the product with *Eco*RI-*Pst*I, and its insertion into MCS of pCattTrE18.	This study
pCattTrE18 rI.1 rIII	As pCattTrE18 rIII but bearing, in addition, *rI.1*. The *rI.1* gene was cut out from pCattTrE18 rI.1 with *Eco*RI-*Pst*I and inserted into MCS of pCattTrE18 rIII	This study
pCattTrE18 rI-1 rIII	As pCattTrE18 rIII but bearing, in addition, *rI.-1*. The *rI-.1* gene was cut out from pCattTrE18 rI.-1 with *Eco*RI-*Pst*I and inserted into MCS of pCattTrE18 rIII	This study
pCattTrE18 rI rI.1 rIII	As pCattTrE18 rIII but bearing, in addition, *rI and rI.1*. The *rI* and *r.1.1.* genes were cut out from pCattTrE18 rI rI.1 with *Eco*RI-*Pst*I and inserted into MCS of pCattTrE18 rIII	This study
pCattTrE18 rI rI-1 rIII	As pCattTrE18 rIII but bearing, in addition, *rI and rI.-1*. The *rI* and *rI-.1* genes were cut out from pCattTrE18 rI-rI.-1 with *Eco*RI-*Pst*I and inserted into MCS of pCattTrE18 rIII	This study
pCattTrE18 lacZ	As pCattTrE18, but bearing the *lacZ* gene. *LacZ* was cut out from pET24 lacZ (M.Sektas, Dept. Microbiology, UG, personal communication) with *Bam*HI-*Hind*III and inserted into MCS of plasmid pCattTrE18	This study

generated during slow growth of bacterial cells by delaying the cell lysis [8]. This indicates that superinfection is not the only event which may trigger LIN or LIN-like response.

The *rI* gene (location 59594–59202 on genetic map) and its neighbors, *r1.1* and *r1.-1*, form an operon. Genes from this operon are transcribed from two promoters: the late promoter located between *tk* and *rI.1* genes and the early promoter located 3.3 kb upstream of the late promoter. All genes from this operon were suggested to be highly conserved among T-even phages [1]. The RI.-1 protein is hydrophilic, with calculated $pI = 5.6$. It is a 129 aa long polypeptide with predicted molecular mass of 14.6 kDa [1, 2]. The *rI.1* gene encodes a 70 aa long protein, with calculated $pI = 10.2$ and molecular mass of predicted 8.3 kDa [2]. Similarly to RI.-1, its function is unknown. However, the localization of RI.1 and RI.-1 in one operon, and conservation of sequences of genes coding for these proteins, may suggest their role in LIN [1].

A gene coding for RIII, a protein also involved in LIN, is located in a different region of the T4 genome than the *rI* operon (location 131033–130785 on genetic map). This protein, with calculated $pI = 8.5$, is 82 aa long with predicted molecular mass of 9.3 kDa [1]. It is supposed to localize in the cytoplasm, and it has been proposed that it stabilizes the RI-T interaction [1]. Due to its localization, it was assumed that it interacts with the cytoplasmic domain of the T holin [9] (gene location 160221–160877 on genetic

Table 2 Primers

Primers	Sequences
rIII Hindend	5′-GCTAAAGCTTACGGCTCCTCGATTTAGCTC-3′
rIII Pststart	5′-GCCTGCAGTTCAGAAGAACGTGACTACC-3′
rI.1 Pstend	5′-GCCTGCAGGCTTCAATCGATGGAGATAAAAC-3′
rI.1 Ecostart	5′-GCGAATTCAAGCTCGGTTAGTGCAAAAG-3′
rI.-1 Pstend	5′-GCCTGCAGGTACCACAGCTCAAACGGAAAG-3′
rI.-1 Ecostart	5′-GCGAATTCTGTATAGAGCGAGGCGCGGAGATG-3′
rI Pstend	5′-GCCTGCAGGTCGCTGAATTTCATTCAGTCTCC-3′
rI Ecostart	5′-GCGAATTCTAAGGCCGTGCATCGGCACAAAG-3′
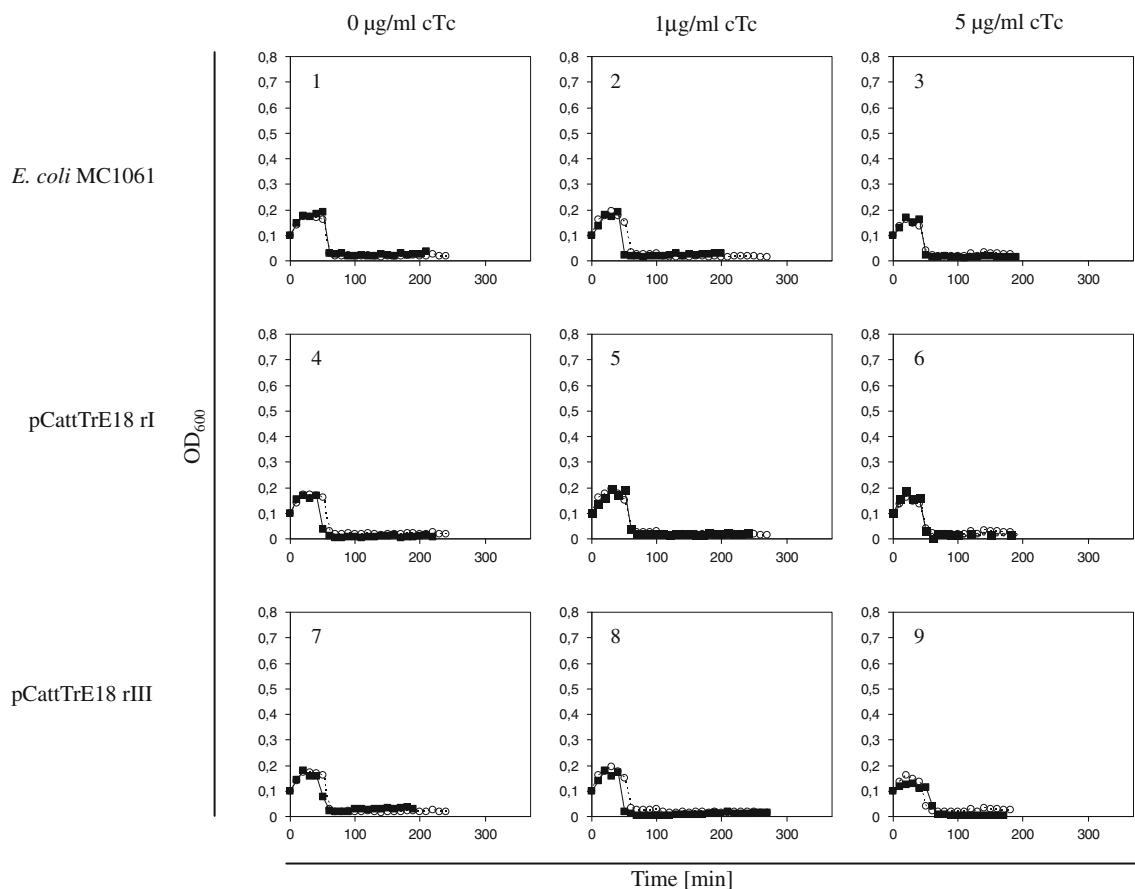
Fig. 1 Lysis of bacterial cultures by bacteriophage T4*rI*. The plasmids present in bacterial cells are indicated on *left side* of the figure, the concentration of inducer is indicated on the *top*. *Filled squares* show lysis curves of bacterial cells with indicated plasmid. *Open circles* show lysis curves of a control experiment with pCattTrE18 lacZ. Experiments which are not shown in the figure have given results identical with other experiments in all concentrations of the inducer tested. For the construct of each plasmid, see Table 1

map). It is likely that transcription of *rIII* can be initiated at three independent promoters: early, middle, and late [1].

The model proposed by Ramanculov and Young [10] assumes that RI interacts (in the periplasm) with the T holin, which is embedded in cell membrane. This interaction is very unstable, but it is able to block the opening of a lesion in the membrane made by the holin. This unstable interaction was proposed to be stabilized by RIII interacting with the cytoplasmic domain of the T holin [9].

The aim of this study was to investigate a possible role for accessory genes *rI.-1* and *rI.1* (in combination with *rIII*) in the regulation of LIN.

Materials and methods

Bacterial and phage strains

Escherichia coli MC1061 [11] was used as a bacterial host in all experiments. Bacteriophage T4D was obtained from Dr. Józef Nieradko, Department of Microbiology, University of Gdansk, Poland. Phages T4*rI* (r48) and T4*rIII* (r67), mutants of phage T4D were obtained from Prof. Karin Carlson, University of Uppsala, Sweden.

Plasmids and primers

Plasmids used in this work are listed in Table 1, and primers are listed in Table 2.

Lysis profiles (LIN collapse)

A culture of a bacterial strain bearing indicated plasmid was grown overnight at 37°C with shaking in LB medium supplemented with 34 µg/ml of chloramphenicol. Bacteria were centrifuged, the pellet was washed three times with a fresh LB medium, and then inoculated (1:100) into a fresh LB medium without chloramphenicol, but supplemented with indicated amounts of autoclaved chlortetracycline (cTc). Autoclaved cTc is commonly used as inducer for tetracycline promoter, as it is not toxic for bacterial cells. In the case of strains bearing plasmids containing the *rI.1* gene and lacking the *rIII* gene, the induction of gene expression was performed at the time of addition of phages. The culture was grown to $OD_{600} = 0.1$ at 37°C with shaking, then it was divided into three shaking flasks, cultivated in the same conditions, and T4 phage lysate was added to m.o.i. = 0.2 to each flask. OD_{600} was measured in 10 min intervals.

Results

In order to investigate the influence of the overexpression of phage genes on phage development, we constructed a series of plasmids (Table 1) based on pCattTrE18 [12]. As a control, we used the same plasmid with the *lacZ* gene cloned under the control of the tetracycline promoter (p_{tet}). Interestingly, the pCattTrE18 plasmid without any insert ceased LIN completely and slowed down the growth of the bacterial culture whereas introduction of any DNA fragment into MCS resulted in total abolition of these effects (data not shown). Moreover, all phage strains used in the study gave pin-point plaques when titrated on the strain bearing pCattTrE18, contrary to normal plaques on the plasmid-less strain. However, inserting any DNA sequence into the MCS also caused reversion of this
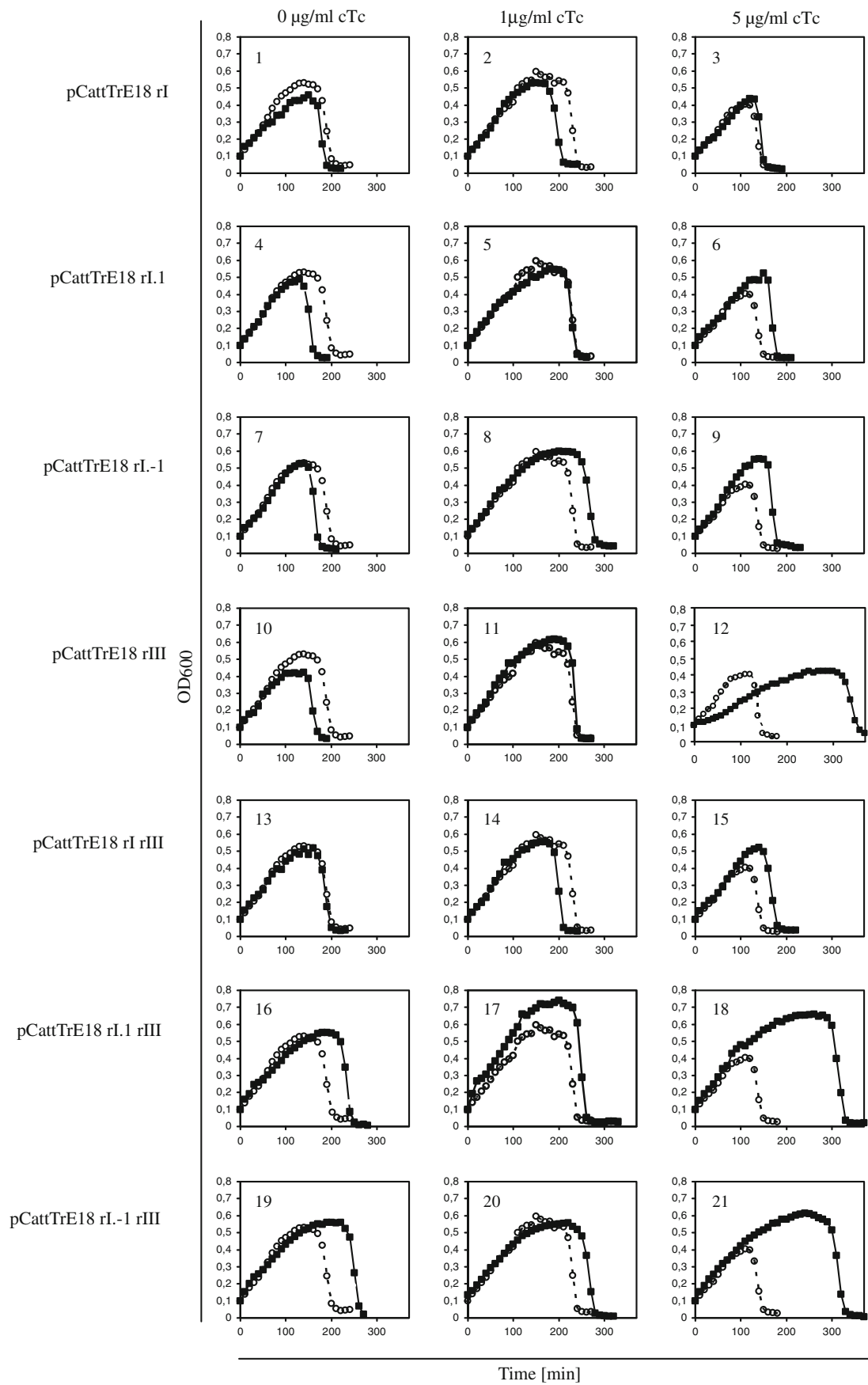
Fig. 2 Lysis of bacterial cultures by bacteriophage T4D. The ▶ plasmids present in bacterial cells are indicated on *left side* of the figure, and the concentration of inducer is indicated on the *top*. *Filled squares* show lysis curves of bacterial cells with indicated plasmid. *Open circles* show lysis curves of a control experiment with pCattTrE18 lacZ. For the construct of each plasmid, see Table 1
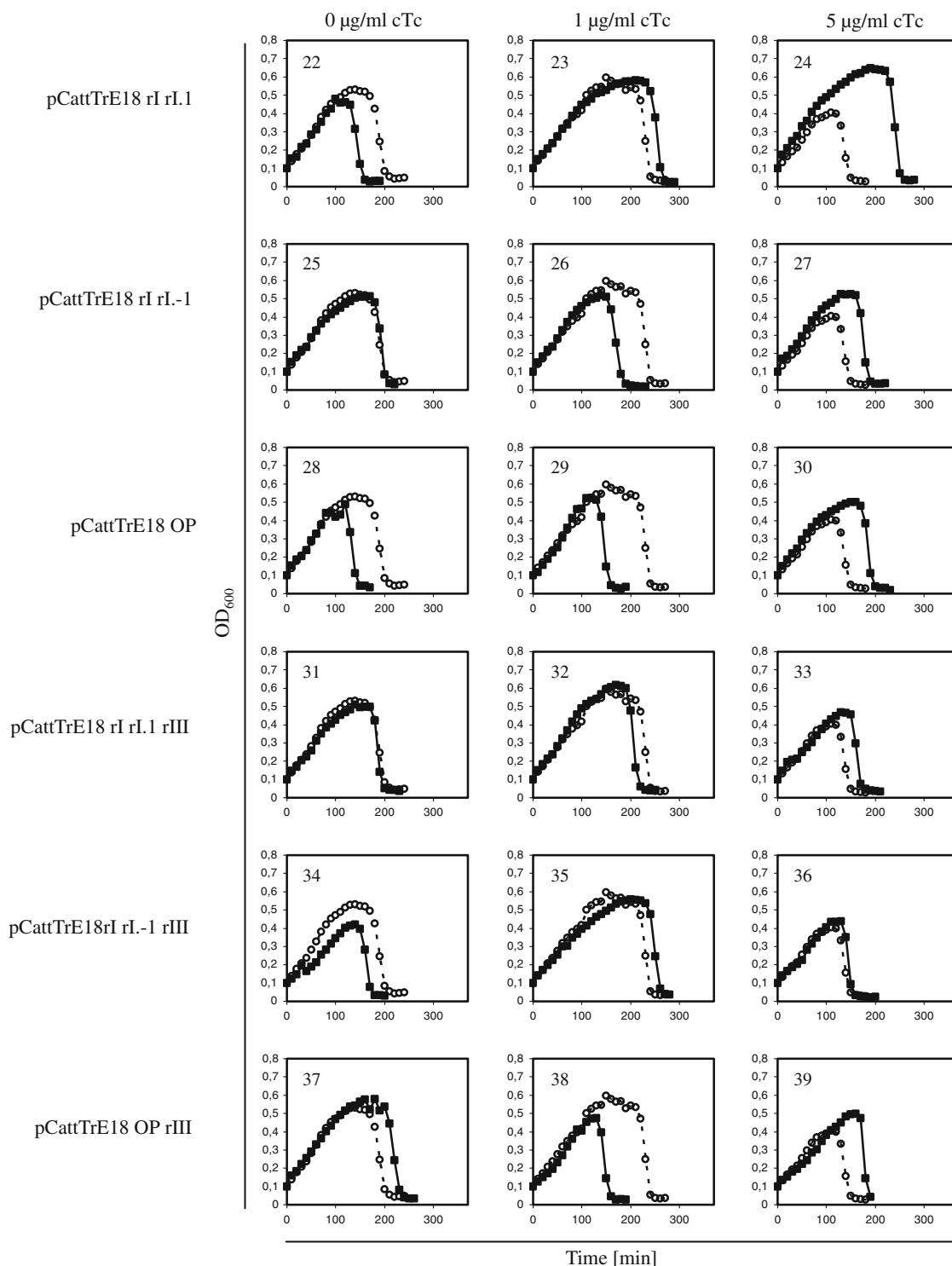
phenotype (data not shown). We decided to use, in the control experiments, bacteria bearing a plasmid with the *lacZ* gene, because an induction of a promoter in the plasmid could potentially cause a shift in the lysis timing. Thus, it would be inappropriate to compare plasmid-bearing cells with those devoid of a plasmid, as overproduction of protein(s) from the plasmid may change the lysis profile. Moreover, the use of bacterial cells devoid of plasmid as a control would result in introduction of another parameter, which might be confusing.

Induction of gene expression caused prolongation of lysis profiles in *E. coli* MC1061 (pCattTrE18 *lacZ*). This behavior may be attributed to extensive protein production and thus a decrease in availability of the protein synthesis system and amount of precursors in early stages of bacteriophage development. Thus, these cells cannot be directly compared to strain *E. coli* MC1061, which was not affected by the inducer. Nevertheless, experiments with plasmidless cells were performed by us, and generally all gave curves similar to those of *E. coli* MC1061 (pCattTrE18 *lacZ*) without induction (data not shown).

We performed experiments using two different concentrations of the inducer (autoclaved cTc): 1 and 5 µg/ml. In experiments without the inducer, a leakiness of the promoter allowed for a negligible expression level of a cloned gene. The p_{tet} activity under conditions of various inducer concentrations was investigated previously. Those studies demonstrated a negligible promoter activity without the inducer, and then, an increase in the p_{tet} activity that was proportional to the inducer concentration [12–14].

We observed that overproduction of RI.1, or overexpression of the whole *rI* operon, had a strong toxic effect on host cells. When bacteria bearing plasmids encoding RI.1 were induced, the only survivors were cells with spontaneous IS1 transposon insertion between the promoter and the *rI.1* gene, which was found after sequencing of plasmids from several colonies (data not shown). The overproduction of RI.1 resulted in filamentation of bacterial cells (data not shown). The toxicity of *rI*, but not of *rI.1* was suggested by Ramanculov and Young [10]. We did not observe any toxic effects of overproduction of the RI protein. Due to RI.1 toxicity, induction of expression in cells harboring plasmids which contain the *rI.1* gene, but not *rIII*, was provoked at the moment of phage addition. Otherwise, lysis profiles were significantly affected by the toxic effect, or even impossible to obtain, as bacteria

Time [min]

	0 µg/ml cTc	1 µg/ml cTc	5 µg/ml cTc
pCattTrE18 rI rI.1	22	23	24
pCattTrE18 rI rI.-1	25	26	27
pCattTrE18 OP	28	29	30
pCattTrE18 rI rI.1 rIII	31	32	33
pCattTrE18rI rI.-1 rIII	34	35	36
pCattTrE18 OP rIII	37	38	39

OD$_{600}$

Time [min]

Fig. 2 continued

ceased growth before reaching $OD_{600} = 0.1$, or were growing extremely slowly (data not shown).

Induction of expression during phage addition was used also for plasmids in which *rI.1*, *rI*, and *rIII* genes were located together, as expression of *rI* removed protec-

tion given by *rIII* against toxic effect caused by sole over-production of RI.1. Contrary to wild-type T4D phage, infection of bacterial culture by the T4*rI* or T4*rIII* mutants resulted in a very fast lysis of the whole culture. LIN collapse experiments with phage T4*rI* showed that using

plasmids constructed in this work, including those over-expressing the *rI* gene, we could not obtain any phenotype reversion and all lysis profiles were exactly matching those obtained in control experiments with *lacZ* overexpression and with plasmid-less cells (Fig. 1, 1–3). This is consistent with the results of other researchers [1 and references therein], indicating that *rI* is absolutely necessary for LIN. The lack of reversion of the phenotype, even when *rI* was overexpressed from a plasmid, was probably due to a very low stability of this protein [2], which, together with the very fast silencing of the host gene expression, caused a lack of this protein in later stages of phage development.

Contrary to T4*rI*, T4D lysis profile was sensitive to overexpression of several genes, and in most of cases the lysis profile was also dependent on the dose of the inducer (Fig. 2). The most pronounced elongation of the lysis profile was obtained by overproduction of RIII (Fig. 2, 10–12), especially together with RI.-1 (Fig. 2, 19–21) or RI.1 (Fig. 2, 16–18). When *rIII* was present on the plasmid together with *rI.-1* or *rI.1*, even a very low level expression, resulting from a leakiness of the promoter, was sufficient to prolong the lysis profile considerably (Fig. 2, 16, 19). When the efficient overexpression system was used, *rIII* alone caused slowing down of bacterial growth. This effect was not observed when *rIII* was co-expressed with *rI.-1* or *rI.1*. Efficient overexpression of *rIII*, *rIII* together with *rI.1*, and *rIII* together with *rI.-1* caused a loss of the rapid lysis phenotype, when T4*rIII* phage was used for infection (Fig. 3, 4–6, 10–12, 13–15). In the same experimental system, an effective prolongation of LIN was observed after infection with T4D (Fig. 2, 10–12, 16–18, 19–21). Results of this set of experiments showed a synergistic effect of *rI.1* and *rI.-1* in prolongation of LIN when these genes were co-expressed with *rIII*. Low expression level of *rIII* alone gave no effect on LIN caused by T4D or T4*rIII* infection.

In the set of experiments with lysis profiles of T4D, we also observed more subtle effects caused mainly by overproduction of RI.1 (Fig. 2, 4–6) and RI.-1 (Fig. 2, 7–9) proteins, which was not accompanied by overproduction of RIII. Among them, RI.-1 caused prolongation of the lysis at both tested concentrations of the inducer. A slight shortening of the time of lysis profile was observed in the control experiment. Similar phenomenon was observed when *rI.1* was overexpressed alone (Fig. 2, 4). When *rI* was co-expressed with *rI.1*, prolongation of LIN profile was observed, especially when 5 μg/ml of cTc was used (Fig. 2, 24). In experiments with 1 μg/ml of cTc, the prolongation was slight in relation to the control experiment, and when no inducer was added the LIN profile showed premature termination (Fig. 2, 23, 22, respectively). Interestingly, co-overexpression of *rIII* in this system caused a cease of the effect obtained with *rI* and

rI.1 alone (Fig. 2, 31–33). However, in experiments where T4 *rIII* was used, we observed no effect of co-overproduction of RI and RI.1 (data not shown). Moreover, the overexpression of whole *rI* operon resulted in only a slight prolongation of LIN at 5 μg/ml of cTc, and under other conditions the lysis profile was terminated prematurely (Fig. 2, 28–30). This profile of lysis was not affected by parallel overexpression of the *rIII* gene (Fig. 2, 37–39).

Discussion

Lysis inhibition is already known for over 60 years, and it was used for elucidation of fundamental biological processes. On the other hand, the mechanism of LIN remained mostly unraveled. It is also not established which genes are involved in this process. In this study we tested a possible role for accessory genes *rI.-1* and *rI.1* in the regulation of LIN.

Overproduction of phage proteins from a plasmid (or from the bacterial chromosome) has one disadvantage—the production ceases shortly after T4 infection. This is due to the modification of the host RNA polymerase by the product of the phage gene *asiA* and then the fragmentation of host DNA. Thereby, all interferences we could observe were caused by stable proteins, which could act *in trans*. This would explain a lack of effects of *rI* overexpression on both plaque morphology and LIN collapse profiles.

In recent studies on *rI*, a very short, about 5 min, delay in the lysis was observed by Tran et al. [3], which was explained by a short half-life period of the RI protein. Thus, it seems that this protein have to be produced constantly in order to provide proper pattern of LIN. Opposite effect was observed while overproducing RIII. This overproduction can restore wild-type phenotype in *rIII* mutant, thus, the RIII protein is stable enough to allow for prolonged LIN, but only in the case of efficient overproduction. When *rIII* was overexpressed from a plasmid, the reversion of the phenotype of T4*rIII* plaques was also observed (data not shown).

Lysis inhibition collapse experiments, performed in this study, showed that the action of the proteins in phage development cycle may be dose-dependent. In the case of infection of the host harboring a plasmid with the *rI* gene by phage T4*rIII*, a weak effect on lysis prolongation was visible only in non-induced cultures. This may suggest that a low level expression of *rI*, resulting from residual activity of the p_{tet}, may influence phage T4*rIII* development more significantly than efficient overexpression of this gene.

We were unable to obtain a reversion of the *rI* phenotype, which causes inability to form wild-type plaques and
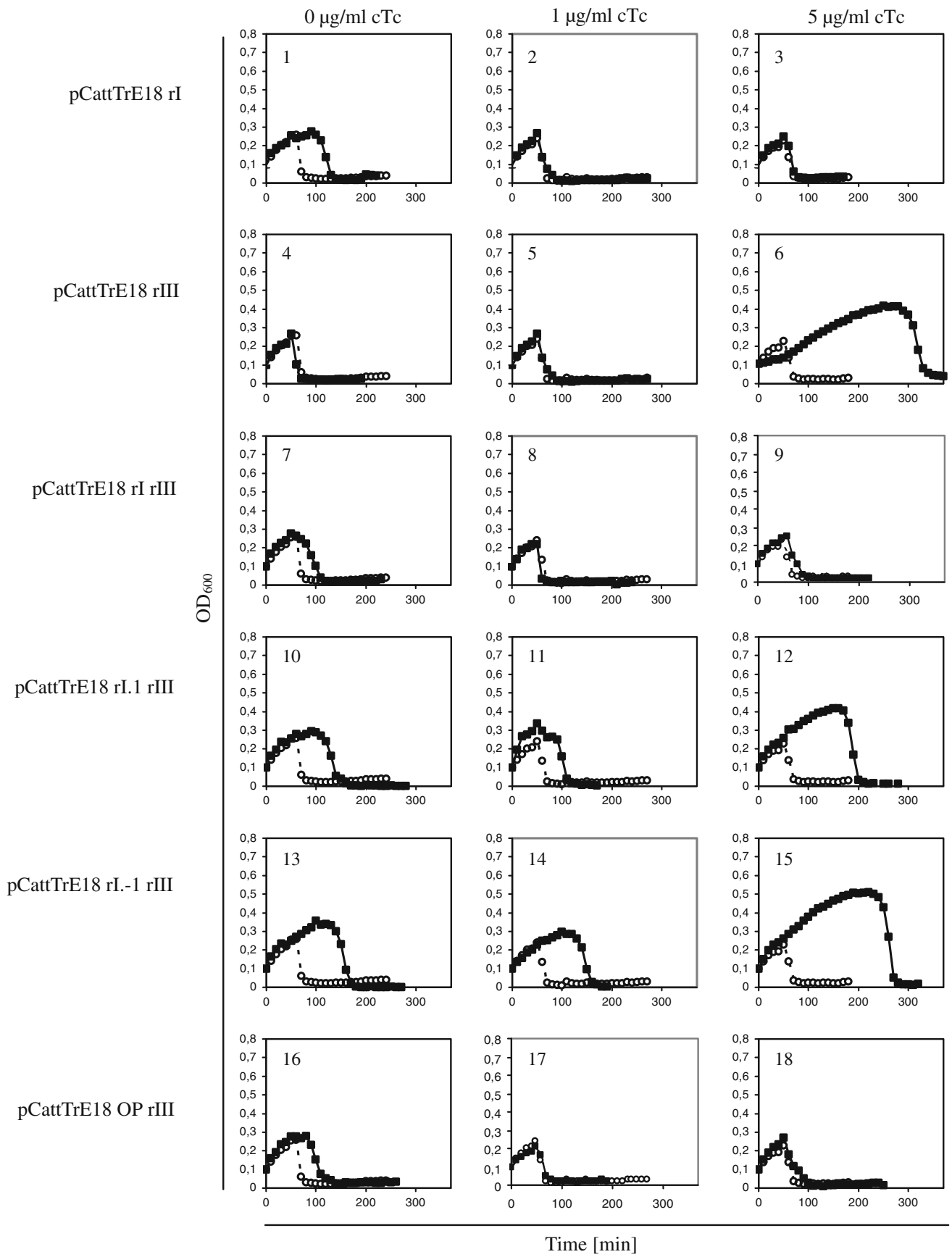
◀ **Fig. 3** Lysis of bacterial cultures by bacteriophage T4*rIII*. The plasmids present in bacterial cells are indicated on *left side* of the figure, and the concentration of inducer is indicated on the *top*. *Filled squares* show lysis curves of bacterial cells with indicated plasmid. *Open circles* show lysis curves of a control experiment with pCattTrE18 lacZ. Experiments were performed with all plasmids constructed in this work. Experiments which are not shown in the figure have given results identical with control experiments at all concentrations of the inducer tested. For the construct of each plasmid, see Table 1

to establish LIN in response to superinfection, by overproduction of any of the investigated proteins. This supports the thesis about a key role of this protein in LIN [1, 9].

Results of overexpression of the *rIII* gene suggest its supportive role in stabilizing RI–T interaction, as it was not only able to reverse the effect of the *rIII* mutation (Fig. 3, 4–6), but also to prolong LIN caused by T4D (Fig. 2,

10–12). The side effect of *rIII* overexpression was a slight toxicity, which caused a slower growth of host cells. Interestingly, parallel overexpression of *rI.-1* (Figs. 2, 19–21; 3, 13–15) or *rI.1* (Figs. 2, 16–18; 3, 10–12) not only strengthened the effect of prolonging LIN by *rIII*, but also ceased RIII toxicity, as growth of bacterial cells reached the normal level. This suggests that proteins RI.-1 and RI.1 play a supportive role in LIN by direct or indirect interactions with RIII. This suggestion is supported by the fact that overexpression of *rI.-1* or *rI.1* prolonged LIN of T4D development (Fig. 2, 4–9), but had no effect during T4*rIII* infection (data not shown), even though fully functional RI protein was encoded by the phage genome.

Another interesting observation is a lack of any effects of overproduction of RIII when *rI* was co-expressed (Figs. 2, 13–15, 31–39; 3, 7–9, 16–18). These results suggest a direct interaction between both proteins, RI and
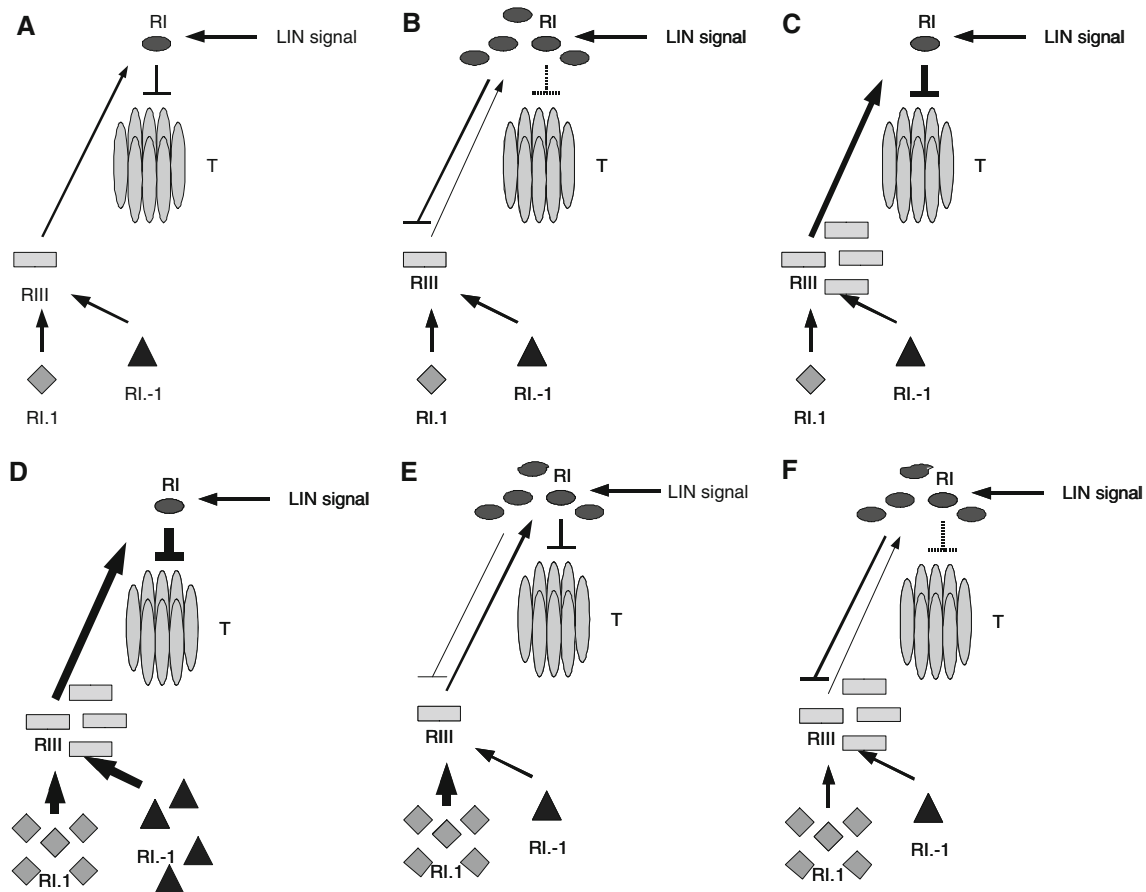
Fig. 4 A putative model for functions of R.I.1 and RI.-1 in LIN. *Arrows* indicate stimulation and *blunt-ended lines* indicate inhibition. The thicknesses of *arrows* and *lines* are proportional to efficiencies of corresponding processes. **a** In wild-type cells RI inhibits T-mediated lysis of host cells, when LIN signal is present. RIII stimulates RI function. RI.1 and RI.-1 modulate RIII action. **b** Overproduction of RI causes less efficient LIN possibly by titrating off the RIII protein. **c** Overproduction of RIII increases efficiency of LIN. **d** Overproduction of RIII together with RI.1 or RI.-1 strongly increases efficiency of LIN. **e** Overproduction of RI.1 diminishes the effect of increased levels of RI. **f** When RI, RI.1, and RIII are overproduced, the LIN efficiency is decreased, similarly to the effect of overproduction of RI alone. This may suggest that RI:RIII:RI.1 ratio is important for LIN regulation

RIII, which may lead to inactivation of RIII (Fig. 4b). This suggestion seems to be supported by a toxicity of overexpression of genes *rI.1*, *rI*, and *rIII* from one plasmid, where the presence of *rI* ceased the effect of the *rI.1–rIII* mutual antitoxicity. Moreover, the toxic effect was of the type caused by RI.1 not by RIII, i.e., the growth inhibition was significantly more severe. The parallel overexpression of *rI* together with *rIII* ceased also an effect of an efficient prolongation of LIN profile obtained when *rI* was co-expressed with *rI.1* (Fig. 4d, e) and weak prolongation of LIN profiles obtained during parallel overexpression of *rI* and *rI.-1* or overexpression of the whole *rI* operon. Even though *rIII* overexpression ceased the above mentioned effects, it seems to be likely that action of accessory proteins—RI.1 and RI.-1 require functional RIII protein, as no effect of the overproduction (beside the toxicity of RI.1 to host cells) was observed when T4*rIII* was used and RIII was not supplied *in trans*. This raises the question about a possible role for stoichiometric proportions of both proteins, RI and RIII.

On the basis of our results we propose a model for the role of accessory genes *rI.1* and *rI.-1* in LIN maintenance (Fig. 4). According to this model, in wild-type cells, RI inhibits T-mediated lysis of host cells when LIN signal is present, and RIII stimulates RI function, which has already been known. However, we propose that RI.1 and RI.-1 can modulate RIII action. This proposal is supported by observations that (i) overproduction of RI causes less efficient LIN, possibly by titrating off the RIII protein, (ii) overproduction of RIII increases efficiency of LIN, (iii) overproduction of RIII together with RI.1 or RI.-1 strongly increases efficiency of LIN, (iv) overproduction of RI.1 diminishes the effect of increased levels of RI, and (v) when RI, RI.1, and RIII are overproduced, the LIN efficiency is decreased, similarly to the effect of overproduction of RI alone. Therefore, we hypothesize that the RI:RIII:RI.1 ratio is important for LIN regulation.

Prolongation of LIN, observed in this work, has also another aspect, which may be helpful to understand the lysis process and the LIN collapse caused by bacteriophage T4. Since the presence of an excess of some phage proteins made bacterial culture less prone to LIN collapse; it is possible that the causative agent of LIN collapse is not only the accumulation of damages done by superinfecting phages, but also optimal amounts and activities of some phage proteins.

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