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

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Received 4 October 2012
Accepted 11 December 2012

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 the 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 (T4rI) and rIII (T4rIII) mutants in slowly growing bacteria, where T4 did not initiate LIN or pseudolysogeny. We determined eclipse periods, phage propagation times, latent periods and burst sizes of T4wt, T4rI and T4rIII. We also compared intracellular proteomes of slowly growing Escherichia coli infected with either T4wt or the mutants. Using two-dimensional PAGE analyses we found 18 differentially expressed proteins from lysates of infected cells. Proteins whose amounts were different in cells harbouring 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 phage T4 development in slowly growing bacteria. This regulation may be more complicated than previously anticipated, with many factors 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 min, releasing 100–200 progeny virions per infected cell (Abedon, 1994). The aim of this development, referred to as the short-latent-period 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 the 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 the exclusion of a superinfecting phage, lysis inhibition (LIN) 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, 2002). Using different media to control the bacterial growth rate, it was revealed that with a decreasing growth rate, 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 LIN (Bode, 1967). This phenomenon is employed when the already infected bacterial cell is again infected by another T-even phage at least 3 min 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 pseudolyisogeny (Golec et al., 2011; Kutter et al., 1994; Łos et al., 2003; Łos & 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. The destruction of cells is connected to 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, 2007). Holin T interacts with phage antiholin RI and this is necessary to start the LIN mechanism (Tran et al., 2005, 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, RI1 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 also involved in pseudolyisogeny.

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 the environment (Abedon, 1994; Abedon et al., 2001, 2003; Golec et al., 2010, 2011; Hadas et al., 1997; Kutter et al., 1994; Łos et al., 2003; Łos & Węgrzyn, 2012; Paddison et al., 1998; Rabinovitch et al., 1999, 2002). While RI and RIII are known to play a role in LIN and in the pseudolyisogeny 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 the intracellular assembly of phage particles), latent period and burst size in a slowly growing bacterial culture where neither LIN nor pseudolyisogeny were initiated. We investigated whether the products of rI and rIII genes, known to be involved in LIN and pseudolyisogeny, also participate in the regulation of phage development in slowly growing host cells.

**RESULTS**

**Kinetics of development of wild-type T4 (T4wt) and its mutants in slowly growing bacterial cells**

Bacteriophage T4 development depends on the physiology of its host *Escherichia coli*. T4wt is able to adapt to the growth rate of a bacterial culture by prolonging its development or by initiating LIN or pseudolyisogeny mechanisms (Golec et al., 2010, 2011; Hadas et al., 1997; Kutter et al., 1994; Łos et al., 2003; Łos & Węgrzyn, 2012; Paddison et al., 1998; Rabinovitch et al., 1999, 2002; Tran et al., 2005). Previously, we found differences in the rapidity of lysis of a slowly growing bacterial culture triggered by T4wt and its rI (Łos et al., 2003) and rIII mutants (Golec, 2010). It was hypothesized that T4rI and T4rIII mutants cannot precisely regulate their development in response to the host metabolic status (Golec, 2010; Łos et al., 2003). In this study, we analysed the development of T4wt, T4rI and T4rIII in detail, determining their eclipse periods, phage propagation times, latent periods and burst sizes in a slowly growing bacterial culture, where neither LIN nor pseudolyisogeny 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 an m.o.i. of 5, followed by incubation for 1 min. It should be noted, however, that within the period of incubation only about 30% of the 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 the 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 set up conditions for collecting samples from the chemostat cultures for proteomic studies.

The parameters of phage T4wt, T4rI and T4rIII infection of slowly growing hosts are presented in Table 1. We found that the development of phage T4wt differs from that of its mutants with respect to the eclipse period, phage propagation time, latent period and burst size. Moreover, the lower the growth rate of the host, the bigger the differences were between the developmental parameters of T4wt and the mutants T4rI and T4rIII. 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 T4wt and its mutants in slowly growing bacterial cells

In order to further characterize differential developments of phage T4wt and its mutants, we decided to employ two-dimensional (2D) gel-based proteomics (Görg et al., 1999; Thürmer et al., 2011). A bacterial growth rate of 0.05 was chosen for the proteomic analysis, since at this rate, the biggest differences were observed between the development rates of T4wt and the mutants (Table 1). We added phage to chemostats to a final m.o.i. of 1 at time 0. We analysed proteomes from samples collected 10 and 50 min after phage infection, which correlated with the eclipse period and the end of the latent period of mutants, respectively. The decision to collect the samples 10 min after the infection was also justified by the observation that the adsorption of the majority of phage particles occurred within 5 min 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 T4wt and those infected with T4rI or T4rIII phage mutants, which corresponded to 18 different proteins, as some of the spots were variants of the same protein (Figs 1 and 2). Seventeen of the detected differences represented spots of increased intensity with T4rI and T4rIII infections in comparison with T4wt infection. Three protein spots were of decreased intensity after infection with rI and rIII phage mutants. Tables 2 and 3 present the identified phage-encoded and bacterial proteins, respectively, displaying differential expression between T4wt- and mutant-infected cells.

As mentioned above, the amounts of three bacterial proteins (i.e. GatZ, RpoA and AccD) were decreased in T4rI- and T4rIII-infected cells, relative to the levels in T4wt-infected cells. It should be noted, however, that RpoA was identified in two of the analysed protein spots (Fig. 1). The intensity of one of these spots was decreased after infection with rI and rIII phage mutants. At the same time, the intensity of the other RpoA spot increased in T4rI- and T4rIII-infected cells. This could reflect a modification of the alpha subunit of RNA polymerase, which occurred significantly faster in both phage mutants than in the T4wt phage.

The viral protein Gp23 (major head protein) was also identified in two different positions on 2D gels (Fig. 1). It

<table>
<thead>
<tr>
<th>Phage</th>
<th>µ</th>
<th>Eclipse period (min)</th>
<th>Phage propagation time* (min)</th>
<th>Latent period (min)</th>
<th>Burst size</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4wt</td>
<td>0.3</td>
<td>33 ± 2</td>
<td>47 ± 5</td>
<td>80 ± 5</td>
<td>13.1 ± 2</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>35 ± 3</td>
<td>55 ± 5</td>
<td>90 ± 5</td>
<td>10 ± 1</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>52 ± 5</td>
<td>93 ± 10</td>
<td>145 ± 10</td>
<td>4.8 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>65 ± 5</td>
<td>110 ± 10</td>
<td>175 ± 10</td>
<td>4.2 ± 0.5</td>
</tr>
<tr>
<td>T4rI</td>
<td>0.3</td>
<td>40 ± 2</td>
<td>27 ± 3</td>
<td>67 ± 3</td>
<td>12 ± 3</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>42 ± 3</td>
<td>30 ± 5</td>
<td>72 ± 5</td>
<td>7.7 ± 1</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>50 ± 4</td>
<td>33 ± 5</td>
<td>83 ± 5</td>
<td>5 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>53 ± 3</td>
<td>37 ± 5</td>
<td>90 ± 5</td>
<td>1.5 ± 0.5</td>
</tr>
<tr>
<td>T4rIII</td>
<td>0.3</td>
<td>41 ± 3</td>
<td>24 ± 4</td>
<td>65 ± 4</td>
<td>10 ± 1.2</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>45 ± 3</td>
<td>27 ± 5</td>
<td>72 ± 5</td>
<td>6 ± 0.9</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>50 ± 4</td>
<td>32 ± 5</td>
<td>82 ± 5</td>
<td>3 ± 1</td>
</tr>
<tr>
<td></td>
<td>0.05</td>
<td>57 ± 5</td>
<td>33 ± 5</td>
<td>90 ± 5</td>
<td>1 ± 0.5</td>
</tr>
</tbody>
</table>

*The phage propagation time (period for intracellular assembly of phage particles) was calculated by subtracting the length of the eclipse period from the length of the latent period.

Table 1. Parameters of the development of phages T4wt, T4rI and T4rIII in E. coli MG1655 cells growing at different growth rates (µ)
was reported previously that a precursor of Gp23 exists in the form of three intermediates of different molecular masses, i.e. 43, 48.7 and 56 kDa (Miller et al., 2003). Detection of only two out of three Gp23 intermediates may result from transiency of expression of the 43 kDa intermediate. Fifty minutes after T4wt infection, we noticed a slight increase in the intensity of one of the two spots identified as the Gp23 protein, which corresponded to a molecular mass of 48.7 kDa. In the case of rI and rIII mutants, the intensity of the corresponding 48.7 kDa spot was already increased 10 min following the infection. Then, 50 min after infection with mutant phages, we observed a possible transformation of the 48.7 kDa species into the 56 kDa species (Fig. 2).

Some other proteins (phage: A-gt, RIIB, E.6, Gp32 and Gp47; bacterial: EF-Tu2) that were expressed after 50 min in T4wt-infected cells appeared after 10 min in mutant-infected cells (Fig. 2). The intensities of the corresponding protein spots in gels separating proteins derived from mutant-infected cells 10 min post-infection were higher than, or similar to, those spots observed in gels separating proteins derived from T4wt-infected cells 50 min post-infection. These proteins reached high levels of expression 150 min after infection with T4wt (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 phage. Therefore, we conclude that the expression of genes encoding these proteins is directly or indirectly controlled by RI and RIII proteins.

Interestingly, two of the visualized viral proteins (vs6 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 indeed efficiently expressed, 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,
Table 2. Identified proteins encoded by phage T4

The levels of all proteins listed in the table increased with T4rI or T4rIII cell infection relative to the levels in T4wt cell infection. ND, Not determined; pI, isoelectric point.

<table>
<thead>
<tr>
<th>Protein name (short name)</th>
<th>Accession no.</th>
<th>Mr (kDa)</th>
<th>pI</th>
<th>Sequence coverage (%)*</th>
<th>Protein score†</th>
<th>Functional category</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha glucosyltransferase (A-gt)</td>
<td>NP_049673.1</td>
<td>46.7</td>
<td>6.11</td>
<td>71</td>
<td>581</td>
<td>Host or phage interactions</td>
</tr>
<tr>
<td>Protector from prophage-induced early lysis (RIIB)</td>
<td>NP_049889.1</td>
<td>35.5</td>
<td>6.04</td>
<td>57</td>
<td>477</td>
<td>Host or phage interactions</td>
</tr>
<tr>
<td>Conserved hypothetical protein (E.6)</td>
<td>NP_049742.1</td>
<td>22</td>
<td>6.06</td>
<td>71</td>
<td>814</td>
<td>ND</td>
</tr>
<tr>
<td>DNMP kinase (Gp1)</td>
<td>NP_049752.1</td>
<td>27.3</td>
<td>5.06</td>
<td>56</td>
<td>134</td>
<td>Nucleotide metabolism</td>
</tr>
<tr>
<td>Major head protein (Gp23)</td>
<td>NP_049787.1</td>
<td>55.9</td>
<td>5.34</td>
<td>52</td>
<td>625</td>
<td>Virion protein</td>
</tr>
<tr>
<td>RecA-like recombination protein (UvsX)</td>
<td>NP_049656.2</td>
<td>43.9</td>
<td>5.31</td>
<td>63</td>
<td>504</td>
<td>DNA replication, recombination, repair and processing</td>
</tr>
<tr>
<td>Single-stranded DNA-binding protein (Gp32)</td>
<td>NP_049854.1</td>
<td>33.5</td>
<td>4.82</td>
<td>52</td>
<td>328</td>
<td>DNA replication, repair and recombination</td>
</tr>
<tr>
<td>Recombination endonuclease subunit (Gp47)</td>
<td>NP_049672.1</td>
<td>39.1</td>
<td>5.04</td>
<td>36</td>
<td>158</td>
<td>DNA replication, repair and recombination</td>
</tr>
<tr>
<td>Sliding clamp, DNA polymerase accessory protein (Gp45)</td>
<td>NP_049666.1</td>
<td>24.8</td>
<td>4.89</td>
<td>41</td>
<td>261</td>
<td>DNA replication, repair and recombination</td>
</tr>
<tr>
<td>Conserved hypothetical protein (vs6)</td>
<td>NP_049730.1</td>
<td>13.8</td>
<td>5.71</td>
<td>88</td>
<td>314</td>
<td>ND</td>
</tr>
<tr>
<td>DsDNA-binding protein (DsbA)</td>
<td>NP_049858.1</td>
<td>10.4</td>
<td>5.04</td>
<td>93</td>
<td>249</td>
<td>Transcription</td>
</tr>
<tr>
<td>Protector from prophage-induced early lysis (RIIA)</td>
<td>NP_049616.1</td>
<td>82.8</td>
<td>5.97</td>
<td>31</td>
<td>592</td>
<td>Host or phage interactions</td>
</tr>
</tbody>
</table>

*The sequence coverage gives the percentage of the protein sequence covered by the peptides measured for the specific protein.
†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 increases (↑) or decreases (↓) in the level of a protein after infection of cells with T4 *rI* or *rIII* mutants relative to that of T4wt. 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.

<table>
<thead>
<tr>
<th>Protein (short name)</th>
<th>Effect</th>
<th>Accession no.</th>
<th>M_r (kDa)</th>
<th>pI</th>
<th>Sequence coverage (%)*</th>
<th>Protein score†</th>
<th>Protein function/category</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-tagatose 1,6-bisphosphate aldolase 2, subunit (GatZ)</td>
<td>↓</td>
<td>NP_416598.1</td>
<td>47.1</td>
<td>5.5</td>
<td>11</td>
<td>196</td>
<td>Catalytic activity, catabolism of galactitol</td>
</tr>
<tr>
<td>RNA polymerase, alpha subunit (RpoA)</td>
<td>↑↓</td>
<td>NP_417754.1</td>
<td>36.5</td>
<td>4.98</td>
<td>77</td>
<td>620</td>
<td>Transcription</td>
</tr>
<tr>
<td>Protein chain elongation factor Tu 2 (EF-Tu2)</td>
<td>↑</td>
<td>NP_418407.1</td>
<td>43.3</td>
<td>5.3</td>
<td>62</td>
<td>541</td>
<td>GTP binding, GTPase activity, translation elongation factor activity</td>
</tr>
<tr>
<td>DNA-binding response regulator in two-component regulatory system with ArcB or CpxA (ArcA)</td>
<td>↑</td>
<td>NP_418818.1</td>
<td>27.3</td>
<td>5.21</td>
<td>60</td>
<td>354</td>
<td>Global regulatory functions, DNA binding</td>
</tr>
<tr>
<td>Global DNA-binding transcriptional dual regulator (H-NS)</td>
<td>↑</td>
<td>NP_415753.1</td>
<td>15.5</td>
<td>5.43</td>
<td>70</td>
<td>342</td>
<td>Regulation of transcription, DNA binding</td>
</tr>
<tr>
<td>Acetyl-CoA synthetase (AccD)</td>
<td>↓</td>
<td>NP_418493.1</td>
<td>72</td>
<td>5.5</td>
<td>48</td>
<td>512</td>
<td>Fatty acid and phosphatidic acid biosynthesis</td>
</tr>
</tbody>
</table>

*The sequence coverage gives the percentage of the protein sequence covered by the peptides measured for the specific protein.
†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).
we characterized, in detail, the differences in development between phage T4 and its \( rI \) and \( rIII \) mutants. Under conditions which support only the slow growth of host cells and which prevent the bacteriophages from initiating LIN or pseudolysogeny, the phage mutants tended to develop as if they were infecting fast-growing hosts, contrary to T4wt, whose intracellular development was significantly slower in slowly growing \( E. \) \( coli \) cells. In line with our expectations, the burst sizes of all tested phage strains decreased with an increase in the 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 that of T4wt suggests that functional \( RII \) 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 prolonging the 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 (six 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 T4\( rI \) and T4\( rIII \) mutants, relative to the levels in the T4wt-infected cells. 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 the \( RII \) 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 of the 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 with that of T4wt (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 in those infected 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 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 the energy necessary to finalize the production of viral proteins and assemble these components. Contrary to the \( rI \) and \( rIII \) mutants, T4wt 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 host resources by developing T4 phage. 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 vs6, had never before been identified during T4 development. Their identification during the 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 vs6 proteins and the corresponding genes revealed the 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 Shf12 and SP18) and with unclassified phages (e.g. \( Escherichia \) phage ECML-134 and \( Yersinia \) phage phiD1). Protein vs6 is highly similar to a protein from T4-like phages: e.g. \( Enterobacteria \) phages AR1, Bp7, IME08, ime09, JS10, JS98 and RB69; \( Shigella \) phages Shf12 and SP18) and with unclassified phages (e.g. \( Escherichia \) phage ECML-134 and \( Yersinia \) phage phiD1). Protein vs6 is highly similar to a protein from T4-like phages: e.g. \( Enterobacteria \) phages AR1, Bp7, IME08, ime09, JS10, JS98 and RB69; \( Shigella \) phages Shf12 and SP18) and with unclassified phages (e.g. \( Escherichia \) phage ECML-134 and \( Yersinia \) phage phiD1).
in previous studies carried out under standard laboratory conditions.

**METHODS**

**Bacterial and phage strains.** *E. coli* MG1655 strain (Jensen, 1993) was used in all experiments. Bacteriophage T4wt (our collection) and its otherwise isogenic frameshift mutants, T4rI (148) (Doermann & Hill, 1953) and T4rIII (667) (Edgar et al., 1962), were also used.

**Culture media and growth conditions.** Bacterial cultures for phage titration were grown overnight in Luria–Bertani (LB) medium at 37 °C with shaking. LB agar (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 °C. FB-mineral salt medium was prepared according to Teich et al. (1998), by autoclaving the mineral salts [in g l⁻¹: Na₂SO₄, 2; (NH₄)₂SO₄, 2.468; K₂HPO₄, 14.6; NaH₂PO₄, 2H₂O, 4; NH₄Cl, 0.3; (NH₄)₂-H-citrate, 1.0] in a 10 l flask and the subsequent addition of 2 ml l⁻¹ trace elements (stock buffer in g l⁻¹: CaCl₂, 6H₂O, 0.74; ZnSO₄, 2H₂O, 0.18; MnSO₄, H₂O, 0.1; EDTA, 20.1; FeCl₃, 6H₂O, 16.7; CuSO₄, 0.1; CoCl₂, 10.4) 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.** The numbers of bacteriophages (p.f.u.) and infected cells (infective centres, ICs) were estimated using a standard plaque technique on disposable plastic Petri dishes (90 mm diameter; Merck). Twenty-five millilitres 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 °C. Serial dilutions (2.5 μl) of samples from the chemostat, either untreated (in the case of ICs) or treated with chloroform (in the case of p.f.u.), were spotted onto a bacterial lawn prepared in a top, soft (0.7 %) agar. Plaques were counted after an overnight incubation at 37 °C.

**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 °C, with stirring on a magnetic stirrer (cylindrical shape, 3 cm length) at 300 r.p.m., aerated by a sterile air flow. A batch culture was started with an initial glucose concentration of 0.5 g l⁻¹ and the subsequent addition of 2 ml l⁻¹ trace elements (stock buffer in g l⁻¹: CaCl₂, 6H₂O, 0.74; ZnSO₄, 2H₂O, 0.18; MnSO₄, H₂O, 0.1; EDTA, 20.1; FeCl₃, 6H₂O, 16.7; CuSO₄, 0.1; CoCl₂, 10.4) glucose (0.25 g l⁻¹) and thiamine (10 mg l⁻¹) through a 0.22 μm syringe filter.

**Kinetics of phage development in the chemostat.** Five millilitres of the bacterial culture from the stabilized chemostat culture was infected with T4 phage (either wild-type or mutant) at an m.o.i. of 5. After 1 min of incubation at 37 °C, free phage particles were removed by a washing procedure, repeated three times (centrifugation at 4500 g for 1 min at room temperature and then resuspension in FB medium pre-warmed to 37 °C). Next, the infected bacteria were added into the chemostat culture. The p.f.u. (samples treated with chloroform) was estimated 10, 15 and 20 min after infection and then every 10 min for 300 min. At the end of eclipse and latent periods, samples were estimated every 5 min. The samples for estimating the number of ICs (samples untreated with chloroform) were collected at 10, 15 and 20 min after infection. The real number of ICs was calculated by subtracting the number of plaques that were formed by free phage from the total number of plaques of all phage (i.e. phage present inside and outside of the bacterial cells).

**Sample preparation for 2D gel electrophoresis.** Chemostat cultures at μ=0.05 were infected with T4 (either wild-type or mutant) at an m.o.i. of 1. Samples for proteomic analyses (80 ml) were collected both before infection and 10 and 50 min after infection. Bacterial cells were harvested by centrifugation (4500 g, 5 min, 4 °C). The pellet was washed three times with a buffer containing 10 mM Tris/HCl pH 7.0 and 250 mM sucrose at 4 °C and was resuspended in a 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) in an ice bath. The soluble protein fraction was separated from cell remnants by centrifugation (20000 g for 30 min at 20 °C).

**2D SDS-PAGE and computer analysis.** The concentration of proteins was determined using Roti-Nanoquant (ROTH). Isoelectric focusing (IEF) was performed in a Multiphor II system (GE Healthcare) with commercially available 18-cm immobilized pH gradient (IPG) strips (GE Healthcare) in the pH range of 4–7. IPG strips were passively rehydrated at 20 °C with 300 μl 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 50 μg protein. The following program was employed for IEF: 1 kWh (500 V), 3 kWh (gradient 500–3500 V), 22.5 kWh (3500 V) at 20 °C. 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 %, v/v, glycerol; 2 % SDS; and 2 % DTT or 2.5 % iodoacetamide with 0.005 % bromophenol blue, respectively). SD-PAGE was carried out in 25 × 25 cm gels (12.5 % resolving gel, 4 % stacking gel) using the following program: 4 W per gel for 1 h; 2 W per gel until 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), 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 2D gels manually. Cut-out spots were then transferred into 96-well microtitre plates. The tryptic digest with subsequent spotting on a matrix-assisted laser desorption/ionization (MALDI) target was carried out automatically with the Ettan Spot Handling Workstation (Amersham Biosciences) using the following protocol. Gel pieces were washed twice with 100 μl 50 % CH₃OH and 50 % 50 mM NH₄HCO₃ solution, for 30 min and once with 100 μl 75 % CH₃CN for 10 min. After drying at 37 °C for 17 min, 10 μl trypsin solution containing 20 ng trypsin (Promega) μ⁻¹ was added and incubated at 37 °C for 120 min. For extraction, gel pieces were covered with 60 μl 0.1 % TFA in 50 % CH₃CN and incubated for 30 min at room temperature. The peptide-containing supernatant was transferred into a new microtitre plate and the extraction was repeated with 40 μl of the same solution. The supernatant was dried completely at 40 °C for 220 min. The dry residue was dissolved in 0.9 ml c-cyano-4-hydroxycinnamic acid matrix [3.5 mg ml⁻¹ in 50:49.5:0.5 % (by vol.) CH₃CN/H₂O/TFA] and 0.7 μl of this solution was directly spotted on the MALDI target plate. The samples were allowed to dry on the target 10–15 min before measurement in a MALDI-time-of-flight (TOF) mass spectrometer.

**Mass spectrometry (MS).** MALDI-TOF measurements were carried out on the 4800 MALDI TOF/TOF Analyser (Applied Biosystems). This instrument is designed for high-throughput measurement, being...
ACKNOWLEDGEMENTS

This work was supported by the Polish Ministry of Science and Higher Education via a Iuventus Plus grant (IP2011 015071) to P. Golec and by the European Union within the European Regional Development Fund, through the Innovative Economy grant (POIG.01.01.02-00-008/08). J. K. G. was supported by the Pomeranian Special Economy Zone scholarship for young scientists. P. G. thanks Anjie Gardebrecht for introducing him to the use of the Delta 2D software and Susanne Gebrauer for excellent technical assistance.

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