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Abstract

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Summary
Phage T4, the archetype of lytic bacterial viruses, needs only 62 genes to propagate under standard laboratory conditions. Interestingly, the T4 genome contains more than 100 putative genes of unknown function, with few detectable homologues in cellular genomes. To characterize this uncharted territory of genetic information, we have identified several T4 genes that prevent bacterial growth when expressed from plasmids under inducible conditions. Here, we report on the various phenotypes and molecular characterization of 55.1, one of the genes of unknown function. High-level expression from the arabinose-inducible P_{BAD} promoter is toxic to the bacteria and delays the intracellular accumulation of phage without affecting the final burst size. Low-level expression from T4 promoter(s) renders bacteria highly sensitive to UV irradiation and hypersensitive to trimethoprim, an inhibitor of dihydrofolate reductase. The delay in intracellular phage accumulation requires UvsW, a T4 helicase that is also a suppressor of 55.1-induced toxicity and UV sensitivity. Genetic and biochemical experiments demonstrate that gp55.1 binds to FolD, a key enzyme of the folate metabolism and suppressor of 55.1. Finally, we show that gp55.1 prevents the repair of UV-induced DNA photoproducts by the nucleotide excision repair (NER) pathway through interaction with the UvrA and UvrB proteins.

Introduction
As the number of sequenced genomes is growing at a rapid pace, our understanding of the functions of the newly discovered open reading frames (ORFs) is lagging far behind. Even in the case of Escherichia coli, probably the best-studied model organism, only 54% of the estimated 4452 gene products could be functionally annotated based on experimental evidence (Riley et al., 2006). When computational prediction of possible function was included, a further 32% could be annotated, leaving 14% of the gene products without any functional annotation whatsoever.

The large lytic phages also contain many potential genes of unknown function. Most of them represent ‘orphan’ genes, as they have no detected homologues in known cellular genomes. For example, the genomes of five T4-like phages contain 750 ORFs that lacked T4 orthologues. Among them, only 64 either resembled a protein of known function or matched a known functional domain (Nolan et al., 2006). Given that phages are probably the most abundant organisms on earth (Brüssow and Hendrix, 2002; Chibani-Chennoufi et al., 2004), these ORFs of unknown function represent an enormous source of uncharted genetic information that has been referred to as the ‘dark matter’ of life (Comeau et al., 2008). In addition to its importance in the evolution of life, this ‘dark matter’ constitutes a reservoir of potential new functions that could serve as useful tools for molecular and synthetic biology (Liu et al., 2004).

Phage T4, the archetype of the T-even phages, is one of the seven E. coli phages selected originally by Max Delbrück and the phage group in the 1940s as model organisms to study the basic principles of life (Delbrück, 1945; Abedon, 2000). Studies of the T-even phages led to the formulation of many modern fundamental biological concepts (Cairns et al., 1966; Mosig and Eiserling, 2006). Thanks to the isolation of two large collections of conditional lethal mutants – nonsense, amber (am) and temperature-sensitive (ts) mutants – T4 provided the first example of a systematic characterization of all genes essential for the development of an organism (Epstein et al., 1964; Edgar, 1966). The genome of T4 consists of 168 903 bps that code for 156 genes that have been characterized by mutations and/or by the predicted properties of their gene products (Miller et al., 2003). Among these genes, 62 are essential for T4 growth under standard laboratory conditions and the rest serve either auxiliary or
nonessential roles. Surprisingly, 20% of the T4 genome is tightly packed with 126 potential protein-coding genes (Miller et al., 2003; Nolan et al., 2006). The regions containing several of these uncharacterized ORFs can be deleted without significantly affecting phage propagation under standard laboratory conditions. Nevertheless, these ORFs are likely to represent bona fide T4 genes since their protein products can be detected (Kutter et al., 1994). Furthermore, most have been retained in at least some T4-related phages (Repola et al., 1994; Nolan et al., 2006; Petrov et al., 2010). Most of these uncharacterized genes are transcribed from *E. coli* promoters and can thus be expressed immediately after phage infection (Miller et al., 2003). Therefore, they could reprogram host metabolism and/or various specific host functions to somehow favour intracellular phage growth. These genes may play an important role in allowing phage growth in certain hosts, under certain environmental conditions, or to furnish a small growth advantage, which, though difficult to detect experimentally, may nevertheless be evolutionally important.

We have begun an extensive study of the T4 genes of unknown function, by initiating a screen based on the toxicity of some of these genes when expressed ectopically in *E. coli*. Here, as a proof of principle, we describe in detail the characterization of gene 55.1.

**Results**

**Identification of 55.1 and 55.2, two of the T4 ORFs with unknown function whose overexpression is toxic to *E. coli* growth**

In order to identify phage T4 genes that inhibit *E. coli* growth when expressed from a multicopy plasmid, we used a T4 genomic library of random DNA fragments cloned in pBAD18KKn, a vector with a pBR origin (~50 copies per cell). In this vector, expression of the DNA inserts is under the tight control of the arabinose-inducible P_{BAD} promoter (Guzman et al., 1995). The T4 DNA plasmid library was electroporated and transformants were screened for inserts that prevented bacterial growth in the presence of arabinose. Approximately 5% of the T4 DNA inserts conferred sensitivity to arabinose (Ara^® phenotype). Here, we will focus on the insert found in pDB21, a plasmid that completely abolished bacterial growth under inducing conditions.

The 1.4 kb insert of plasmid pDB21 contains two ORFs of unknown function (55.2 and 55.1) and an essential gene, gene 55. To pinpoint the toxic gene(s), we generated four plasmids that expressed either 55, 55.1 and 55.2 (55.1+2), 55.1 alone, or 55.2 alone (Fig. S1). Colony formation assays demonstrated that overexpression of either 55.1 or 55.2 was toxic, while 55 overexpression was not (Fig. 1A).

When the hypothetical protein sequences encoded by 55.1 and 55.2 were compared with the UniProt knowledgebase by using blastp (Altschul et al., 1997), the only significant hits were hypothetical proteins of other T4-related phages (data not shown, see also Petrov et al., 2010). Thus, the function of genes 55.1 and 55.2 and the mechanism of their toxicity cannot be deduced from the properties of any known cellular or phage homologues.

**UV sensitivity conferred by a plasmid containing both 55.1 and 55.2**

We discovered that strains carrying pDB21 were extremely UV sensitive (UV^®) in the absence of P_{BAD} induction. UV sensitivity assays showed that this phenotype requires both 55.1 and 55.2 but not 55. We performed bacterial viability assays to quantify this effect. At 48 J m^{-2}, a UV dose that decreased viability 10-fold in non-sensitive wild-type bacteria, cells carrying the 55.1+2 insert were 10^4-fold more sensitive than cells carrying the corresponding empty vector, a 55.1 insert alone, or a 55.2 insert alone (Figs 1B and S2).

**55.1 expression from T4 early promoters located in 55.2 is responsible for the UV^® phenotype**

Two hypotheses can explain why both 55.1 and 55.2 are required to confer UV sensitivity. First, both gene products could act synergistically to confer this phenotype. Because the UV^® phenotype was observed without activation of the P_{BAD} promoter, this hypothesis assumes that very low levels of expression of the two genes cause the UV^® phenotype. Alternatively, a T4 promoter located in 55.2 could drive 55.1 expression. To distinguish between these two possibilities, we performed a cis-trans assay of UV sensitivity. Whereas cells with both genes in the same insert (in cis) were UV^®, cells carrying 55.1 and 55.2 on two different compatible plasmids (in trans) were not (Fig. 1C). This result is compatible with the presence of at least one T4 promoter in 55.2.

In order to map the putative promoter(s), we performed RNase protection assays (Fig. 1D). Two RNA transcripts were detected in uninduced cells carrying 55.1+2 or a related plasmid that lacks the P_{BAD} promoter (55.1+2 ΔP_{BAD}), whereas with pYM5 (P_{T7,55.1}) only the smaller transcript was observed (Fig. 1D). The size of the smaller protected fragment suggests that the transcriptional start site is located extremely close to the putative AUG initiation codon of 55.1. The larger protected fragment was the same size as the one seen with an induced culture containing 55.1 and 55.2 (55.1+2). Further RNase protection assays performed with a probe containing the full length 55.2 demonstrated that the 5’ end of this second transcript is located ~290 bps upstream of the 55.1 AUG initiation codon (Fig. 1D and data not shown). Thus, two T4 pro-
motors (P₁ and P₂), both located in the 55.2-coding sequence, can drive 55.1 expression (Fig. 1D, lower panel). It is noteworthy that pDB2112, which contains both P₁ and P₂ promoters, induced a stronger UVS phenotype than pYM5, which has only the P₁ promoter (Fig. S2).

55.1 encodes a 9 kDa protein that causes both the UV$^\alpha$ and Ara$^\beta$ phenotypes

Radiolabelling experiments showed that these two genes are translated as proteins. A ∼ 9 kDa protein was detected
in extracts of uninduced bacteria containing a plasmid with the P T1 55.1 insert (Fig. 2A). This protein was also observed with a 55.1+2 insert and its expression was stronger in accordance with the presence of two T4 promoters in this insert (P T1 and P T2). Expression of the ≈9 kDa protein was strongly increased following arabinose induction; in this case a ≈13 kDa protein, corresponding to gp55.2, was also observed (Fig. 2A).

Although a protein was translated from 55.1, this did not prove that this protein was responsible for the Ara S and UVS phenotypes. For example, the small RNAIII of S. aureus is both an mRNA that encodes haemolysin d and a regulatory RNA that acts as an intracellular effector of the quorum-sensing system (Benito et al., 2000; Boisset et al., 2007). In order to determine whether the protein encoded by 55.1 was indeed responsible for the Ara S and UV S phenotypes, we introduced into pDB2112 a single base substitution that changed the tyrosine codon at position 14 of 55.1 (UAU) into an amber stop codon (UAG). The resulting plasmid, pYM18, did not induce UV sensitivity in DHB3, a sup+ strain that does not suppress amber mutations. However, when pYM18 was transformed in an isogenic supF strain that inserts tyrosine at amber stop codons, the UV S phenotype was restored (Fig. 2B). Arabinose sensitivity experiments showed that the Ara S phenotype also required a full-length gp55.1 (data not shown). Taken together, these results clearly demonstrate that the phenotypes of 55.1 are due to its protein product and not to its nucleotide sequence.

**Preexisting gp55.1 delays the intracellular accumulation of T4 virions**

Next, we asked whether 55.1 overexpression could also affect the T4 infection cycle. We found that in cells harbousing a pBAD plasmid with 55.1, induction of 55.1 for 10 min before infection had no detectable effect on the final burst size (data not shown). In order to assess whether 55.1 exerts a more subtle effect on the T4 growth cycle, we followed the kinetics of intracellular accumulation of phage particles. Low-level expression of 55.1 from P T1 before infection exerted no detectable effect on the accumulation of intracellular phage particles (Fig. 3A). However, induction of 55.1 expression from P BAD for 10 min before infection caused a marked delay in intracellular phage accumulation. Similar results were obtained with infections at a low multiplicity (data not shown), where the initiation of phage DNA replication could be more dependent on origin-mediated initiation. Thus, gp55.1 accumulated prior to infection causes a significant delay in intracellular phage accumulation.

We also asked whether the absence of gp55.1 could affect T4 infection cycle. Using the T4 I/S system (Selick et al., 1988), we replaced in the K10 T4 strain genome the ATG initiation codon of 55.1 with a GTT valine codon; an analogous substitution on a plasmid completely abolished 55.1-induced UV S and Ara S phenotypes (data not shown). Under standard laboratory conditions, the 55.1 mutant phage exhibited the same kinetics of intracellular particles accumulation as the parent strain and achieved a similar burst size (Fig. 3B). We conclude that 55.1 is a bona fide non-essential gene.

**Identification and characterization of multicopy suppressors of 55.1-induced toxicity**

We searched for multicopy suppressors of the Ara S phenotype of 55.1 using E. coli and T4 DNA genomic libraries. Plasmid libraries were electroporated into cells carrying 55.1 on a compatible plasmid and transformants were
directly selected on LB plates containing 0.2% arabinose. Library plasmids from each isolate were purified and tested for their suppressor phenotype. Non-specific suppressors, commonly found in selections based on the PBAD promoter (Bost et al., 1999), were eliminated because they were shown to also suppress the AraS phenotype induced by a non-homologous toxic chimeric protein containing the uncleaved signal sequence of plasminogen activators inhibitor-2 (PAI2) fused to alkaline phosphatase (Bost et al., 2000). The plasmid DNA inserts of the specific suppressors were identified by sequencing and BLAST searches. Finally, the identity of the individual gene responsible for the suppressor phenotype of each plasmid was confirmed by subcloning onto the parental vector and re-testing its ability to suppress the 55.1-induced toxicity.

**Ectopic expression of UvsW, a phage T4 helicase, suppresses 55.1-induced phenotypes**

A T4 genomic DNA library (pBR origin) was used to select for plasmids that suppressed the AraS phenotype induced by 55.1 expression from pDB2113-33 (p15A origin). We tested more than 100 T4 genome equivalents and isolated four different plasmids conferring specific resistance to 55.1. All of the inserts overlapped with the region coding for UvsW, an ATP-dependent DNA helicase that represses the origin-dependent replication initiation during late times of phage infection and that is also implicated in DNA repair, and uvsW.1, a recently described downstream ORF that encodes an 8.8 kDa protein of unknown function (Carles-Kinch et al., 1997; Dudas and Kreuzer, 2001; Kerr et al., 2007; Nelson and Benkovic, 2007). Expression of uvsW and uvsW.1 suppressed 55.1 toxicity but was partially toxic when expressed in the absence of 55.1 (Fig. 4A). Furthermore, a pBAD plasmid with a uvsW and uvsW.1 insert also
suppressed the UV\(^5\) phenotype induced by 55.1 expression from P\(_T\), in the absence of arabinose (Fig. 4B). Further subcloning showed that *uvsW* expression alone was able to suppress 55.1-induced UV\(^5\) and Ara\(^5\) phenotype although to a lesser extent (Fig. S3). In contrast, expression of *uvsW*.1 alone had no demonstrable effect on the 55.1-induced phenotypes (data not shown).

*uvsW* suppresses 55.1 independently of its effect on plasmid copy number

UvsW is a functional homologue of *E. coli* RecG (Carles-Kinch *et al.*, 1997), a DNA helicase whose overexpression reduces the copy number of plasmids containing ColEI-type origins of replication (Fukuoh *et al.*, 1997). Because *recG* was repeatedly isolated as a non-specific suppressor of the Ara\(^5\) phenotype induced by 55.1, we asked whether overexpression of *uvsW* could also affect plasmid copy number (PCN). We determined the plasmid DNA content of cells harbouring a pBAD plasmid with a *uvsW* and *uvsW*.1 insert or an empty control vector, as well as a compatible vector. In the absence of arabinose, the same amount of plasmid DNA was detected under all conditions. However, in the presence of arabinose, the total plasmid DNA content of cells expressing *uvsW* and *uvsW*.1 was significantly reduced compared with that of cells harbouring the empty vector (Fig. S4A). Since plasmids with a pSC101 origin are not affected by *recG* overexpression (Harinarayanan and Gowrishankar, 2003; our unpublished data), we cloned 55.1 into pBAD101, a low-copy vector that contains a pSC101 origin (Bieler *et al.*, 2006). Cells harbouring this plasmid stopped growing in the presence of 0.2% arabinose, and this phenotype was suppressed by *uvsW* but not by *recG* (Fig. S4B and data not shown). We conclude that expression of *uvsW* can reduce PCN but that this is not sufficient to explain the suppression of the Ara\(^5\) phenotype of 55.1. Finally, the suppression of the UV\(^5\) phenotype by a pBAD plasmid with a *uvsW* and *uvsW*.1 insert (Fig. 4B) occurred in the absence of P\(_BAD\) induction, a condition where the basal, low expression of *uvsW* had no detectable effect on PCN (Fig. S4A).

The suppression of 55.1-induced UV sensitivity requires UvsW helicase activity

During a phage T4 infection, a *uvsW*-K141R mutant lacks the UvsW helicase activity and has the same phenotypes as a *uvsW* deletion (Carles-Kinch *et al.*, 1997; Dudas and Kreuzer, 2001). We investigated whether the helicase activity of UvsW was also required for the suppression of UV\(^5\) phenotype induced by low-level expression of 55.1. As shown in Fig. 4B, the introduction of the K141R mutation into a pBAD plasmid with a *uvsW* and *uvsW*.1 insert almost completely abolished its suppressing ability. This helicase mutation also abolished the ability of *uvsW* to suppress 55.1-induced Ara\(^5\) phenotype, although it also eliminated the partial toxicity of overexpressed UvsW (data not shown).

The overexpression of 55.1 does not affect the intracellular accumulation of phage particles in a T4 *uvsW*\(\Delta\) mutant strain

The results presented above show that 55.1 and *uvsW* interact genetically when they are ectopically expressed in *E. coli*. We tested whether 55.1 and *uvsW* could also interact in the context of T4 infection. Bacteria overex-

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Fig. 4. The T4 *uvsW* gene suppresses 55.1-induced toxicity and UV sensitivity in a helicase-dependent manner.

A. DB503 cells transformed with pBAD33 (vector) or pDB213-33 (55.1) and one of the compatible plasmids, pBAD22K (vector) or pBAD-*uvsW* (*uvsW*), were streaked on LB plates with or without 0.2% arabinose. *uvsW* means that both *uvsW* and *uvsW*.1 are expressed by the plasmid.

B. UV sensitivity assays. DHB3 cells were transformed with pBAD33 (−) or pYM5-33 (+) and one of the compatible plasmids, pBAD22K (vector), YM53 (*uvsW*) or YM52 [(*uvsW*(K141R)]. Dilutions of overnight cultures were streaked on LB plates before irradiation with the indicated doses of UV light.
Fig. 5. Overexpression of the E. coli folD gene suppresses 55.1-induced toxicity, TMP hypersensitivity and UV sensitivity independently of its enzymatic function in the folate cycle. A. Left panel: DB503 cells transformed with pDB2113-33 (55.1) and one of the compatible plasmids, pMPM-A2 (vector) or pYM15 (folD), were streaked on LB plates with or without 0.2% arabinose. Right panel: UV sensitivity assay. DHB3 cells were transformed with pBAD33 (-) or pBB2112-33 (+) and pMPM-A2 (vector) or pYM15 (folD). Dilutions of overnight cultures were streaked on LB plates before irradiation with the indicated doses of UV light.
B. Left panel: MG1655 ΔfolP::kan (ΔfolP) cells transformed with pBAD33 (vector) or pBB2113-33 (55.1) were streaked on LB plates supplemented with thymidine plus or minus 0.2% arabinose and incubated for 40 h at 37°C. Right panel: UV sensitivity assay. MG1655 ΔfolP::kan (ΔfolP) cells were transformed with pBAD33 (-) or pYM5-33 (+) and pMPM-A2 (vector) or pYM15 (folD). Dilutions of equal amounts of overnight culture were plated on LB plates supplemented with thymidine. Plates were irradiated with the indicated doses of UV light and incubated for 26 h at 37°C.
C. Antibiotic disc tests. Left panel: Overnight cultures of DHB3 cells transformed with pBAD22K (vector) or pYM5 (P1,55.1) were used to measure the sensitivity to TMP (165 μg), nalidixic acid (NAL, 165 μg) and rifampicin (RIF, 330 μg). Right panel: DHB3 cells were transformed with pBAD33 (vector) or pYM5-33 (P1,55.1) and pMPM-A2 (vector) or pYM15 (folD); overnight cultures were used to measure the sensitivity to 330 μg of TMP. Data represent mean areas of inhibition and standard errors of three independent cultures.
D. In vivo cross-link and Ni2+ pull-down. Exponentially growing DB503 cells transformed with pBAD33-K (vector) or pYM5-33-K (55.1) were induced with 100 μM IPTG and 0.2% arabinose and cross-linked with 0.6% formaldehyde. His-tagged proteins were pulled-down on Ni-NTA agarose beads. Whole-cell extracts (W) and eluted proteins (P) were analysed by immunoblotting with anti-HA. Positions of uncross-linked gp55.1-HA and of the molecular weight protein standards (kDa) are indicated on the left.

Pressing or not 55.1 were infected with T4 uvsWΔ mutant or control phage strains and the kinetics of intracellular phage accumulation was followed. In the absence of uvsW, the eclipse period was longer and the burst size reduced by 10-fold (Fig. 3C). The overexpression of 55.1 had no effect on these phenotypes. These results show that uvsW is required for the effect of 55.1 on intracellular phage accumulation and suggest that uvsW is, genetically, a target of 55.1.

A host enzyme of the folate metabolism suppresses the phenotypes of 55.1

Next, we searched for E. coli multicopy suppressors of the Ara5 phenotype induced by 55.1 expression from pDB2113-33 (p15A origin) using a genomic library prepared from random DNA fragments cloned into a vector with a pBR origin. After testing the equivalent of ~20 E. coli genomes, we found that only two independently isolated plasmids conferred specific resistance to 55.1 expression. Both plasmid inserts contained folD, a gene coding for a bifunctional enzyme implicated in the folate cycle (D’Ari and Rabinowitz, 1991). The results displayed in Fig. 5A show the extent to which the folD insert suppressed both the Ara5 and UV5 phenotype induced by 55.1.

Low-level expression of 55.1 renders E. coli hypersensitive to trimethoprim

Escherichia coli cannot use exogenous folate and it relies on endogenous synthesis to generate folate and its derivatives, which are essential for both nucleotide and amino acid synthesis, as well as numerous methylation reactions (Fig. S5). Since FoIId is a bifunctional enzyme central to the folate cycle (FC), we asked whether 55.1 expression could somehow interfere with folate metabolism. To do this, we tested whether low-level expression of 55.1 could affect E. coli sensitivity to trimethoprim (TMP), a competitive inhibitor of FolA, the main bacterial dihydrofolate reductase (Hawser et al., 2006). Bacteria harbouring a P1,55.1 plasmid were much more sensitive to TMP than cells carrying the control empty vector (Fig. 5C). This enhanced sensitivity to TMP is highly specific since P1,55.1 did not alter bacterial sensitivity to other antibiotics tested (Figs 5C and S6B). Furthermore, using compatible 55.1 and folD plasmids, we showed that folD suppressed the TMP hypersensitivity caused by low-level expression of 55.1 (Fig. 5C). Multicopy expression of folD by itself also decreased the basal TMP sensitivity of bacteria that did not express 55.1.

The UV5 and Ara5 phenotypes of 55.1 are not dependent on folates

Because folate derivatives are necessary for the synthesis of numerous metabolites, perturbations of the folate cycle may result in very indirect effects (Das et al., 2008). Therefore, the toxicity and UV sensitivity caused by 55.1 could result from an alteration of the folate cycle. To investigate this possibility, we used strains harbouring deletions of either the folP or folE genes, which code for enzymes that catalyse early steps in the synthesis of folates and pterins (Fig. S5). As a consequence, these mutant strains contain no detectable levels of folate species but are viable in rich media supplemented with thymidine (Waller et al., 2010). The deletion of either folP or folE did not suppress the UV or arabinose sensitivities induced by 55.1 (Fig. SB and data not shown). We conclude that 55.1 UV5 and Ara5 phenotypes are not the consequence of an effect on the folate cycle.

Gp55.1 forms a complex with FoIId in vivo

Interestingly, overexpression of folD was still able to suppress 55.1-induced UV sensitivity in bacteria devoid of
folates (Fig. 5B). This suggested that FolD suppressed the UV (and Ara) sensitivity by directly binding to gp55.1. To determine whether such an interaction really occurred, we performed in vivo formaldehyde cross-linking experiments. Bacteria carrying an IPTG inducible His-folD expression plasmid from the ASKA library (Kitagawa et al., 2005) and a compatible pBAD vector with a 55.1-HA insert were sequentially induced with 100 μM IPTG and 0.2% arabinose. After cross-linking with 0.6% formaldehyde, cell lysate were analysed by Ni²⁺ pull-down followed by Western blot analysis with an anti-HA antibody. In the absence of His-tagged protein expression, several specific HA reactive bands were detected in whole-cell lysate but not in the eluate of the Ni²⁺-NTA column. In presence of His-FolD, a strong band corresponding to a ~40 kDa complex was observed both in the whole cell lysate and in the pull-down eluate (Fig. 6D); weaker higher-molecular-weight complexes were also present in the eluate. None of these complexes was present in cells expressing another His-tagged protein (AdK). We conclude that gp55.1 and FolD interact in vivo.
55.1 interferes with DNA repair

In addition to an increased sensitivity to UV, cells expressing low levels of gp55.1 also exhibited increased sensitivity to mitomycin C and hydroxyurea but not to hydrogen peroxide (Fig. S6A). This result prompted us to examine the effect of 55.1 on the repair of (6-4) pyrimidine–pyrimidone photoproducts (6-4PPs), the most prevalent UV-induced DNA lesions, along with cyclobutane pyrimidine dimers (CPDs) (Franklin and Haseltine, 1984; Goosen and Moolenaar, 2008).

We took advantage of the fact that the addition of an N-terminal His6 tag generated a hypomorphic allele of 55.1 (His-55.1). Bacteria carrying a plasmid with this allele under the control of the P_BAD promoter exhibited an Ara^R phenotype but they also exhibited an arabinose-inducible UV^S phenotype (Fig. S7). Bacteria harbouring the His-55.1 plasmid, induced or not with 0.2% arabinose, were exposed to 48 J m^-2 of UV light and genomic DNA was extracted at various times before and after irradiation. These DNA samples were then probed with a monoclonal antibody to 6-4PPs (Mori et al., 1991). In induced cells carrying the empty vector, DNA damage was almost completely repaired within 20 min of UV exposure. Rapid repair of DNA damage was also observed in uninduced cells carrying the His-55.1 plasmid. In striking contrast,
6-4PPs were still detectable 2 h after UV irradiation when expression of His-55.1 was induced with arabinose (Fig. 6A, left panel).

A quantitative time-course of DNA repair is shown in the right panel of Fig. 6A. No DNA damage was detected before UV irradiation under either condition. Immediately after UV exposure, similar amounts of 6-4PPs were detected under both conditions; the slightly lower amount observed in uninduced cells might be due to DNA repair initiated during the time necessary to process the samples. In uninduced cells, DNA repair occurred quickly and efficiently so that after 20 min only 2% of the initial damage was detected. When His-55.1 expression was induced, 6-4PPs levels remained almost constant during 40 min, indicating that DNA repair was severely impaired under these conditions. Thus, gp55.1 blocks DNA repair after UV irradiation.

The 55.1-induced UV pheno- nce is dependent on uvrA, uvrB and uvrC

In E. coli, most UV-induced DNA lesions are repaired by the nucleotide excision repair (NER) pathway (Franklin and Haseltine, 1984). NER requires six proteins: UvrABC, the three core proteins, recognize the damage and incise the DNA strand on both sides of the lesion; helicase II (UvrD) then removes the oligonucleotide containing the lesion; finally DNA polymerase I (PolA) and ligase (LigA) are required to fill the single-strand gap. The almost complete block of 6-4PPs repair in bacteria expressing His-55.1 suggested that gp55.1 interfered with the function of at least one of the NER proteins. In order to determine which of the Uvr proteins might be the target of gp55.1, we have tested for genetic interactions between 55.1 and deletions of uvrA, uvrB, uvrC or uvrD. Cells with single deletions in either uvrA, uvrB or uvrC and carrying an empty vector were very sensitive to UV irradiation. For these mutants, a single UV dose of 20 J m⁻² led to a ≈ 10⁻²-fold decrease in colony-forming units (cfu) number while isogenic wild-type bacteria carrying an empty vector showed almost no loss in viability after the same irradiation (Fig. 6B). Cells with a uvrD deletion showed a ≈ 10⁻³-fold decrease in cfu number after UV irradiation. The intermediate UV sensitivity of the ΔuvrD strain is explained by the fact that these cells retain a limited NER capacity (Crowley and Hanawalt, 2001). In wild-type cells, 55.1 expression from P₄ₐ and P₄₂ led to ≈ 10⁻²-fold decrease in viability after UV irradiation. 55.1 expression did not further increase the UV sensitivity of uvrA, uvrB or uvrC deletion mutants. However, ΔuvrD mutant carrying the 55.1 expressing plasmid showed a ≈ 10⁻³-fold increase in UV sensitivity compared with ΔuvrD mutant carrying the corresponding empty vector (Fig. 6B). The additive effect of 55.1 expression on the UV sensitivity of the uvrD deletion mutant was observed at different UV dose (data not shown). Taken together, these results suggest that 55.1-induced UV sensitivity is not due to a block of UvrD function per se but rather to a partial inhibition of the UvrABC machinery. However, it is also possible that gp55.1 has broad-spectrum anti-helicase activity and that it can inhibit both UvrD and the helicase(s) that are responsible for the residual NER activity in ΔuvrD mutants.

UvrA overexpression suppresses the 55.1-induced UV sensitivity

To discriminate between these two hypotheses, we attempted to rescue the UV sensitivity induced by low level of 55.1 by overexpressing an NER component. Cells harbouring a plasmid with the P₄ₐ,55.1 insert and IPTG inducible expression vectors with the indicated uvr genes were irradiated with progressive doses of UV light in the presence of 100 μM IPTG (Kitagawa et al., 2005). Overexpression of uvrA, but not uvrB, uvrC or uvrD, almost completely abolished the UV sensitivity induced by 55.1 expression (Fig. 6C). Interestingly, high levels of UvrD also strongly enhanced cell UV sensitivity. UvrA suppression was specific to the UV pheno- type of 55.1, as the overexpression of uvrA did not suppress the Ara pheno- type (data not shown).

Gp55.1 interacts with both UvrA and UvrB

During NER repair UvrA and UvrB form a complex (UvrA₂B) that recognizes the damaged DNA. In the following steps, the UvrA dimer detaches from UvrB. UvrB remains bound to the damaged DNA and interacts with UvrC that incises the DNA on both sides of the damaged DNA strand (Truglio et al., 2006). We asked whether it was possible to detect a direct interaction of gp55.1 with one of these three proteins. We used an in vivo formaldehyde cross-linking strategy followed by Ni²⁺ pull-down and anti-HA Western blot analysis, as described above for the FolD–gp55.1 interaction. We were able to detect an interaction of gp55.1-HA with UvrA and UvrB but not with UvrC. In the case of UvrA, both a ≈ 100 kDa and a high-molecular-weight complex were observed. For UvrB, a single ≈ 80 kDa HA reactive band was detected. Additional experiments showed that the three proteins were efficiently expressed and bound to Ni²⁺ (Fig. S10). In conclusion, the gp55.1 protein physically interacts with UvrA and UvrB but not detectably with UvrC.

Discussion

Since it is known that deletion of many of the genes coding for unknown function does not appear to affect phage production under standard laboratory conditions (Miller
et al., 2003), a loss-of-function strategy is unlikely to identify their function. In addition, the majority of the 126 T4 genes of unknown function do not show any significant similarity to genes in databases of cellular genomes (Nolan et al., 2006; O. Rubin and D. Belin, unpubl. data). As an alternative strategy, we devised a genetic screen to identify those T4 genes whose ectopic expression in E. coli strongly interferes with bacterial growth. To do so, we used random T4 genomic DNA fragments to generate a library in pBAD18, an inducible expression vector (Guzman et al., 1995). We found that 5% of the inserts conferred sensitivity to arabinose (AraS phenotype) and that some of them contained more than one toxic gene. Among the genomic fragments whose expression is toxic, several contained genes already known to interfere with bacterial growth, including rII58 (Selzer et al., 1981) and gene 57 (Hashemolhosseini et al., 1996).

The excessive basal expression of some toxic T4 genes in the absence of arabinose could prevent their isolation. In addition, unregulated expression can be due to the presence of T4 early promoters that are recognized by the bacterial transcription machinery. For example, a promoter adjacent to gene 32 prevents its cloning in E. coli (Belin et al., 1987). Thus, in both cases, a loss of bacterial viability would prevent the detection of some toxic T4 genes by our system.

Among the inserts that conferred sensitivity to arabinose, two contained 55.1 and 55.2, two genes of unknown function. Further analyses defined three different phenotypes: toxicity (AraS phenotype) induced by high expression of either 55.1 or 55.2 from the PBAD promoter, and UV sensitivity (UV phenotype) induced by low expression of 55.1 alone from one or two T4 early promoters localized in the upstream 55.2-coding sequence. The AraS phenotype induced by 55.1 or 55.2 results from high levels of expression, and these phenotypes may be, entirely or partially, artefactual. For example, it has been demonstrated that proteins that have a high intrinsic disorder content tend to be harmful when overexpressed because they engage in promiscuous interactions when their concentration is increased (Vavouri et al., 2009). However, the UV phenotype, which is induced by low levels of gp55.1, is more likely to reflect a physiological function of 55.1.

The UV phenotype of 55.1 results from a drastically reduced capacity to repair UV-induced DNA lesions. Mutants of uvrA, uvrB or uvrC are totally unable to repair 6-4PPs or CPD (Franklin and Haseltine, 1984; Kiyosawa et al., 2001), raising the possibility that gp55.1 blocks the function of at least one of the proteins that are central to the NER pathway. To define more precisely the target(s) of gp55.1, we measured the combined effect of uvr mutations and 55.1 expression on UV survival. Since polA mutants can excise UV-induced DNA lesions (Katsuki and Sekiguchi, 1975; Cooper, 1977), we did not test the combined effect of 55.1 and polA. 55.1 expression further increased the UV sensitivity of the uvrD deletion mutant, while it had essentially no effect on the UV sensitivity of the uvrA, uvrB or uvrC deletion mutants. These data suggest that the target of gp55.1 is the core NER machinery (UvrABC) rather than its DNA helicase II (UvrD) component. This hypothesis is supported by the suppression of 55.1-induced UV sensitivity by uvrA overexpression. Finally, we have demonstrated an interaction between gp55.1 and UvrA and UvrB by in vivo formaldehyde cross-linking. We conclude that gp55.1 inhibits the NER pathway by interacting with UvrA and UvrB.

We have demonstrated that the target of 55.1 must be present in the absence of SOS induction (Fig. S8). This is compatible with an inhibition of UvrAB by gp55.1. Indeed, even if the expression of uvrA and uvrB is increased during the SOS response, the NER is already active in the absence of SOS induction. It has been shown that 6-4PPs, whose repair is blocked by 55.1, are repaired efficiently in bacteria unable to induce the SOS response (Crowley and Hanawalt, 1998). Interestingly, we have shown that low-level expression of 55.1 leads to a moderate activation of the SOS response and a small increase in mutation rate (Fig. S9). Many mutants with defects in DNA metabolism and repair show a partially constitutive SOS response (McCool et al., 2004; O’Reilly and Kreuzer, 2004). However, deletions of uvrA, uvrB or uvrC are not reported to induce the SOS response and strains carrying these deletions possess a slightly decreased mutation frequency (Hasegawa et al., 2008). This discrepancy could be explained if gp55.1 blocks only some function of UvrABC, leading to the formation of small patches of single-strand DNA that can trigger an SOS response. The fact that bacteria expressing 55.1 are less UV sensitive than uvrA, uvrB or uvrC deletion mutants also supports a partial inhibition of the UvrABC machinery by gp55.1. Alternatively, there may be not enough gp55.1 to inhibit all UvrABC complexes present in the cells.

The major advantage of non-directed approaches is the possibility of discovering unexpected functional linkages. We have identified folD as an E. coli gene whose multicopy expression can suppress both the AraS and UV phenotypes of 55.1. FolD is a bifunctional enzyme that catalyses the two steps of the reversible conversion of 5,10-methylene-THF to 5-formyl-THF (10f-THF) (D’Ari and Rabinowitz, 1991). These reactions are part of the FC pathway (Fig. S4) that generates reduced folate derivatives used as methyl donors for purine, thymidine and amino acid synthesis; 10f-THF is also essential for formylation of initiator tRNA5m1 (Matthews, 1996). The fact that low levels of gp55.1 render bacteria hypersensitive to TMP, an inhibitor of the E. coli DHFR, confirms a link between 55.1 and the FC. Nevertheless, the UV and AraS phenotypes are unlikely to be an indirect conse-
sequence of the effect of 55.1 on the FC. First, the supplementation of the culture media with the full complement of folate end-products (Herrington and Chirwa, 1999) did not alleviate the UV8 and Ara8 phenotypes (data not shown). Second, despite the complete lack of folates, Δfol/E and ΔfolP mutants were as sensitive to 55.1 as are their isogenic fol+ strains. Overexpression of folD still suppressed 55.1 phenotypes in these mutant strains. Moreover, we have detected a direct binding of gp55.1 to FolD. Thus, we propose that FolD acts as a molecular ‘sponge’ that binds gp55.1 preventing it from interacting with other targets. The TMP hypersensitivity is likely to be caused by the binding of gp55.1 to the endogenously expressed FolD.

UvsW was identified in this study as a phage T4 gene whose ectopic expression in E. coli suppresses the UV8 and Ara8 phenotype induced by 55.1. UvsW is one of the three known helicases encoded by phage T4; its helicase activity is DNA and ATP-dependent and, in vitro, it can unwind a variety of substrates including branched DNA, D-loops and R-loops. In vivo, UvsW triggers the transition from the early, origin-dependent, mode of DNA replication initiation to the late, more efficient, recombination-dependent mode by unwinding R-loop at phage origins (Derr and Kreuzer, 1990; Carles-Kinch et al., 1997; Dudas and Kreuzer, 2001).

Experiments using a K141R inactive mutant showed that the helicase activity of UvsW is required for suppression of the UV8 and Ara8 phenotypes induced by 55.1. Interestingly, multicopy expression of RecG does not specifically suppress 55.1 phenotypes. Despite being functional analogues, these two proteins share no significant sequence homology outside of their conserved helicase motifs (Carles-Kinch et al., 1997; Kerr et al., 2007; Nelson and Benkovic, 2007). This suggests that suppression by UvsW may not be due entirely to its helicase function. At least two possibilities could account for the requirement of UvsW helicase activity: the active site may be necessary for the conformation of the domain that may interact with gp55.1; gp55.1 may only interact, directly or indirectly, with UvsW when it is engaged in a helicase activity. Both the deletion of uvsW and the overexpression of 55.1 cause a delay in intracellular phage accumulation, yet 55.1 overexpression does not further delay a T4 uvsW mutant. Hence, the delay induced by 55.1 could be caused by an alteration of UvsW activity by gp55.1 that would delay the onset of recombination-driven replication initiation. It is tempting to speculate that in addition to inactivating the T4 phage origins of replication, UvsW may directly interact with some of the early gene products including gp55.1. In conclusion, a direct reciprocal inhibitory interaction of UvsW and gp55.1 is the most straightforward explanation for our results.

The physiological role of gp55.1 during the T4 infection cycle remains an open question. Under standard laboratory conditions, the lack of 55.1 has no detectable effect on T4 infection. However the conservation of 55.1 in all the T-even type phages as well as in some more distant relatives of the T-even phages (Petrov et al., 2010) argues that it endows the phage with some growth advantage in nature. It is known that T4 infection leads to a rapid inactivation of NER (Strike, 1978) and 55.1 is expressed immediately after infection (Luke et al., 2002). Thus, a potential role for gp55.1 is the inhibition of the NER pathway, although the significance of this inactivation is not known. The increased sensitivity of bacteria to TMP caused by 55.1 and the suppressing effect of folD suggest that gp55.1 could modulate folate metabolism during the T4 infection cycle. Indeed, folates are essential cofactors for two T4 encoded enzymes: thymidylate synthase and dCMP hydroxymethylase. Furthermore, T4 encodes its own DHFR (Johnson and Hall, 1973) that is also part of a T4 dNTPs synthetase complex (Mathews, 1993; Murthy and Reddy, 2006). Finally, six molecules of a hexaglutamate derivative of DHF are structural components of the tail baseplate of the phage particles (Kozloff, 1983).

In conclusion, this article characterized 55.1, a T4 gene with previously unknown functions. We showed that its gene product perturbs the folate cycle of E. coli and that it binds FolD, one of the enzymes of this cycle. In addition, we have demonstrated that gp55.1 interacts with UvrA and UvrB, and blocks the repair of UV-induced DNA damage. More generally, our study demonstrates that ectopic expression and genetic suppression are powerful tools to study genes of unknown functions with no characterized homologues.

**Experimental procedures**

**E. coli strains, plasmids and growth conditions**

The bacterial strains and phage strains used in this study are listed in Table 1. The plasmids are listed in Table S1. The construction of the strains, plasmids and genomic DNA libraries are described in supplementary experimental procedures in Supporting information. Unless otherwise stated, all E. coli strains were cultivated at 37°C in LB medium. For growth on solid medium, 1.5% bacteriological agar was included. Antibiotics were used at the following concentrations: ampicillin (Ap), 200 μg ml⁻¹; chloramphenicol (Cm), 30 μg ml⁻¹; kanamycin (Kn), 40 μg ml⁻¹; and tetracycline (Tc), 7.5 μg ml⁻¹. When indicated, thymidine was added at 300 μg ml⁻¹. Compared with the DNA sequence of 55.1 found in the complete genome of phage T4 (GenBank: AF158101.6), we found that the plasmids used in this study have a single deletion at position G210 of 55.1, resulting in two amino acids changes (K70N and C71A) and a C-terminal truncation of the predicted protein at position 72. We found the same deletion in two clones of two independent T4 genomic libraries. Furthermore, the predicted protein sequences of the 55.1 homologues found in six T4-related phages match the shorter version of 55.1. Therefore, we conclude that our 55.1 sequence is the correct one.
**Table 1.** *E. coli* and T4 strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Prototrophic</td>
<td>Daegelen et al. (2009)</td>
</tr>
<tr>
<td>CR63</td>
<td>F&lt;sup&gt;+&lt;/sup&gt;, supD, lamB63</td>
<td>Lab collection</td>
</tr>
<tr>
<td>DB503</td>
<td>MC4100 malE16-1 ara714</td>
<td>Boyd et al. (1987)</td>
</tr>
<tr>
<td>DHB3</td>
<td>MC1000 malF3 phoA3(PvuII) phoR</td>
<td></td>
</tr>
<tr>
<td>MC1000</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; araD139 (araA-leu) (lac)X74 rpsL150 galE15 galK16 relA1 thi</td>
<td>Casadaban and Cohen (1980)</td>
</tr>
<tr>
<td>MC4100</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; araD139 (argF-lac)U169 thi5D301 fruA25 relA1 rpsL150 rbsR22 (flmB-fm-lec) decoC1 thi</td>
<td>Casadaban (1976)</td>
</tr>
<tr>
<td>MG1655</td>
<td>F&lt;sup&gt;−&lt;/sup&gt; rph-1</td>
<td>ATCC</td>
</tr>
<tr>
<td>MG1655 ΔfolE</td>
<td>MG1655 ΔfolE::kan</td>
<td>Klaus et al. (2005)</td>
</tr>
<tr>
<td>MG1655 ΔfolP</td>
<td>MG1655 ΔfolP::kan</td>
<td>Waller et al. (2010)</td>
</tr>
<tr>
<td>YM22</td>
<td>DHB3 oppC506::Tn10 supF</td>
<td>This study</td>
</tr>
<tr>
<td>YM42</td>
<td>DHB3 ΔuvrA753::kan</td>
<td>This study</td>
</tr>
<tr>
<td>YM43</td>
<td>DHB3 ΔuvrB751::kan</td>
<td>This study</td>
</tr>
<tr>
<td>YM44</td>
<td>DHB3 ΔuvrC759::kan</td>
<td>This study</td>
</tr>
<tr>
<td>YM45</td>
<td>DHB3 ΔuvrD769::kan</td>
<td>This study</td>
</tr>
<tr>
<td>T4D</td>
<td>Wild type</td>
<td>Epstein et al. (1964)</td>
</tr>
<tr>
<td>T4 K10</td>
<td>38amB262 51amS29 nd28 (denA) rIIPT8 (denB-rII deletion)</td>
<td>Selick et al. (1988)</td>
</tr>
<tr>
<td>T4 K10-uvWaA</td>
<td>K10 uvaWaA1</td>
<td>Derr and Kreuzer (1990)</td>
</tr>
<tr>
<td>T4 K10-55.1</td>
<td>K10 55.1 (ATG -&gt; GTT)</td>
<td>This study</td>
</tr>
</tbody>
</table>

a. All strains are *E. coli* K-12 derivatives except B<sup>e</sup>, which is an *E. coli* B derivative.

**UV irradiation: viability assays and sensitivity assays**

For UV viability assays, cells were grown with aeration to mid-logarithmic phase, harvested by centrifugation at 4°C and resuspended at 0.4 A<sub>600</sub> ml<sup>−1</sup> in cold 100 mM MgSO<sub>4</sub>. Two millilitres of samples were placed in 35 mm plates and irradiated with gentle shaking under a germicidal UV lamp (TUV 15W/G15T8, Philips, the Netherlands). After irradiation, cells were placed back on ice, diluted in 100 mM MgSO<sub>4</sub> and aliquots were plated on appropriate LB plates. Following overnight incubation, cfu were counted and the titres calculated according to the dilution factor. For UV sensitivity assays, overnight cultures were diluted 1:500 (or less for strains that grew slowly) in fresh media and 40 μl were immediately streaked on appropriate LB plates. Plates were covered with a metal lid and placed under the UV lamp. Parts of the plate were exposed to increasing amount of UV light by sliding the metal lid. All assays were performed in the dark to avoid DNA repair by the photolysis. The power of the UV lamp was measured with a UVX radiometer equipped with a UVX-25 sensor (UVP, Upland, CA).

**RNA extraction and RNase protection assay**

Cells were grown with aeration to A<sub>600</sub> = 0.5. RNA was extracted as described earlier (Pogliano et al., 1997). RNase protection assays were performed as previously described (Belin, 1997) with 2 μg of total RNA and a 32P-labelled RNA probe generated with T3 RNA polymerase on pYM3 plasmid linearized with HindIII.

**Protein pulse-labelling experiments**

Pulse-labelling was performed as described elsewhere (Belin, 2010). Samples were centrifuged and directly resuspended in SBB [50 mM Tris pH 6.8, 5% (v/v) β-mercaptoethanol, 1% SDS, 0.0025% (w/v) bromophenol blue and 8.5% (v/v) glycerol] at a concentration of 10 A<sub>600</sub> ml<sup>−1</sup>. Samples were lysed by freezing and thawing followed by sonication and centrifugation. The supernatants were boiled 3 min and equal volumes were loaded onto 19% SDS-polyacrylamide gels. Gels were fixed, dried and autoradiographed.

**Phage infection and intracellular phage growth**

*Escherichia coli* B<sup>e</sup> (for T4D) or CR63 (for K10 strains) transformed with the indicated plasmids were grown to a concentration of ~1 × 10<sup>8</sup> ml<sup>−1</sup>. When indicated, cells were treated with 0.2% arabinose, and grown 10 more min. Bacteria were centrifuged, resuspended at 4 × 10<sup>8</sup> ml<sup>−1</sup> in cold LB media supplemented with tryptophan (100 μg ml<sup>−1</sup>) and infected with T4 strains at the indicated multiplicity of infection (moi). After 10 min on ice, the infected bacteria were transferred to prewarmed tubes (30°C) containing equal volume of LB media supplemented with 0.4% glucose (time of transfer is referred to as t = 0). Phage growth was carried at 30°C with vigorous agitation. At indicated times, 0.2 ml of samples were removed and immediately lysed with CHCl<sub>3</sub> to ice to determine intracellular phage content. Samples taken at 5 min with and without CHCl<sub>3</sub> were used to determine free unadsorbed phages and infective centres. After appropriate dilution in M9 salts, the samples were mixed with indicator bacteria, molten 0.7% top H agar, and plated on H media agar. Plaques-forming units (pfu) were scored after overnight incubation at 37°C.

**Antibiotic/DNA-damaging agent disc sensitivity test**

For antibiotic disc sensitivity test, 0.1 ml aliquots of overnight cultures were diluted in 3 ml of warm LB top agar and poured on LB agar plate. Six-millimetre filter paper discs (Nr. 321260, Schleicher & Schüll, Germany) were placed in the centre of
the plates and the indicated amount of antibiotics added to each disc. The plates were incubated at 37°C and the zones of inhibition were measured after 4–5 h. For DNA-damaging agent, the procedure was the same except that 12.5 μl of overnight culture was used and that the zones of inhibition were measured after overnight incubation.

**In vivo formaldehyde cross-link and Ni²⁺ pull-down**

Cells were grown with aeration to A₆₀₀ = 0.3, induced with 100 μM IPTG for 1 h, then 0.2% arabinose was added for an additional 20 min. Formaldehyde [fresh 4% (w/v) solution in PBS] was added to 0.6% final and cells were fixed for 15 min at room temperature with agitation; cold glycine (125 mM) was added to 0.6% final and cells were fixed for an additional 20 min. Formaldehyde [fresh 4% (w/v) solution in PBS] was added to 0.6% final and cells were fixed for 15 min at room temperature with agitation; cold glycine (125 mM) was added to 0.6% final and cells were fixed for 15 min at room temperature with agitation; cold glycine (125 mM) was added to 0.6% final and cells were fixed for an additional 20 min. Formaldehyde [fresh 4% (w/v) solution in PBS] was added to 0.6% final and cells were fixed for 15 min at room temperature with agitation; cold glycine (125 mM) was added to 0.6% final and cells were fixed for an additional 20 min. 

Detection of 6-4PPs by immunodot blot analysis

Cells were grown with aeration to A₆₀₀ = 0.3, then half of the culture was treated with 0.2% arabinose for 1 h while the rest was left untreated. Cells were then harvested and irradiated as described above with 48 J m⁻² of UV light. After irradiation, cells were centrifuged, resuspended in warm LB media plus or minus 0.2% arabinose, and incubated at 37°C with aeration. Aliquots were removed at various times after irradiation and DNA was isolated using the Puregene method (Gentra systems) according to the manufacturer's instructions. Immunodot blot were performed with 150 ng of heat-denatured DNA as described elsewhere (Smit et al., 2001; Clément et al., 2006). Detection of 6-4PPs was performed by using the mouse monoclonal antibody 64 M-2 (Mori et al., 1991), a secondary horseradish peroxidase-linked anti-mouse goat antibody (Bio-Rad) and chemiluminescence visualization. To verify equal loading and normalize extracts were mixed with 25 μl of Ni-NTA agarose beads (Quiagen) and incubated on a rotary wheel for 45 min at 4°C. The beads were washed thrice with cold wash solution [50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, 1 mM PMSF, 0.5 g l⁻¹ lysozyme, pH 7.85]. Cell extracts were obtained by sonication on ice and centrifugation to remove unsoluble material. One hundred and eighty microlitres of cell extracts were mixed with 25 μl of Ni-NTA agarose beads (Quiagen) and incubated on a rotary wheel for 45 min at 4°C. The beads were washed thrice with cold wash solution [50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, 0.8% (v/v) Nonidet P 40 (74385, Sigma), pH 7.85] and bound proteins were eluted with SB⁺ containing 20 mM EDTA for 10 min at 37°C. Eluted samples were heated 5 min at 70°C, separated on 19% SDS-polyacrylamide gels, and proteins were transferred to nitrocellulose membranes. Proteins were detected with chemiluminescence using mouse monoclonal antibody from MRL lpr/lpr mice that develop systemic lupus erythematosus. To verify equal loading and normalize extracts were mixed with 25 μl of Ni-NTA agarose beads (Quiagen) and incubated on a rotary wheel for 45 min at 4°C. The beads were washed thrice with cold wash solution [50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, 0.8% (v/v) Nonidet P 40 (74385, Sigma), pH 7.85] and bound proteins were eluted with SB⁺ containing 20 mM EDTA for 10 min at 37°C. Eluted samples were heated 5 min at 70°C, separated on 19% SDS-polyacrylamide gels, and proteins were transferred to nitrocellulose membranes. Proteins were detected with chemiluminescence using mouse monoclonal antibody from MRL lpr/lpr mice that develop systemic lupus erythematosus.

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Supporting information

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