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Proteomic profiles and kinetics of development of bacteriophage T4 and its *rI* and *rIII* mutants in slowly growing *Escherichia coli*

Running title: Proteomics and kinetics of T4 development in *E. coli*

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Summary

Bacteriophage T4 survival in its natural environment requires adjustment of phage development to the slow bacterial growth rate or the initiation of mechanisms of pseudolysogeny or lysis inhibition (LIN). While phage-encoded RI and probably RIII proteins seem to be crucial players in pseudolysogeny and LIN phenomena, the identity of proteins involved in regulation of T4 development in slowly growing bacteria has remained unknown. In this work, using a chemostat system, we studied the development of wild-type T4 (T4wt) and its *rI* (T4*rI*) and *rIII* (T4*rIII*) mutants in slowly growing bacteria, where T4 initiated neither LIN nor pseudolysogeny. We determined eclipse periods, phage propagation times, latent periods and burst sizes of T4wt, T4*rI* and T4*rIII*. We also compared intracellular proteomes of slowly growing *Escherichia coli* infected with either T4wt or the mutants. Using 2-D PAGE analyses we found 18 differentially expressed proteins from lysates of infected cells. Proteins whose amounts were different in cells harboring T4wt and the mutants are involved in processes of replication, phage-host interactions or they constitute virion components. Our data indicate that functional RI and RIII proteins - apart from their already known roles in LIN and pseudolysogeny - are also necessary for the regulation of development of phage T4 in slowly growing bacteria. This regulation may be more complicated than previously anticipated, with many players influencing T4 development in its natural habitat.

Introduction

Bacteriophage T4 is a model organism in molecular biology. Its development in rich medium, under so called standard laboratory conditions, is a well-studied process. Under these conditions, phage T4 develops in about 25 – 30 minutes releasing 100 – 200 progeny virions per infected cell (Abedon, 1994). The aim of this development, referred to as *short latent-period* (SLP) strategy, is to quickly produce progeny particles (Abedon *et al.*, 2003). Interestingly, under standard laboratory conditions, phage T4 requires for its development the activity of only 62 out of its ~ 300 predicted genes (Miller *et al.*, 2003). Proteins encoded by these genes are involved in processes of replication, transcription and translation or they are structural proteins building the phage's capsid. The other ~ 240 genes encode nucleases; inhibitors of host replication, transcription and protease activity; enzymes responsible for nucleotide biosynthesis, recombination and DNA repair and proteins involved in exclusion of a superinfecting phage, lysis inhibition, and other membrane changes (Miller *et al.*, 2003).

Previous findings indicated that in the natural environment of phage T4 – the mammalian intestine - bacteria grow significantly slower than under laboratory conditions or they stop growing completely (Hadas *et al.*, 1997; Koch 1971, Kutter *et al.*, 1994). T4 has adapted to such conditions – it is capable of adjusting its development to the bacterial growth rate (μ) (Abedon *et al.*, 2001; Hadas *et al.*, 1997; Rabinovitch *et al.*, 1999; Rabinovitch *et al.*, 2002). Using different media to control the bacterial growth rate, it was revealed that with decreasing μ, the rate of phage release and the burst size decrease while the eclipse and latent periods increase (Hadas *et al.*, 1997; Rabinovitch *et al.*, 2002). The molecular basis of this adaptation has remained unknown.

Apart from the adaptation described above, T4 may also use two other mechanisms to survive in its natural habitat. When there are more phage particles than bacterial cells in the environment, the phage can initiate the mechanism of lysis inhibition (LIN) (Bode, 1967). This phenomenon is employed when the already infected bacterial cell is again infected by another T-even phage at least 3 minutes after the first infection. LIN enables the phage to prolong its development from minutes to hours and to increase the phage yield to about 1000 progeny particles per infected cell (Abedon, 1994; Bode, 1967; Doermann, 1948; Tran *et al.*, 2005). When host cells do not grow, the phage can turn on the mechanism of pseudolysogeny (Golec *et al.*, 2011; Kutter *et al.*, 1994; Łoś *et al.*, 2003; Łoś & Węgrzyn, 2012). In this case, T4 adsorbs to the cell, injects its DNA, expresses some of the early genes and eventually stops its development until environmental conditions improve (Kutter *et al.*, 1994).

Development of phage T4 ends with the lysis of bacterial cells. Destruction of cell is connected with the activity of two phage-encoded proteins: holin T which triggers the disruption of the cytoplasmic membrane and endolysin E which enters the periplasm and attacks the peptidoglycan (Miller *et al.*, 2003; Ramanculov & Young, 2001; Tran *et al.*, 2005; Tran *et al.*, 2007). Holin T interacts with phage antiholin RI and this is necessary to start the LIN mechanism (Tran *et al.*, 2005; Tran *et al.*, 2007). Mutants in the *rI* gene are unable to start LIN and are called "rapid lysis" mutants (Burch *et al.*, 2011). The interactions between T and RI proteins are probably stabilized by the phage RIII protein (Golec *et al.*, 2010; Paddison *et al.*, 1998). Furthermore, RI.1 and RI.-1 proteins, encoded by genes which form an operon with the *rI* gene, also seem to be involved in the regulation of T4 development (Golec *et al.*, 2010). Apart from their roles in LIN, functional RI and RIII proteins were shown to be essential for phage T4 to

survive in a starved bacterial culture (Golec *et al.*, 2011), suggesting that both of these proteins are involved also in pseudolysogeny.

To date, literature has suggested that the adaptation of T4 phage development to the growth rate of bacteria plays a pivotal role in maintaining phage particles in environment (Abedon, 1994; Abedon *et al.*, 2001; Abedon *et al.*, 2003; Golec *et al.*, 2010; Golec *et al.*, 2011; Hadas *et al.*, 1997; Kutter *et al.*, 1994; Łoś *et al.*, 2003; Łoś & Węgrzyn, 2012; Paddison *et al.*, 1998; Rabinovitch *et al.*, 1999; Rabinovitch *et al.*, 2002). While RI and RIII are known to play a role in LIN and pseudolysogeny of T4, the question has remained if they regulate the phage development in slowly growing bacteria and what other proteins contribute to this regulation. In this study, we aimed to determine developmental parameters of phage T4, i.e.: eclipse period, phage propagation time (defined as a period for intracellular assembly of phage particles), latent period and burst size in a slowly growing bacterial culture where neither LIN nor pseudolysogeny were initiated. We asked if functions of *rI* and *rIII* genes, known to be involved in LIN and pseudolysogeny, participate also in regulation of phage development in slowly growing host cells.

Results

Kinetics of development of wild-type T4 and its mutants in slowly growing bacterial cells

Bacteriophage T4 development depends on the physiology of its host *E. coli*. Wild-type T4 is able to adapt to the growth rate of a bacterial culture by prolonging its development or by

initiating LIN or pseudolysogeny mechanisms (Golec *et al.*, 2010; Golec *et al.*, 2011; Hadas *et al.*, 1997; Kutter *et al.*, 1994; Łoś *et al.*, 2003; Łoś & Węgrzyn, 2012; Paddison *et al.*, 1998; Rabinovitch *et al.*, 1999; Rabinovitch *et al.*, 2002; Tran *et al.*, 2005). Previously we found differences in the rapidity of lysis of a slowly growing bacterial culture triggered by wild-type T4 and its *rI* (Łoś *et al.*, 2003) and *rIII* mutants (Golec, 2010). It was hypothesized that T4*rI* and T4*rIII* mutants cannot precisely regulate their development in response to the host metabolic status (Golec, 2010; Łoś *et al.*, 2003). In this study, we analyzed development of T4wt, T4*rI* and T4*rIII* in detail, determining their eclipse periods, phage propagation times, latent periods and burst sizes in a slowly growing bacterial culture where neither LIN nor pseudolysogeny were initiated. For this purpose we used chemostat cultivations which enabled us to obtain reproducible conditions in which the bacterial growth rate was the only differential factor (Hoskisson & Hobbs, 2005). The experimental procedure included infection of the slowly growing bacterial culture by T4wt or the mutants at m.o.i. of 5, followed by incubation for 1 minute. It should be noted, however, that within the time of incubation only about 30% of phages adsorbed to the cells (data not shown). This means that one bacterial cell was infected most probably by one phage; therefore, the LIN mechanism was not initiated. Furthermore, we did not observe significant differences in adsorption of T4wt and the mutants to the slowly growing *E. coli* cells (data not shown). The data collected in this experiment allowed us to setup conditions for collecting the samples from the chemostat cultures for proteomic studies.

The effects of infection of slowly growing hosts with phage T4 and mutants in *rI* and *rIII* genes are presented in Table 1. We found that the development of phage T4 differs from that of its mutants with respect to eclipse period, phage propagation time, latent period and burst size. Moreover, the lower the growth rate, the bigger the differences between the developmental

parameters of wild-type T4 and *rI* and *rIII* mutants. Additionally, in our experimental approach T4wt was not able to lyse the slowly growing bacterial culture, whereas both *rI* and *rIII* mutants caused lysis after several hours at each of the tested growth rates (data not shown).

Proteomic analysis of development of wild-type T4 and its mutants in slowly growing bacterial cells

In order to further characterize differential developments of phage T4 and its mutants, we decided to employ 2D gel-based proteomics (Görg *et al.*, 1999; Thürmer *et al.*, 2011). The bacterial growth rate of 0.05 was chosen for the proteomic analysis since at this μ the biggest differences between development of T4wt and the mutants were observed (Table 1). We added phage to chemostats to a final m.o.i. of 1 at time 0. We analyzed proteomes from samples collected 10 and 50 minutes after the phage infection, which correlated with eclipse period and the end of the latent period of mutants, respectively. The decision to collect the samples 10 minutes after the infection was also justified by the observation that adsorption of the majority of phage particles occurred within five minutes after infection, reaching about 70% (data not shown).

We were able to visualize and estimate the relative amounts of approximately 700 proteins, of both bacterial and phage origin. We found 20 major differences in spot intensities, between gels derived from *E. coli* cultures infected with wild-type T4 and with *rI* or *rIII* phage mutants, which corresponded to 18 different proteins as some of the spots were variants of the same protein (Fig. 1 and 2). 17 of the detected differences represented spots of increased intensity after T4*rI* and T4*rIII* infections in comparison with wild-type T4. Three protein spots were of

153 decreased intensity after infection with *rI* and *rIII* phage mutants. Tables 2 and 3 present the
154 identified phage-encoded and bacterial proteins, respectively, displaying differential expression
155 between wild-type T4 and mutant-infected cells.

156 As mentioned above, the amounts of three bacterial proteins (i.e. GatZ, RpoA, AccD**)**
157 were decreased in T4 *rI*- and *rIII*-infected cells relative to wild-type T4-infected ones. It should
158 be noted, however, that RpoA was identified in two of the analyzed protein spots (Fig. 1). The
159 intensity of one of these spots was decreased after infection with *rI* and *rIII* phage mutants. At the
160 same time, the intensity of the other RpoA spot increased in T4 *rI*- and *rIII*-infected cells. This
161 could reflect a modification of the alpha subunit of RNA polymerase, which occurred
162 significantly faster in both phage mutants than in the wild-type T4 phage.

163 The viral protein Gp23 (major head protein) was also identified in two different positions
164 on 2-D gels (Fig.1). It was reported previously that a precursor of gp23 exists in the form of three
165 intermediates of different molecular weights, i.e. 43, 48.7 and 56 kDa (21). Detection of only two
166 out of three gp23 intermediates may result from transiency of expression of the 43-kDa
167 intermediate. 50 minutes after T4wt infection, we noticed a slight increase in the intensity of one
168 of the two spots identified as the gp23 protein, with a molecular weight of 48.7 kDa. In the case
169 of *rI* and *rIII* mutants, the intensity of the corresponding 48.7-kDa spot was already increased 10
170 minutes following the infection. Then, 50 minutes after the infection with mutant phages, we
171 observed a possible transformation of the 48.7-kDa species into the 56-kDa species (Fig. 2).

172 Some other proteins (phage: A-gt, RIIB, E.6, Gp32 and Gp47; bacterial: EF-Tu 2) which
173 were expressed after 50 min in wild-type T4-infected cells, appeared already 10 min after
174 infection with the phage mutants (Fig. 2). The intensities of the corresponding protein spots in
175 gels separating proteins derived from cells infected with mutants for 10 min were higher than, or

similar to, those observed in gels separating proteins derived from cells infected with the wild-type phage for 50 min. These proteins reached high levels of expression 150 min after infection with wild-type T4 (displayed by higher spot intensities in the gels, data not shown). This indicates that the amounts of these proteins increased more slowly in cells infected with wild-type phages. Therefore, we conclude that expression of genes coding for these proteins is directly or indirectly controlled by RI and RIII proteins.

Interestingly, two of the visualized viral proteins (Vs.6 and E.6) have so far been referred to only as hypothetical proteins on the basis of T4 DNA sequence analyses for potential ORFs. Here, we provide evidence that the corresponding genes are efficiently expressed indeed, but under specific growth conditions supporting slow growth of the host.

Discussion

Bacteriophage T4 development in slowly growing host cells is still relatively poorly understood. In the presented work, we characterized in details the differences in development between phage T4 and its mutants in *rI* and *rIII* genes. Under conditions which support only slow growth of host cells and prevent the bacteriophages from initiating LIN or pseudolysogeny, the phage mutants tended to develop as if they were infecting fastly growing hosts, contrary to wild-type T4, whose intracellular development was significantly slower in slowly growing *E. coli*. In line with our expectations, the burst sizes of all tested phage strains decreased with an increase in doubling time of bacterial cultures. Hadas *et al*. (1997) suggested that the burst size is limited by the rates of synthesis and assembly of phage components and by the time of lysis but not by the bacterial cell size or DNA composition. In our study the greater reduction of burst sizes of

mutants relative to T4wt suggests that functional RI and RIII proteins may be necessary to control the timing of T4 development and the yield of T4. RI and RIII seem to be directly or indirectly responsible for the prolongation of: eclipse period, phage propagation time and latent period and for an increase in the number of progeny particles, which could reflect evolutionary adaptations to conditions encountered by T4 in its natural environment.

To learn more about T4 development and the effects of *rI* and *rIII* dysfunctions, we performed proteomic analyses of phage-infected cells. We found significant differences in the levels of proteins encoded by T4 phage (12 proteins) and the host (6 proteins) between slowly growing *E. coli* cells infected with wild-type and mutant phages. Most of these proteins were expressed either earlier or in higher amounts in cells infected by *rI* and *rIII* mutants of T4, relative to the wild-type phage. The only exceptions were bacterial proteins GatZ, RpoA and AccD. Nevertheless, only one of two forms of RpoA was less abundant in the mutants, suggesting a more rapid modification of this protein in the absence of RI and RIII. Interestingly, among the proteins differentially expressed in cells infected by wild-type and mutant phages there are two replisome components (Gp32, Gp45) and proteins involved in replication and nucleotide metabolism (RpoA, Gp47, UvsX, Gp1). So far, RI and RIII proteins have been considered as directly or indirectly associated with the regulation of phage development based on the interaction of these proteins with holin T. RI binding to T was shown to inhibit the lethal hole-forming function of T (Tran *et al.*, 2005). It was proposed that RIII protein stabilizes this interaction (Golec *et al.*, 2010; Paddison *et al.*, 1998). The results of this study suggest that functional RI and RIII proteins are also necessary to precisely regulate, directly or indirectly, the timing of production of proteins involved in replication and nucleotide metabolism.

Due to the fact that the development was relatively similar in the case of both mutant phages, we hypothesize that both mutants have the same defects in the regulation of T4 development. Most of the differentially expressed proteins are products of middle and late phage genes, however they were expressed relatively early during development in mutant phages. This is perhaps the effect of an early switch to expression of middle and late genes in the mutants. Interestingly, many of the differentially expressed proteins are involved in DNA metabolism and phage morphogenesis (Gp1, Gp32, Gp45, Gp47, UvsX, Gp23). Furthermore, a possible modification of the bacterial RNA polymerase was observed earlier during the development of the mutant phages when compared to the wild-type T4 (compare results of the RpoA analysis). The intensity of the spots corresponding to the above mentioned proteins was higher in cells infected with mutant phages than with the wild-type viruses. Therefore the question arises: why does an excess of DNA replication proteins and capsid components, present shortly after infection of the cell with *rI* and *rIII* mutant phages, result in a reduced burst size? One may speculate that the mutant phages are deficient in an effective resource management. Such phages may consume a large part of the cellular energy and resources for the initial developmental stages, including DNA replication and capsid protein production. Thus, when all phage components are eventually ready to form progeny virions, the host cell may be deprived of energy necessary to finalize production of viral proteins and assemble these components. Contrary to the *rI* and *rIII* mutants, wild type T4 phage may show a less greedy approach. A prolonged development may allow the coordination of the consumption of bacterial resources by the phage with the growth rate of the host cell. Therefore, RI and RIII proteins appear to be important components of the regulatory mechanism devoted to the optimal use of the host resources by developing T4 phages. Regulation of this machinery may be controlled directly or indirectly by RI and/or RIII proteins and influenced by other proteins identified in this work.

The results of proteomic analysis revealed a number of changes in bacterial and phage proteomes after infection. Two of the identified T4 proteins: E.6 and Vs.6 had never been identified before during T4 development. Their identification during development of T4 in slowly growing bacteria in this study suggests that they play some role in this process. Database searches based on both amino acid and nucleotide sequences of E.6 and Vs.6 proteins and the corresponding genes, respectively, reveal conservation of these sequences across various phages. Protein E.6 shares strong similarity with a protein from T4-like phages: e.g. *Enterobacteria* phages AR1, Bp7, IME08, ime09, JS10, JS98 and RB69, *Shigella* phages Shfl2 and SP18 and to unclassified phages: e.g. *Escherichia* phage ECML-134 and *Yersinia* phage phiD1. Protein Vs.6 is highly similar to a protein from T4-like phages: e.g. *Enterobacteria* phages AR1, CC31, ime09, RB14, RB16, RB32, RB43, RB51 and RB69, *Klebsiella* phage KP15 and *Shigella* phage Shfl2. Identification of E.6 and Vs.6 proteins in a slowly growing, infected host may suggest that their expression is devoted to the development of T4-like phages under conditions encountered rather in their natural habitat, thus explaining why the proteins could not have been experimentally identified in previous studies carried out under standard laboratory conditions.

Materials and methods

Bacterial and phage strains

Escherichia coli MG1655 strain (Jensen, 1993) was used in all experiments. Bacteriophage T4wt (our collection), and its otherwise isogenic frameshift mutants: T4*rI* (r48) (Doermann & Hill, 1953) and T4*rIII* (r67) (Edgar *et al.*, 1962) were employed.

Culture media and growth conditions

Bacterial cultures for phage titration were grown overnight in Luria–Bertani (LB) medium at 37 ºC with shaking. Luria–Bertani agar (LA) (Sambrook *et al.*, 1989) was used as a solid medium (1.5% agar in regular plates and 0.7% agar in "top agar" for phage titration). Bacterial cultures used in chemostats were grown in phosphate-buffered (FB) minimal medium with stirring at 37 ºC. FB-mineral salt medium was prepared according to Teich *et al*. (1998) by autoclaving the mineral salts (in g l^{-1}: Na_2SO_4, 2; $(NH_4)_2SO_4$, 2.468; K_2HPO_4, 14.6; Na_2HPO_4 x 2 H_2O, 4; NH_4Cl, 0.5; $(NH_4)_2$-H-citrate, 1.0) in a 10-l flask and subsequent addition of trace elements (stock buffer in g l^{-1}: $CaCl_2$ x 6 H_2O, 0.74; $ZnSO_4$ x 2 H_2O, 0.18; $MnSO_4$ x H_2O, 0.1; EDTA, 20.1; $FeCl_3$ x 6 H_2O, 16.7; $CuSO_4$, 0.1; $CoCl_2$, 0.104) (2 ml l^{-1}), glucose (0.25 g l^{-1}) and thiamine (10 mg l^{-1}) through a 0.22-μm syringe filter.

Titration of bacteriophages and estimation of the number of infected cells

Number of bacteriophages (plaque-forming units, PFUs) and infected cells (infective centers, ICs) were estimated using a standard plaque technique on disposable plastic Petri dishes (diameter, 90 mm) (Merck, Germany). Twenty-five milliliters of bottom LB agar was used. The top agar (4 ml) containing 200 μl of an overnight bacterial culture was poured onto the plate. The plates were used immediately or were stored at 4 ºC. 2.5 μl of serial dilutions of samples from chemostat either untreated (in the case of ICs) or treated with chloroform (in the case of PFUs) were spotted onto a bacterial lawn prepared in a top, soft (0.7%) agar. Plaques were counted after an overnight incubation at 37 ºC.

Chemostat culture conditions

Following inoculation of a fresh mineral salt medium with an overnight culture (1:100), bacteria were grown in 1000 ml of the medium in water-jacketed glass fermenters at 37 ºC, with stirring on a magnetic stirrer (cylindrical shape, 3 cm length) at 300 rpm, aerated by a sterile air flow. A batch culture was started with an initial glucose concentration of 0.5 g l^{-1}. After the initially added glucose was consumed, the glucose-limited chemostat mode was initiated by starting the feed pump at a controlled rate. Volume constancy was maintained by removing excess medium by a faster pump from the surface of the culture. Phage infection experiments were started at steady-state conditions after at least five changes of the total reactor volume. Reaching of steady-state growth by bacterial cultures was verified by monitoring their optical density. The dilution rates (equal to growth rates, μ) used, were: 0.3, 0.2, 0.1 and 0.05 h^{-1}, which was equivalent to the generation time of 2.5, 3.5, 7 and 14 h, respectively.

Kinetics of phage development in the chemostat

5 ml of the bacterial culture from the stabilized chemostat culture were infected with T4 phage (either wt or the mutants) at a multiplicity of infection (m.o.i.) of 5. After 1 min of incubation at 37 ºC, free phage particles were removed by a washing procedure, repeated three times (centrifugation at 4500 x *g* for 1 min at room temperature (RT), resuspension in FB medium pre-warmed to 37 ºC). Next, the infected bacteria were added into the chemostat culture. PFUs (samples treated with chloroform) were estimated 10, 15 and 20 min following the infection and next every 10 min for 300 min. At the end of eclipse and latent periods, samples were estimated every 5 min. The samples for estimation of the number of ICs (samples untreated with chloroform) were collected 10, 15 and 20 min following the infection. Real number of ICs

was calculated by subtracting the number of plaques that were formed by free phages from the total number of plaques of all phages (i.e. phages present inside and outside of the bacterial cells).

Sample preparation for two-dimensional gel electrophoresis

Chemostat cultures at μ = 0.05 were infected with T4 (either wt or mutants) at m.o.i. of 1. Samples for proteomic analyses (80 ml) were collected both before and 10 as well as 50 min after infection. Bacterial cells were harvested by centrifugation (4500 x *g*, 5 min, 4 ºC). The pellet was washed 3 times with a buffer containing 10 mM Tris-HCl pH 7.0 and 250 mM sucrose, at 4 ºC and resuspended in an urea buffer (8 M urea and 2 M thiourea). Cells were then disrupted by ultrasonication for 3 min in an Omni-Ruptor 4000 (OMNI International Inc., Kennesaw, GA, USA) in an ice bath. The soluble protein fraction was separated from cell remnants by centrifugation (20000 x *g* for 30 min at 20 ºC).

2-DE SDS-PAGE and computer analysis

Concentration of proteins was determined using Roti®-Nanoquant (ROTH, Germany). Isoelectric focusing (IEF) was performed in a Multiphor II system (GE Healthcare, UK) with commercially available 18 cm-IPG strips (GE Healthcare, UK) in the pH range of 4 – 7. IPG strips were passively rehydrated at 20 ºC with 300 µl of IEF buffer (8 M urea, 2 M thiourea, 1% w/v CHAPS, 20 mM DTT and 0.5% v/v Bio-Lyte 3/10 Ampholyte) containing 500 µg of protein. The following program was employed for IEF: 1 kVh (500 V), 3 kVh (gradient 500 V – 3500 V), 22.5 kVh (3500 V) at 20 ºC. After IEF, strips were incubated for a total of 30 min in reduction and alkylation buffers (6 M urea; 50 mM Tris, pH 8.8; 30% glycerol; 2% SDS and 2% DTT or 2.5% iodoacetamide with 0.005% bromophenol blue, respectively). SDS-PAGE was carried out

in 25 x 25 cm gels (12.5% resolving gel, 4% stacking gel) using the following program: 4 W per gel for 1 hr; 2 W per gel till the end of electrophoresis. Gels were stained by a modified Coomassie staining procedure according to Kang *et al*. (2002). Image analysis was performed with the use of the DECODON Delta 2D software, version 4.0 (DECODON GmbH, Germany), which is based on the dual-channel image analysis technique described by Bernhardt *et al*. (1999).

In Gel digest

Protein spots were excised from stained 2-D gels manually. Cut spots were transferred into 96-well microtiter plates. The tryptic digest with subsequent spotting on a MALDI-target was carried out automatically with the Ettan Spot Handling Workstation (Amersham Biosciences, Uppsala, Sweden) using the following protocol. Gel pieces were washed twice with 100 µl of a solution of 50% CH_3OH and 50% 50 mM NH_4HCO_3 for 30 min and once with 100 µl 75% CH_3CN for 10 min. After drying at 37 ºC for 17 min, 10 µl trypsin solution containing 20 ng/µl trypsin (Promega, Madison, WI, USA) was added and incubated at 37 ºC for 120 min. For extraction, gel pieces were covered with 60 µl 0.1% TFA in 50% CH_3CN and incubated for 30 min at RT. The peptide-containing supernatant was transferred into a new microtiter plate and the extraction was repeated with 40 µl of the same solution. The supernatant was dried completely at 40 ºC for 220 min. The dry residue was dissolved in 0.9 µl of α-cyano-4-hydroxycinnamic acid matrix (3.3 mg/ml in 50%/49.5%/0.5% (v/v/v) CH_3CN/H_2O/TFA) and 0.7µl of this solution was directly spotted on the MALDI target plate. The samples were allowed to dry on the target 10 to 15 min before measurement in MALDI-TOF.

Mass Spectrometry

MALDI-TOF measurements were carried out on the 4800 MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA). This instrument is designed for high-throughput measurement, being automatically able to measure the samples, calibrate the spectra and analyze the data using the 4800 Explorer^{TM} Software V3.6. The spectra were recorded in a mass range from 900 to 3700 Da with a focus mass of 2000 Da. For one main spectrum, 25 sub-spectra with 100 shots per sub-spectrum were accumulated using a random search pattern. If the autolytic fragment of trypsin with the mono-isotopic (M+H)+ m/z at 2211.104 reached a signal to noise (S/N) ratio of at least 10, an internal calibration was automatically performed as a one-point calibration using this peak. The standard mass deviation was less than 0.15 Da. If the automatic mode failed (in less than 1%) calibration was carried out manually. MALDI-TOF-TOF measurements were also carried out on the 4800 MALDI TOF/TOF Analyzer (Applied Biosystems, Foster City, CA, USA). From the TOF-spectra, the three strongest peaks were measured. For one main spectrum, 20 sub-spectra with 125 shots per sub-spectrum were accumulated using a random search pattern. Internal calibration was automatically performed as one-point calibration with the mono-isotopic Arginine (M+H)+ m/z at 175.119 or Lysine (M+H)+ m/z at 147.107, if it reached an S/N ratio of at least 15. The peak lists were created using GPS Explorer™ Software Version 3.6. The following settings were used for TOF-MS: mass range, 900–3700 Da; peak density, 20 peaks per 200 Da; minimum S/N ratio of 15 and maximum 65 peaks per spot. The TOF-TOF-MS settings were: a mass range from 60 to Precursor - 20 Da; a peak density of 50 peaks per 200 Da and maximum 65 peaks per precursor. The peak list was created for a S/N ratio of 10. For database search, the Mascot search engine Version: 2.1.04 (Matrix Science Ltd, London, UK) with a specific *E. coli* MG1655 sequence database was used.

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Table 1. Parameters of the development of phages T4wt, T4*rI* and T4*rIII* in *E. coli* MG1655 growing at different growth rates [μ]. The presented numbers are mean numbers from 3 experiments ± SD.

μ	Eclipse period [min]	Phage propagation time[a] [min]	Latent period [min]	Burst size
		Bacteriophage T4wt		
0.3	**33** ± 2	47 ± 5	**80** ± 5	**13.1** ± 2
0.2	**35** ± 3	55 ± 5	**90** ± 5	**10** ± 1
0.1	**52** ± 5	93 ± 10	**145** ± 10	**4.8** ± 0.7
0.05	**65** ± 5	110 ± 10	**175** ± 10	**4.2** ± 0.5
		Bacteriophage T4*rI*		
0.3	**40** ± 2	27 ± 3	**67** ± 3	**12** ± 3
0.2	**42** ± 3	30 ± 5	**72** ± 5	**7,7** ± 1
0.1	**50** ± 4	33 ± 5	**83** ± 5	**5** ± 0.2
0.05	**53** ± 3	37 ± 5	**90** ± 5	**1.5** ± 0.5
		Bacteriophage T4*rIII*		
0.3	**41** ± 3	24 ± 4	**65** ± 4	**10** ± 1.2
0.2	**45** ± 3	27 ± 5	**72** ± 5	**6** ± 0.9
0.1	**50** ± 4	32 ± 5	**82** ± 5	**3** ± 1
0.05	**57** ± 5	33 ± 5	**90** ± 5	**1** ± 0.5

[a] Phage propagation time (period for intracellular assembly of phage particles) was calculated by subtraction of the length of the eclipse period from the length of the latent period.

Table 2. Identified proteins encoded by phage T4. Arrow ⇧ indicates proteins whose amounts increased after cell infection with T4 *rI* or *rIII* mutants relative to wild-type T4. ND, not determined.

Protein name, (short name)	Effect	Accession number	M_r (kDa)	pI	Sequence Coverage %[a]	Protein Score[b]	Functional category
Alpha glucosyl transferase, (**A-gt**)	⇧	NP_049673.1	46.7	6.11	71	581	Host or phage interactions
Protector from prophage-induced early lysis, (**RIIB**)	⇧	NP_049889.1	35.5	6.04	57	477	Host or phage interactions
Conserved hypothetical protein, (**E.6**)	⇧	NP_049742.1	22	6.06	71	814	ND
DNMP kinase, (**Gp1**)	⇧	NP_049752.1	27.3	5.06	56	134	Nucleotide metabolism
Major head protein, (**Gp23**)	⇧	NP_049787.1	55.9	5.34	52	625	Virion protein
RecA-like recombination protein, (**UvsX**)	⇧	NP_049656.2	43.9	5.31	63	504	DNA replication, recombination, repair and processing
Single-stranded DNA binding protein, (**Gp32**)	⇧	NP_049854.1	33.5	4.82	52	328	DNA replication, repair and recombination
Recombination endonuclease subunit, (**Gp47**)	⇧	NP_049672.1	39.1	5.04	36	158	DNA replication, repair and recombination
Sliding clamp, DNA polymerase accessory protein, (**Gp45**)	⇧	NP_049666.1	24.8	4.89	41	261	DNA replication, repair and recombination
Conserved hypothetical protein, (**Vs.6**)	⇧	NP_049730.1	13.8	5.71	88	314	ND
DsDNA binding protein, late transcription, (**DsbA**)	⇧	NP_049858.1	10.4	5.04	93	249	Transcription
Protector from prophage-induced early lysis,(**RIIA**)	⇧	NP_049616.1	82.8	5.97	31	592	Host or phage interactions

[a] The sequence coverage gives the percentage of the protein sequence covered by the peptides measured for the specific protein.

[b] The protein score is a measure of certainty for the identification of a protein calculated by Mascot. For this experiment, protein scores greater than 49 were significant ($p<0.05$).

Table 3. Identified proteins encoded by *E. coli*. Arrows indicate proteins whose amount increased (⇧) or decreased (⇩) after infection of cells with T4 *rI* or *rIII* mutants relative to wild-type T4. Two arrows (⇧⇩) indicate proteins displaying an increase in protein amount after infection with T4 mutants in one corresponding spot and a decrease in the second corresponding spot.

Protein name, (short name)	Effect	Accession number	M_r (kDa)	pI	Sequence Coverage %[a]	Protein Score[b]	Protein function/category
D-tagatose 1,6-bisphosphate aldolase 2, subunit, (**GatZ**)	⇩	NP_416598.1	47.1	5.5	11	196	Catalytic activity, catabolism of galactitol
RNA polymerase, alpha subunit, (**RpoA**)	⇧⇩	NP_417754.1	36.5	4.98	77	620	Transcription
Protein chain elongation factor Tu 2, (**EF-Tu2**)	⇧	NP_418407.1	43.3	5.3	62	541	GTP binding, GTPase activity, translation elongation factor activity
DNA-binding response regulator in two-component regulatory system with ArcB or CpxA, (**ArcA**)	⇧	NP_418818.1	27.3	5.21	60	354	Global regulatory functions, DNA binding
Global DNA-binding transcriptional dual regulator, (**H-NS**)	⇧	NP_415753.1	15.5	5.43	70	342	Regulation of transcription, DNA binding
Acetyl-CoA synthetase, (**AccD**)	⇩	NP_418493.1	72	5.5	48	512	Fatty acid and phosphatidic acid biosynthesis

[a] The sequence coverage gives the percentage of the protein sequence covered by the peptides measured for the specific protein.

[b] The protein score is a measure of certainty for the identification of a protein calculated by Mascot. For this experiment, protein scores greater than 49 were significant ($p<0.05$).

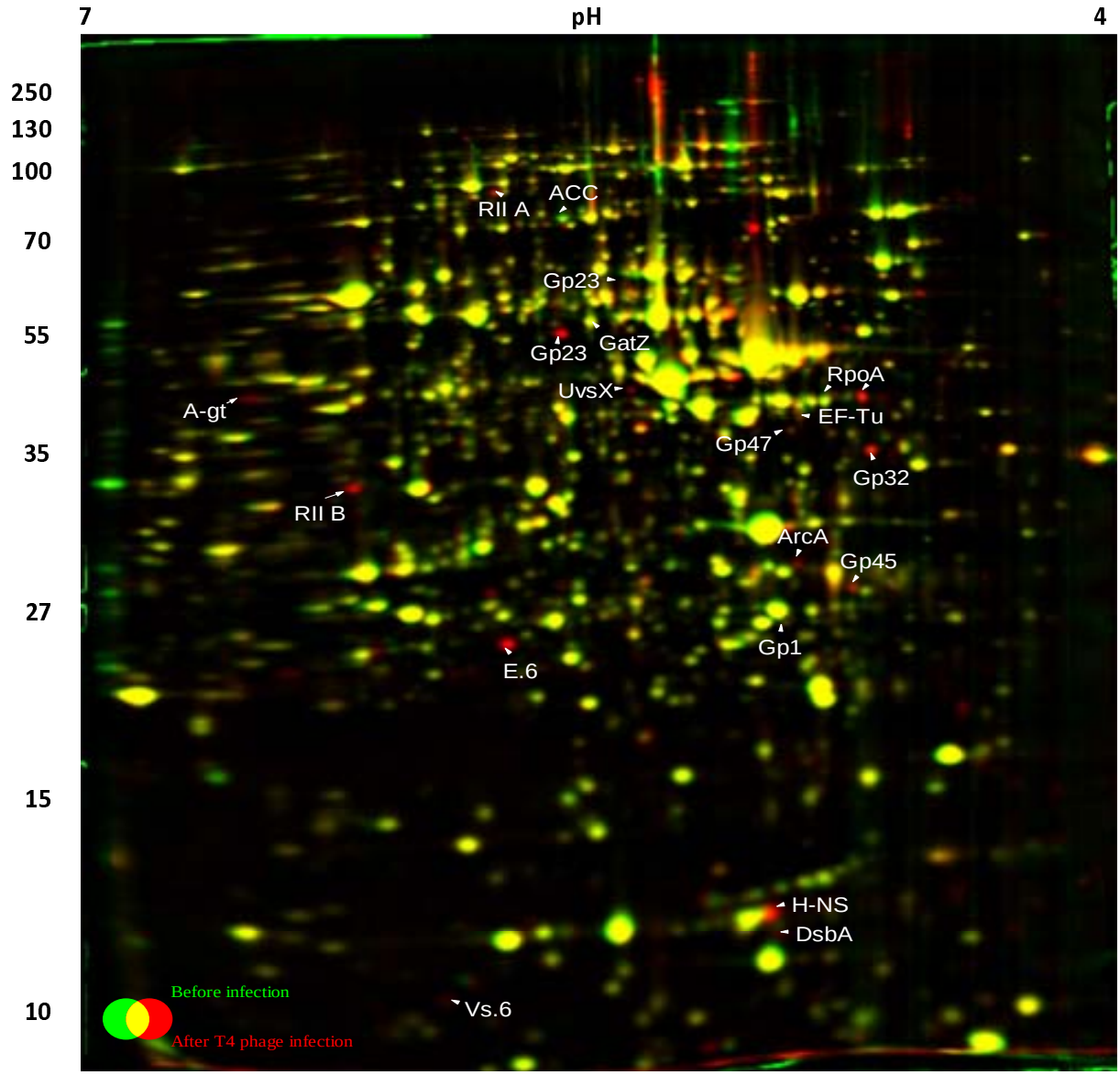

Figure 1. Representative dual-channel image of 2-D gels before infection (green) and after infection with T4*rI* phage (red) of slowly growing *E. coli* MG1655 cells (μ = 0.05, generation time = 14 h). IPG strips with pH 4 – 7 were used for separation of the intracellular soluble protein fraction in the first dimension. Molecular masses (in kDa) are indicated on the left side of the image. Arrows indicate positions of differentially expressed proteins. Names are the same as short names in Tables 2 and 3.

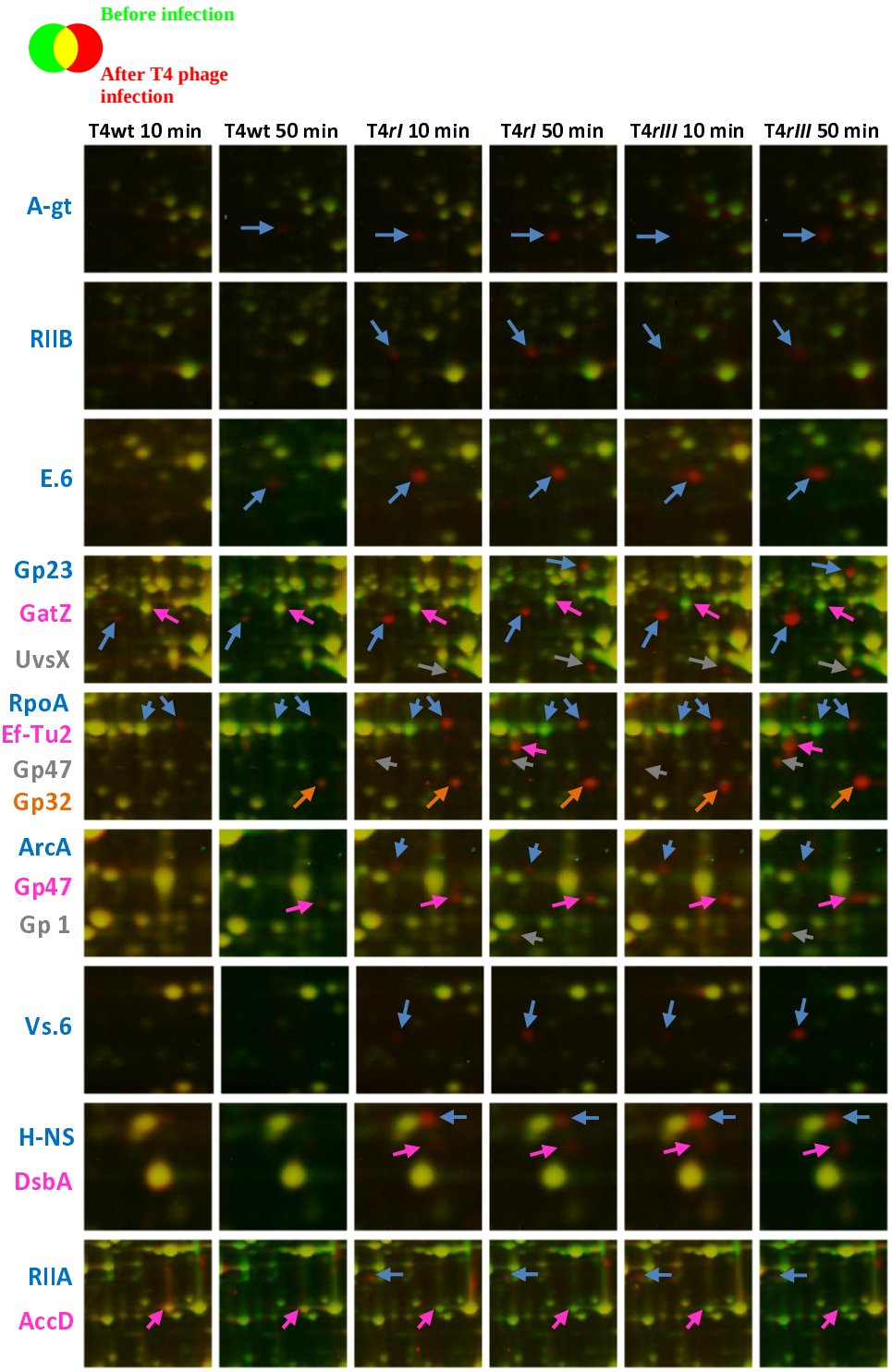

Figure 2. Fragments of dual-channel images of 2-DE gels before infection (green) and 10 and 50 minutes after infection with T4wt and *rI* and *rIII* mutants (red) of slowly growing *E. coli* MG1655 (μ = 0.05, generation time = 14 h). Names of proteins are the same as short names in Tables 2 and 3. Identified proteins are indicated by arrows of the same color.