Supplementary data for article:

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Supporting Information - Experimental Procedures

Mutant generation

The polyP kinase (*ppk)* gene of *P. putida* KT2440 with flanking regions (700 bp) was amplified using specific primers ppk_seq_(F) and ppk_seq_(R) (Supporting Table 2) The PCR product was gel purified and ligated to the pGEM-T Easy Vector (Promega, Madison, WI) cloning system to generate the pGEM/*ppk*±700 plasmid. Two HinDIII restriction sites were introduced to either end of the coding region of the *ppk* gene of pGEM/*ppk*±700 by successive rounds of site directed mutagenesis PCR using QuikChange® II XL Site-Directed Mutagenesis Kit (Agilent Technologies, Ireland) and specifically designed primer sets *ppk*_HinDIII_beg (F/R) and *ppk*_HinDIII_end_(F/R) (Supporting Table 2). Plasmids were introduced into chemically competent *E. coli* XL10 Gold by heat shock according to manufacturer's specifications (Stratagene, Agilent Technologies, Santa Clara, CA).

The construct p-GEM/*ppk*±700_H3_BE was digested with HinDIII enzyme to excise the central (2122 bp) coding region of the *ppk* gene. The linearised plasmid subsequently ligated to an 1100 bp DNA fragment containing a gentamicin resistance cassette. The ligation product pGEM/*ppk*/GM was transformed to *E. coli* XL10 Gold cells and verified by sequencing at Source Bioscience (Dublin, Ireland). The confirmed construct was subsequently introduced to electrocompetent *P. putida* KT2440 prepared using the method described by Choi *et al*., 2006 to generate a *P. putida* KT2440 Δ*ppk* mutant.

Putative knockouts were confirmed by Southern blot. Genomic DNA from wild-type and mutant strains were digested to completion by either a double restriction digest of NcoI and NdeI or a single digest of NcoI. Digests were resolved in a 0.8 % (w/v) agarose gel for 1.5 h at 60 V. DNA was transferred to a positively charged nylon membrane using the Turboblotter Rapid Downward Transfer System according to manufacturer's instructions (Schleicher and Schuell, Inc., Dassel, Germany). The gentamicin cassette was digoxigenin (DIG) labelled and used as the probe for the blot. Mutant genomic DNA developed a band at 4874 bp when digested with NdeI and NcoI enzymes and at 7545 bp when digested with NcoI alone (Supporting Fig. 5). The specific nature of the digest allows for accurate determination of the site of incorporation by comparing the probed fragment size to theoretical band sizes

yielded by the digest. An unsuccessful single crossover event would have yielded bands 6747 bp larger in both cases due to the incorporation of the full pGEM/*ppk*/GM plasmid. No band developed for the negative control of wild-type genomic DNA, indicating the absence of the gentamicin resistance cassette. To further confirm the exact location of the gentamycin cassette in the genome of *P. putida* KT2440 a PCR product from genomic DNA using primers the ppk_seq_(F) and ppk_seq_(R) (Supporting Table 2). The sequence (Supporting Fig. 6) shows the areas flanking the ppk gene, the residual *ppk* bases and the cassette containing the gentamycin resistance gene.

Mutant complemention

The *ppk* gene was amplified with specific primers ppkrbs_comp_(F/R) (Supporting Table 2) to include an appropriate ribosomal binding sequence (RBS) and specifically determined restriction sites at the beginning (KpnI) and end (EcoRI) of the PCR product. The product was introduced to pGEM-T Easy vector and following transformation into *E. coli* XL10 Gold cells, was digested with the specific restriction enzymes. This sticky ended fragment was ligated to pJB861 broad host range expression vector (Blatny *et al*., 1997) which was cut by an identical digest. This yielded a pJB861/*ppk* plasmid which was verified by sequencing and then introduced to electrocompetent *P.putida* KT2440 *Δppk* as described previously (Choi *et al*., 2006). Positive transformants were screened for kanamycin resistance and plasmid re-isolation and sequencing (Source BioScience, Dublin, Ireland, data not shown). The resulting *ppk* gene was now under the control of a Pm promoter which is stimulated by the XylR protein in the presence of an *m*-toluic acid inducer.

Culture conditions

All strains (Supporting table 3) were maintained on LB agar plates (Sigma-Aldrich, St Louis, MO) supplemented with appropriate antibiotic. *P. putida* KT2440 wild-type strains were maintained using carbenicillin (50 mg l^{-1}), *P. putida* KT2440 *Δppk* plates were supplemented with gentamicin (50 mg l^{-1}), and *P. putida* KT2440 *Δppk*pJB/ppk with kanamycin (40 mg l^{-1}).

Cultures were routinely grown in 250 ml Erlenmeyer flasks containing 50 ml of Minimal Salt Medium (MSM) (Schlegel *et al*., 1961). Cultures were supplemented with either 1 g l^{-1} or 0.25 g l^{-1} ammonium chloride (NH_4Cl) as nitrogen source to create excess or limited nitrogen conditions (required for PHA accumulation). Flasks were incubated at 30 °C and shaking at 200 rpm for 48 h unless otherwise stated.

Polyphoshate accumulation assay

P. putida KT2440 wild-type and *Δppk* strains were grown in 250 ml Erlenmeyer flasks containing 50 ml of a modified minimal media containing 50 mM 3-Morpholinopropanesulfonic acid (MOPS-KOH) (pH 6.9) supplemented with 1 g l^{-1} ammonium chloride, and 1 ml l^{-1} trace elements solution (Pfennig, 1974). Glucose was added as sole carbon and energy source to a concentration of 20 mM. Phosphate was added as KH_2PO_4 to final concentrations 0.07, 1, 10, 36 mM to evaluate polyP accumulation at varying phosphate concentrations.

PolyP levels were assessed following 15, 24 and 48 h of incubation at 30 °C shaking at 200 rpm. 1 ml samples were withdrawn and washed in ice-cold 50 mM4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid (HEPES-KOH) (pH 7.0). Pellets were frozen at -20 °C for several hours prior to assay. The cell pellet was resuspended in assay buffer (150 mM KCl, 20 mM HEPES-KOH, pH 7.0). 4',6-diamidino-2-phenylindole (DAPI) was added to suspension at a final concentration of 10 μM (Kulakova *et al*., 2011). Fluorescence was measured with excitation/emission filters 420/550 nm and compared to a P45 phosphate glass standard (Sigma-Aldrich, St. Louis, MO). Values are given as relative fluorescence units (RFU) per mg of dry bacterial biomass.

PHA polymer analysis

Following incubation, cells were harvested by centrifugation at 4,000 rpm for 10 min in benchtop 5810R centrifuge (Eppendorf, Hamburg, Germany). The pellet was then lyophilised. Approximately 5 mg of dried cells was subjected to acidic methanolysis according to previously described protocols

(Brandl *et al*. 1988; Lageveen *et al*. 1988). Cell material was resuspended in 2 ml acidified methanol (15 % H_2SO_4, v/v) and 2 ml of chloroform containing 6 mg l^{-1} methyl benzoate as an internal standard. The mixture was placed in 15 ml Pyrex test tubes and incubated at 100 °C for 3 h. The solution was extracted with 1 ml of water and the lower organic phase containing PHA monomers was removed for gas chromatography analysis.

Monomer determination was performed using an Agilent 6890N series GC fitted with a 30 m x 0.25 mm x 0.5 μm HP-INNOWaxcolumn (Hewlett-Packard, Palo Alto, CA). The oven method employed was 110 °C for 5 min, increasing by 3 °C/min to 130 °C for 1 min and increasing again by 5 °C/min to 250 °C. Peak areas were compared to that of the internal standard, methyl benzoate, for concentration determination.

Stress assays

P. putida KT2440 wild-type and *Δppk* strains were grown in 250 ml Erlenmeyer flasks containing 50 ml of MSM as previously described. Glucose was added to a concentration of 20 mM as the carbon and energy source. Flasks were incubated for 24 and 48 h and harvested by centrifugation. Cell pellets were lyophilised using a FreeZone 2.5 freeze dryer (Lab Conco, Kansas, MO). Cell pellets were weighed to provide cell dry weight (CDW) data and analysed for PHA content as well as polyP as previously described. For temperature variation study, flasks were incubated in orbital shakers set at 25 °C, 30 °C or 37 °C. For oxidative stress study flasks were maintained at 30 °C with the addition of H_2O_2 solution to a final concentration of 0, 0.5 or 1 mM at time 0.

Motility assay

Motility assays were carried out based on the procedure of Tittsler and Sandholzer (1936). LB plates solidified with 0.25% agar (semi solid) were inoculated with liquid LB culture by stabbing the centre of each plate. Plates were then incubated at 20 °C, 30 °C or 37 °C and monitored for diffuse growth at 24 and 48 h.

Biofilm assay

Biofilm assays were carried out based on the procedure of O'Toole and Kolter (1999). *P. putida* KT2440 wild-type and *Δppk* strains were grown to stationary phase in 2 ml liquid LB medium. Cultures were diluted 1:100 and used to inoculate wells in a 96 well plate. Cells were incubated at 30 °C for 24 h and planktonic cells were removed. Remaining biofilm was stained with a solution of 0.4 % (w/v) crystal violet. Stain was solubilised with 95 % (v/v) ethanol and the absorbance of solubilised stain was read at 600 nm on a Spectra Max 340 multi-well plate reader (Molecular Devices, Sunny Vale, CA).

Carbon substrate analysis and time course assays

P. putida KT2440 wild-type and *Δppk* strains were grown in 250 ml Erlenmeyer flasks containing 50 ml of MSM (reduced nitrogen) as previously described. Flasks were supplemented with 20 mM of either glucose or glycerol. For preliminary analysis cultures were harvested and analysed at 48 h. For time course analysis flasks were harvested every 6 h for 48 h and checked for PHA and polyP content.

Protein sample preparation for proteomic analysis

Changes in metabolic pathway proteins were monitored using quantitative proteomics. Total protein samples were obtained from wild-type KT2440 and Δ*ppk* strains at early-(OD_{540} 0.2), mid-(OD_{540} 0.6), and late-log (OD_{540} 1.0) phase and analyzed by Orbitrap mass spectrometry using label-free quantitation. Over 1700 proteins were identified. Using Extracted ion Currents (XIC) data it was possible to determine significance of observed differences of protein expression at $P < 0.05$ and $P < 0.01$ (Fig. 3).

Cultures of *P. putida* KT2440 wild-type and Δ*ppk* were grown in 250 ml shake flaks containing limited nitrogen MSM supplemented with 20 mM glycerol. Cells were harvested by centrifugation (4000 rpm for 10 min at 4 °C) and resuspended in 5 ml lysis buffer containing 50 mM sodium phosphate monobasic, 300 mM sodium chloride, 10 μM imidazole and 1 mg ml^{-1} lysozyme. Resuspended cells were sonicated at 50 % amplitude for 30 s (3 x 10 s intervals) using a Sonic Dismembrator FB120 (Thermo

Fisher Scientific Inc., Waltham, MA). Cell debris was removed by centrifugation at 20000 rpm using a Sorval RC5c Plus centrifuge. Protein concentration of the supernatant was determined a bicinchoninic acid method (Smith *et al*., 1985) using bovine serum albumin BSA as protein standard. Protein samples (30 μl) were resuspended in double the volume of 2 × non-reduced SDS-PAGE sample buffer (80 mM Tris/HCl, pH 6.8, 10% *β*-mercaptoethanol, 2% SDS, 10%, v/v, glycerol, 0.1% Bromophenol Blue) and heated at 100 °C for 5 min. Denatured proteins were separated on a 10% (w/v) polyacrylamide SDS gel and stained with Coomassie Brilliant Blue (Sigma-Aldrich, St Louis, MO).

Mass spectromic peptide analysis

SDS-PAGE gels were cut into bands (10 bands per time point) and the proteins digested in-gel with trypsin according to the method of Shevchenko *et al.* (1996). The resulting peptide mixtures were resuspended in 1% formic acid and analysed by nano-electrospray liquid chromatography MS (nano-LC MS/MS).

Transcript analysis

Cultures of *P. putida* KT2440 wild-type and Δ*ppk* were grown in 250 ml shake flaks containing limited nitrogen MSM supplemented with 20 mM glycerol. Cells were harvested by centrifugation (4000 rpm for 10 min at 4 °C) at optical density (OD 540 nm) of 0.2 approximately corresponding to early log phase of growth. Cell pellets were immediately frozen at -80 °C. Total RNA was extracted using High Pure RNA Isolation Kit (Roche Applied Science, Bavaria, Germany) according to manufacturer's instructions. RNA integrity was checked on 1 % (w/v) agarose gel run at 100 V for 15 min. Samples were DNAse treated using Ambion DNAse treatment and removal kit (Life Technologies). Reverse transcription of RNA sample was carried out using Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Bavaria, Germany). Samples were used to carry out quantitative PCR (qPCR) reactions for glycerol related genes *glpK*, *glpF* and *glpD* using Light Cycler 480 SYBR Green 1 Master (Roche

Applied Science, Bavaria, Germany) on a Light Cycler 480 System. Relative transcription levels were determined by the $2^{-\Delta\Delta C}{}_{T}$ method (Livak and Schmittgen, 2001)

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