Expression of a Novel P22 ORFan Gene Reveals the Phage Carrier State in Salmonella Typhimurium

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Abstract

We discovered a novel interaction between phage P22 and its host Salmonella Typhimurium LT2 that is characterized by a phage mediated and targeted derepression of the host dgo operon. Upon further investigation, this interaction was found to be instigated by an ORFan gene (designated pid for phage P22 encoded instigator of dgo expression) located on a previously unannotated moron locus in the late region of the P22 genome, and encoding an 86 amino acid protein of 9.3 kDa. Surprisingly, the Pid/dgo interaction was not observed during strict lytic or lysogenic proliferation of P22, and expression of pid was instead found to arise in cells that upon infection stably maintained an unintegrated phage chromosome that segregated asymmetrically upon subsequent cell divisions. Interestingly, among the emerging siblings, the feature of pid expression remained tightly linked to the cell inheriting this phage carrier state and became quenched in the other. As such, this study is the first to reveal molecular and genetic markers authenticating pseudolysogenic development, thereby exposing a novel mechanism, timing, and populational distribution in the realm of phage–host interactions.

Introduction

Due to billions of years of co-evolution and their overpowering abundance in the biosphere, viruses of bacteria (i.e. bacteriophages or phages) have a profound impact on the conduct and ecology of their hosts [1,2]. Lytic proliferation of phages for example can affect host mutation rates [3], structure microbial consortia [4], and contribute significantly to the global biogeochemical carbon flux [5]. Lysogenic proliferation as stable prophages, on the other hand, increases the genetic repertoire and genome plasticity of the host, thereby often extending its adaptive potential in terms of virulence and ecological fitness [6].

While the basic molecular events and genetic circuitry behind lytic and lysogenic development have traditionally received a lot of attention and are reasonably well understood for a number of model phages [7–9], the increasing wealth of novel phage genes with no known homologs and function nevertheless suggests an unforeseen intricacy in phage – host interactions [1,10]. Furthermore, in many ecological niches phage – host associations often appear to defy the classical bifurcation into strict lytic or lysogenic development, as a large number of reports indicate a lysogeny-independent but stable co-existence between phages and their hosts. These phenomena are often vaguely referred to as pseudolysogeny, and hypothesize the existence of stable “phage carrier” cells in which the incoming phage has temporarily refrained from lytic or lysogenic development [11]. This suspended state is believed to play an important role in the long term survival strategy of viruses, as it might (i) prevent poor replication or even degradation of the phage chromosome in a host that is too starved to support further steps in lytic or lysogenic development, and/or (ii) provide a transient intracellular refuge for the phage chromosome in environments characterized by low host densities and short capsid half-lives [12,13]. Despite its ecological importance [11,14], however, no formal molecular evidence currently exists for the presence of such a state, let alone its possible impact on the physiology of the cell.

In this study, we extend on the intricacy of phage – host interactions and provide both genetic and direct cell biological evidence for the existence of a dedicated pseudolysogenic state in the Salmonella Typhimurium – phage P22 model system.

Results

MudK mutagenesis of Salmonella Typhimurium LT2 reveals a clone that responds to infection by phage P22

During routine screening of a MudK based lacZ promoter-trap library in Salmonella Typhimurium LT2 on LB X-Gal agar plates, our attention was drawn to a colony displaying an inhomogeneous distribution of LacZ activity (i.e. blue coloration; Figure 1A) that was neither symmetrical, nor sectorial. Moreover, after streaking...
out on new LB X-Gal plates, this particular clone segregated both into plain white colonies and colonies with an irregular blue coloration similar to that of the parent colony (Figure 1B). Interestingly, however, when the latter colonies were replicated on green indicator agar, the blue patches on LB X-Gal agar overlapped perfectly with the dark green sites of cell lysis that were revealed by the green indicator agar (compare Figure 1B and 1C). As we reasonably assumed this cell lysis to stem from infection by residual P22 HT105/1 int201 transducing phage that was initially used to deliver the MuΔK element during construction of the library, we hypothesized LacZ activity of the isolated clone to be triggered by exposure to phage P22.

In order to further examine this phenotype, the MuΔK insertion of the corresponding clone was transduced into a fresh LT2 strain and a phage-free transductant (designated LT2K7) was streaked across wild-type P22 (P22 wt) on LB X-Gal agar (Figure 1D). As a result, we found LT2K7 to turn from white to blue upon encountering P22, suggesting that phage P22 is causally involved in triggering lacZ expression in LT2K7.

MuΔK of LT2K7 maps to the dgoT gene

The MuΔK insertion site of LT2K7 was mapped to the dgoRK1T operon. DNA sequence analysis revealed that the MuΔK insertion resulted in a translational fusion of the lacZ reporter gene to dgoT (Figure 2A). The dgoR gene located at the beginning of the operon is predicted to function as an antiterminator [15] and indeed LT2K7 ΔdgoK constitutively expressed the dgoT::MuΔK fusion (Figure 2B), regardless of infection by P22 wt. Furthermore, increasing the level of DgoR by providing the corresponding gene on a multicopy plasmid (pFPV-dgoRK) was able to abolish induction of dgoT::MuΔK by P22 wt, but had no obvious effect on phage infection per se (Figure 2C). These data suggest that infection by P22 interferes with autorepression of the dgo operon in LT2.

A P22 moron locus is responsible for specifically triggering dgo expression in LT2

Subsequently, we noticed that derepression of dgoT::MuΔK upon phage infection was a feature supported by P22, but not by another S. enterica specific temperate phage such as ES18 (Figure S1). This raised the possibility that induction of the dgo operon stemmed from a genetic circuit in P22, rather than from a generic host response to phage infection. To examine this, a plasmid library of random P22 genomic fragments was screened for loci able to render the LT2K7 indicator strain blue on LB X-Gal. As such, a 521 bp P22 fragment could eventually be obtained that triggered dgoT::MuΔK upon conditional expression (using the arabinose inducible PBAD promoter) in LT2K7. More specifically, this fragment was found to correspond to a small and unannotated locus situated between arf2J and arf8J in the late region of the P22 genome [16,17] (Figure 3A, boxed region), and was subsequently designated as pid (for phage P22 encoded instigator of dgo expression).

Interestingly, close inspection of the pid region revealed it to be a genuine moron locus [6], as it is integrated at a site where related phages have either no (cfr. PS34 in Figure 3B) or another insert (Figure 3B). In addition, the pid locus is further characterized (i) by the fact that it is divergently transcribed relative to its surrounding genes, indicating that its regulatory control might deviate from that of the late region, and (ii) by a 3’ Rho-independent transcriptional termination site.

The P22 pid locus encodes a small ORFan protein that triggers expression of the LT2 dgo operon

During our efforts to discriminate whether the pid locus encoded a small regulatory RNA or a small protein, we discovered the appearance of a distinct low molecular weight protein band on SDS-PAGE upon triggering transcription of the locus from a plasmid (pFPV-pLurpid) (Figure 4A). Moreover, sequencing of this
protein indeed revealed peptide signatures encoded by one of the possible reading frames of the moron locus (Figure 4B). While the stop codon of this open reading frame could be inferred, the start codon was predicted by the presence of an upstream canonical Shine-Dalgarno sequence (AAGGAG) [18] (Figure 4C). Importantly, introduction of a −1 frame shift in the start codon (Figure 4C) simultaneously abolished both expression of the characteristic protein band and induction of dgo::MuK in LT2K7 (Figure 4D), establishing this 86 amino acid and 9.23 kDa derivative grown on LB X-Gal agar. (C) Overexpression of dgo interferes with activation of the dgo::MuK fusion by P22 infection, as a plaque of P22 wt grown on a lawn of LT2K7 pFPV-dgo::MuK fails to display LacZ activity (i.e. blue color) on LB X-Gal agar (left panel). A plaque of P22 wt grown on LT2K7 pFPV25 (i.e. empty vector) was included as a control. A similar experiment performed on green indicator agar (GI; right panel) confirms the actual infection of both strains by P22 wt.

doi:10.1371/journal.pgen.1003269.g002

Figure 2. The dgoRKAT operon and its derepression in LT2. (A) Scheme showing the position of the MuK element (not drawn to scale) generating a translational lacZ reporter fusion to the dgoT gene in strain LT2K7 (i.e. dgoT::MuK). Please note that the grey arrow corresponds to an open reading frame compromised by a −1 frame shift at the position marked with an asterisk (*). (B) Deletion of the dgoR gene in LT2K7 yields constitutive expression of the dgoT::MuK fusion, as shown by the difference in LacZ activity (i.e. blue color) between a spot of LT2K7 and its ΔdgoR derivative grown on LB X-Gal agar. (C) Overexpression of dgoT interferes with activation of the dgoT::MuK fusion by P22 infection, as a plaque of P22 wt grown on a lawn of LT2K7 pFPV-dgoR fails to display LacZ activity (i.e. blue color) on LB X-Gal agar (left panel). A plaque of P22 wt grown on LT2K7 pFPV25 (i.e. empty vector) was included as a control. A similar experiment performed on green indicator agar (GI; right panel) confirms the actual infection of both strains by P22 wt.

The Pid/dgo interaction is not supported during strict lytic or lysogenic propagation of P22

Since upon infection the propagation of P22 wt can either proceed lytically or lysogenically, we wondered which of these two distinct developmental routes would actually mount the Pid/dgo interaction (Figure 5A) in the cell. Surprisingly, however, dgoT::MuK expression was completely absent both when LT2K7 was subjected to obligatory lytic infection with P22 c2 (Figure 5C) or when the reporter strain carried P22 wt as a prophage (Figure 5D). The latter finding is in fact consistent with our initial observation of the Pid/dgo interaction being fully supported by the P22 HT105/1 mt-201 transducing phage (Figure 1A, 1B and Figure 5B) despite its inability to integrate in the host chromosome as a prophage.

To further corroborate this finding, we extended the P22 pid open reading frame with a strep-tag encoding sequence (leading to P22 pid-strep) to facilitate Pid detection by western blot, and checked whether the observed absence of dgoT::MuK expression also correlated with attenuated levels of Pid. In agreement with the results above (Figure 5), Pid production was abundant in LT2 infected with P22 pid-strep (Figure 6A), while it was severely attenuated in LT2 infected with the obligate lytic P22 c2 pid-strep derivative (Figure 6B) and completely absent in LT2 carrying P22 pid-strep as a prophage (Figure 6C).

To determine whether or not compromised Pid production stemmed from attenuated pid transcription, the pid open reading frame of P22 was replaced with the yfp fluorescent reporter gene, and the resulting phage (i.e. P22 Δpid::yfp, carrying yfp under the control of the native pid promoter) was used to interact with LT2. In agreement with our previous findings (Figure 5 and Figure 6), cells infected with an obligate lytic derivative of P22 Δpid::yfp (i.e. P22 c2 Δpid::yfp) only displayed very faint fluorescence in the few minutes before cell lysis (Figure 7C), while cells carrying P22 Δpid::yfp as a prophage displayed no detectable fluorescence (Figure 7D). On the contrary, cells infected with P22 Δpid::yfp (Figure 7A) or its int derivative (i.e. P22 Δint Δpid::yfp) (Figure 7B) clearly showed a plethora of cells exhibiting YFP expression to different extents.

Interestingly, the finding that expression of pid and subsequent derepression of the dgo operon are not supported during lytic or lysogenic propagation of P22 strongly suggests that the Pid/dgo interaction might be dedicated to a different state of P22 development.

pid expression is tightly linked with cells in the phage carrier state

Spurred by the above observations, time-lapse fluorescence microscopy was used to more closely examine the timing and dynamics of pid expression during infection of LT2 with P22 Δpid::yfp at single cell resolution. While this approach demonstrated that the pid locus indeed became expressed in lineages emerging from non-lytic infection with the reporter phage, it also revealed that this expression was a feature that subsequently segregated...
asymmetrically between siblings (Figure 8). Surprisingly, in fact, only one individual within the growing lineage consistently displayed the ability to express pid, thereby revealing an unprecedented timing and populational distribution of this phage – host interaction. It should also be noted that disruption of the int gene in P22 Δpid::yfp did not affect the timing nor the asymmetric distribution of pid expression (Figure S2), corroborating that the actual chromosomal integration event leading to the establishment of a prophage was not required for this phenomenon.

In order to more closely examine the possible role of the P22 chromosome in this peculiar asymmetric segregation phenotype, P22 Δpid::yfp was equipped with a parS site (resulting in P22 Δpid::yfp parS), allowing its whereabouts during infection to become fluorescently tractable in an LT2 strain expressing the ParB protein fused to mCherry (i.e. LT2 pCW-mCherry-parB). Interestingly, soon after infection of LT2 pCW-mCherry-parB with P22 Δpid::yfp parS, a single and coherent mCherry cloud appeared in cells destined for non-lytic infection (Figure 9A and 9B), indicative for the presence of one (or possibly more) P22 chromosome(s). Furthermore, upon subsequent cell divisions, this cloud became asymmetrically segregated between siblings, with pid expression remaining tightly linked to the cell inheriting and carrying the unintegrated P22 chromosome(s) (Figure 9). The gradual dilution of YFP molecules in siblings not inheriting this phage carrier state is consistent with the heterogeneity in YFP fluorescence in liquid cultures of LT2 infected with P22 Δpid::yfp observed earlier (cfr. Figure 7).

**Discussion**

Given the penetration and importance of bacteriophages in global ecology, understanding their possible associations with a host is of tremendous importance. In this report, the S. Typhimurium – phage P22 model system yielded both molecular and genetic evidence authenticating the existence of a dedicated phage carrier state in which an unintegrated phage chromosome is stably maintained in the cell and asymmetrically inherited by only one of the siblings upon further divisions. This behavior differs fundamentally from cells undergoing lytic or lysogenic phage development, which are forced either to lyse after the production of new virions or to symmetrically segregate the prophage chromosome (integrated in the host chromosome or existing as a stable episome) among siblings [19,20], respectively.

The phage carrier (or pseudolysogenic) state is believed to have a tremendous impact on phage ecology, as the ability to postpone the commitment to lytic or lysogenic development might improve phage survival in inhospitable environments [11–14]. Specifically with regard to the biology of phage P22, our findings at the single
cell level are in remarkable agreement with very early observations made by Zinder, who anticipated that upon infection P22 could be maintained in a pseudolysogenic form during several generations before integrating itself as a prophage [21]. Despite the long-standing assumption of its alleged existence and its ecological importance, however, the phage carrier state has so far hardly been documented from a molecular or genetic point of view. In fact, although it has been proposed that the phage remains idle or inert while being in this state [12,13], our results on the contrary provide the first evidence that a dedicated phage – host interaction (as exemplified by Pid/dgo) can be mounted in phage carrier cells.

Clearly, the existence of dedicated genetic programs that are executed solely in phage carrier cells substantiates their biological significance and allows them to differentiate from uninfected cells or cells destined for lytic or lysogenic development.

On itself, the induction of the LT2 dgo operon by the P22 Pid ORFan protein is also peculiar, since only a very limited number of phage – host interactions have so far been discovered in which the phage deliberately and specifically interferes with host gene expression. Indeed, in currently recognized interactions, phage encoded functions either (i) hijack cellular machinery and generally shut down host gene expression to support phage reproduction during lytic proliferation [22], or (ii) contribute virulence factors that support the pathogenicity of the host during lysogenic development [6,23]. A notable exception was only recently described for λ lysogens of E. coli, in which the λ CI repressor was shown to compromise cellular gluconeogenesis by physically obstructing the host pckA promoter [24].

Interestingly, the dgo operon encodes proteins involved in the uptake and metabolism of D-galactonate, which is considered to be an important source of carbon and energy during intracellular survival and proliferation of Salmonella spp. [25]. Moreover, a dgo T knock-out was correspondingly found to attenuate the virulence of S. enterica serovar Choleraesuis in pigs [26]. It remains to be established, however, how exactly the Pid/dgo interaction is mounted within the carrier state, and whether it would endow carrier cells with increased virulence or rather constitutes a way for the phage to decide on how long to maintain this state.
In summary, our results authenticate the existence of the phage carrier state as a distinct developmental route in phage biology that differs from strict lytic or lysogenic propagation. The phenotypic consequences of the interactions taking place in phage carrier cells are likely to provide the missing link in the proper and accurate interpretation of phage – host dynamics occurring throughout microbial ecosystems.

Materials and Methods

Strains and growth conditions

Bacterial strains, phages and plasmids used throughout this study are listed in Table 1. For culturing bacteria, Lysogeny Broth (LB; [27]) medium was used either as a broth or as agar plates after the addition of 15% (for spreading plates) or 7% (for soft-agar plates) agar. Cultures were grown in LB broth for 15–20 h at 37°C under well-aerated conditions (200 rpm on a rotary shaker) to reach stationary phase. Exponential phase cultures were in turn prepared by diluting stationary phase cultures 1/100 or 1/1000 in fresh pre-warmed broth, and allowing further incubation at 37°C. When appropriate, the following chemicals (Applichem, Darmstadt, Germany) were added to the growth medium at the indicated final concentrations: ampicillin (100 μg/ml; Ap100), chloramphenicol (30 μg/ml; Cm30), kanamycin (50 μg/ml; Km50), tetracycline (20 μg/ml; Tc20), glucose (0.02%), L-arabinose (0.02%), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 40 μg/ml).

Figure 5. The Pid/dgo interaction is not supported during strict lytic or lysogenic development of P22. LT2K7 (A, B, C) and a P22 wt lysogen of LT2K7 (D) was streaked (in the direction of the arrow) across a suspension (indicated by a dashed line) of P22 wt (A, D), P22 HT105/1 int-201 (B) and P22 c2 (C) on either LB X-Gal agar (upper panels) in order to visualize LacZ activity caused by induction of the dgoT:MuK fusion, or green indicator agar (lower panels) in order to visualize phage infection. doi:10.1371/journal.pgen.1003269.g005

Figure 6. Expression of Pid protein during P22 infection. Western blot analysis of strep-tagged Pid produced during the indicated time after infecting exponential phase cultures of LT2 at MOI = 0.1 with (A) P22 pid-strep or (B) P22 c2 pid-strep, or (C) in early exponential phase cultures of LT2 carrying P22 pid-strep as a prophage. The position of Pid is indicated by a black arrow, while the molecular marker (MM) shows the position of the 16 kDa reference. doi:10.1371/journal.pgen.1003269.g006

Figure 7. Evidence for pid transcription during P22 infection at the single cell level. Phase contrast (left panels) and corresponding YFP epifluorescence (right panels) micrographs of exponential phase cultures of LT2 either (A) 3 h after infection with P22 Δpid:yfp, (B) 3 h after infection with P22 Δint Δpid:yfp, (C) 2 h after infection with P22 c2 Δpid:yfp (just before general cell lysis occurred), or (D) carrying P22 Δpid:yfp as a prophage. Infections with P22 (A, B and C) were performed at MOI = 0.1. doi:10.1371/journal.pgen.1003269.g007
Phages were propagated on S. Typhimurium LT2 as plaques in LB soft-agar or as lysates in LB broth as described previously [28]. Phage stocks were filter sterilized with 0.2 μm filters (Fisher Scientific, Aalst, Belgium) and chloroform was added to maintain sterility. Generalized transduction was performed with phage P22 HT105/1 int-201 as described previously [28,29]. This mutant is unable to integrate into the host chromosome as a prophage due to the lack of integrase (Int) activity. To discriminate phage infected from uninfected colonies, plates containing green indicator (GI; [28]) agar were used to indicate cell lysis. The latter medium contains glucose as a carbon source, and a pH indicator dye that turns dark green at sites where phage infection causes cell lysis and the concomitant release of organic acids.

Please note that for clear visualization of spotted or (cross-)streaked bacterial and/or phage populations, agar plates were printed to Whatman filter papers (GE Healthcare, Diegem, Belgium) before photographing.

β-galactosidase assay
Expression of β-galactosidase (LacZ) was inferred from the hydrolysis of either 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) or o-nitrophenyl-β-D-galactoside (ONPG). X-Gal was typically added to agar plates (40 μg/ml), where its hydrolysis by β-galactosidase yielded an insoluble blue precipitate. For quantitative measurements of lacZ expression, Miller units were determined as described previously [30] using the CHCl₃-sodium dodecyl sulfate permeabilization procedure.

Construction and screening of a P22 shotgun library
Particles of phage P22 were purified by passing a lysate through a 0.45 μm pore-size filter, after which particles were concentrated by centrifugation (4,000 xg, 20 min) in the presence of polyethylene glycol (PEG) 8,000 (8%, w/v) and 1 M NaCl. Subsequently, further purification was attained by ultracentrifugation (140,000 xg, 3 hours) using a layered CsCl step gradient of 1.33,
1.45, 1.50 and 1.70 g/ml. This resulted in a distinct blue band containing the concentrated P22 particles. This band was subsequently collected and dialysed against phage buffer (10 mM Tris-HCl pH 7, 10 mM MgSO\(_4\), 150 mM NaCl) three times using a Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL, USA). For DNA extraction, the purified and dialysed phage particles were incubated at 56°C for 1 h in the presence of 0.5% SDS (w/v), 20 mM EDTA and 2 mg/ml proteinase K. Subsequently, DNA was extracted and purified from this mixture by phenol/chloroform [27] and precipitated with Na-acetate/ethanol. Finally, the sample was treated with RNase A (0.1 mg/ml) (Fermentas, St. Leon-Rot, Germany) for 1 h at room temperature to remove any residual RNA. Next, the resulting purified P22 genomic DNA was partially digested with the blunt 4 bp-cutter BsuRI restriction enzyme (Fermentas) and separated by agarose gel electrophoresis (1% agarose), after which fragments between 1–2 kb were isolated from the gel using the GeneJET Gel Extraction Kit (Fermentas).

Parallel to this, pFPV-P\(_{BAD}\)-gfp (pAA100; [31]) was digested with XbaI and HindIII (Fermentas) to remove gfp, and treated with calf intestinal alkaline phosphatase (Fermentas) to prevent self-ligation. The genomic P22 DNA fragments and the cut pFPV-P\(_{BAD}\) vector were subsequently ligated after blunting with T4 ligase and Klenow polymerase (Fermentas), and transformed by electroporation into LT2K7. After plating on LB Ap\(_{100}\) this random P22 shotgun library was replica-plated on LB Ap\(_{100}\) X-Gal with and without 0.02% arabinose to screen for plasmids able to trigger LacZ expression in LT2K7.

Whole-genome alignment
Phages containing homologous regions to the region surrounding pid were selected with nucleotide Blast [32]. Whole genome alignment was performed manually and was based on the Blast-search results. The resulting conclusions were later confirmed by a progressive Mauve alignment [33] on the full genomes using default settings.

Construction of bacterial and phage mutants
Strain LT2K7 stems from a random Mu\(_d\)K library, generated as described previously [34], and harbors a translational lac\(_\gamma\) fusion to the LT2 dgo\(_T\) gene (i.e. dgo\(_T::\)Mu\(_d\)K). In strain LT2 Δdgo\(_R\), the dgo\(_R\) gene was deleted via recombineering [35], using

Figure 9. Pid expression is linked to phage carrier cells. Exponential phase cultures of LT2 pCW-mCherry-parB were infected with P22 Δpid:yfp parS (MOI = 0.1) and chased after 30 minutes with the virulent P22 H5 mutant (MOI = 20) to lyse cells not destined for non-lytic development of P22 Δpid:yfp parS. Two independent time-lapse fluorescence microscopy image sequences (A and B) of an LT2 pCW-mCherry-parB cell destined for non-lytic development of P22 Δpid:yfp parS are shown. The corresponding phase contrast images are either superimposed with YFP epifluorescence images visualizing pid expression (left panels) or mCherry epifluorescence images visualizing the presence of the P22 chromosome(s) (right panels), and the time after infection is indicated on the frames. Please note that only the carrier cell produces new YFP, while non-carrier segregants passively acquire preformed YFP by cytoplasmic diffusion, and further dilute it upon subsequent cell divisions.

doi:10.1371/journal.pgen.1003269.g009
an ampiclon (Phusion DNA polymerase; Fermentas) prepared on pKD3 [35] with the primers dgoR_pkd3_Fw and dgoR_pkd3_Rev (Table 2). The cat cassette replacing dgoR was flipped out using pCP20-borne Flp to recombine the two frt-sites [36], resulting in a small flt-scar followed by a new ribosome binding site [35]. Strain LT2K7 ΔdgoR was subsequently constructed by translocating dgoT::MudK to LT2 ΔdgoR.

For the construction of P22 Δp2::yfp, the yfp-frt-cat-frt cassette was PCR amplified (Phusion DNA polymerase; Fermentas) from plasmid pAc [37] with primers pid_YFP_Cm_Fw and pid_YFP_Cm_Rev (Table 2), and used to replace the pid gene in LT2 lysogenized with wild-type P22 via recombineering [35]. Subsequently, the cat cassette was flipped out using pCP20-borne Flp to recombine the two frt-sites [36], and the resulting P22 Δp2::yfp phage was isolated and purified from the corresponding lysogen.

For the construction of P22 Δint Δp2::yfp, the integrase gene (int) was deleted by the recombination, using a PCR ampiclon prepared on pKD3 with primers P22_Int_Fw and P22_Int_Rev (Table 2) [35]. Please note that the frt-flanked cat cassette was not removed by site specific Flp recombination, since this would interfere with the flt-scar already present in the pid locus. The resulting P22 Δint Δp2::yfp phage could be released by amplifying rare excision events through growth on wild-type LT2 in order to allow detection and purification of plaques. Please note that these phages produced

| Name | Characteristic | Source or reference |
|------|----------------|---------------------|
| DH5α | E. coli F−, F′-80lacZAM15 Δ(lacZYA-argF) U169 endA1 recA1 hisD17 deoR thi-1 supE44Δ  tyrA969 relA1 Laboratory collection |
| LT2 | Salmonella Typhimurium LT2 wild-type [46] |
| LT2K7 | LT2 ΔdgoR Laborotory collection |
| LT2ΔΔdgoR | LT2 ΔdgoR This study |
| LT2K7ΔΔdgoR | LT2 ΔdgoRΔdgoR This study |
| LT2[P22 Δp2::yfp] | LT2 P22 Δp2::yfp lysogen This study |
| LT2[P22 pid-strep] | LT2 P22 pid-strep lysogen This study |

### Strains

| Name | Characteristic | Source or reference |
|------|----------------|---------------------|
| P22 wt | Wild-type P22 phage SGSC* |
| P22 c2 | Clear mutant of P22 affected in C2 repressor SGSC* |
| P22 H5 | Virulent derivative of P22Kelly Hughes (University of Utah, USA) |
| P22 HT105/1 int-201 | Integration deficient mutant of P22 Kelly Hughes (University of Utah, USA) |
| P22 Δp2::yfp | pid replaced by yfp This study |
| P22 Δint Δp2::yfp | pid replaced by yfp, int deletion This study |
| P22 c2 Δp2::yfp | pid replaced by yfp, truncated C2 repressor This study |
| P22 pid-strep | C-terminal fusion of strep-tag to pidThis study |
| P22 c2 pid-strep | C-terminal fusion of strep-tag to pid, truncated C2 repressor This study |
| P22 Δp2::yfp parS-cat | pid replaced by yfp, parS-cat inserted between gtrC and 9 This study |

### Plasmids

| Name | Characteristic | Source or reference |
|------|----------------|---------------------|
| pFPV25 | Encodes promoterless gfp [48] |
| pFPV-P_adGfp | Encodes GFP under control of an arabinose-inducible promoter [31] |
| pFPV-P_adPid | Encodes Pid under control of an arabinose-inducible promoter This study |
| pFPV-P_adPid² | As in pFPV-P_adPid but harboring a frame shift in the start codon of pidThis study |
| pFPV-dgoR | Encodes DgoR under its native promoter This study |
| pAc | yfp-frt-cat-frt template for recombineering of yfp [37] |
| pCP20 | Encodes Flp for recombining frt sites [36] |
| pKD46 | Encodes Lambda red genes under control of arabinose inducible promoter [35] |
| pKD3 | Harbors frt-cat-frt site for construction of deletions by recombineering [35] |
| pALA2705 | Encodes GFP-ParB under control of the lac promoter [41] |
| pRSB-mCherry | Used as template for PCR amplification of mCherryRoger Tsien, (University of California, USA) |
| pCW-mcherry-ParB | Encodes mCherry-ParB under control of the lac promoter. Derived from pALA2705This study |
| pGBKD3-parS | Harbors parS-cat-frt site for insertion of the parS site [38] |

*http://people.ucalgary.ca/~kesander/#

DOI:10.1371/journal.pgen.1003269.t001
null
Protein identification and Western blotting

Samples were lysed in standard lysis buffer containing 50 μl/ml Bugbuster (Novagen, Darmstadt, Germany). Total protein concentration was assessed by the BCA protein assay kit (Novagen) and SDS-PAGE was performed as described previously by Sambrook and Russell [27]). Finally, gels were stained with coomassie [27] and when necessary, silver staining was employed as previously described [43].

For protein identification, the corresponding protein band was excised and trypsin-digested according to the method described earlier [44]. Subsequently, the digested peptides were identified by LC–ESI MS/MS (Thermo Electron, San Jose, CA) and further analyzed using Mascot (Matrix Sciences, London, UK) against the NCBI database (http://www.ncbi.nlm.nih.gov/).

For western-blotting, equal amounts of proteins were separated with PAGE and transferred to a nitrocellulose membrane (Hybonet-C Extra; GE Healthcare) by semi-dry electroblotting for 1 hour at 0.15 A using a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad Laboratories) and transfer buffer (50 mM Tris; 40 mM glycine; 0.075% SDS; 20% Methanol). Strep-tagged Pid was subsequently detected by Strep-MAB-Classic, an anti-strep monoclonal antibody conjugated with Horse radish peroxidase (IBA, Gottingen, Germany). Horse radish peroxidase activity was assessed with Pierce ECL Western Control of the blotting process.

Fluorescence microscopy

Fluorescence microscopy and time-lapse fluorescence microscopy were performed with a temperature controlled (Okolab Ottaviano, Italy) Ti-Eclipse inverted microscope (Nikon, Cham-pigny-sur-Marne, France) equiped with a TI-CT-E motorised condensor, a YFP filter (Ex 500/24, DM 520, Em 542/27), an mCherry filter (Ex 562/40, Dm 593, Em 641/75), and a CoolSnap HQ2 FireWire CCD-camera. For imaging, cells were placed between LB agar pads and a cover glass, essentially as described previously [45], and incubated at 37°C. Please note that for experiments involving LT2 pCW-mCherry-yfp, cells were grown on agar pads of AB-minimal media supplemented with 0.02% D-glucose, 100 μg/ml Uracil and 100 μg/ml Thiamine, Ap<sup>100</sup> and incubated at 30°C, as described previously [42]. Images were acquired using NIS-Elements (Nikon) and resulting pictures were further handled with open source software ImageJ (Downloadded from http://rsbweb.nih.gov/ij/).

Supporting Information

Figure S1 Phage ES18 fails to activate the dqoT::MadK fusion in LT2K7. A plaque of phage ES18 grown on a lawn of LT2K7 fails to display Lac<sup>Z</sup> activity (i.e. blue color) on LB X-Gal agar (left panel), while a similar experiment performed on green indicator agar (GI; right panel) confirms the actual infection of LT2K7.

Figure S2 Expression of P22 p<sup>id</sup> segregates asymmetrically between siblings and does not require integration of P22. Exponential phase cultures of LT2 were infected with P22 Δint Δpid::yfp (MOI = 0.1) and chased after 30 minutes with the virulent P22 H5 mutant (MOI = 20) to lyse cells not destined for non-lytic development of P22 Δint Δpid::yfp. Images A and B depict clonal microcolonies displaying asymmetrical segregation of yfp expression. Phase contrast (left panels) and corresponding YFP epifluorescence (right panels) images are shown, and the time after infection with P22 Δint Δpid::yfp is indicated on the frame.

Acknowledgments

The authors would like to thank Kelly Hughes, Lionello Bossi, Nora Figueroa-Bossi, Sherwood Casjens, Stanley Maloy, Roger Tsien, and Olivier Espéli for providing strains, constructs, protocols, and/or helpful feedback, Ariel Lindner, Ni Ming, and Marianne De Paepe for help with time-lapse fluorescence microscopy; Geert Baggerman for technical assistance with mass-spectrometry; and Anne-Sophie Delatitre for technical assistance with Western blot experiments.

Author Contributions

Conceived and designed the experiments: WC AA. Performed the experiments: WC MTM AM AA. Analyzed the data: WC MTM AA. Contributed reagents/materials/analysis tools: P-JC RL RVH FT. Wrote the paper: WC MTM AA.

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