The phage growth limitation system in *Streptomyces coelicolor* A(3)2 is a toxin/antitoxin system, comprising enzymes with DNA methyltransferase, protein kinase and ATPase activity

Paul A. Hoskisson,1 Paul Sumby,2 Margaret C.M. Smith

1 Department of Molecular and Cell Biology, University of Aberdeen, Institute of Medical Science, Foresterhill, Aberdeen AB25 2ZD, UK
2 Department of Genetics, University of Nottingham, Queens Medical Centre, Nottingham NG7 2UH, UK

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**A B S T R A C T**

The phage growth limitation system of *Streptomyces coelicolor* A(3)2 is an unusual bacteriophage defence mechanism. Progeny φC31 phage from an initial infection are thought to be modified such that subsequent infections are attenuated in a Pgl+ host but normal in a Pgl− strain. Earlier work identified four genes required for phage resistance by Pgl. Here we demonstrate that Pgl is an elaborate and novel phage restriction system that, in part, comprises a toxin/antitoxin system where PglX, a DNA methyltransferase is toxic in the absence of a functional PglZ. In addition, the ATPase activity of PglY and a protein kinase activity in PglW are shown to be essential for phage resistance by Pgl. We conclude that on infection of a Pgl+ cell by bacteriophage φC31, PglW transduces a signal, probably via phosphorylation, to other Pgl proteins resulting in the activation of the DNA methyltransferase, PglX and this leads to phage restriction.

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**Introduction**

Bacteria have evolved a plethora of diverse mechanisms to evade killing by bacteriophages. The mechanisms can act at any stage of the phage life cycle from preventing phage adsorption right through to inhibition of cell lysis and release of progeny phage (Hoskisson and Smith, 2007). The phage growth limitation (Pgl) system of *Streptomyces coelicolor* confers resistance against the temperate bacteriophage φC31 and its homoimmune relatives (Chinenova et al., 1982; Laity et al., 1993). This system is called ‘growth limitation’ as phage infecting a Pgl+ strain for the first time undergoes a normal single burst to produce progeny phage but the progeny is attenuated for growth in a second round of infection. The progeny is however able to form normal plaques on a Pgl− host (Fig. 1). The mechanistic explanation of the Pgl phenotype proposed by Chinenova et al. (1982) is that during the initial round of infection the progeny phage are modified and then restricted in the second round of infection. This system differs fundamentally from typical R−M systems, where, if the phage DNA becomes modified, there can be an escape from restriction and rapid spread of infection through sensitive bacteria. In Pgl, however, even if modification fails during the first burst, it is likely that it will occur in subsequent cycles thereby severely limiting spread of infection (Fig. 1; Sumby and Smith, 2002). Furthermore Pgl may confer an added advantage to a clonal population as being Pgl+ amplifies phage that might infect and kill phage-sensitive competitors.

Previous work has identified four genes, located in two operons (pglWX and pglYZ) 6 kbp apart, required for Pgl in *S. coelicolor* (Bedford et al., 1995; Laity et al., 1993; Sumby and Smith, 2002). Bioinformatic searches on the products of the pgl genes revealed several protein motifs (Fig. 2). The product of PglX is predicted to bind AdoMet and has an N6-adenine DNA methyltransferase motif, PglW is a putative serine/threonine protein kinase that contains a putative Walker A and Walker B motifs for the binding and hydrolysis of ATP/GTP and PglZ is predicted to have a conserved alkaline phosphatase-like fold (PFam: PF08665). While the predicted function of PglX as a DNA methyltransferase fits well with the proposed mechanism by Chinenova et al. (1982) the Pgl system appears to involve additional activities that are novel to R−M systems, not least the putative kinase activity of PglW.

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Transcriptional analysis of pgl genes indicates that both operons are transcribed, even in the absence of phage infection (Bedford et al., 1995; Sumby and Smith, 2002). While transcriptional up-regulation of these genes is plausible during infection, we cannot rule out a role for the control of protein function through phosphorylation given that PglW is a predicted protein kinase. Indeed it has been shown that two isoforms of PglZ were detected with different isoelectric points in 2D-PAGE gels, suggesting that this protein could be post-translationally modified (Bedford et al., 1995; Sumby and Smith, 2003). Switching the phage resistance on and off may help to ease the strong defence phenotype (Fig. 2). The strategy used was to generate knock-out or null mutations in each pgl gene and then complement these null mutants with either wild type (wt) or mutant alleles introduced ectopically into the chromosome using the pSC31 int/attP system (Bierman et al., 1992). Where possible we obtained further proof of Pgl protein function by heterologous expression of Pgl protein in Escherichia coli and biochemical assay of the partially purified extracts.

PglX is a DNA methyltransferase

Bioinformatic analysis of the PglX sequence suggested the presence of an N6-adenine methyltransferase motif (NPPY) at 378–381 aa (Roth et al., 1998; Scavetta et al., 2000; Fig. 2). A ΔpglX null mutant, SPHX, was constructed using REDIRECT technology that involves recombination of the kanamycin-resistant cosmid SCIF2, replacing the pglX coding sequence (SC06627) with an apramycin resistance gene. The apramycin resistance gene is flanked by loxP sites and also contains an origin of transfer (oriT) to enable conjugation of the cosmid into the Pgl+ S. coelicolor strain, M145 (Gust et al., 2003; Redenbach et al., 1996). Double crossovers that retain the apramycin marker but had lost the kanamycin resistance marker were then infected with Cre-phage in a transient infection to remove the apramycin resistance marker (Khodakaramian et al., 2006). SPHX was sensitive to 4°C1 and could be complemented by the introduction of PPS8003 encoding a His-tagged version of PglX (Fig. 3A; Sumby and Smith, 2002).

To test whether the putative methyltransferase domain of PglX was necessary for Pgl function, the tyrosine residue (Y381) of the conserved NPPY motif was targeted by site directed mutagenesis (Sumby and Smith, 2002). This tyrosine residue in other methyltransferases is structurally essential for catalysis where it is required for flipping out the target base from the DNA double helix prior to methylation (Roth et al., 1998). The resulting plasmid, pH1002 (encoding PglXY381A–His6), was conjugated into SPHX (ΔpglX) to test in vivo activity of the mutated pglX allele. PglXY381A did not restore phase resistance to SPHX suggesting that the putative methyltransferase motif is essential for the Pgl system (Fig. 3A).

To assay DNA methyltransferase activity of PglX, attempts were made to express a C-terminally His-tagged PglX fusion in E. coli and to enrich extracts by affinity chromatography. The 136 kDa PglX–His6 from E. coli was barely detectable using a 6×His antibody in Western blots of the enriched proteins (data not shown). Nevertheless, low levels of methyltransferase activity using ϕC31 DNA as a substrate were observed (Fig. 3B–D). In a time course of methyltransferase activity, incorporation of 3H-methyl groups into TCA precipitable material increased, the period of 60 min (Fig. 3C) and the level of incorporation was dependent on the amount of protein added (data not shown). An extract of PglXY381A–His6 was prepared in an identical procedure to the expression and enrichment of PglX–His6, but methyltransferase activity was almost undetectable (Fig. 3B), ruling out the possibility that the observed activity was due to endogenous E. coli enzymes. Further controls confirmed that incorporation of label into TCA precipitable material was dependent on DNA addition and could be competed by addition of unlabelled AdoMet (Fig. 3D).
These data strongly suggest that PglX can methylate DNA in an in vitro assay.

A *pglZ* null mutation is lethal in strains with a wild type *pglX* gene

*In silico* predictions on the function of PglZ are limited to a region (527–704 aa) annotated as a ‘PglZ domain’ (Pfam: PF08665), which falls within a family of proteins called the ‘alkaline phosphatase clan’ (Finn et al., 2010; Fig. 2). We attempted to create a knockout mutant of *pglZ* in M145. REDIRECT technology was used to replace the *pglZ* ORF in the cosmid SC4G2, with the apramycin marker generating a cosmid SC4G2::Δ*pglZ::apra*. When this cosmid was introduced into M145 by conjugation, an extremely low frequency of double recombinants (kanamycin sensitive, apramycin resistant) was obtained (Table 2). Three putative M145::Δ*pglZ* colonies were propagated and were found to be phage sensitive as expected. However, introduction of *pglZ*:His6 encoded by the integrating plasmid, pPH1001, was unable to complement the phage sensitive phenotype of any of these recombinants (Fig. 4A).

To demonstrate that pPH1001 was able to complement a *pglZ*/C0 defective allele, the plasmid was introduced into J1934, a *Pgl*/C0 strain constructed by Bedford et al. (1995) by insertional inactivation resulting in the deletion of the 30 end of *pglZ* encoding the C-terminal 130 amino acids (referred to as *pglZ1−834*). The data are the mean of two replicate experiments. (D) Dependence of methyltransferase activity of PglX–His6 on protein, *ϕC31*Δ25 DNA and competition by addition of 10 nM unlabelled AdoMet to indicate specificity of activity (data are the mean of two replicate experiments).

**Fig. 3.** PglX methyltransferase activity is required for the Pgl phenotype. (A) SPHX (Δ*pglX*) was challenged with 3 × 10⁵ pfu/ml of *ϕC31*Δ25, grown on J1929 (pglY °) Bedford et al. (1995). Wild type Streptomyces coelicolor M145 (pglX °), SPHX pPS8003 (Δ*pglX*/pglX °), and SPHX pPH1002 (Δ*pglX*/pglX*1−834°*) were challenged with 1 × 10⁵ pfu ml⁻¹ *ϕC31*Δ25. (B) Methyltransferase activity of heterologously expressed PglX–His6 (squares) and PglX*1−834°*–His6 (circles) using 3H-AdoMet as the methyl donor to *ϕC31*Δ25 DNA isolated from J1929 (pglY °) Bedford et al. (1995). Data are the mean of two replicate experiments. (C) Time dependent methyltransferase activity of PglX–His6 on *ϕC31*Δ25 DNA isolated from J1929 (pglY °). Data are the mean of three replicate experiments and error bars represent the SD of the data. (D) Dependence of methyltransferase activity of PglX–His6 on protein, *ϕC31*Δ25 DNA and competition by addition of 10 nM unlabelled AdoMet to indicate specificity of activity (data are the mean of two replicate experiments).
Pgl (Sumby and Smith, 2003). Sequencing through the G-tract indicated that in two of the three M145ΔpglZ strains the number of G residues had contracted (from 8 to 7 G nucleotides; Fig. 4B (i) and (ii)), while in the third strain the G-tract was as for the Pgl+ wild type M145 (8 G nucleotides; Fig. 4B (i) and (ii)), however this strain was also not complemented by pPH1001 indicating that the secondary mutation must be elsewhere in the genes required for Pgl. The provision (via integration of pPH1001) of a second copy of pglZ in the Pgl+ wild type strain M145 enabled the disruption of the native pglZ at a frequency of recombination that is typical when an inessential gene is targeted, such as pglW by the cosmid SCIF2:ΔpglW::apra (Table 2; Gust et al., 2003; Khodakaramian et al., 2006).

To confirm the essentiality of an intact pglZ, we first performed alignments of related PglZ proteins from the sequence databases to identify conserved residues that we could target for mutagenesis. Alignment of PglZ homologues indicated the presence of conserved residues D535 and D694. These residues fall within the predicted alkaline phosphatase fold (525–792 aa; Fig. 2; Quevillon et al., 2005). D535 and D694 in PglZ were both changed to alanine by site directed mutagenesis in the plasmid pPS5045 and the mutant alleles were subcloned into the integrating vector pIJ6902 (Huang et al., 2005) to generate pPH1007 (encoding PglZD535A–His6) and pPH1008 (encoding PglZD694A–His6). After conjugation of pPH1007 and pPH1008 into J1934, containing the pglZ1-834 allele, the exconjugants failed to complement the Pgl−/C0 phenotype indicating that both pglZD535A and pglZD694A alleles were defective (Fig. 4C). Plasmid, pPH1008 (encoding PglZD694A–His6), was then conjugated into M145 and exconjugants were used as recipients for the SC4G2:ΔpglZ::apra cosmid, but the formation of the pglZ knockout strains was again prevented (Table 2). These experiments confirmed that the pglZD694A allele was unable to confer antitoxin activity in the presence of an otherwise intact Pgl system.

These observations indicate that PglZ may be interacting with PglX to inhibit a toxic activity. To test whether mutations in other pgl genes would permit the disruption of pglZ, the cosmid SC4G2:ΔpglZ::apra, was conjugated into strains SPHX (ΔpglX), SPHW (ΔpglW) and SLMY (ΔpglY) (Tables 1 and 2). Deletions of pglZ were obtained at low frequency, similar to that observed for the deletion of pglZ in a wild type background, except in SPHX (ΔpglX), where deletion of pglZ was obtained at the normal frequency (Table 2). The G tracts in one SPHW:ΔpglZ and one SLMY:ΔpglZ strains were sequenced and they had suffered a contraction and an expansion,
respectively (Fig. 4B (iii) and (iv)). These data are indicative of a specific suppression of a toxic activity of PglX by PglZ. We hypothesise that the pgliW<sup>Δ816</sup> allele in J1934 might retain the protective activity required against an intact pgliX.

**PgliW has kinase activity**

If PglZ prevents the toxic activity of PglX, then the presence of a signalling mechanism that regulates Pgl activity would enable tight control of the system and prevent unwanted toxicity. Bioinformatic analysis of the PglW sequence reveals the presence of two putative protein kinase domains; a tyrosine kinase domain at 195-490 aa (Prosite; PS00108) and a putative serine/threonine protein kinase domain (Prosite; PS00109) at 530-816 aa (Fig. 2). However only the putative STPK domain has a predicted terminal nuclease-related domain (NERD) motif (Fig. 2).

Examination of the codon usage at the start of the coding sequence of PglW<sup>Δ816</sup> showed the presence of several codons that are rarely used in E. coli, although PglW has a putative, but as yet uncharacterised, N-terminal nuclease-related domain (NERD) motif (Fig. 2). This suggests that PglW<sup>Δ816</sup> is highly expressed in a cell-free system. Therefore, the C-terminal NERD domain of PglW may be involved in the regulation of Pgl activity.

**Table 1**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Pgl status</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>M145</td>
<td>Prototrophic</td>
<td>+</td>
<td>Kieser et al. (2000)</td>
</tr>
<tr>
<td>J1934</td>
<td>Insertional mutation of PglZ</td>
<td>–</td>
<td>Bedford et al. (1995)</td>
</tr>
<tr>
<td>SLMW</td>
<td>ΔpgiW M145 isogenic</td>
<td>–</td>
<td>Khodakaramian et al. (2006)</td>
</tr>
<tr>
<td>SPHX</td>
<td>ΔpgiX M145 isogenic</td>
<td>–</td>
<td>This work</td>
</tr>
<tr>
<td>SLMY</td>
<td>ΔpgiY M145 isogenic</td>
<td>–</td>
<td>Khodakaramian et al. (2006)</td>
</tr>
<tr>
<td>SPHZ</td>
<td>ΔpgiZ M145 isogenic G-tract mutant</td>
<td>–</td>
<td>This work</td>
</tr>
<tr>
<td>SPHWZ</td>
<td>ΔpgiWZ M145 isogenic G-tract mutant</td>
<td>–</td>
<td>This work</td>
</tr>
<tr>
<td>SPIXZ</td>
<td>ΔpgiXZ M145 isogenic G-tract mutant</td>
<td>–</td>
<td>This work</td>
</tr>
<tr>
<td>SPHYZ</td>
<td>ΔpgiYZ M145 isogenic G-tract mutant</td>
<td>–</td>
<td>This work</td>
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**Table 2**

Conjugation efficiency in various strains upon introduction of the PglZ deletion cosmid.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Conjugation efficiency per recipient spore</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>M145</td>
<td>Parent</td>
<td>1.5 × 10&lt;sup&gt;−8&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>SLMW</td>
<td>ΔpgiW M145 isogenic</td>
<td>2 × 10&lt;sup&gt;−7&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>SPHX</td>
<td>ΔpgiX M145 isogenic</td>
<td>2 × 10&lt;sup&gt;−4&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>SLMY</td>
<td>ΔpgiY M145 isogenic</td>
<td>10&lt;sup&gt;−7&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>M145 (pPH1001)</td>
<td>Parent, carrying an integrated ectopic copy of pgliZ-His6</td>
<td>2 × 10&lt;sup&gt;−4&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>M145 (pPH1008)</td>
<td>Parent, carrying an integrated ectopic copy of mutant pgliZ&lt;sup&gt;Δ816&lt;/sup&gt;-His6</td>
<td>3 × 10&lt;sup&gt;−7&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

The resulting plasmid pPH1012 (encoding Pgl<sup>Δ816</sup>W<sup>Δ816</sup>–His<sub>6</sub>) was conjugated into SLMW (ΔpgliW) to test whether the pgliW<sup>Δ816</sup>–His<sub>6</sub> allele could complement for the deletion of pgliW and restore phage resistance. The Pgl<sup>Δ816</sup>W<sup>Δ816</sup>–His<sub>6</sub> mutation resulted in a phage-sensitive phenotype, indicating that the putative Hanks-like protein kinase domain is both functional and required for a Pgl<sup>+</sup> phenotype (Fig. 5A).

The gene encoding Pgl<sup>Δ816</sup>W<sup>Δ816</sup>–His<sub>6</sub> was cloned into the E. coli expression vector pT7-7 to generate pPS5012 and introduced into E. coli BL21 DE3 (pLysS). No expression of Pgl<sup>Δ816</sup>W<sup>Δ816</sup>–His<sub>6</sub> was detected. Examination of the codon usage at the start of the pgliW ORF showed the presence of several codons that are rarely used in E. coli. The first 19 codons were therefore optimised for expression in E. coli generating the expression plasmid pPS5025. The frequency of transformation of E. coli BL21 DE3 (pLysS) by pPS5025 was very low and expression trials of the few colonies that were obtained indicated the presence of insoluble and truncated proteins (data not shown). It seems likely that Pgl<sup>Δ816</sup>W<sup>Δ816</sup>–His<sub>6</sub> is toxic in E. coli BL21 and only plasmids that have suffered mutations can be established, explaining the poor transformation frequencies. At this stage we do not understand the basis for the toxicity of PglW although PglW has a putative, but as yet uncharacterised, N-terminal nuclease-related domain (NERD) motif (Fig. 2).

The plasmid pPS5025 was used as a template in an in vitro expression system to generate sufficient full length Pgl<sup>Δ816</sup>W<sup>Δ816</sup>–His<sub>6</sub> to test for autokinase activity (Molle et al., 2003). Incubation of...
PglW–His6 with [γ-32P] ATP resulted in autokinase activity of the protein, resulting in incorporation of radioactive phosphate (Fig. 5B). A control reaction, incorporating 35S-methionine into in vitro expressed PglW–His6, showed that the autophosphorylating band had the same mobility as PglW–His6 (Fig. 5B). The K677A mutant allele of PglW was also tested in the same assay for its ability to autophosphorylate; however, no radioactive signal could be detected, indicating that this residue is essential for the autophosphorylation reaction (Fig. 5B). These data are consistent with PglW forming part of a signal transduction system that senses the modification state of phage DNA during infection of a Pgl+ cell.

**PglY has ATPase activity**

Bioinformatic analysis of the PglY sequence suggested the presence of a Walker A (ATPase) motif (GSFGSGKS) at 75–82 aa (Bedford et al., 1995). It has previously been shown that these motifs are involved in nucleotide binding, and is found in many protein families (Karata et al., 1999; Ramakrishnan et al., 2002). We decided to test whether the ATPase motif is required for resistance to $\phi$C31 as a means to determine the role PglY might have in the Pgl phenotype.

The essential consecutive lysine and serine residues (K81, S82) were targeted by site directed mutagenesis in the plasmid pPS8008, which encodes PglY (Sumby and Smith, 2002). The resulting plasmid pH1003 (encoding His6-PglY$^{K81A/S82A}$) was conjugated into SLMY (ΔpglY). The K81A/S82A double substitution in PglY resulted in a severely inhibited rate of hydrolysis of ATP, indicative of functional ATPase. PglY nucleotidase activity was specific for ATP, and was found not to hydrolyse the other nucleotides tested (AMP, ADP, GMP, GDP, and GTP; data not shown). The non-metabolisable ATP analogue, ADP-NP was tested for its ability to inhibit the activity of PglY in vitro. The addition of equivalent molar amounts of ADP-NP and ATP, and subsequent 2-fold dilutions of ADP-NP in each assay resulted in a severely inhibited rate of hydrolysis of ATP, indicative of ADP-NP binding and inhibition of activity.

Taken together these data indicate an essential role for the ATPase activity of PglY in the Pgl system.

**Discussion**

Previous work describes the phenotype of Pgl in which an infection of *S. coelicolor* Pgl+ by phage $\phi$C31 undergoes a single burst but subsequent infections by progeny phage of *S. coelicolor* Pgl+ are attenuated (Bedford et al., 1995; Laity et al., 1993). A logical mechanistic explanation of these observations is that the phage is modified in the first infection but the second infection is restricted. Modified phage is able to proceed through a normal infection cycle in a Pgl− strain. In this work we demonstrated that PglX is indeed a DNA methyltransferase, as predicted by the bioinformatics searches. A strong genetic interaction between pglX and pglZ implies that PglX is toxic and that toxicity is suppressed in strains that are pglZ+ or contain the truncated pglZ1-834 allele in
target for the methyltransferase encoded by the *B. cereus* PglX homologue was elucidated by PacBio sequencing. The target, TAGGACG (the underlined A is methylated), is modified in uninfected cells, but infecting phage DNA was not modified despite containing multiple target sites. In the BREX system phage replication is prevented in the first infection, apparently through cessation of phage DNA replication. Thus although BREX and the Pgl systems are mediated by a core of orthologous proteins (PglX, PglY and PglZ in *S. coelicolor* and PglX, BrxC and PglZ in *B. cereus*) it seems there are significant differences in their mechanisms of resistance. We hypothesise that Pgl is adapted to cause phage resistance in the context of a host in which there is no detectable DNA methylation (using antibodies to DNA containing either C5- methylcytosine or N6-methyladenine; data not shown) and in which there appears to be general methyl-specific restriction (Gonzalez-Ceron et al., 2009; MacNeil, 1988). In this context host DNA might be unmethylated during growth of uninfected host cells and the Pgl system would be inherently toxic unless the methyltransferase activity is highly regulated. We propose therefore that any DNA methylation occurs only during phage infection. The ATPase activity of PglY was also shown to be required for phage resistance, possibly implying the need for a motor to drive a processive activity, similar to Type I R–M systems.

A model for the mechanism of Pgl

The model proposed by Chinenova et al. (1982) suggested that Pgl would differ from all known methyl-specific restriction systems owing to the proposed marking of phage or phage DNA and flagging it for restriction in later rounds of infection. We propose a model as to how the activities of the Pgl proteins could mediate the Pgl phenotype *in vivo* (Fig. 7).

The model resembles a Type I R–M system in which the modification and restriction activities are governed by the modification status of the DNA (Dryden et al., 2001; Murray, 2002). We propose that the Pgl proteins switch between three activity states: resting, modifying and restricting. In uninfected cells we propose that the Pgl proteins are in a resting, modifying and restricting. In uninfected cells we propose that the Pgl proteins are in a resting, modifying and restricting complex, dependent on whether the incoming signal that is specific to phage *ϕC31* and its relatives) to become either a DNA modifying complex or a restricting complex, dependent on whether the incoming phage is unmodified or modified, respectively. The modifying complex is likely to have N6-adenine methylation activity through the activity of PglX (blue). We propose that the restricting complex is activated by infection by modified phage. Candidate Pgl proteins that could mediate restriction are indicated in red. PglWP and PglZP are putatively phosphorylated isoforms due to the kinase activity of PglW that transduce a signal to the cells of phage infection (more details are provided in the text).

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**Fig. 6.** The Walker A motif of PglY is required for Pgl function. (A) Strains (indicated) were challenged with 1 x 10⁶ pfu ml⁻¹ of *ϕC31::ΔC25* harvested following J1929 infection (*PglY⁻*). (B) Release of Pi from ATP as a function of the PglY ATPase domain in wild type PglY and the PglYK81A/S82A mutant. Wild-type PglY (squares) and PglYK81A/S82A (circles). Data are mean of 3 replicate experiments. (C) Hydrolysis of ATP in the presence of ADP-NP. 4 mM ATP (black triangles; 3 mM ATP, 1 mM ATP-NP (circles); 2 mM ATP, 2 mM ATP-NP (grey triangles); 1 mM ATP, 3 mM ATP-NP (squares). Data are mean of 3 replicate experiments.

**Fig. 7.** Proposed two step model for the Pgl mechanism by modification and then restriction. In uninfected cells Pgl proteins assemble into a ‘resting complex’ in which the toxic activities of PglX is inhibited by an interaction between PglX and PglZ. Following infection the resting complex changes (presumably triggered by a signal that is specific for phage *ϕC31* and its relatives) to become either a DNA modifying complex or a restricting complex, dependent on whether the incoming phage is unmodified or modified, respectively. The modifying complex is likely to have N6-adenine methylation activity through the activity of PglX (blue). We propose that the restricting complex is activated by infection by modified phage. Candidate Pgl proteins that could mediate restriction are indicated in red. PglWP and PglZP are putatively phosphorylated isoforms due to the kinase activity of PglW that transduce a signal to the cells of phage infection (more details are provided in the text).
the S. coelicolor proteome (Bedford et al., 1995; Hesketh et al., 2002; Sumby and Smith, 2002). In the model infection by phage coming from a Pgl− host causes a change in the activities of Pgl proteins to modify progeny phage, most likely by N6-adenine methylation through the activity of PglX (Fig. 7). The putative N6-adenine methyltransferase activity could modify all the DNA in the infected cell or just targets in $\Phi C31$ and its relatives. The trigger for switching between resting and modifying activities of the Pgl proteins might also be specific to infection by $\Phi C31$ and its relatives. To avoid restriction of the modified phage DNA in the first infection cycle, we propose that Pgl proteins in the modifying state cannot switch directly to a restricting state or be reversed back to the resting state. We also propose that host methyl-specific restriction enzymes either do not recognise the modification in progeny phage or that the modification occurs late in the phage replication cycle. Thus, in agreement with the observations of Chinenova et al. (1982), modified phage progeny are released in a normal burst. Infection of Pgl strain with Pgl+ strains with this modified phage triggers the activation of the restricting activity of Pgl (Fig. 7). The mechanism of restriction is not clear but could be mediated by the PglW NERD domain, the host cell methyl-specific restriction endonucleases or an unidentified motif in PglX that is responsible for conferring toxicity. If the latter is the case, PglX could resemble some Type IIS restriction/modification systems that demonstrate yet further biological novelty in the cellular mechanisms of defence against bacteriophages.

Materials and methods

Bacterial strains, plasmids, growth conditions and conjugal transfer from E. coli to Streptomyces

The S. coelicolor strains used in this study are summarised in Table 1. All strains were cultivated on mannitol and soya flour (MS) agar (Hobbs et al., 1989). Plaque assays were performed as in Kieser et al. (2000) on Difco nutrient agar. Bacteriophage $\Phi C31$ cΔ25 was used throughout this work as described in Kieser et al. (2000). Plasmids were conjugated into Streptomyces from the E. coli strain ET12567 (dam− dcm− hsdS) containing pUZ8002 to provide the transfer functions (MacNeil, 1988). Plasmids used are summarised in Table 2. Plasmids for expression of the His-tagged Pgl proteins in E. coli were made as follows starting with the PglX–His6 expression plasmid, pP50032: A 5772 bp BamHI fragment encoding PglX from pPS1001 (Sumby and Smith, 2002) was inserted into BamHI cut pB λ19 (Parke, 1990) to generate pPS2002. An Ndel site was introduced at the PglX–His6 through the activity of PglX (Fig. 7). The putative N6-adenine methylation activity could modify all the DNA in the infected cell or just targets in $\Phi C31$ and its relatives. To avoid restriction of the modified phage DNA in the first infection cycle, we propose that Pgl proteins in the modifying state cannot switch directly to a restricting state or be reversed back to the resting state. We also propose that host methyl-specific restriction enzymes either do not recognise the modification in progeny phage or that the modification occurs late in the phage replication cycle. Thus, in agreement with the observations of Chinenova et al. (1982), modified phage progeny are released in a normal burst. Infection of Pgl strain with Pgl+ strains with this modified phage triggers the activation of the restricting activity of Pgl (Fig. 7). The mechanism of restriction is not clear but could be mediated by the PglW NERD domain, the host cell methyl-specific restriction endonucleases or an unidentified motif in PglX that is responsible for conferring toxicity. If the latter is the case, PglX could resemble some Type IIS restriction/modification systems that demonstrate yet further biological novelty in the cellular mechanisms of defence against bacteriophages.

Disruption of Pgl genes and marker removal in Streptomyces

The PglX and PglZ null mutants were created according to the protocol of Gust et al. (2003) using the pJ774 apramycin resistance cassette, flanked with loxP sites. In the double mutants (Table 1) the marker was removed as described (Khodakaramian et al., 2006) prior to disruption of the second target gene. Oligonucleotides for creating the disruptions are listed in Table S1.

Site-directed mutagenesis

Point mutants in the Pgl proteins were created using site-directed mutagenesis, performed using the QuikChange XL site-directed mutagenesis kit (Stratagene): primer sequences are detailed in Table S1. The exception to this protocol was in pglW, where the parental vector (pPS8002) was too large for Quickchange. The PglW K677A allele was produced by asymmetric PCR of a 900 bp region using the primers in Table S2. A first round of PCR created a product containing the mutant allele, with a flanking natural Stul site (pglW K677A F and pglW Asym FlankR), and a second PCR created the mutant allele with a flanking SrfI site (pglW K677A R and pglW Asym FlankF). The products were then mixed and a final round of PCR using the outer PCR primers (pglW...
Asym FlankF and pgIW Asym FlankR resulted in a final product of 900 bp representing a region of pgIW with the desired mutation. This fragment was cloned into pGEM-T-Easy, and the mutation confirmed by sequencing. The resulting plasmid, pPH1004, was cut with SrfI and Stul, giving a 460 bp product, which was used to replace the natural fragment in pPS8002, creating the vector pPH1012 (pgIW K677A).

Overproduction of Pgl proteins

Pgl proteins were overproduced as His6-tagged fusions. The plasmids were introduced into E. coli Rosetta (DE3) (Novagen) and protein expression induced by the addition of 0.1 M IPTG (isopropyl-β-D-thiogalactopyranoside) in exponentially growing cells (optical density 0.5 at 600 nm). The resulting His-tagged proteins were purified by nickel affinity chromatography, and their identity confirmed by MALDI–TOF mass spectrometry.

The exception to this was pgIW, which could not be produced in any DE3 lysogens. The protein was expressed from the plasmid pPS5025 using the EcoPro T7 coupled in vitro transcription-translation system (Merck Biosciences), with the mass of the expressed protein confirmed by incorporation of 35S-methionine in to the translation product according to the manufacturers instructions.

In vitro kinase assay

In vitro phosphorylation of PgIW was carried out according to Molle et al. (2003). Briefly, PgIW was incubated for 1 h at 30 °C in a 20 μl reaction containing 25 mM Tris–HCl, pH7.0, 1 mM DTT, 5 mM MgCl2, 1 mM EDTA, 2 μg protein and 200 μCi ml–1 [γ-32P] ATP. The reaction was stopped by the addition of an equal volume of 2× sample buffer, followed by heating at 98 °C for 5 min. The protein was visualised by autoradiography following separation on 4–12% SDS PAGE gels (Invitrogen).

ATPase assays

The ability of wild-type and mutant PglY proteins to hydrolyse nucleotides was assayed at 30 °C in 10 mM MgCl2, 10 mM dithiothreitol, and 0.1 mg ml–1 bovine serum albumin. Nucleotides and protein was added at the indicated concentrations. Reactions (10 μl) were pre-incubated at 30 °C for 10 min with PgIW prior to the addition of the nucleotide. Reactions were stopped by the addition of 2.5 μl of stop buffer (100 mM Tris–HCl (pH 7.5), 5% SDS, 200 mM EDTA, and 10 mg ml–1 proteinase K) and incubation at 37 °C for 20 min. The hydrolysis of ATP was detected by measurement of the release of inorganic phosphate using acidic ammonium molybdate and malachite green according to the method of McGlynn et al. (2000).

In vitro methylation assays

The reaction mixture contained 50 mM Tris–HCl (pH7.5), 7 mM 2-mercaptoethanol, 1 mM EDTA. 12 μM [3H]-5-adenosyl-l-methionine (GE Healthcare; specific activity 84 Ci mmol–1), 2 μg 4C31 DNA and 2 μg protein. Reactions were incubated for 1 h at 30 °C. Protein and unincorporated label were removed by phenol extraction and ethanol precipitation. Labelled was detected by liquid scintillation counting.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.12.036.

References

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