Freeing *Pseudomonas putida* KT2440 of its proviral load strengthens endurance to environmental stresses

by

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Summary

2.6% of the genome of the soil bacterium *Pseudomonas putida* KT2440 encodes phage-related functions, but the burden of such opportunistic DNA on the host physiology is unknown. Each of the 4 apparently complete prophages borne by this strain was tested for stability, spontaneous excision and ability to cause lysis under various stressing conditions. While prophages P3 (PP2266-PP2297) and P4 (PP1532-1586) were discharged from the genome at a detectable rate, their induction failed otherwise to yield infective viruses. Isogenic *P. putida* KT2440 derivatives bearing single and multiple deletions of each of the prophages were then subject to thorough phenotypic analyses, which generally associated the loss of proviral DNA with an increase of physiological vigour. The most conspicuous benefit acquired by prophage-less cells was a remarkable improvement in tolerance to UV light and other insults to DNA. This was not accompanied, however, with an upgrade of *recA*-mediated homologous recombination. The range of tolerance to DNA damage gained by the prophage-free strain was equivalent to the UV resistance endowed by the TOL plasmid pWW0 to the wild type bacterium. While the *P. putida*'s prophages are therefore genuinely parasitic, their detrimental effects can be offset by acquisition of compensatory traits through horizontal gene transfer.

Introduction

Although viruses that prey on bacteria have been known since the onset of modern microbiology, only recently their diversity and abundance has been realized owing to the ease of genomic and metagenomic sequencing (Chibani-Chennoufi *et al.*, 2004; Rosario and Breitbart, 2011). Typically, bacteriophages either run into lytic cycles or they lysogenize (i.e. temperate phages), in which case their DNA sequences are passively transmitted to the bacterial progeny as prophages. In the last scenario, prophages often remain altogether silent until cells encounter stresses such as DNA damage -or just because of stochastic events that trigger the start a new lytic cycle (Casjens, 2003). At first sight, the presence of one or more prophages in a bacterial chromosome (where they may make up to 20% of the whole genome; Casjens, 2003) would be expected to be detrimental, as they can be turned on any time and kill its host. Should this be the case, evolutionary logic would make such lysogens to first undergo a pseudogenization process followed by eventual removal (Fong *et al.*, 1995). Certainly, prophages found in most bacterial genomes frequently appear to be defective and display a state of mutational decay that

makes them unable to produce new infective viruses (Casjens, 2003). But, intriguingly, many of the encoded genes are still expressed and functional, thus raising the question of why such active or semi-active –but otherwise burdensome DNA is kept rather than quickly deleted (Minot *et al.*, 2013). One simple explanation is that lysogens provide the host with immunity and super-infection exclusion against other viral infections (Canchaya *et al.*, 2003). A second aspect is that phage genomes may carry beneficial genes that ultimately contribute to the genetic repertoire and activity portfolio of the host. This scenario includes e.g. metabolic diversity (Sharon *et al.*, 2011), stress endurance (Wang *et al.*, 2010) and, in the case of pathogens, virulence factors as well (Winstanley *et al.*, 2009; Wagner and Waldor, 2002). Finally, phages (as any other mobile genetic elements) may bring about genetic diversity that helps adaptive evolution of the host (Gardner and Welch, 2011). But in other cases, either active or defective prophages clearly impose a burden to the carrier by interfering with its metabolic network (Chen *et al.*, 2005) or its ability to colonize competitive niches (e.g. the rhizosphere; Quesada *et al.*, 2012).

During the course of our efforts to edit the extant genome of the soil bacterium *Pseudomonas putida* KT2440 as an optimal chassis for industrial and environmental biotechnology (Nikel *et al.,* 2014) we faced the dilemma of either keeping or deleting the 6 regions that are annotated in the ACLAME database of mobile prokaryotic DNA (http://aclame.ulb.ac.be; Leplae *et al.*, 2004) as *bona fide* proviral sequences. These six regions seem to actually encode 4 prophages, as two of them lay adjacent to the others in contiguous operons (Canchaya *et al.*, 2003; Wu *et al.* 2011; Hayashi and Nakayama, 2004). Taken together these prophage regions comprise approximately 2.6 % of the genome. According to the preamble above, one cannot ascertain upfront (i.e. by looking at the DNA) whether these segments are necessarily unfavourable to the robustness of the host or they instead contribute to its physiological qualities. On this background we set out to examine the phenotypic and physiological consequences of having or not such prophage sequences as part of the genetic complement of *P. putida* KT2440 both as individuals and as whole proviral load. To this end, we generated a collection of strains accurately deleted of each of the corresponding DNA segments and examined the cognate bacteria for a large number of stress-related traits. As shown below, removal of the whole proviral contents of *P. putida* consistently resulted in cells more tolerant to environmental hardships, in particular DNA damage. Moreover, the detrimental effects of the proviral load can be fully compensated by the UV resistance

rulAB genes borne by the TOL plasmid pWW0 (Tark *et al.*, 2005), as is the case in the environmental precursor of *P. putida* KT2440, namely, the strain *P. putida* mt-2.

Results and Discussion

The prophages of the genome of P. putida KT2440.

P. putida KT2440 has four prophage elements in its chromosome which have been numbered 1 to 4 (Canchaya *et al.*, 2003; Wu *et al.*, 2011). The distribution of these prophages throughout the strain's genome is indicated in Fig. 1A. A detailed inspection of these gene clusters revealed the following composition: prophage 1 (PP3849-PP3920), prophage 2 (PP3026-PP3066), prophage 3 (PP2266-PP2297), and prophage 4 (PP1532-PP1586; Fig. 1B). The prophage 1 (P1) cluster is 54.7-kb long and contains 72 ORFs; it is a mosaic element with a Sfi21-like (phiE125) siphovirus head gene cluster and a Mu-like (Sfv) tail gene operon (Canchaya *et al.*, 2003). We have included PP3849 gene within the sequence of this provirus, which was not considered as such neither in the ACLAME database nor in Wu *et al.* (2011). P1 encodes two integrases, an endolysin, two holin genes, a toxin-antitoxin system and one repeats-in-toxin exoprotein (RTX). Prophage 2 (P2) consists of 35.6-kb with 41 ORFs; it is also a chimeric element with a with a Sfi21-like siphovirus head gene cassette with a P2-like myovirus tail gene cluster (Canchaya *et al.*, 2003). P2 encodes a serine recombinase, a chitinase-lytic enzyme and a holin. Prophage 3 (P3) is closely related to T7-like phages (Canchaya *et al.*, 2003) and comprises 39.1-kb including 32 coding sequences that, excepting two of them, are all oriented in the same direction. In contrast to other T7-type lytic phages, P3 encodes an integrase (PP2297) and a muralytic enzyme, but no holins. Finally prophage 4 (P4) has a size of 40-kb and it includes 53 ORFs; this genetic element is similar to the *Pseudomonas* phage D3 (Canchaya *et al.*, 2003). P4, also named Gi28 by (Quesada *et al.*, 2012) has been shown to excise from the genome at an unknown frequency. Interestingly, it encodes an excisionase, two integrases and one holin but surprisingly not a lytic enzyme. The inclusion or not of PP1585 and PP1586 (encoding a toxin-antitoxin system) in the P4 element was dubious as their functionality has been recently described (Tamman *et al.*, 2014) but it is not necessarily associated to the prophage (see below). Taken together, the whole proviral DNA contents of *P. putida* KT2440 comprises about 2.58% of its genome. Fig. 1C shows the percentage of the total of ORFs of these four

prophages grouped in a gross functional classification. Note that the account reveals ~ 40% of the corresponding ORFs encoding proteins of unknown function.

Prophage excision frequencies

At the time of starting this work, the activity of some components of the proviral load of *P. putida* KT2440 had been observed. Quesada *et al.* (2012) reported that the so-called GI28 genomic island that overlaps with the P4 prophage (see above; Fig. 1B) was lost spontaneously and originated structures reminiscent of phage capsides. Further inspection of *P. putida* KT2440 stocks of various origins corroborated that the spontaneous loss of P4 was a frequent occurrence. One of such spontaneous ΔP4 strains (named *P. putida* EM371; Table 1) was chosen as a reference for quantifying the excision rates of the other prophages and to define accurately the boundaries of their insertions. To this end, we generated a series of diagnostic PCR primers (Supplementary Table S1) aimed at detecting and quantifying the presence of each of the proviruses in the cognate genome. As a reference for P4, we amplified the region predicted to be left behind in the genome of *P. putida* EM371 after prophage excision (i.e., the TS1-TS2 segment of Supplementary Fig. S1A), using primers P1532-XmaI-P1586-BamHI (Table S2) and sequenced the resulting DNA product. This allowed us to confirm that the active P4 prophage encompasses exactly ORFs PP1532 to PP1584, a segment that does not include the toxin-antitoxin system (PP1585-PP1586; Tamman *et al.*, 2014) entertained above. Analysis of the PCR products shown in Supplementary Fig. S1 revealed that amplification of the genomic DNA of the wild type *P. putida* KT2440 with primers designed for detection of P4 excision resulted in a faint band of the same size as that coming from the ΔP4 strain *P. putida* EM371. This verified that the loss of P4 does occur at a detectable spontaneous frequency, a fact that was instrumental to set positive and negative controls to measure the frequency of excision of each prophage under a suite of stress conditions.

To this end, we resorted to quantitative PCR (qPCR) of bacterial DNA amplified from cognate *P. putida* cultures using primers flanking the each of the proviral gene clusters (Supplementary Table S1) and adopting the *rpoA* gene sequence as an internal control (see Experimental Procedures for details). Prophage stability was tested in each case under five physiological scenarios (Fig. 2): exponential growth, stationary phase, irradiation with UV, inhibition of DNA replication (i.e. addition of nalidixic acid) and prolonged starvation. As expected, the most active prophage was P4 (Fig. 2B), which was lost at a

frequency of □10^{-3} in stationary phase, in cells stressed with UV, added with Nal or subject to protracted starvation. P4 was also excised, albeit at a lower frequency (□10^{-4}) in exponentially growing cells. The next more active prophage was P3, which was excised with starvation (□10^{-4}) and, to a lesser extent (□10^{-5}) with DNA damaging agents. In contrast, we could not detect with this procedure the loss of either P1 or P2 at frequencies above the detection threshold (<10^{-7}, not shown). This in intriguing, as some the genes encoded in their sequences become up-regulated in *P. putida* cells treated with mitomycin C (Abella *et al.*, 2007). We cannot rule out that they become active under other growth conditions or at lower frequencies. But whether all or some prophages are actively excised or they are in the way to pseudogenization they may still burden the host cells with diverting metabolic resources into expression of detrimental –if not directly killer functions. The nature of such a burden and the individual impact of each prophage to the physiological vigour of *P. putida* was addressed next.

Gross metabolic traits of prophage-less strains

In order to investigate separately and as a whole the role of the four prophages in the cellular physiology of *P. putida* KT2440 we exploited the I-SceI-based method of targeted genomic deletions (Martinez-Garcia and de Lorenzo, 2011) for constructing a panel of isogenic strains that differed only in their proviral content (Table 1). This panel included *P. putida* variants individually deleted of each of the prophage segments P1-P4 as well as an entirely prophage-free specimen, thereafter named *P. putida* Δall-Φ. With this strain collection in hand, we first evaluated the overall effect of lacking all prophages by comparing the growth of the wild type strain *vs.* the Δall-Φ counterpart in nutrient-rich LB medium and minimal M9 with gluconeogenic (citrate) and glycolytic (glucose) carbon (C) sources. As shown in Supplementary Fig. S2A, both strains reached similar maximum OD_{600} values in such growth conditions. In order to determine whether the ratio of live/dead bacteria in stationary phase was influenced by the presence of the prophages, cells from the cognate overnight cultures were treated with propidium iodide (PI), a fluorescent dye that binds damaged membranes only (Williams *et al.*, 1998). As shown in Supplementary Fig. S3, flow cytometry of these samples suggested that the prophage-free strain tends to produce less dead bacteria in non-growing, stationary cultures. Although divergences in the PI-stained counts become more pronounced in glucose than in the other two media tested, they were still relatively minor and within the same range. In any case, they did not translate into

any longer-term consequences, because both wild type and ΔΔall-Φ strains maintained a prolonged viability even after a period of starvation of up to 14 days (Supplementary Fig. S2B; Budzik *et al.*, 2004).

Phenotypic consequences of deleting 4 prophages from the P. putida's genome

The gross metabolic characteristics of *P. putida* seem therefore not to change much by the presence or absence of the proviruses. The situation varied however when the same strains were passed through the series of > 1000 growth conditions included in the Biolog phenotypic microarray system (Bochner *et al.*, 2001; Bochner, 2009). Specifically, plates PM1 to PM20 (http://www.biolog.com) allowed us to examine different carbon, nitrogen, phosphorous, and sulphur sources, together with the influence of nutrient supplements, osmolytes, pH values and sensitivity to a broad panel of chemicals and stressors. A mere visual inspection of the corresponding graphs (Supplementary Fig. S4) immediately indicated the predominance of the gain-of-function phenotypes (visualized as green colour in the individual plots) versus the loss of them (in red). Supplementary Table S1 lists the 19 traits more significantly influenced by the deletion of the four prophages, all of them gained rather than lost. Among them, the changes include improvements of glutamine and dipeptides valine-valine, tyrosine-histidine and valine-histidine as a sole nitrogen source, increased utilization of various growth supplements and higher tolerance to caffeine (a cAMP phosphodiesterase inhibitor) and the oxidizing agent 2-hydroxy-1,4-naphtoquinone. Taken together, however, the consistent trend was that of a better physiological performance of bacteria freed of the proviral load, i.e. the general vigour of cells was coherently enhanced without the prophages. Some environmentally relevant traits were then examined in more detail.

UV light super-sensitivity of P. putida KT2440 *can be traced to its proviral load*

Environmental bacteria such as *P. putida* KT2440 are frequently exposed to the ultraviolet (UV) irradiation of sunlight as part of their ordinary lifetime. Since proviruses are often induced by DNA-damaging agents we set out to judge the ability of the wild type and the prophage-free strains to face UV light. In order to have suitable references, we first ran comparisons between the endurance of a wild type *E. coli* strain and its *recA*-minus derivative to 254 nm UV light along with *P. putida* KT2440 and two directed mutants in its *recA* and *recB* genes. The simple test shown in Fig. 3A revealed that *P. putida* KT2440 was far more sensitive to this type of DNA damage than the *E. coli* strain employed as a

standard. Although the growth of the *recA* variants of each species under UV was roughly similar, the wild type parental strain displayed a marked difference that could be detected by mere visual inspection of the irradiated plates (Fig. 3A). To quantify such a disparity the same experiments were performed as quantitative spot assays applying controlled doses of UV light to the samples under study (see Experimental procederes). As shown in Fig. 3B, the contrast between the two strains was considerable: while a radiation of 30 J/m2 left *E. coli* virtually intact, the same dose decreased survival of *P. putida* KT2440 by >4 orders of magnitude. On this background, the next question was on the role of the proviral load in such sensitivity.

To this end, we first grossly estimated the UV tolerance of the different prophage-deleted strains in the same plate assay used before, where cells are exposed to a gradient of $UV^{245\ nm}$ irradiation (Fig. 4A). Perusal of the plate indicated some informative results: [i] None of the individual prophages accounted individually for the full UV sensitivity of the wild type strain, [ii] the prophage-free strain was noticeably more resistant to UV damage than the wild type, and [iii] in relative terms, the P1 element seemed to contribute more than the other prophages to the UV sensitivity of *P. putida* KT2440. These qualitative observations were faithfully reproduced when the same strains and others with some combinations of deletions were subject to experiments in liquid medium with precise UV light doses. As shown in Fig. 4B, the Δall-Φ strain showed to be more resistant than the wild type, especially at the highest UV intensity (15 to 30 J m^{-2}). Note that single, double and triple deleted strains still failed to explain the phenotype of the Δall-Φ bacteria. Still, in comparative terms, the ΔP1-only strain survived better at the higher UV dose. The benefit of accumulating deletions can be deduced from the superior performance of the ΔP1ΔP4 and ΔP1ΔP4ΔP3-deleted strains, although much of the gain can still be traced to the loss of P1. In any case, the accumulative resistance pattern to UV exposure was fully observed only when all the parasitic elements of the wild type strain had been eliminated.

UV light sensitivity of P. putida KT2440 is not due to the malfunction of the SOS system

The strong effects of UV radiation on viability of *P. putida* KT2440 (Fig. 3 and Fig. 4) resemble phenotypically those caused by a faulty performance of the *recA*-based SOS system of repair of DNA damage that is shared by virtually all bacteria (Erill *et al.*, 2007). Otherwise, the same sensitivity to UV might be the result of the lethal induction of the prophages by the SOS response to the DNA damage

entered by UV. To examine these possibilities we compared the functioning of the *recA*-based homologous recombination system and the spontaneous mutagenesis rates of the wild type strain with those of the Δall-Φ *P. putida*. Although the SOS system of *P. putida* has not been entirely characterized (Abella *et al.*, 2007) we took these simple assays as a proxy of the overall performance of the recombination machinery. To this end we first tested integration of non-replicative plasmids bearing DNA sequences homologous to genomic regions. For this we separately electroporated three recombinant pEMG plasmids carrying different DNA segments (□500, □600, □800 bp) that matched chromosomal sequences of the wild type cells and the ΔallΦ strain. The results shown in Fig. 5A failed to reveal any significant difference in the ability of these bacteria to co-integrate the test plasmids. That such integration was mediated by *recA* was accredited by the use of an isogenic *recA P. putida* as receptor of the same plasmids: as expected, this control strain failed to get hold of the same test plasmids, at least within the range of frequencies covered by the test. Note that this assessment was made with cells not subject to any stress. In a second approach, we re-examined the same question but with a more demanding homologous recombination assay. This was run on wild type (prophage$^+$) and ΔallΦ (prophage$^-$) strains that had been inserted in their chromosomal Tn*7* attachment site with a promoterless phenol monooxygenase *pheBA* operon, the expression of which (and thus, growth on phenol as carbon source) occurs only upon homologous recombination of a plasmid that restores transcriptional activity (Tavita *et al.*, 2012). Unlike the plain cointegration test shown in Fig. 5A, emergence of recombinants on selective minimal medium plates with phenol happens in the midst of the oxidative stress associated to stationary phase (Tavita *et al.*, 2012) and to the chemical injury caused by the substrate itself. As shown in Fig. 5B, Phe$^+$ colonies were detected after 7 days of incubation and their numbers grew steadily in both strains tested. However, there were not significant differences in the figures whether the bacteria at stake had prophages or not. Finally the wild type and ΔallΦ *P. putida* strains were subject to a test of spontaneous mutagenesis by plating cultures on media with or without rifampicin (Rosche and Foster, 2000). The results of Fig. 5C show that the two strains had comparable mutation rates and that the presence of absence of the prophages made little difference to this parameter. In sum, each of the 3 tests shown in Fig. 5 consistently indicated that the recombination and DNA damage repair of *P. putida* KT2440 is not influenced by the presence of the prophages in its genome. Instead, the phenotype of DNA damage hypersensitivity exposed in the experiments of Fig. 3 and Fig. 4 can be explained as consequence of the activation of the SOS system by UV light and ensuing lethal expression of proviral genes.

The proviral load of P. putida KT2440 *amplifies the physiological cost of DNA damage*

The data above made evident the role of prophages P1-P4 in the unusual response of *P. putida* KT2440 to UV. While this type of insult brings about formation of T^T dimers which leads to fatal mutagenesis, there are other forms of triggering RecA and LexR-dependent expression of proviral genes (Abella *et al.*, 2007). For instance, damage to DNA can result from oxidative stress and ensuing generation of 8-oxo-G (van Loon *et al.*, 2010). Furthermore, treatment with β-lactam antibiotics has been reported also to elicit an SOS response (Miller *et al.*, 2004; Gutierrez et *al.,* 2013). On this basis we set out to compare the effects of compounds known to cause each a distinct type of stress in the growth of *P. putida* KT2440 in parallel with its prophage-free equal. The reference conditions were set in this case with nalidixic acid, a quinolone that inhibits DNA gyrase and DNA topoisomerase IV and that traps cleavage complexes (Malik *et al.*, 2006; Pommier *et al.*, 2010). As expected, the drop dilution test of Fig. 6A and 6B made evident the increased tolerance of the prophage-less strain to this agent in respect to the parental wild type strain. Note also that consistently with the data above (Fig. 3), deletion of P1 suffices to detect an improvement of the tolerance to this DNA-damaging antibiotic (Supplementary Fig. S5). Using the same procedure we next tested the general alkylating agent *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine, which produces a wide spectrum of DNA damage both *N*- and *O*-methylation (Beranek, 1990). As shown in Fig. 6C, cells without prophages stood much better in the face of such a chemical insult. In contrast both strains had the same sensitivity to ethyl methanesulfonate (which produces O6-ethyl-G) and methyl methanesulfonate (generates N7-dG and N3-dA; data not shown). Similarly, both strains deployed the same level of sensitivity to paraquat (Fig. 6D). The plate test was repeated in liquid medium with different concentration of the oxidative agent, but with similar results (not shown). Paraquat is predicted to be first reduced *in vivo* with NADPH followed by reacting with O_2 to produce $^0O_2^-$, a reactive oxygen species (ROS) that acts on DNA. Yet, responses to superoxide stress in *P. putida* seem to be different from other bacteria (Park *et al.,* 2006), that could account for this somewhat unexpected result. In contrast, another ROS producing agent, 4-nitroquinolone-1-oxide (4NQO; Nunoshiba and Demple, 1993) had a pronounced toxic effect on the wild type *P. putida* KT2440 as compared to the prophage-free strain (Fig. 6E). That the insult caused by 4NQO originated in ROS production was indicated by the increase of tolerance to this agent when plates were added with 50 mM thiourea a scavenger of hydroxyl radicals (Tavita *et al.*, 2012; data not shown). The match between the

gain of tolerance to UV (see above) and the increased endurance to 4NQO / ROS in the prophage-free strain observed in this case makes sense in light of the connections between the two types of DNA injury (Krisko and Radman, 2010). However, exposure to sublethal ampicillin concentrations (50 μg ml^{-1}) did not translate in detectable differences between the two *P. putida* strains at stake (Fig. 6F). Taken together, the qualitative data of Fig. 6 strengthens the view that the loss of the proviral burden of *P. putida* KT2440 improves these bacteria to withstand archetypal environmental hardships such as DNA damage and oxidative stress.

Proviral elements do not develop into production of infective phages

Once the activity of the prophages under DNA injury conditions could be demonstrated with the experiments above, we wondered whether the same process could result in formation of functional viruses able to re-infect cells devoid of such elements. To this end we collected lysates and supernatants of *P. putida* KT2440 cells treated with mitomycin C (a typical inducer of lytic cycle in lysogens, Otsuji *et al.*, 1959) and from cultures subject to a shorter (2 days) or longer (7 days) period of nutrient starvation (see Experimental procedures). These supernatants were assayed for their ability to generate lysis plaques on lawns of the wild type and the Δall-Φ strains. Since the Δall-Φ bacteria are altogether deleted of proviral genes they should have lost the immunity towards re-infection by the same phages -provided they were functioning as such. However, we were unable to detect any sign of activity in the lysates, whether applied on the lawn as such (not shown). Since the lysates from the mitomycin C-treated cultures were generated under conditions in which the detrimental effect of the prophages was manifest, we provisionally concluded that infective phage particles were not produced.

Alas, since bacteriophages that prey on *P. putida* KT2440 have not been described, we lacked a suitable positive control in these tests. To overcome this problem we resorted to an alternative approach for addressing the same question that involved co-culture of wild type and Δall-Φ cells in the presence of sublethal concentrations of ciprofloxacin. This quinolone is known to trigger a strong SOS response in pseudomonads, thereby causing induction of potential prophages (Fothergill *et al.*, 2011). On this basis, the rationale of the experiment below is that infective viruses released during prophage activation in *P. putida* KT2440 should immediately pass onto the Δall-Φ cells of the co-culture, thereby decreasing its viable count. On the contrary, if ciprofloxacin causes lethal induction of prophagic genes but no release

of infective viruses, the antibiotic should give a growing advantage to the prophage-free cells. To test these alternative scenarios we tagged the wild type strain with a mCherry fluorescent protein by means of a hybrid Tn*7* while we labelled the Δall-Φ bacteria with a similar transposon encoding the green tag of GFP (see Experimental procedures for details). Insertion of these Tn*7* variants happens in a neutral location of the *P. putida*'s genome, so the fluorescent markers have no impact on cell fitness. Equal amounts of cells were adjusted to an OD_{600} ~ 0.005, mixed in M9-citrate medium amended of not with ciprofloxacin and their viable count followed during two 24 h passes. As shown in Fig. 7, the prophage-less strain quickly prevailed over the wild type bacteria in the presence of the SOS-triggering agent. This was considered a sound indication that none of the prophages present in *P. putida* KT2440 matures to the point of becoming infective to other prophage-free cells. In light of these data, it seems that the capsides observed upon induction of P4 (named Pspu28 by Quesada *et al.*, 2012) do not develop into infective viruses. The results also confirm the gain of a fitness advantage of the prophage-less strain under environmental conditions of DNA damage.

The TOL plasmid offsets the detrimental effect of prophages in P. putida *strain* mt-2

All the results observed so far introduce a new angle on the role of the TOL plasmid pWW0 to bring about a degree of UV tolerance to strain *P. putida* mt-2, the environmental isolate from which *P. putida* KT2440 derives (Regenhardt *et al.*, 2002). pWW0 encodes not only a complete catabolic plasmid for biodegradation of *m*-xylene, but also carries the UV resistance genes *rulAB* encoding an error-prone DNA polymerase Pol V which increases survival under conditions of DNA damage (Tark *et al.*, 2005). In view of the data above, it looks intriguing to have a plasmid that protects its natural carrier from UV light while the same host is very sensitive to the UV radiation alike. On this bases, we set out to determine whether the TOL plasmid balances the disadvantage of having the proviral load, or the encoded *rulAB* genes provide an inherent, additive advantage to the host regardless of containing prophages. The simple UV irradiation experiment on a plate shown in Fig. 8 gives an answer to this question: both the Δall-Φ strain and the natural isolate *P. putida* mt-2 (= *P. putida* KT2440 with the TOL plasmid; Regenhardt *et al.*, 2002) displayed the same level of UV tolerance (streaks 2 and 3 of the plate of Fig. 8). This suggested that under natural conditions the TOL plasmid endows its ordinary host with a degree of protection towards DNA damage that is roughly equivalent to that lost by having acquired the 4 prophages. On the other hand, introduction of pWW0 in the Δall-Φ strain (streak 4) further increased its

already higher tolerance to the same insult. We can thus conclude that [i] UV-tolerance trait borne by the TOL plasmid has an activity in its own which is manifested independently of the UV sensitivity of the host and [ii] the coexistence of the TOL plasmid and 4 prophages in *P. putida* mt-2 creates a factual scenario of *molecular mutualism* in which the host benefits from a default property delivered by the plasmid for the sake of the complete system. It thus seems that plasmid-mediated transfer of DNA damage-fixing genes (e.g. *rulAB*) might be one more component of the intricate interplay between mobile elements bacteria and phages that is required for homeostasis of the environmental niches frequented by *Pseudomonas putida* (Srinivasiah *et al.*, 2008; Weinbauer, 2004).

Conclusion

The results shown in this paper consistently indicate the parasitic nature of the prophages found in the genome of *P. putida* KT2440. But at the same time, the data raise evolutionary questions that are not trivial to tackle. On one hand, the sensitivity to DNA damage, whether direct (UV light) or indirect (ROS) endowed by the whole proviral load bears witness of the clear activity of the elements, with little indication of pseudogenization or vestigialization (Fong *et al.*, 1995). But on the other hand, prophage activity caused by the same stress fails to produce infective viruses and thus does not result in propagation of their sequences –as would be expected from selfish genetic elements (Doolittle and Sapienza, 1980; Werren *et al.*, 1988). There must thus be a different *raison d'être* for their maintenance. One possibility is that the prophages encode thus far unidentified genes that confer advantages under unknown conditions. This is the case of some inactive prophages of *E. coli* and *Pseudomonas* proviruses that enter conspicuously valuable traits in their carriers (Wang *et al.*, 2010; Winstanley *et al.*, 2009). Despite our numerous efforts we did not detect anything of the like in *P. putida* KT2440, but we cannot prove otherwise either. A second possibility is that stress-related prophage induction and ensuing cell death of part of the bacteria could enrich the nearby environment with common goods, thereby enabling a pseudo-altruistic strategy of survival in harsh environmental scenarios. This would be reminiscent of the so-called *Eagle effect* under which the lysis of a small part of the population caused by antibiotics becomes beneficial to the whole (Eagle and Musselman, 1949). Released metabolites would then become a nutritional asset that the non-lysing population can use for their own sake. By the same token, having a cell death program afforded by the proviral pack might ultimately facilitate survival of part of the population under DNA-damaging stress. A similar mechanism of

nutritional altruism has been proposed to justify the maintenance of the *mazEF* toxin-antitoxin system in *E. coli* (Ramisetty *et al.*, 2013). Finally, the hazardous effects of the prophages could be compensated (and thus become evolutionarily neutral) by another acquired element that takes DNA sensitivity back to the situation prior to being infected by the cognate ancestral viruses. This would bring about co-existence of two functionally neutralizing gene sets, both acquired through horizontal gene transfer. The data of Fig. 8 is consistent with such a setting: the UV hyper-sensitivity of *P. putida* KT2440 is restored to that of the prophage-free strain in the natural isolate *P. putida* mt-2 by virtue of the UV protection deployed by the TOL plasmid pWW0 in the prophage-containing host. While all these evolutionary scenarios are worth to explore from a fundamental perspective, the main outcome of the work above is the production of a more robust *P. putida* strain which adds to the value of this microorganism as a platform of choice for a variety or biotechnological and environmental applications (Nikel *et al.,* 2014).

Experimental procedures

Strains, plasmids, media and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. A Δ*recA* derivative of P. putida KT2440 was generated by integrating/excising the delivery plasmid pEMG-*recA* (Table 1) with the procedure of Martinez-Garcia and de Lorenzo (2011). Bacteria were grown routinely in LB (10 g l^{-1} of tryptone, 5 g l^{-1} of yeast extract and 5 g l^{-1} of NaCl). M9 medium with 10 mM of the carbon sources indicated in each case, was used as minimal medium (Sambrook *et al.*, 1989) and added whenever necessary with 1% (w/v) casamino acids. *P. putida* was cultured at 30ºC while *E. coli* cells were grown at 37ºC. Antibiotics, when needed, were added at the following final concentration: 150 μg ml^{-1} ampicillin (Ap) for *E. coli* cells and at 500 μg ml^{-1} for *P. putida*; 50 μg ml^{-1} kanamycin (Km); 15 μg ml^{-1} nalidixic acid (Nal), 250 μg ml^{-1} rifampicin (Rif). Other supplements were added to the media when required as follows: 10 μM paraquat; 300 μM 4-nitroquinolone-1-oxide (4-NQO); 0.1 mM hydrogen peroxide; 2 mM methyl methanesulfonate (MMS); 25 mM ethyl methanesulfonate (EMS); 0.08 mM N-methyl-N'-nitro-N-nitrosoguanidine **(**MNNG**)**. The growth kinetics of the different strains was determined by following the OD_{600} of the cultures in 96-well microtiter plates using a SpectraMax $M2^e$ plate reader (Molecular Devices, Sunnyvale, CA, USA). Data from three biological samples was used to average the resulting figures. Stationary phase survival of the strains under examination was performed by following

the CFU ml^{-1} of cultures maintained at 30ºC for a total period of 14 days without addition of fresh nutrients. CFUs were determined by serial dilution of the cultures in PBS and 10 μl of each dilution spotted onto LB agar plates. To explore global physiological differences between the wild type and Δall-Φ strains we used the Biolog Phenotypic Microarray™ technology (Biolog Inc, Hayward, CA, USA). Phenotypic analyses for examining physiological activity under 1187 growth conditions was done by Biolog Inc with plates PM1 to PM20 (http://www.biolog.com).

DNA techniques and plasmid construction

Manipulation of DNA was done following routine laboratory procedures as described in Sambrook *et al.*, (1989). Plasmid DNA was prepared using Wizard Plus SV Miniprep kit (Promega, Wi, USA). DNA amplified with polymerase chain reactions (PCR) was purified with NucleoSpin Extract II (Macherey-Nagel, Düren, Germany). Oligonucleotides were purchased from SIGMA. Colony PCR was performed using a single colony from a fresh agar plate and transferred directly into the PCR reaction tube. The correctness of all constructs was verified by DNA sequencing. Supplementary Table S2 lists the oligonucleotides used in this work.

Directed deletion of all prophages from the genome of P. putida KT2440

The 4 chromosomal segments spanning the proviral sequences borne by the extant genome of P. putida KT2440 were sequentially removed (ΔP1→ΔP1/ΔP4→ΔP1/ΔP4/ΔP3→ΔP1/ΔP4/ΔP3/ΔP2) using the I-SceI methodology described in (Martinez-Garcia and de Lorenzo, 2011; Martinez-Garcia and de Lorenzo, 2012). To this end, 800 bp of the DNA upstream (TS1) and downstream (TS2) regions of homology flanking the termini of each predicted prophages (Fig. 1B) regions were amplified and the resulting products joined *in vitro* by SOEing-PCR (Horton *et al.*, 1989). In the case of P1, P2 and P3, each of the 1.6 kb segments were then purified, digested with EcoRI / BamHI and ligated to conditional replication vector pEMG, which bears I-SceI sites flaking its polylinker (Martinez-Garcia and de Lorenzo, 2011). For P4, the PCR product was digested with XmaI-BamHI and cloned to the same vector. The ligations were transformed into *E. coli* DH5αλ*pir* strains and positive clones confirmed by sequencing the entire TS1-TS2 segment of each construct. The thereby generated plasmids pEMG-P1, pEMG-R2, pEMG-R3 and pEMG-P4 (Table 1) were mobilized either by triparental mating or electroporation to *P.*

putida KT2440 pre-transformed with the I-SceI conditional expression plasmid pSW-I (Table 1) as described in Martinez-Garcia and de Lorenzo (2012). Resolution of cointegrates was performed by induction of the I-SceI enzyme with 3-methylbenzoate followed of appropriate dilutions and plating of the induced cultures onto LB-Ap (500 μg ml^{-1}) plates. Colonies growing in this medium were checked for the loss of the Km^R marker and then analyzed by PCR to verify the deletion. The pSW-I plasmid was cured after growth of the strains without selective pressure and its loss confirmed by testing sensitivity to Ap and diagnostic PCR (Supplementary Table S2).

Phage infection tests

To obtain samples containing potentially infective viral capsides overnight-grown cultures of *P. putida* KT2440 in LB medium were added treated for 6 h with 2 μg ml^{-1} mitomycin C. Alternatively, once non-treated cultures had reaches stationary phase, we continued the incubation in the same conditions for 2 and 7 days, what place the cells in a state of protracted starvation. Whether induced with mitomycin C or depriving bacteria of nutrients (what generates mutagenic oxidative stress; Kasak *et al.*, 1997), 1 ml of each culture was treated with chloroform, vortexed, centrifuged and the supernatant stored at 4ºC. 25 μl of thereby produced lysates were then spotted onto bacterial lawns of Δall-Φ and wild type *P. putida* prepared by dispersing 20 μl of overnight cultures of each strain in 3 ml of LB 0.7% (w/v) top agar and pouring the mixture onto LB agar plates supplemented with 5 mM $CaCl_2$ and 5 mM $MgCl_2$. A second infection test involved the co-culture of wild type and Δall-Φ *P. putida* cells in a medium added with the DNA gyrase inhibitor ciprofloxacin. In this assay, one strain was chomosomally tagged for expression of an mCherry fluorescent protein (Rochat *et al.*, 2010) at an intergenic and neutral site (Koch *et al.*, 2001; Lambertsen *et al.*, 2004) while the other was tagged likewise with a Gfp green protein (Koch *et al.*, 2001). Both fluorescent proteins were expressed constitutively. Co-cultures were started by adjusting OD_{600} at 0.005 in 3 ml of M9-citrate amended where indicated with 20 ng ml^{-1} ciprofloxacin. Cultures were subject to two cycles of regrowth for 24 h, during which dilutions were plated on LB, and green and red fluorescent colonies counted after overnight incubation at 30ºC

Flow cytometry

For preparing the samples subject to cytometry analyses cells from overnight cultures in LB of the strain under examination were diluted 1:5 in phosphate-buffered saline (PBS, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 3 mM KCl, 137 mM NaCl, pH 7.0). In the case of bacteria grown in M9-based minimal media, cells were directly examined without any pre-treatment. Prior to loading, samples were treated with propidium iodide (PI) at a final concentration of 1 $\mu g\ ml^{-1}$. Single-cell quantification of PI exclusion (a proxy of cell vitality; Nebe-von-Caron *et al.*, 1998) were performed in a GalliosTM flow cytometer (Beckman Coulter Inc., Indianapolis, IN, USA) equipped with an argon ion laser of 15 mW at 488 nm as the excitation source and following protocols well established in our Laboratory (Martinez-Garcia et al., 2014). Fluorescence emission was detected using a 530/30-nm band pass filter set, $\geq$ 100,000 events were counted and the percentage of PI-stained cells calculated.

Stress resistance

A gross test of tolerance to UV irradiation was set up as follows. Cells from overnight cultures of the strains under examination pregrown in LB medium were diluted to an OD_{600} ~ 0.1. 30 µl drops of each dilution were then let to slide from one of the extremes of LB agar plates for shaping an even line, dried and exposed at 15 cm of distance to UV light from a 254 nm lamp (VL-6MC, Vilber Lourmat, France; 400 $\mu W/cm^2$) at various time intervals for 20-140 s. The plates were then wrapped with aluminium foil to avoid photoreactivation, incubated overnight at 30ºC and photographed. Quantitative UV tolerance studies were performed by modifying the procedure previously described by Sidorenko *et al.* (2011). Briefly, bacteria were grown on M9 medium supplemented with glucose and casamino acids to mid-exponential growth phase. Bacterial cultures were then serially diluted into the same medium and 5 µl of the resulting samples spotted onto M9 agar plates containing glucose as sole carbon source. The plates were then subjected to UV (254 nm) irradiation at doses ranging from 10 to 40 $J\ m^{-2}$ using the UV Crosslinker model CX-2000 manufactured by UVP (USA). The figures of colonies surviving UV exposure were transformed to common logarithm but avoiding zero values (whose logarithm is - ∞) by replacing no-colonies by 1×10^{-8}. Survival was expressed as the number of common logarithm taken from equation where the number of colony forming units (CFU) detected after irradiation was divided by CFU of dose 0 $J\ m^{-2}$. At least 11 independent measurements were performed for each strain and for each UV intensity applied. The factorial ANOVA method and Tukey's HSD (*honest significant difference*) test were used to assess the UV-tolerance of different strains. For statistical tests the

significance level was set at $P < 0.05$. The calculations were done using the Statistica 7 software. Spot assays were used also to test the survival of the bacteria under study to various genotoxic and/or oxidative stressors. For this, overnight cultures of bacteria in LB were serially diluted in PBS and 5-10 μl of each dilution plated (10^{-2} to 10^{-9}) onto the appropriate plates, incubated for 24 h and photographed.

qPCR

The spontaneous excision frequencies of each of the 4 prophages of the *P. putida* KT2440 genome were calculated from comparing the PCR signals raised from diagnostic primers for the boundaries of each prophage segment (Supplementary Table S2) out the total DNA extracted from cells subject or not to the stresses specified in each case. For this, bacteria were cultured in LB medium until mid-exponential phase (OD_{600} = 0.5) and then alternatively [i] irradiated 60 s with UV light (254 nm), [ii] treated for 30 minutes with 15 μg ml^{-1} nalidixic acid, and [iii] let run out of nutrients and incubated for 5 days at 30ºC to bring about protracted starvation and ensuing stationary phase mutagenic stress. Cells from the different handlings were then collected and their DNA extracted with the UltraClean Microbial DNA isolation kit (Mo Bio Laboratories). To monitor variation in the phage load of the bacteria before and after the various treatments we adopted primers flanking each of the predicted prophages' ends (Fig. 1B; Supplementary Table S2) in such a way that a visible PCR product is generated only in case deletion has occurred (Supplementary Fig. S1). Quantitative PCR (qPCR) for monitoring the process was performed in an ABI PRISM 7900HT Fast Real-Time PCR system (Applied Biosystems). To calculate the relative prophage excision rate we employed the ΔΔCt method (Livak and Schmittgen, 2001; Pfaffl, 2001) using the *rpoA* gene as an endogenous reference to compensate inter-PCR variations. *P. putida* strain EM371 (Table 1), that lacks P4 after an spontaneous deletion was used to generate a reference for maintenance/loss of the corresponding sequence, while the Δall-Φ *P. putida* strain was used as a calibrator for P1, P2 and P3 prophages.

Recombination and mutagenesis tests

To estimate the capacity of recombination between homologous DNA sequences under non-stressed conditions in the wild type *P. putida* KT2440 *vs*. those of the Δall-Φ and Δ*recA* counterparts, each strain was transformed with 400 ng of three non-replicative plasmids derived from vector pEMG-derivative

(Martinez-Garcia and de Lorenzo, 2011) containing chromosomal homology regions covering genomic coordinates 2123827-2124626 / 2133602-2134401, 3008533-3009132 / 3022979-3023578 and 3846376-3846907 / 3852452-3852972 (Supplementary Table S2). Electrocompetent cells were prepared as described (Martinez-Garcia and de Lorenzo, 2012) and recombined clones resulting from plasmid co-integration selected on LB agar plates supplemented with Km. The frequency of recombination was expressed as the number of Km^R colonies normalized to 10^9 cells. To estimate homologous DNA recombination ability under stressed conditions we employed the method of Tavita *et al.* (2012), which is based on the gain of a the capacity to grow on phenol upon prolonged starvation owing to the activation of otherwise silent phenol oxidase gene. Finally, spontaneous mutation rates were determined as described in Rosche and Foster (2000). For this, 100 µl of sequential 10-fold dilutions of overnight cultures of each strain were plated by quadruplicate onto LB agar, and 100 µl of undiluted cultures were spread (by quadruplicate also) onto LB-rifampicin plates and inspected after 48h. Mutation frequencies were calculated as the ratio between the number of Rif^R colonies and the total CFU count.

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Table 1. Strains and plasmids used in this work

Strains	Description / relevant characteristics	Reference
E. coli		
DH5α	Cloning host; *supE44, ΔlacU169, (ϕ80 lacZ Δ M15), hsdR17, (rk^-mk^+), recA1, endA1, thi-1, gyrA, relA*	Grant *et al.*, 1990
DH5α*λpir*	Cloning host; *λpir* lysogen of DH5α	Lab collection
HB101	F^- *mcrB mrr hsdS20 recA13 leuB6 ara-14 Δ*(*proBA*)*2 lacY1 galK2 xyl-5 mtl-1 rpsL20* (Sm^R), *glnV44* λ^-	Sambrook *et al.*, 1989
MG1655	Wild type reference strain, prototrophic	Laboratory collection
MG1655 *recA*	*recA* derivative of MG1655	Laboratory collection
P. putida		
mt-2	Natural isolate, prototrophic, host of the TOL plasmid pWW0 for biodegradation of *m*-xylene	Regenhardt *et al.*, 2002
KT2440	mt-2 derivative cured of the TOL plasmid pWW0	Bagdasarian *et al.*, 1981
KT2440 ΔP1	KT2440 derivative with prophage 1 deleted	This work
KT2440 ΔP2	KT2440 derivative with prophage 2 deleted	This work
KT2440 ΔP3	KT2440 derivative with prophage 3 deleted	This work
KT2440 ΔP4	KT2440 derivative with prophage 4 deleted	This work
KT2440 ΔP1ΔP4	KT2440 derivative with prophage 1, and prophage 4 deleted	This work
KT2440 ΔP1ΔP4 ΔP3	KT2440 derivative with prophage 1, prophage 4, and prophage 3 deleted	This work
KT2440 Δall-Φ	KT2440 derivative with prophage 1, prophage 4, prophage 3, and prophage 2 deleted	This work
KT2440 Δ*recA*	KT2440 derivative with *recA* deleted	This work
recB	mini-Tn*5* insertion in *recB* gene of KT2440	Duque *et al.*, 2007
PP3901	mini-Tn*5* insertion in ORF3901 of prophage P1	Duque *et al.*, 2007

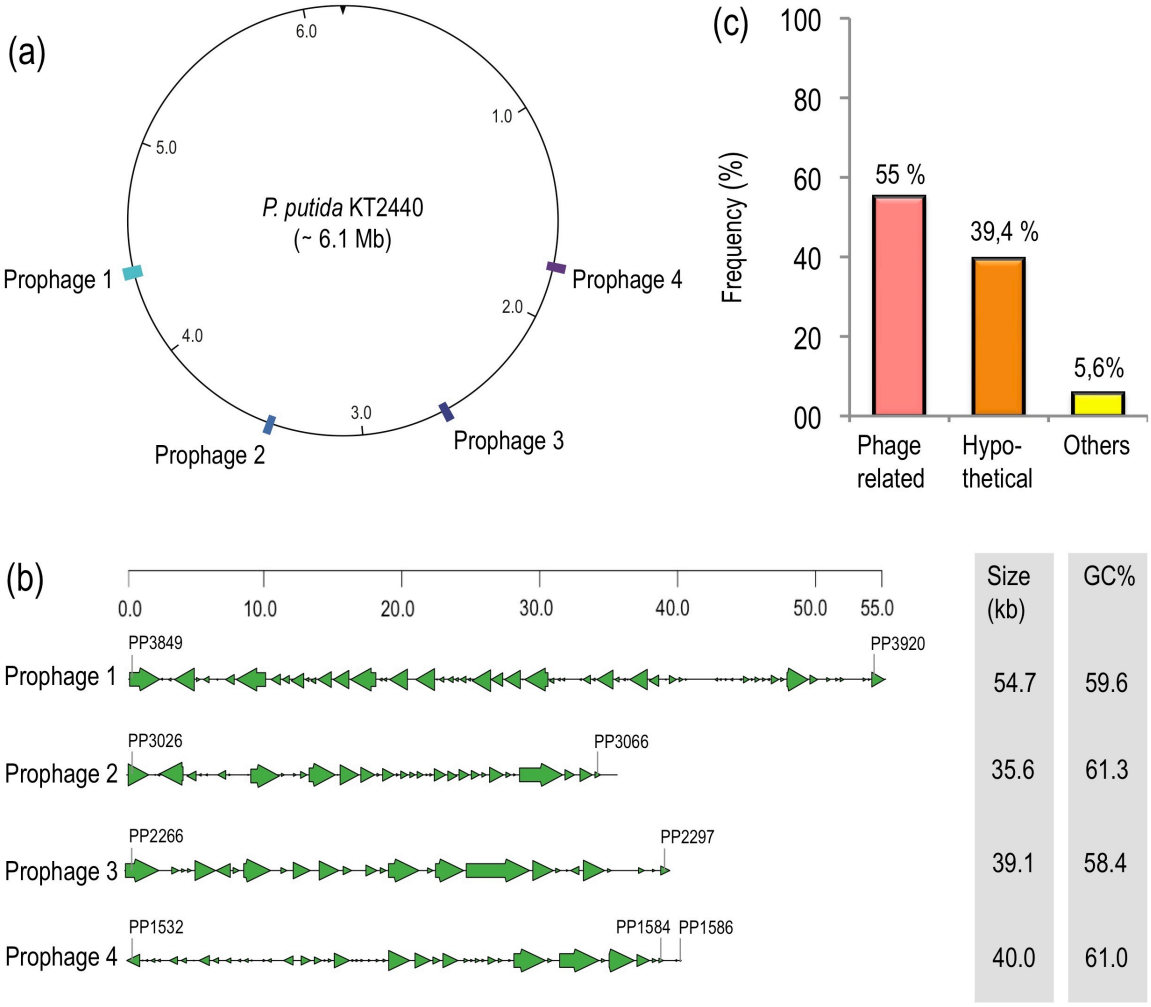
EM371	KT2440 derivative with an spontaneous deletion of prophage 4	Laboratory Collection
Plasmids		
pRK600	Cm^R; *ori*ColE1, RK2 *mob*$^+$, *tra*$^+$	Kessler *et al.*, 1992
pEMG	Km^R, *ori*R6K, *lacZ*α with two flanking I-SceI sites	Martinez-Garcia and de Lorenzo, 2011
pSW-I	Ap^R, *ori*RK2, *xylS*, *Pm*→*I-Sce*I	Wong and Mekalanos, 2000
pEMG-P1	pEMG bearing a 1.6 kb TS1-TS2 EcoRI-BamHI insert for deleting prophage 1 assembled with fragments amplified with primers PP3849-EcoRI-F-PP3849R and PP3920F-PP3920-BamHI-R (Supplementary Table S1)	This work
pEMG-R2	pEMG bearing a 1.6 kb TS1-TS2 EcoRI-BamHI insert for deleting prophage 2 assembled with fragments amplified with primers TS1(RR2)EcoRI-F-TS1(RR2)R and TS2(RR2)F-TS2(RR2)BamHI-R (Supplementary Table S1)	Martinez-Garcia and de Lorenzo, 2011
pEMG-R3	pEMG bearing a 1.6 kb TS1-TS2 EcoRI-BamHI insert for deleting prophage 3 assembled with fragments amplified with primers TS1(RR3)EcoRI-F-TS1(RR3)R and TS2(RR3)F-TS2(RR3)BamHI-R (Supplementary Table S1)	Martinez-Garcia and de Lorenzo, 2011
pEMG-P4	pEMG bearing a 1.6 kb TS1-TS2 XmaI-BamHI insert for deleting prophage 4 assembled with fragments amplified with primers PP1532-XmaI-F-PP1532R and PP1586F-PP1586-BamHI-R (Supplementary Table S1)	This work

pEMG-*recA*	pEMG bearing a 1 kb TS1-TS2 EcoRI-BamHI insert for deleting *recA* assembled with fragments amplified with primers TS1-recA-EcoRI-F-TS1-recA-R and TS2-recA-F-TS2-recA-BamHI-R (Supplementary Table S1)	This work
pEMG-1887[a]	pEMG inserted with DNA segments covering chromosomal coordinates 2123827-2124626 / 2133602-2134401	This work
pEMG-286	pEMG inserted with DNA segments covering chromosomal coordinates 3008533-3009132 / 3022979-3023578	This work
pEMG-298	pEMG inserted with DNA segments covering chromosomal coordinates 3846376-3846907 / 3852452-3852972	This work
pBK-miniTn*7*-*gfp2*	Ap^R, Cm^R, Gm^R, *ori*pUC19, *mob*$^+$, mini-Tn*7*, $P_{A1/04/03}$→*gfp2*	Koch *et al.*, 2001
pME9407	Ap^R, Gm^R, *ori*pUC19, mini-Tn*7*, P_{tac}→*mCherry*	Rochat *et al.*, 2010
pUX-BF13	Ap^R; *ori*R6K, *mob*$^+$, provides the Tn*7* transposition function *in trans*	Bao *et al.*, 1991

[a] The frame of the vector backbone in this case has 6-base DNA changes in respect to pEMG that do not affect either recombination or the activity of the system.

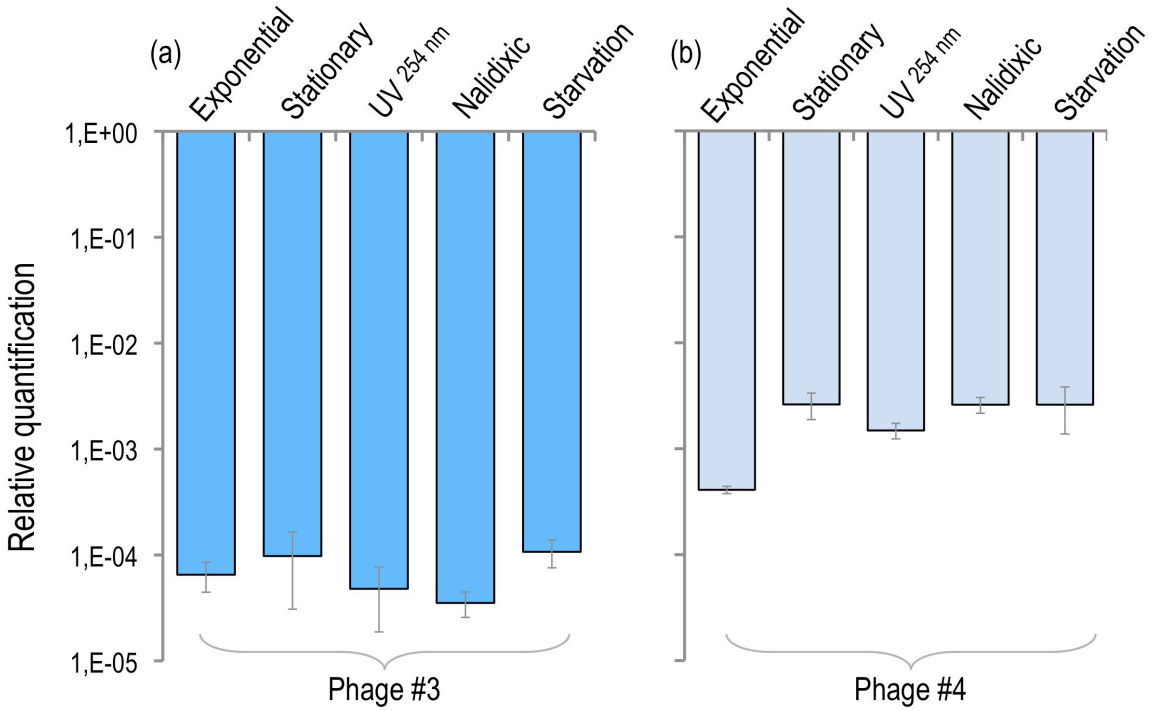
FIGURES

Figure 1. Genomic arrangement and genetic composition of the four prophages of *P. putida* KT2440.



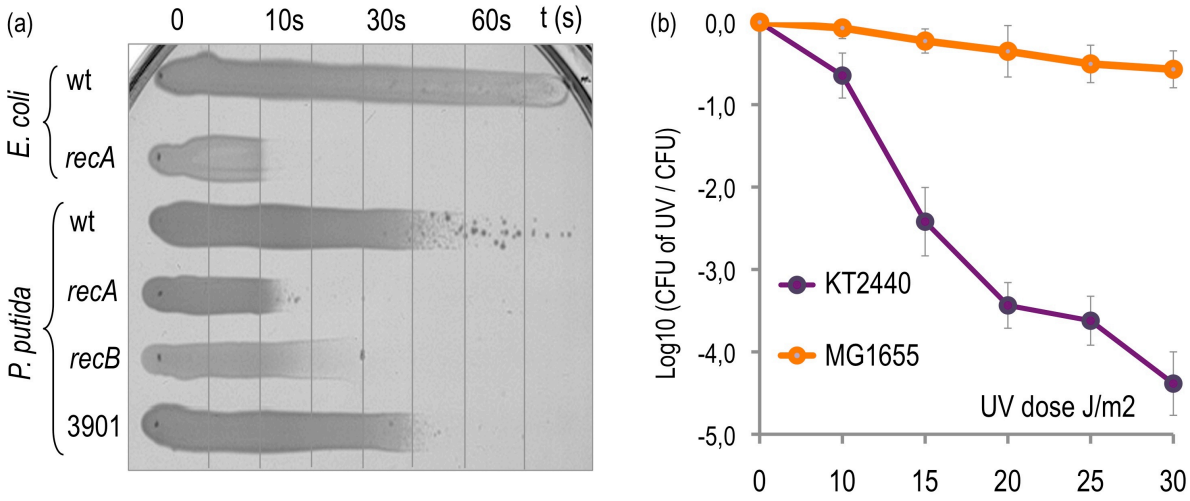
(a) Circular map of the *P. putida* KT2440 chromosome showing the physical location of the prophages in the genome. **(b)** Genetic organization of the four prophages. The total length and the GC% content is indicated along with the sketch of the number and sizes of the genes in each provirus. The overall GC % of *P. putida* KT2440 is 61.6%. **(c)** Distribution and annotations of ORFs found in the proviral sequences. The category *others* includes ORFs with a putative role but alien to known viral functions. Then, the percentage of each category calculated and plotted. The functional classification of the ORFs was based on the *Pseudomonas* Genome Database (http://www.pseudomonas.com; Winsor *et al.*, 2011).

Figure 2. Frequency of spontaneous prophage loss.



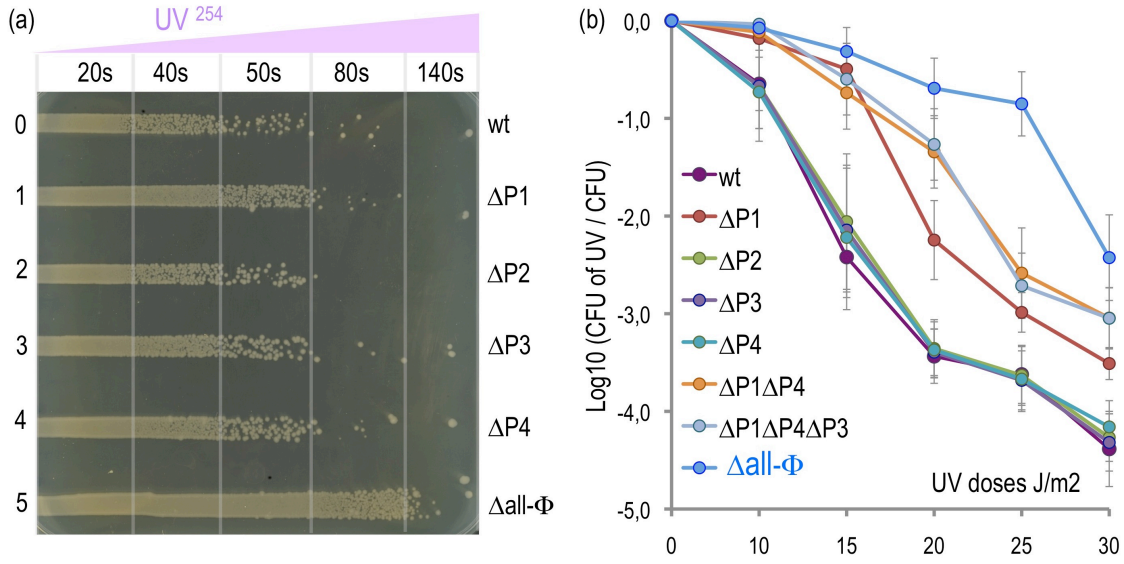
Excision of each of the proviral sequences in the genome of *P. putida* KT2440 was followed by means of qPCR with diagnostic primers that targeted the most external boundaries of each of the prophage sequences (Fig. 1B). The relative quantification of the excision frequencies of prophages P3 (panel A) and P4 (panel B) upon different physiological circumstances is shown: exponential growth, stationary phase, exposure to UV light, addition of nalidixic acid and protracted starvation (see Experimental procedures for details). No loss of P1 and P2 was detected with this method under the same conditions.

Figure 3. Sensitivity of *P. putida* KT2440 to UV irradiation.



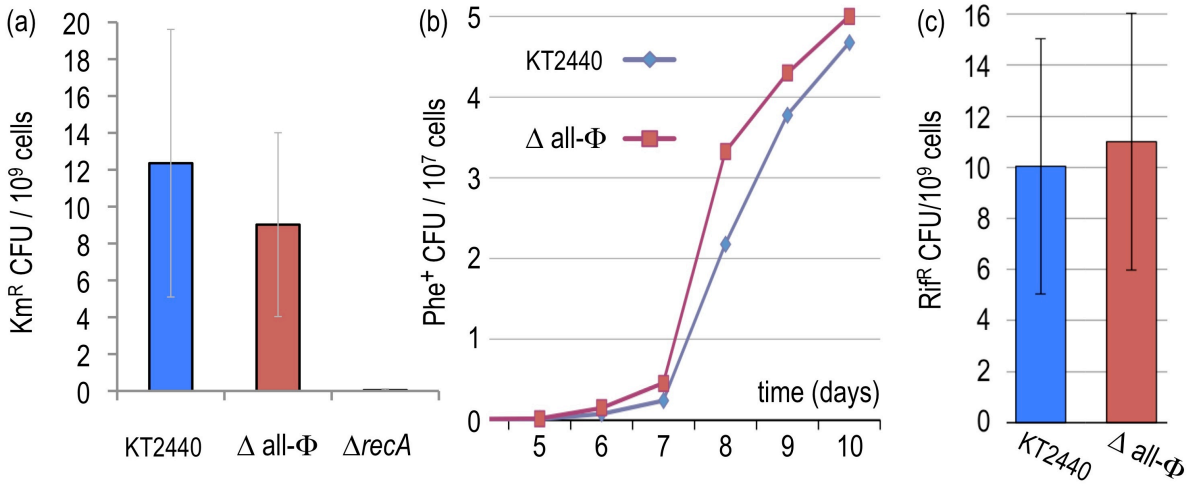
(a) Plate test. The strains subject to the assays were streaked on the surface of an LB plate and exposed to stepwise intensities of UV (254 nm) from top. Controls included wild type and *recA* strains of *E. coli* MG1655 as well as *recA* and *recB* derivatives of *P. putida* KT2440 (Table 1). A control strain bearing a transposon insertion in the gene PP3901 of prophage P1 was examined as well. Note the sensitivity of *P. putida* KT2440 as compared to *E. coli.* **(b)** Quantitation of *E. coli* vs. *P. putida* KT2440 tolerance to UV damage. *E. coli* MG1655 was compared side-by side to *P. putida* KT2440 in their ability to survive increasing doses of UV light with the numerical method explained in Experimental procedures. Data are expressed as $\log_{10}$ [CFU of UV-irradiated cells relative to the CFU of non-irradiated cells]. Error bars specify the 95% confidence intervals.

Figure 4. Separate and combined contribution of prophages P1-P4 to UV hypersensitivity of *P. putida* KT2440.



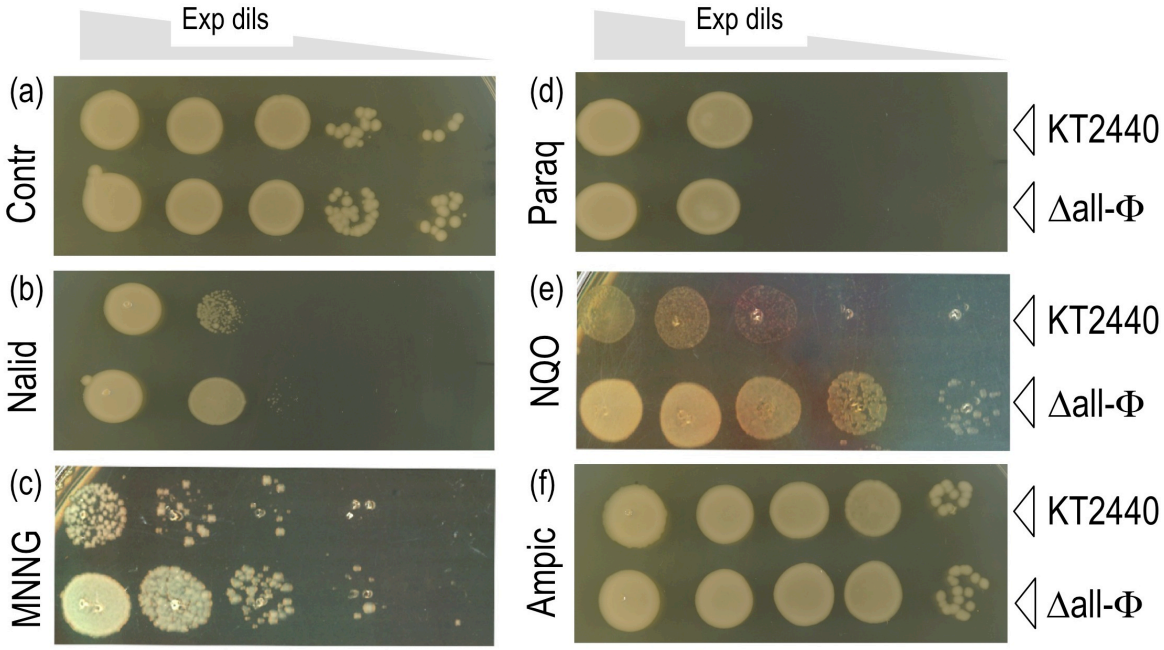
(a) Qualitative estimation of UV tolerance. Individual *P. putida* variants ΔP1-ΔP4 indicated to the right were inoculated on the surface of an LB plate along with one with all the four deletions and the wild type strain as a reference. Plates were then irradiated with UV light (254 nm) for the time indicated and grown overnight in the darkness. **(b)** Tolerance of *P. putida* derivatives with single and multiple prophage deletions to UV damage. Each of the strains indicated was subject to the same procedure indicated in the legend to Fig. 3B. Survival to UV was calculated as explained in Experimental procedures.

Figure 5. Effect of *P. putida* KT2440 prophages in DNA recombination efficiency.



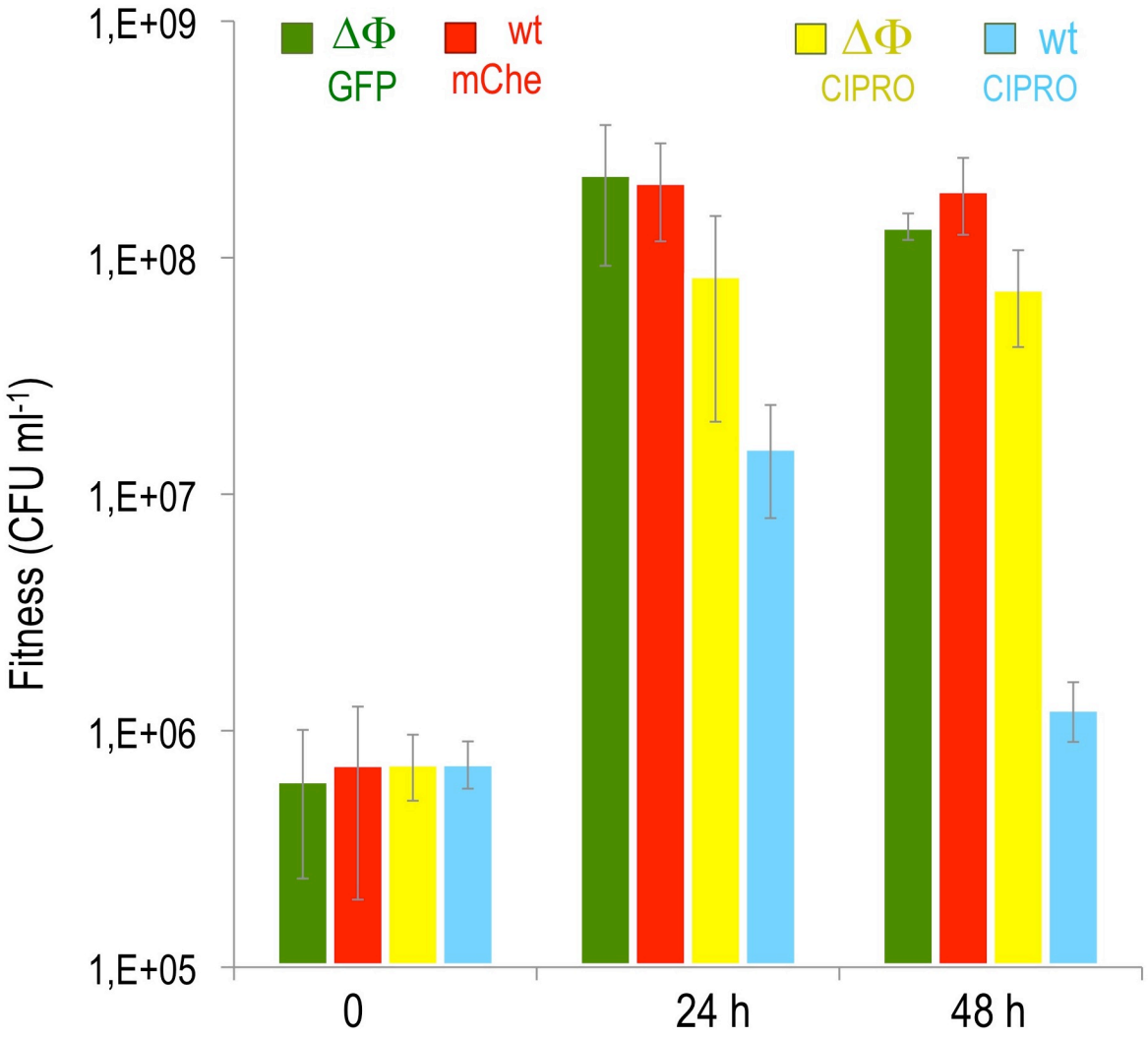
(a) Homologous co-integration under non-stressed conditions. The experiment shows the average frequencies of Km^R clones out of electroporating 400 ng of non-replicative plasmids pEMG-1887, pEMG-286 and pEMG-298 (Table 1) bearing □0.5, □0.6 and □0.8 kb DNA homology with different chromosomal regions of the wild type strain and its prophage-less derivative along with a *recA* strain as a no-recombination control. **(b)** Recombination under stressed conditions. The test is based on conditional transcription of a chromosomally inserted *pheBA* operon upon homologous recombination of a donor plasmid, which results in expression of phenol monooxygenase and thus growth on phenol as sole carbon source (Tavita *et al.*, 2012). Note emergence of Phe^+ colonies out of the two tested strains after 7 days of starvation. **(c)** Spontaneous mutagenesis. The numbers quantify the appearance of Rif^R following plating of each of the two strains indicated on selective medium (Rosche and Foster, 2000).

Figure 6. Effect of proviral load on direct and indirect DNA damage.



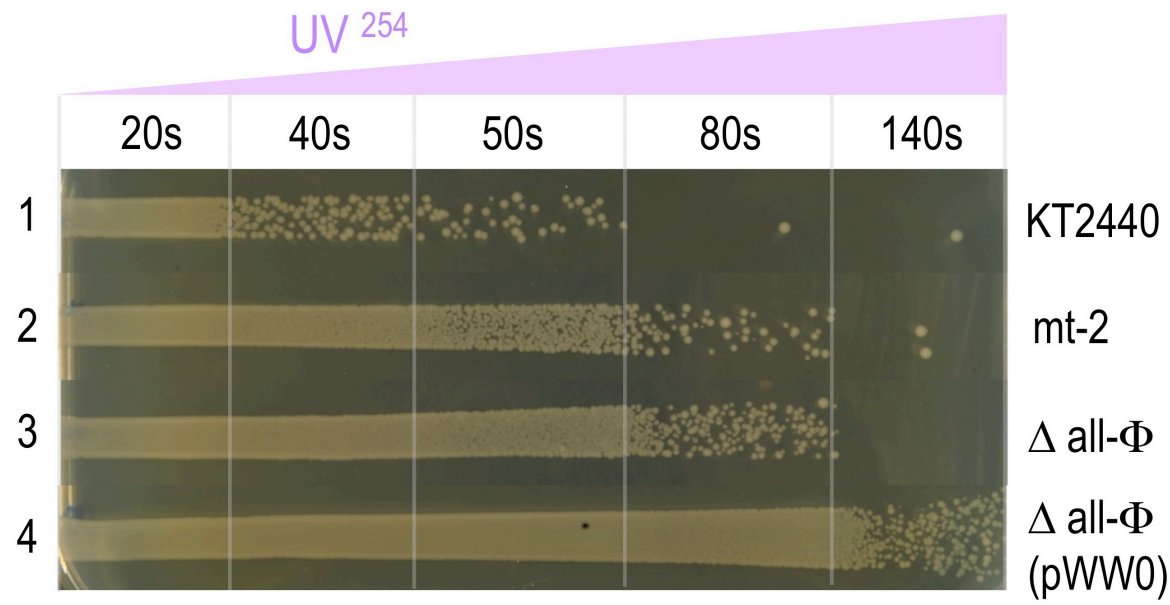
(a) Serial dilutions of overnight cultures of the wild type strain *P. putida* KT2440 and its prophage-less derivative (Δall-Φ) were plated on LB agar media an added with different stressors: **(b)** nalidixic acid, **(c)** *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine **(d)** paraquat, **(e)** 4-nitroquinolone-1-oxide and **(f)** ampicillin. The experimental dilutions for (a), (b), (d), and (f) were 10^{-2}, 10^{-4}, 10^{-6}, 10^{-8} and 10^{-9}; while for (c) and (e) were 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7}.

Figure 7. Dynamics of co-culturing wild type (prophage$^+$) and Δall-Φ (prophage$^-$) variants of *P. putida*.



Equivalent inocula of mCherry-tagged wild type strain and the prophage-less counterpart (labelled with GFP) were mixed at an OD_{600} of 0.005 in M9-citrate supplemented with or not with a non-lethal dose of ciprofloxacin (20 ng ml^{-1}) as indicated. The evolution of the CFU count of each strain was followed though two passes along 48 hours as indicated. Note the sharp decline of the prophage-containing cells in the cultures exposed to the DNA-damaging agent.

Figure 8. Enhancement of the UV-tolerance of *P. putida* strains through acquisition of the *rulAB*$^{+}$ TOL plasmid pWW0.



The conditions of the experiment were identical to those of Fig. 4A. The individual *P. putida* variants indicated to the right were drawn on the surface of an LB plate and irradiated with UV light (254 nm) as indicated. Note the gain of UV tolerance endowed by acquisition of the plasmid.

SUPPLEMENTARY INFORMATION

Supplementary Table S1. Relevant phenotypes of the PM microarray analysis of the Δall-Φ strain compared to the wild-type strain

Plate Panel	Well(s)	Chemical	Category	Phenotype[a]
PM07	A02	L-Glutamine	N-source (aa)	+
PM07	H11	Valine-Valine	N-source (peptide)	+
PM07	G09	Tyrosine-Histidine	N-source (peptide)	+
PM07	H07	Valine-Histidine	N-source (peptide)	+
PM05	E08	D-Pantothenic acid	Nutritional supplement	+
PM05	H08	Choline	Nutritional supplement	+
PM05	D08	D-Glutamic acid	Nutritional supplement	+
PM05	D09	D, L-Diamino-a, e-Pimelic acid	Nutritional supplement	+
PM05	B07	L-Methionine	Nutritional supplement	+
PM05	D06	D-Alanine	Nutritional supplement	+
PM05	B03	L-Histidine	Nutritional supplement	+
PM05	D10	Cytosine	Nutritional supplement	+
PM05	F03	Nicotinamide	Nutritional supplement	+
PM05	B02	Glycine	Nutritional supplement	+
PM05	F06	Hematin	Nutritional supplement	+
PM05	C09	(5)4-Amino-Imidazole-4(5)-Carboxamide	Nutritional supplement	+
PM05	F05	d-Amino-levulinic acid	Nutritional supplement	+
PM19	E11, E12	2-Hydroxy-1,4-Napthoquinone	Oxidizing agent	+
PM17A	H08	Caffeine	cAMP phosphodiesterase inhibitor	+

[a] Gain of function of the Δall-Φ strain in respect to the wild type *P. putida* KT240 manifested as a higher respiration rate under the conditions tested.

Supplementary Table S2. Primers used in this study

Name	Sequence 5' → 3'[a]	Usage
PP3849-EcoRI-F	CG**GAATTC**CGGGCTGCGCCACAGCCGGCCTGA	Deletion of prophage 1
PP3849R	*GAGACCAGTCCCAAAACTTCA*TCAGTGTATATGAGTAAGCCAG	Deletion of prophage 1
PP3920F	TGAAGTTTTGGGACTGGTCTC	Deletion of prophage 1
PP3920-BamHI-R	CG**GGATCC**GCCCCATTCGCCGTACTG	Deletion of prophage 1
TS1(RR2)EcoRI-F	CG**GAATTC**ACCACCGCCCGCCTTAGGTCC	Deletion of prophage 2[b]
TS1(RR2)R	*ATCAGCCCAACTACTCCGGCCGT*ACCCTGATTTCACTCCAACC	Deletion of prophage 2[b]
TS2(RR2)F	ACGGCCGGAGTAGTTGGGCTGAT	Deletion of prophage 2[b]
TS2(RR2)BamHI-R	CG**GGATCC**AATATGATTTGCGTATCTAGAC	Deletion of prophage 2[b]
TS1(RR3)EcoRI-F	CG**GAATTC**CGCCATTAGGCAGCTTTGG	Deletion of prophage 3[b]
TS1(RR3)R	*GAATCAGAAAATGTGTCCCAA*AAAGGGAATCTCTTAGTAATC	Deletion of prophage 3[b]
TS2(RR3)F	TTGGGACACATTTTCTGATTC	Deletion of prophage 3[b]
TS2(RR3)BamHI-R	CG**GGATCC**CACTTCACGGTGCGTTTCCAGG	Deletion of prophage 3[b]
PP1532-XmaI-F	TCCC**CCCGGG**GACCAGGCGGTGCGACAGCA	Deletion of prophage 4
PP1532R	*TAACGTTAAGCATTCAGCTC*TCCTGCGACTAATTTTTGGG	Deletion of prophage 4
PP1586F	GAGCTGAATGCTTAACGTTA	Deletion of prophage 4
PP1586-BamHI-R	CG**GGATCC**CCAACACGAAGCTGAAGCTGGC	Deletion of prophage 4
3864F	ACTTGTACACCCGTGGTTCG	Diagnose P1 deletion

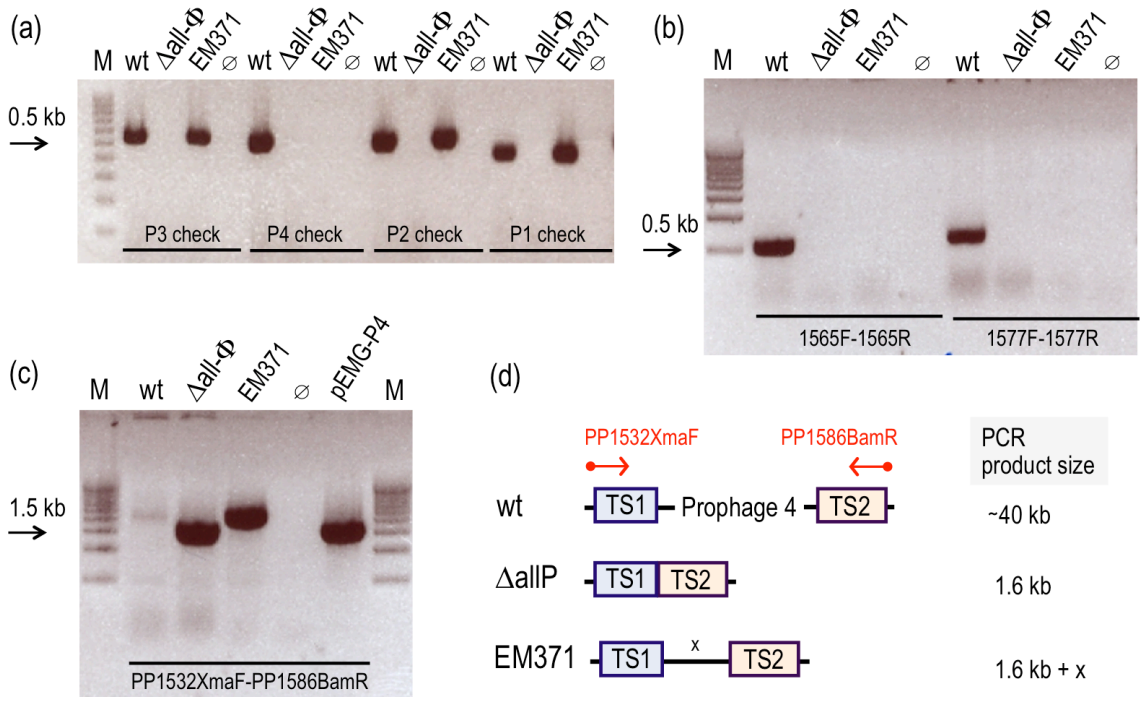
3864R	TGAACCAGCGTTCGATACTG	Diagnose P1 deletion
3065F	GTCGACGAGGTGGAATTGAG	Diagnose P2 deletion
3065R	GCAGAGGTTTTGTTGGGGTA	Diagnose P2 deletion
2277F	GCAGATCGAGGACTTCAAGC	Diagnose P3 deletion
2277R	GCACTCCATAGCACCCTAGC	Diagnose P3 deletion
1565F	CTGACCGAGGATCAGATGGT	Diagnose P4 deletion
1565R	CCGGGTTGAACTTCACGTAG	Diagnose P4 deletion
1577F	CCGCTTCTGAGGAACAACTC	Diagnose P4 deletion
1577R	CTGTGGTCAGCGCAGTAAAA	Diagnose P4 deletion
TS1-recA-EcoRI-F	CG**GAATTC**CGGTGGTGGCATTGCCGAAGC	Deletion of *recA*
TS1-recA-R	*TTCGGCTATCTACTGCGCAA*TGAAATCCTCACGTGTTCGAC	Deletion of *recA*
TS2-recA-F	TTGCGCAGTAGATAGCCGAA	Deletion of *recA*
TS2-recA-BamHI-R	CG**GGATCC**TTCGAGCTTCAATAATCGTCG	Deletion of *recA*
pSW-F	GGACGCTTCGCTGAAAACTA	Curing of pSW-I[b]
pSW-R	AACGTCGTGACTGGGAAAAC	Curing of pSW-I[b]
P1-F	CTGGCATCAAGAACATCGTAGTGTCA	QPCR
P1-R	GCAGGAAAACACCGAAGTCCCAA	QPCR
P2-F	TCCGGTGAAGGGTAATATATACTCTGG	QPCR
P2-R	AACCAAGGCATGCCCACAGCTC	QPCR

P3-F	CCCTTAGAGACATCCTTAGAAATGAT	QPCR
P3-R	GCCTACTCTGCCCGAGCCACG	QPCR
P4-F	AGGCGATGGCTTTGTCTTCGAG	QPCR
P4-R	CAATGTACACAATGACCTACACG	QPCR
rpoA-F	GAGTCGGGTGGCGCAGCTTGC	QPCR
rpoA-R	ACATGAACACCGACCACACCCTC	QPCR

[a] Recognition site for the restriction enzymes specified are in bold in the DNA sequence.

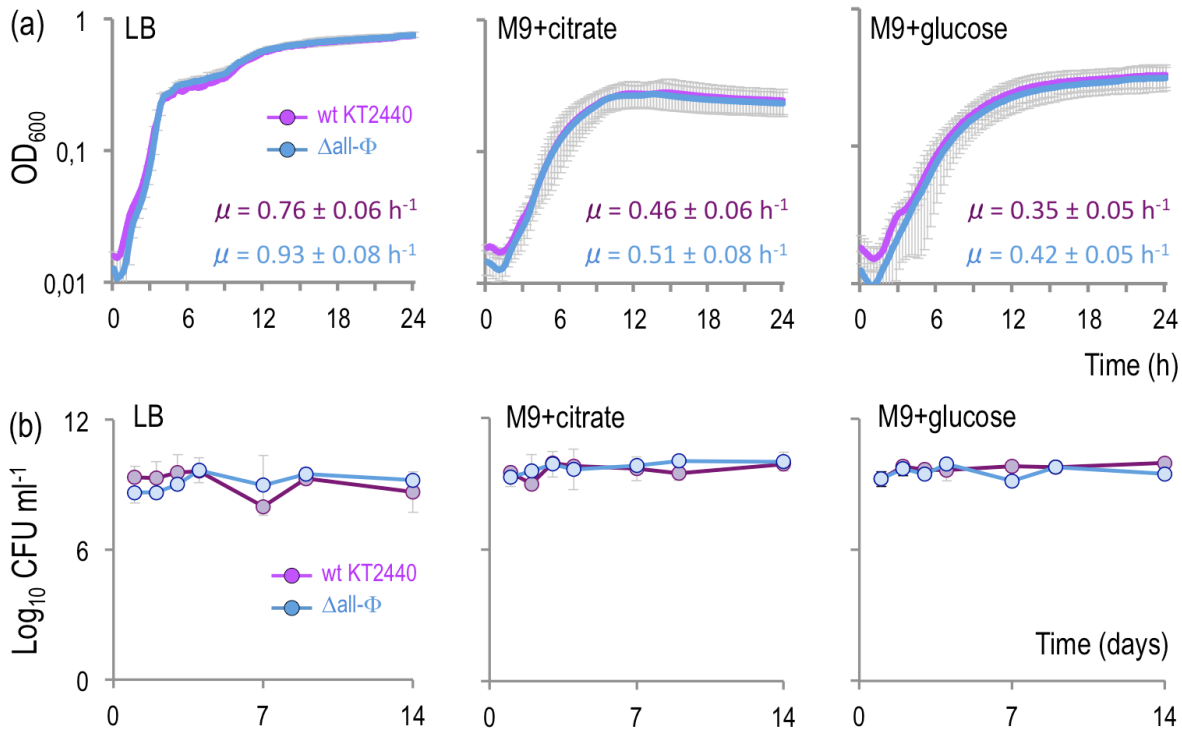
[b] Martinez-Garcia, E. and de Lorenzo, V. (2011) Engineering multiple genomic deletions in Gram-negative bacteria: analysis of the multi-resistant antibiotic profile of *Pseudomonas putida* KT2440. *Env Microbiol* **13**: 2702-2716.

Supplementary Fig. S1. PCR-based diagnose of proviral sequences in the genome of *P. putida* KT2440.



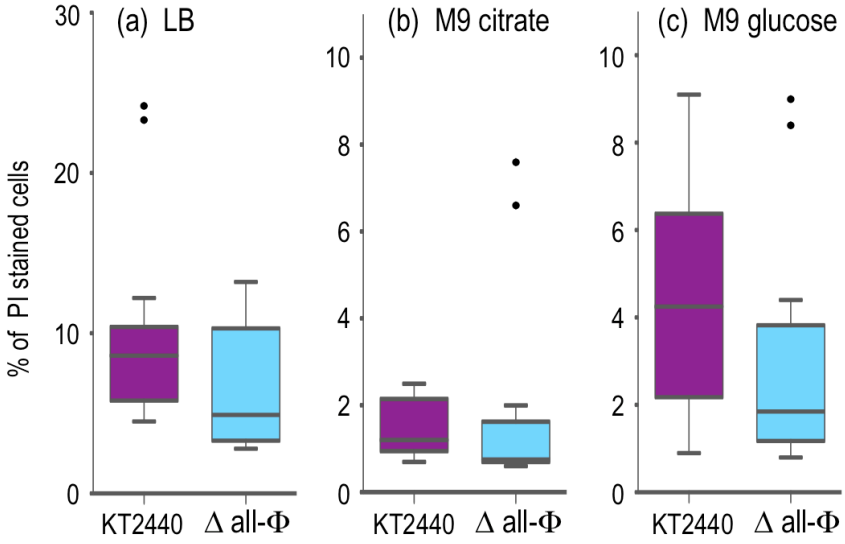
(a) Agarose gel electrophoresis of ~500 bp PCR products resulting from amplifying internal sequences of each of the prophages P1, P2, P3, and P4 as indicated. As a reference, PCR reactions with DNA of the wild type strain (*P. putida* KT2440) and the prophage-free strain (Δall-Φ) were loaded in each case. *P. putida* EM371 (Table 1) has undergone an spontaneous deletion of P4. A DNA ladder (M) with fragments differing by 100-bp was loaded to the sides. Empty Ø lanes are blank samples. **(b)** verification of spontaneous deletion of phage P4. PCR products from DNA of strains indicated on top, raised with primers 1565F/1565R and 1577F/1577R (Supplementary Table S2), which amplify internal P4 genes PP1565 and PP1577, respectively. Note that these genes are absent in both the directed deletion strain (Δall-Φ) and the spontaneous excision counterpart *P. putida* EM371. **(c)** Analysis of spontaneous excision of P4 phage. The gel shows the products resulting from amplifying the strains indicated on top following PCR of their genomic DNA with primers PP1532XmaF/PP1586BamR (Supplementary Table S2), using pEMG-P4 plasmid DNA as a positive control. The band originated in *P. putida* EM371 is bigger than the one from the Δall-Φ strain. **(d)** The boundaries of phage P4. The small size divergence between the PCR products raised by *P. putida* strains Δall-Φ and EM371 when their DNA was amplified with PP1532XmaF and PP1586BamR primers (Supplementary Table S2) is due to the maintenance of genes PP1585-PP1586 after spontaneous excision of P4 –in contrast with its directed removal the prophage-free strain (see main text for explanation).

Supplementary Fig. S2. Physiological characterization of the prophage-free strain.



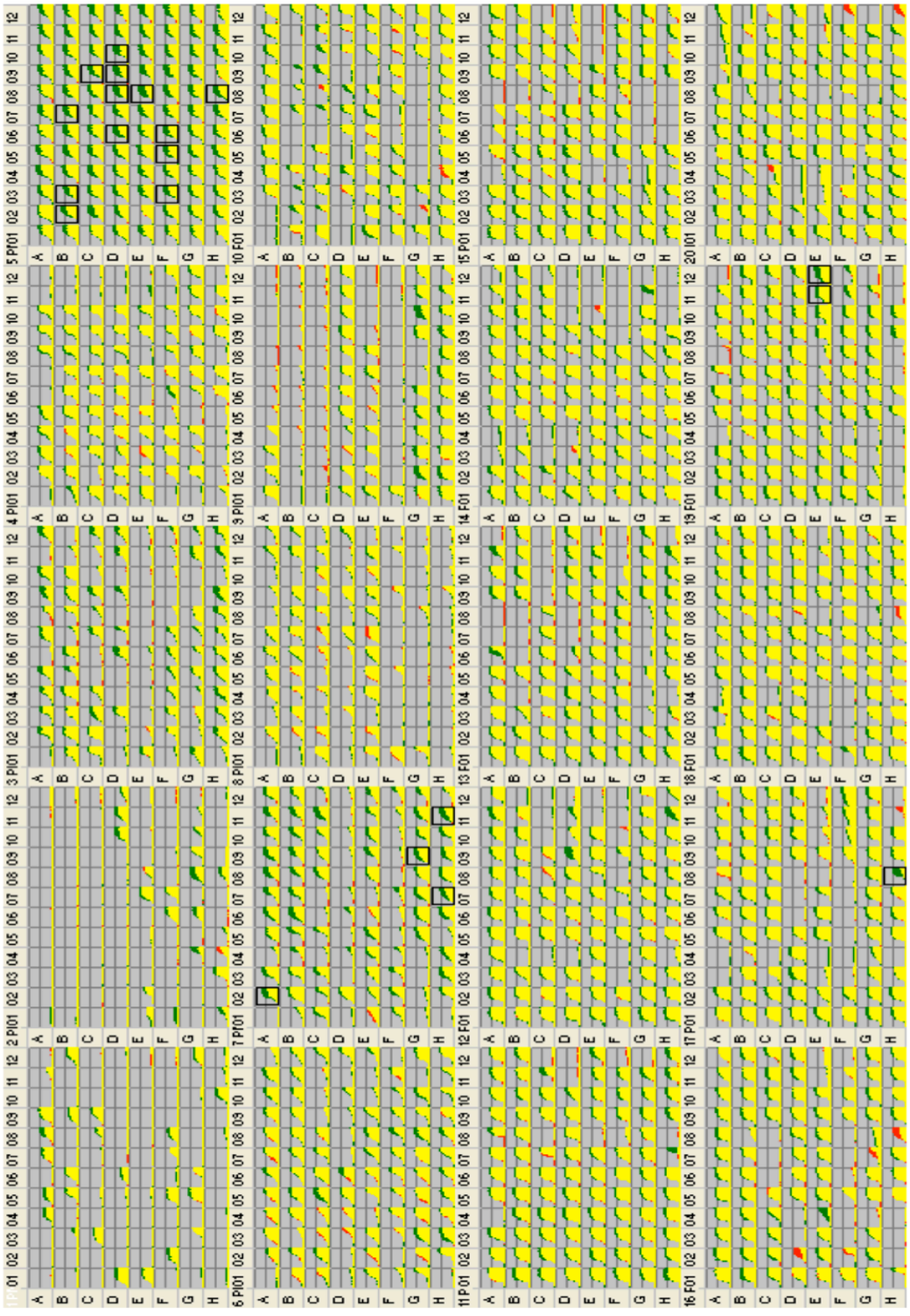
(a) Growth curves of *P. putida* KT2440 ( ) and the Δall-Φ ( ) strains in rich (LB), gluconeogenic (M9-citrate) and glycolytic (M9-glucose) regimes as indicated. Cultures were followed in a 96-well plate reader and μ values for specific growth rates calculated as described in Experimental procedures. The average values and standard deviation of three independent experiments is shown. Note consistently superior –albeit relatively minor growth advantages of the phage-free strain in respect to the wild type *P. putida*. **(b)** Long-term survival. Strains under examination strains were left to run out of nutrients and kept in stationary phase up to 14 days as shown. At the time points indicated sampled were taken and the CFU count for each strain determined.

Supplementary Figure S3. Cell viability in stationary phase.



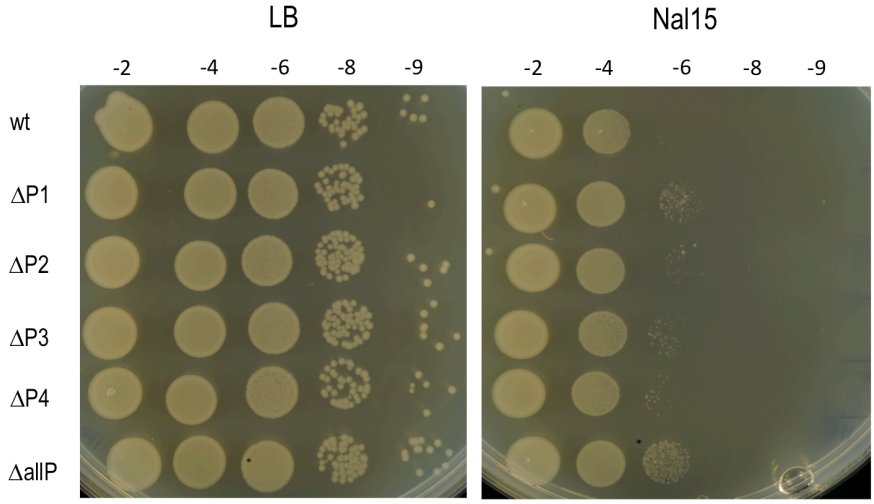
Cells of the wild type (*P. putida* KT2440) and the prophage-free strain (Δall-Φ) were grown overnight in **(a)** LB, **(b)** M9-citrate or **(c)** M9-glucose and then stained with propidium iodide (PI) for quantifying the presence of dead cells by flow cytometry. The data is drawn as box plot chart (Tukey style) where the median is represented as a grey line within the rectangles. The dots outside the rectangles stand for the outliers that were also included in the statistical analyses. The box plot charts summarize the results of at $\geq$ 4 biological replicates.

Supplementary Fig. S4. Consensus image for Biolog phenotypic microarray profiling the prophage-free strain (Δall-Φ) in respect to wild type *P. putida* KT2440.



For each growth condition, *yellow* colour indicates similar respiration rates in both strains; green and red means, respectively, a better or worse performance of the phage-deleted strain in respect to the wild-type control. Specific information on growth conditions in the Biolog plates can be found in http://www.biolog.com/pdf/pm_lit/PM1-PM10.pdf and http://www.biolog.com/pdf/pm_lit/PM11-PM20.pdf. The most conspicuous gain-of-function cases are indicated with boxes.

Supplementary Fig. S5. Plate dilution tests of resistance to nalidixic acid of *P. putida* KT2440 derivatives deleted of individual phages.



Serial dilutions of overnight cultures of the wild type strain *P. putida* KT2440 and each of its single-prophage deletion derivatives were plated on LB agar media added with 15 μg ml^{-1} nalidixic acid. The all-phage free strain (Δall-Φ) was tested also as a reference. The extension of the dilutions is indicated on top of the plates.