Supporting Information

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SI Materials and Methods

Plasmid Construction.

(i) Construction of transposon vectors. *vanR* and the *vanR-vanAB* intergenic region were amplified from plasmid pRVYFPC-2 (1) with primers VanR_R/VanR_F and cloned in pCM62 (2) between PciI/HindIII. The −10 region (AAGATT) of P_{vanAB} was then changed to TAACTG using Quickchange PCR (Agilent) and primers Pvan2_F/Pvan2_R. Subsequently, the ribosome binding site (RBS) of *vanR* was changed by replacing an internal NcoI/MunI fragment with annealed oligonucleotides VanR_RBS2_s/VanR_RBS2_as. *vanR* and the modified *vanR-vanAB* intergenic region were amplified with primers VanR_F/Pvan_R2 and cloned in pAK401 between PciI/HindIII, giving pAK409. *tnp* was then amplified from plasmid pRK27 (3) with primers tnp_Oend_F/tnp_R and cloned in pAK409 between AseI/PvuI, giving pAK409-tnp. Next, *cat* was amplified from pBAD33 (4) with primers CAT_F_SpeI/CAT_R_Iend_PvuI and cloned in pAK409-tnp, giving pAK411, the final mini-Tn5 transposon vector. pAlmar2 (a gift from Urs Jenal, Biozentrum Basel, Basel, Switzerland), was used as a template to generate a derivative of *himar1C9* (5) that encodes, in addition, the hyperactive W119A mutation (6) by overlap PCR using primer pairs Himar1_F/Himar1W119A_R and Himar1W119A_F/Himar1_R. The *himar* mutant allele, which we named *himar1C9W*, was cloned in pAK409, giving pAK409-himar1C9W. Then, *cat* was amplified from pBAD33 using primers CAT_F_SpeI/CAT_himer1ITR_R and cloned between SpeI/PvuI of pAK409-himar1C9W, yielding pAK415, the final *himar*-based transposon vector.

(ii) Plasmids for genetic selection and lacZ reporter plasmids. *rpsL1* was amplified from pAK405 (7) with primers rpsL88_RBS_F/rpsL88_R2 and cloned in pAK127 (8) via BamHI/EcoRI, giving pAK134. Next, the σ^{EcfG}-dependent *ecfG2* promoter was amplified from *S. melonis* Fr1 genomic DNA (gDNA) with primers ecfG2p_F2/ecfG2p_R2 and cloned in pAK134 via HindIII/XbaI, giving pAK145. pAK128 (8) was digested with HindIII/BamHI, and a fragment containing the *nepR* promoter generated by annealing of oligonucleotides nepRpcore_a/nepRpcore_as was inserted upstream of *syfp2*, yielding pAK128-nepRpcore. A fragment containing terminators, *nepRp*, and *syfp2* was amplified from pAK128-nepRpcore using nepR-CAS_F/nepR-CAS_R and inserted in pUC18-mini-Tn7T-Gm (9) via ApaI/XhoI, giving pAK406. Plasmid pAK144 was constructed by amplifying the gene encoding FLP recombinase from pFLP3 (9) using primers FLP_F/FLP_R, partial digestion with EcoRI and full digestion with MluI, and ligation in the same sites of pAK130 (8). The σ^{EcfG}-dependent *nhaA2* promoter was amplified from gDNA with primers nhaA2p_F/nhaA2p_R and cloned in the *lacZ* reporter plasmid pAK501 (8) via HindIII/Acc65I, giving pAK501-nhaA2p. The σ^{EcfG}-independent *rpmB* promoter was amplified from gDNA with primers rpmB_F/rpmB_R and cloned in pAK501 via HindIII/Acc65I, giving pAK501-rpmBp. pAK501-nepRp was described previously (8).

(iii) pENTR and expression plasmids. Full-length *pak* ORFs and truncated ORFs only encoding the kinase domain (for *pakA*, *pakB*, and *pakE*) were amplified by PCR from gDNA and cloned in pENTR4a or pENTR4b as detailed in Table S2. pENTR4a is a derivative of pENTR4 (Invitrogen) obtained by excision of *ccdB* by EcoRI and self-ligation. pENTR4b is a derivative of pENTR4a that includes a sequence encoding a Gly-Ser–rich linker upstream of the multiple cloning site and was obtained by inverse PCR using primers pENTR4_linker_F/pENTR4_linker_R, followed by self-ligation of the PCR product. *sdrG* and *phyR* were similarly cloned in pENTR4b and pENTR4a, respectively. Point mutations were introduced in pENTR plasmids by Quickchange PCR. Primers are listed in Table S2. ORFs were recombined into pQYD (8) for expression in *Sphingomonas* and/or pDEST-MBPHis (10) (Addgene plasmid 11085) for protein expression and purification in *E. coli* using LR Clonase II mix (Invitrogen). For complementation experiments, *sdrG* was cloned in pAK126a (7) using primers BK51comp_F/BK51comp_R via XbaI/Acc65I. Point mutations were introduced by overlap PCR and cloning in the same sites using the same flanking primers and the mutagenic primers listed in Table S2. The constructs for NepR and PhyR overproduction and purification, pET26bII-NepR and pET26bII-PhyR, were described previously (11). *sdrG* was amplified using primers 2886_pET_F/2886_pET_R and cloned in pET26bII (a gift from W. Malaga, Institute of Pharmacology and Structural Biology, Toulouse, France) via NdeI/Acc65I, giving pET26bII-SdrG that was used for protein purification. For bacterial two-hybrid analysis (12), full-length *pak* ORFs were cloned in pUT18 and pKNT25 (Euromedex) with primers listed in Table S2.

(iv) Plasmids for mutant construction. Approximately 750 bp of upstream and downstream regions of genes to be deleted (or the codon to be exchanged in the case of $phyR^{D194A}$) were PCR-amplified and joined using overlap PCR, and then cloned in pAK405. Primers are listed in Table S2.

Strain Construction. All *Sphingomonas* in-frame deletion mutants were constructed by double homologous recombination using pAK405 as described previously (7). For allelic exchange of chromosomal *phyR* for $phyR^{D194A}$, a similar strategy using pAK405 was used. To delete *pakD* with complementation *in trans* on plasmid pQYD-PakD, pAK405-pakD was first integrated in the genome of strain JVZ857, followed by transformation of pQYD-PakD and subsequent counterselection of the resulting strain on LB containing tetracycline, streptomycin, and 5 μM cumate. Colonies were screened for *pakD* WT or mutant genotype by PCR. As a control, the same colonies were used to perform PCR on a chromosomal region amplifying the *pkrF* ORF and a region on the megaplasmid that is positioned approximately 180° apart from *pakD*. For all colonies for which PCR did not amplify the *pakD* region, it also did not amplify the megaplasmid control region but did amplify the chromosomal control region, suggesting loss of the megaplasmid. The strain used for selection (JVZ1919) was obtained in several steps, starting from the WT strain JVZ857. First, the *nepRp-syfp2* transcriptional fusion was inserted in the *att*Tn7 locus of JVZ857 by co-transformation of pAK406 and the helper plasmid pTNS2 (9) and selection on gentamycin (Gm). Next, this strain was transformed with plasmid pAK144 that constitutively expresses the FLP recombinase, leading to excision of the chromosomal Gm-resistance cassette associated with Tn7. Next, this strain was cured from pAK144, which also expresses the dominant streptomycin-sensitive *rpsL1* allele, by plating on streptomycin (Sm), giving strain JVZ1841. Finally, plasmid pAK145 harboring the *ecfG2p-rpsL1* fusion was transformed into JVZ1841, giving JVZ1919.

Western Blots. To follow *pak* expression, 1 mL of bacterial culture before or 2 h after addition of cumate was spun down (12,000 × *g*, 2 min), and cell pellets were resuspended in 1× Lämmli buffer, corresponding to an OD_{600} of 10, and incubated at 99 °C for 15 min. Samples were subjected to SDS/PAGE [12.5% (wt/vol) acrylamide],

proteins were transferred to nitrocellulose membranes by semidry blotting, and proteins were detected using mouse α-GFP antibody (GF28R; Pierce) and goat α-mouse HRP-coupled secondary antibody (Biorad). As a control, the same samples were probed with rabbit α-GroEL (*E. coli*) serum and goat α-rabbit HRP-coupled secondary antibody (Biorad). Chemiluminescence was detected using ECL Western Blotting Detection Reagent (GE Healthcare).

Protein Expression and Purification. PhyR and NepR were overproduced as described previously (11). Overproduction of all other proteins was carried out in LB-Lennox medium in 200- to 400-mL cultures, with the following details. For SdrG overproduction, *E. coli* BL21(DE3)/pET26bII-SdrG was grown to an OD_{600} of 0.8 at 37 °C and induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Cells were harvested after 4 h. All Paks were overproduced as His6-MBP fusions from pDEST-HisMBP. N-terminally truncated PakB (amino acids 297–507) was overproduced in BL21(DE3); the strain was grown to an OD_{600} of 0.6–0.8 at 37 °C, shifted to 25 °C, and induced with 50 μM IPTG. Cells were harvested after 16 h. N-terminally truncated PakA (amino acids 287–488) and PakE (amino acids 535–726) and full-length PakG were overproduced in Rosetta2 (DE3); strains were grown to an OD_{600} of 0.6–0.8 at 28 °, shifted to 15 °, and induced with 100 μM IPTG after 1 h of equilibration, and cells were harvested after 24 h. Full-length PakC was overproduced in the same way, but in BL21(DE3). Full-length PakF was overproduced in BL21(DE3); the strain was grown to an OD_{600} of 0.6–0.8 at 28 °C, shifted to 18 °C, and induced with 50 μM IPTG after 30 min of equilibration, and cells were harvested after 16–24 h.

Cell pellets were washed once in PBS and resuspended in 7.2 mL of wash buffer [20 mM Hepes-KOH (pH 8.0), 0.5 M NaCl, 10% (vol/vol) glycerol, 20 mM imidazole, 0.1% Triton X-100] supplemented with lysozyme (0.1 mg/mL), DNase I (0.01 mg/mL), 800 μL of BugBuster 10× Protein Extraction Reagent (Novagen), and one tablet of EDTA-free Complete protease inhibitor mixture (Roche). Cell lysis was allowed to proceed at room temperature with mild shaking until the suspension was clear and nonviscous. All following steps were performed at 4 °C. Debris was pelleted (12,000 × *g*, 20 min), and the supernatant was filtered (pore size of 0.22 μM), incubated with 800 μL of nickel-nitrilotriacetic acid (Ni-NTA) agarose slurry (Qiagen) for 1 h, and loaded on a polypropylene column (Qiagen). Ni-NTA agarose was washed with 30–50 mL of wash buffer by gravity flow, and proteins were eluted in 2.5 mL of elution buffer [20 mM Hepes-KOH (pH 8.0), 0.5 M NaCl, 10% (vol/vol) glycerol, 250 mM imidazole]. The eluate was immediately loaded on a PD-10 column (GE Healthcare) preequilibrated in kinase buffer [10 mM Hepes-KOH (pH 8.0), 50 mM KCl, 10% (vol/vol) glycerol, 0.1 mM EDTA, 1 mM DTT], and proteins were eluted with 3.5 mL of kinase buffer. Purified proteins were stored at −20 °C, and protein concentrations were determined using a NanoDrop 2000 UV-VIS spectrometer (Thermo Scientific) or a Biorad Protein Assay (Biorad).

PhyR~P-NepR Complex Formation and Native Gels. One hundred-microliter reactions containing combinations (Fig. S4*A*) of PhyR (5 μM), NepR (7.5 μM), and/or acetyl phosphate (AcP; 5 mM) in kinase buffer were incubated at room temperature for 30 min to allow PhyR phosphorylation and complex formation, followed by removal of AcP using Micro Bio-Spin P-6 columns (Biorad) preequilibrated with kinase buffer and elution of proteins with 100 μL of kinase buffer. Eluted proteins/complexes were split into four 25-μL aliquots, and aliquots were either left untreated (control) or NepR alone (final concentration of 20 μM) or NepR and AcP (final concentration of 10 mM) were added (total reaction volume of 50 μL), followed by incubation at room temperature for 15 min to allow complex formation. A total of 5× native loading buffer [310 mM Tris·HCl (pH 6.8), 50% (vol/vol) glycerol, bromophenol blue] was added, and proteins/protein complexes were separated on native gels (to observe complex formation). The same samples (in Lämmli buffer) were also subjected to standard SDS/PAGE. Native PAGE was performed as follows. The separating gel contained 375 mM Tris·HCl (pH 8.8) and 7.5% acrylamide/bis acrylamide solution (30:0.8; Protogel), and the stacking gel contained 125 mM Tris·HCl (pH 6.8) and 3.75% acrylamide/bis acrylamide solution (30:0.8; Protogel); gels were solidified using ammonium persulfate (APS) and N,N,N′,N′-tetramethylethylenediamine (TEMED) (Protogel). Running buffer was 25 mM Tris base and 190 mM glycine, adjusted to pH 8.3. The gel was run at room temperature at 150 V for 45 min and stained with Coomassie Brilliant Blue, and proteins were transferred to nitrocellulose membranes using semidry blotting and blotting buffer containing 20% methanol, 25 mM Tris base, and 190 mM glycine, adjusted to pH 8.3. Immunoblotting was performed using rabbit α-PhyR serum and goat α-rabbit HRP-coupled antibodies (Biorad), followed by chemiluminescence detection using ECL Western Blotting Detection Reagent.

Genetic Selection for "PhyR Cascade Down" Mutants. The "selection strain" JVZ1919 was subjected to random transposon mutagenesis by conjugation of plasmids pAK411 (mTn5) or pAK415 (*himar1C9W*) from *E. coli* S17-1λ*pir* (13), as described previously (7). Mating mixtures were plated on LB-Lennox medium containing kanamycin (50 μg/mL, for selection of transposon insertion mutants), carbenicillin (50 μg/mL, for *E. coli* counterselection), tetracycline (10 μg/mL, for maintenance of plasmid pAK145 carrying the *ecfG2p-rpsL1* fusion), and Sm (60 μg/mL, for selection of "cascade down" mutants). To estimate the efficiency of random mutagenesis, a 10-fold serial dilution was plated on otherwise identical medium lacking Sm, giving an estimate of *ca.* 0.5–2 Mio mutants per mating. Colonies appearing after 3–4 d were purified by restreaking once on the same medium, and they were then screened for reduced activity of the *nepRp-syfp2* fusion using an IVIS Spectrum (Caliper Life Sciences). Transposon insertion sites were mapped using semiarbitrary PCR and sequencing of PCR products. The *sdrG* mutant was isolated using the mTn5 transposon, which has inserted after nucleotide 27 of the annotated ORF.

Transposon Insertion Site Mapping Using Semiarbitrary PCR. A small loop of bacteria was resuspended in 100 μL of 50 mM NaOH and incubated at 99 °C for 10 min. Cell debris was briefly spun down, and 1 μL of the supernatant was used as a template for the subsequent PCR. The first PCR assay was carried out in a total volume of 20 μL using Phusion polymerase (Thermo Scientific) in GC buffer and primers Arb-P1, Arb-P2, Arb-P3, and pAK411-nested1 (0.4 μM each) according to the supplier's recommendations, except that the DMSO concentration was raised to 10% (vol/vol). The cycling parameters were as follows: 98 °C for 2 min; 98 °C for 10 s, 30 °C for 20 s, and 72 °C for 30 s (six cycles); 98 °C for 10 s, 45 °C for 20 s, and 72 °C for 30 s (30 cycles); 72 °C for 1 min; and hold at 10 °C. The PCR assay was column-purified using a NucleoSpin Gel and PCR Clean-Up Kit (Macherey–Nagel) with elution in 50 μL. One microliter of purified PCR product was used as a template for the second PCR assay in a total volume of 50 μL using Phusion polymerase in GC buffer containing 10% (vol/vol) DMSO and primers Anchor-P and pAK411-nested2 (0.4 μM each). The cycling parameters for the second PCR assay were as follows: 95 °C for 2 min; 95 °C for 10 s, 61 °C for 10 s, and 72 °C for 30 s (five cycles); 95 °C for 10 s, 58 °C for 10 s, and 72 °C for 30 s (31 cycles); 72 °C for 30 s; and hold at 10 °C. The PCR product was column-purified as described above, except that the elution volume was 20 μL. Eight microliters of purified PCR product was sent for sequencing (Microsynth) using primer pAK411-nested2.

1. Thanbichler M, Iniesta AA, Shapiro L (2007) A comprehensive set of plasmids for vanillate- and xylose-inducible gene expression in *Caulobacter crescentus*. *Nucleic Acids Res* 35(20):e137.
2. Marx CJ, Lidstrom ME (2001) Development of improved versatile broad-host-range vectors for use in methylotrophs and other Gram-negative bacteria. *Microbiology* 147(Pt 8):2065–2075.
3. Larsen RA, Wilson MM, Guss AM, Metcalf WW (2002) Genetic analysis of pigment biosynthesis in *Xanthobacter autotrophicus* Py2 using a new, highly efficient transposon mutagenesis system that is functional in a wide variety of bacteria. *Arch Microbiol* 178(3):193–201.
4. Guzman LM, Belin D, Carson MJ, Beckwith J (1995) Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *J Bacteriol* 177(14):4121–4130.
5. Lampe DJ, Akerley BJ, Rubin EJ, Mekalanos JJ, Robertson HM (1999) Hyperactive transposase mutants of the Himar1 mariner transposon. *Proc Natl Acad Sci USA* 96(20):11428–11433.
6. Lampe DJ (2010) Bacterial genetic methods to explore the biology of mariner transposons. *Genetica* 138(5):499–508.
7. Kaczmarczyk A, Vorholt JA, Francez-Charlot A (2012) Markerless gene deletion system for sphingomonads. *Appl Environ Microbiol* 78(10):3774–3777.
8. Kaczmarczyk A, Vorholt JA, Francez-Charlot A (2013) Cumate-inducible gene expression system for sphingomonads and other Alphaproteobacteria. *Appl Environ Microbiol* 79(21):6795–6802.
9. Choi KH, et al. (2005) A Tn7-based broad-range bacterial cloning and expression system. *Nat Methods* 2(6):443–448.
10. Nallamsetty S, Austin BP, Penrose KJ, Waugh DS (2005) Gateway vectors for the production of combinatorially-tagged His6-MBP fusion proteins in the cytoplasm and periplasm of *Escherichia coli*. *Protein Sci* 14(12):2964–2971.
11. Kaczmarczyk A, et al. (2011) Role of *Sphingomonas* sp. strain Fr1 PhyR-NepR-σ^{EcfG} cascade in general stress response and identification of a negative regulator of PhyR. *J Bacteriol* 193(23):6629–6638.
12. Karimova G, Pidoux J, Ullmann A, Ladant D (1998) A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proc Natl Acad Sci USA* 95(10):5752–5756.
13. Simon R, Priefer U, Pühler A (1983) A broad-host range mobilization system for in vivo genetic engineering: Transposon mutagenesis in gram-negative bacteria. *Bio/Technology* 1(1):784–791.

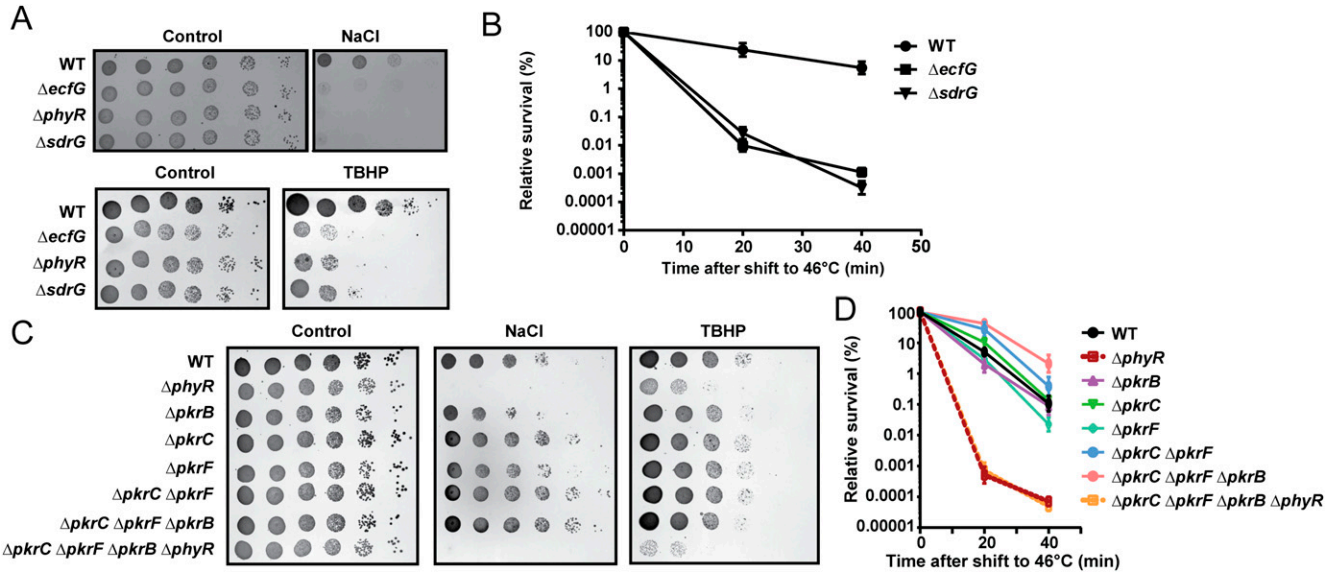

Fig. S1. Sensitivity of the *sdrG* (*A* and *B*) and cognate SDRR mutants (*C* and *D*) toward salt and TBHP stress (*A* and *C*) and HS (*B* and *D*). Tenfold dilution series are shown in *A* and *C*. Mean ± SD of three independent experiments is shown in *B* and *D*. Genetic nomenclature is the same as in the main text.

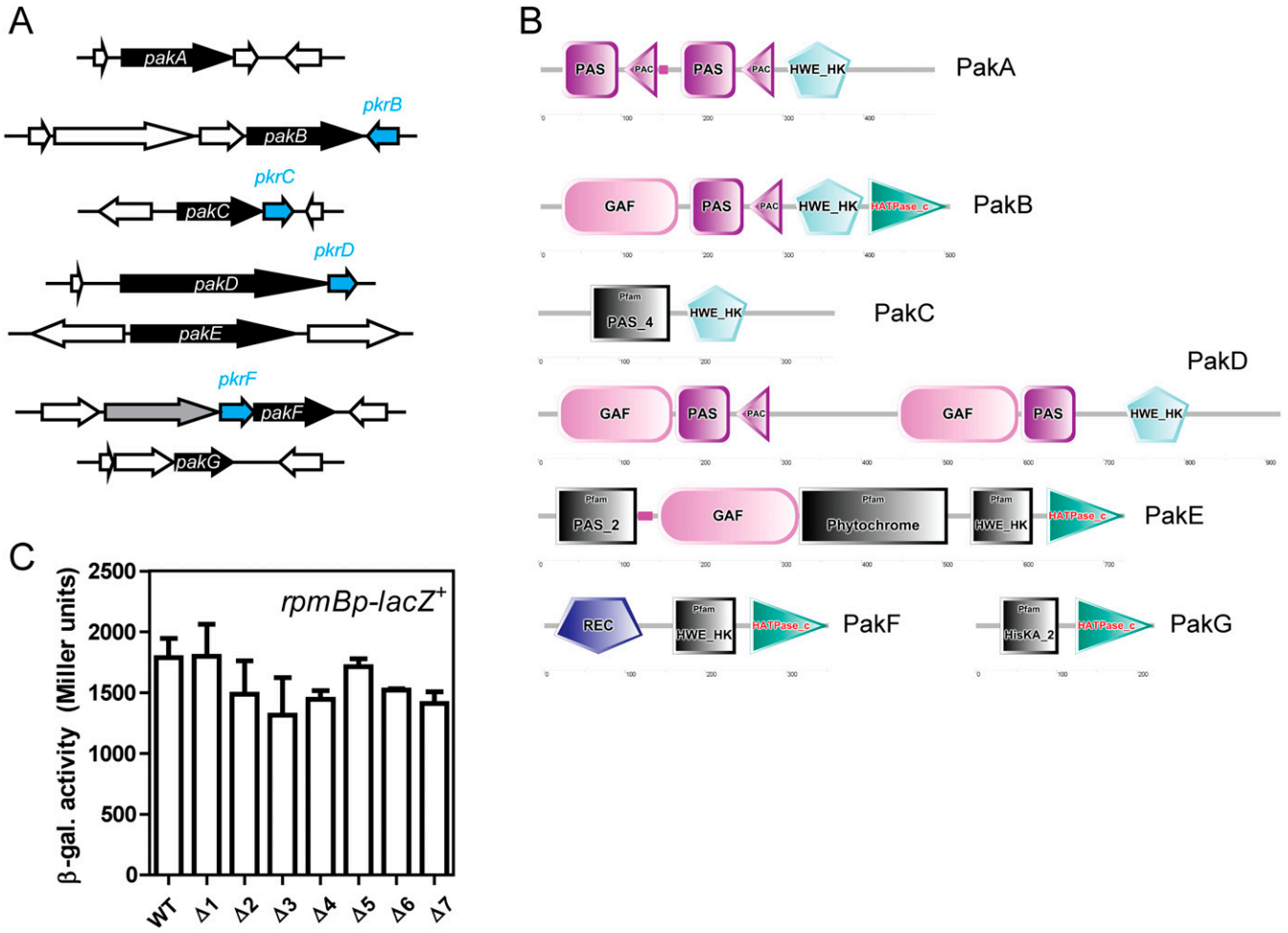

Fig. S2. Genetic organization of *pak* loci (*A*) and domain composition of Paks (*B*). (*A*) *pak* ORFs are indicated by black arrows, and ORFs encoding SDRRs are indicated by blue arrows with their names on top; genes unrelated to two-component signaling are indicated by white arrows. The gray arrow at the *pakF* locus indicates another His kinase-encoding gene. (*B*) Domain composition of Pak kinases predicted by SMART (1) as of May 28, 2014. (*C*) Activity of the *rpmBp-lacZ*$^+$ control fusion in multiple kinase mutants. The nomenclature follows that described in Fig. 2. Mean ± SD of three independent experiments is shown.

1. Letunic I, Doerks T, Bork P (2012) SMART 7: Recent updates to the protein domain annotation resource. *Nucleic Acids Res* 40(Database issue):D302–D305.

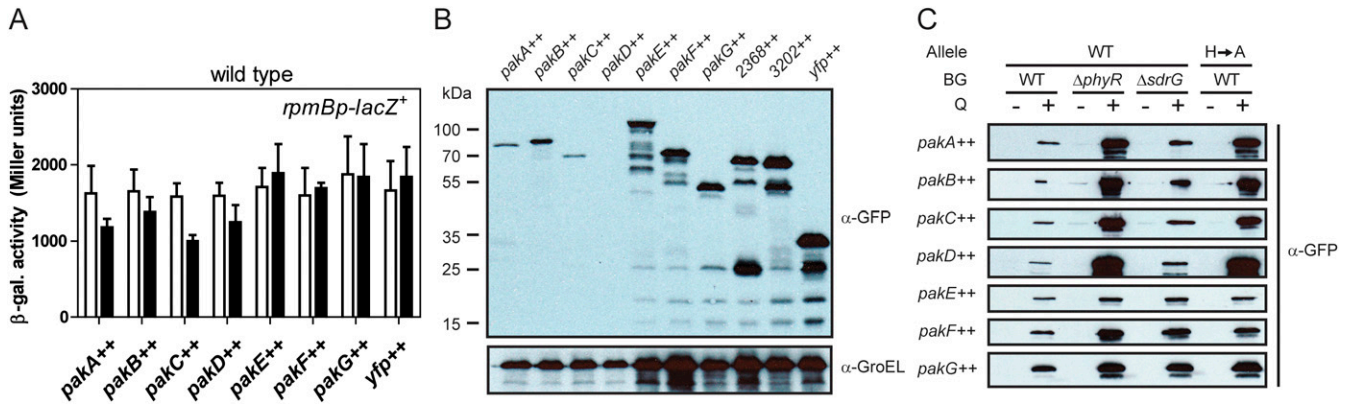

Fig. S3. Control experiments for *pak* overexpression. (*A*) *pak* overexpression does not affect activity of the *rpmBp-lacZ*$^+$ control fusion. *rpmBp-lacZ*$^+$ activity was measured before (white bars) and after 2 h of (black bars) induction of kinase overexpression (++) from promoter P_{Q5} (plasmid pQYD) with 25 μM cumate. (*B*) Western blot of strains after 2 h of overexpression of *paks* in the WT background. (*C*) Western blot of strains before (–) and 2 h after (+) addition of cumate (indicated by Q) to induce *pak* expression in different genetic backgrounds.

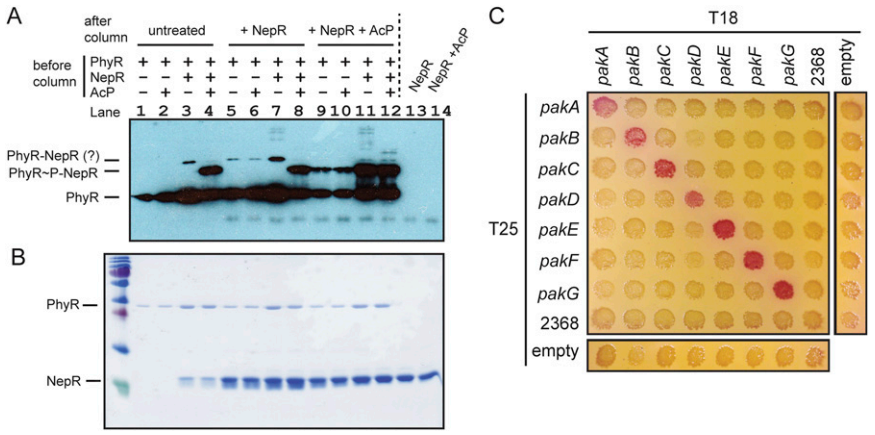

Fig. S4. PhyR~P-NepR complex formation depends on coincubation with NepR and acetyl-phosphate (*A* and *B*), and Paks form homomers. (*A* and *B*) PhyR, NepR, and/or AcP was incubated for 30 min in different combinations ("before column"), followed by removal of AcP. (*A*) Then ("after column"), samples were left untreated, NepR alone or NepR and AcP were added, and protein complexes were resolved on native gels, followed by immunoblotting against PhyR. (*B*) Same samples were subjected to standard SDS/PAGE. As seen in the control (*A*, lane 4), formation of the PhyR~P-NepR complex resulted in a slower migrating band ("PhyR~P-NepR"). Addition of NepR to PhyR preincubated with AcP (*A*, lane 6) caused no such shift, indicating that PhyR is not phosphorylated. When AcP is added in addition to NepR (*A*, lane 10), the PhyR~P-NepR complex is readily formed, indicating that the incubation time with NepR (after column) is sufficiently long, in principle, to allow complex formation. Note that an even slower migrating band is observed in samples containing PhyR and NepR without AcP, indicated by "PhyR-NepR (?)," which might consist of unphosphorylated PhyR and NepR; whether this hypothetical complex has biological relevance or is simply an experimental artifact is currently unknown. (*C*) Bacterial two-hybrid analysis of Pak interactions. *E. coli* cya- (BTH101; Euromedex) carrying fusions of Paks to T18 and T25 fragments of *Bordetella pertussis* adenylate cyclase (1) were spotted on McConkey lactose and incubated at 28 °C for 4 d. Red colonies/spots are indicative of positive interactions.

1. Karimova G, Pidoux J, Ullmann A, Ladant D (1998) A bacterial two-hybrid system based on a reconstituted signal transduction pathway. *Proc Natl Acad Sci USA* 95(10):5752–5756.

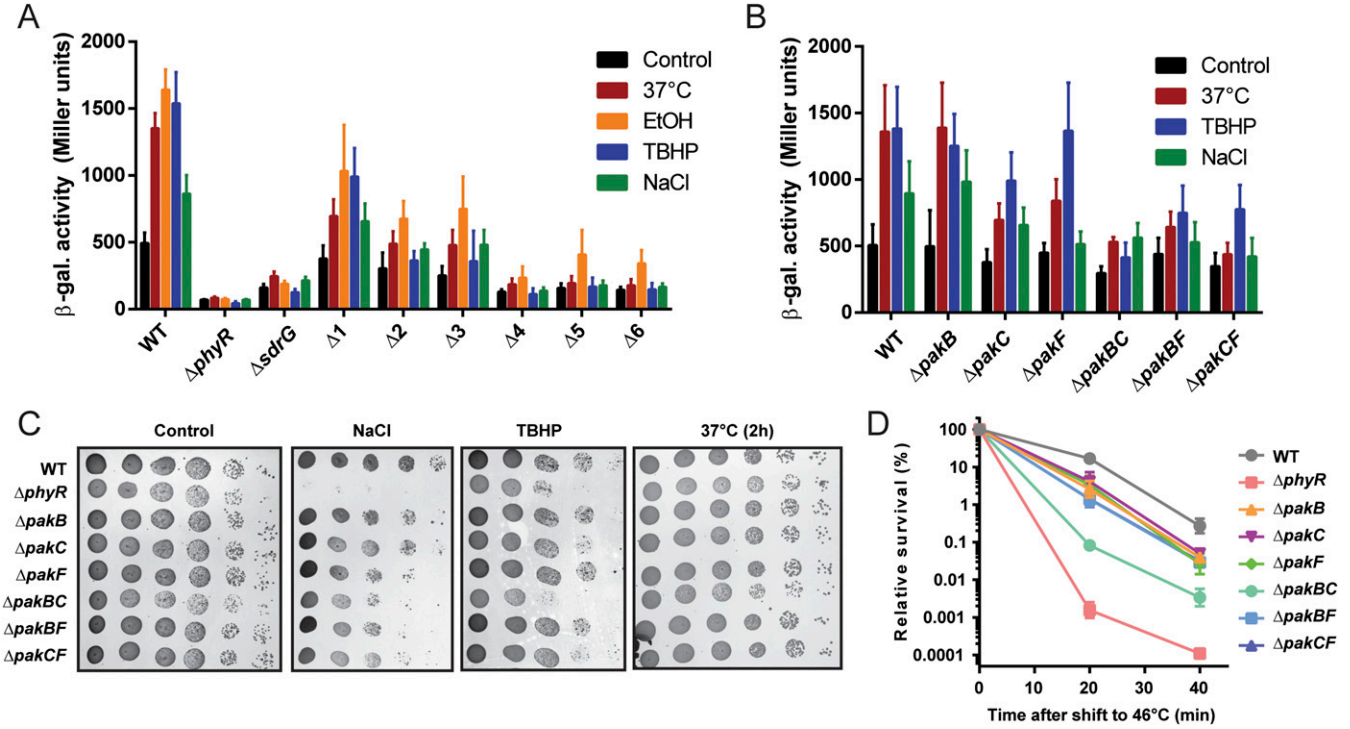

Fig. S5. (*A* and *B*) β-Gal activity of the *nhaA2p-lacZ*$^{+}$ fusion in the multiple kinase and *phyR* and *sdrG* mutants used to calculate RSA shown in Fig. 5. The nomenclature for kinase mutants follows that described in the main text. Mean ± SD of three independent experiments is shown. (*C* and *D*) Stress phenotypes of selected single and double *pak* mutants as indicated. Results in *D* are given as mean ± SD of three independent experiments.

Table S1. Absence of PhyR and HRXXN kinases in selected Alphaproteobacteria

Species	Genome, Mbp	PhyR	HRXXN kinases	Classical HK	HHK	HRR	RR	Others	No. of TCS proteins
Pseudovibrio sp. FO-BEG1	5.9	No	No	25	8	0	40	2	75
Octadecabacter arcticus 238	5.5	No	No	18	2	0	22	0	42
Magnetococcus marinus MC-1	4.7	No	No	29	55	13	71	4	172
Acidiphilium multivorum AIU301	4.2	No	No	14	2	0	21	0	37
Maricaulis maris MCS10	3.4	No	No	22	13	1	41	2	79
Liberibacter crescens BT-1	1.5	No	No	9	1	0	13	0	23

Completed alphaproteobacterial genomes on the MiST2 database (1) were searched for absence of PhyR and then checked for absence of HRXXN kinases. TCS, two-component system.

1. Ulrich LE, Zhulin IB (2010) The MiST2 database: A comprehensive genomics resource on microbial signal transduction. *Nucleic Acids Res* 38(Database issue):D401–D407.

Table S2. *S. melonis* Fr1 strains

Strain	Relevant genotype	Source
JVZ857	*Sphingomonas melonis* Fr1 WT strain	(1)
JVZ1703	JVZ857 Δ*ecfG*	(2)
JVZ1704	JVZ857 Δ*phyR*	(2)
JVZ2520	JVZ857 Δ*sdrG*	This study
JVZ3234	JVZ857 $phyR^{D194A}$	This study
JVZ1817	JVZ857 Δ*pakA*	This study
JVZ2470	JVZ857 Δ*pakB*	This study
JVZ2257	JVZ857 Δ*pakC*	This study
JVZ1818	JVZ857 Δ*pakE*	This study
JVZ1953	JVZ857 Δ*pakF*	This study
JVZ2471	JVZ857 Δ*pakG*	This study
JVZ2521	JVZ857 Δ*pkrB*	This study
JVZ2518	JVZ857 Δ*pkrC*	This study
JVZ2519	JVZ857 Δ*pkrF*	This study
JVZ2509	JVZ857 ΔSphme2DRAFT_2368	This study
JVZ2503	JVZ857 Δ*ecfG sdrG*	This study
JVZ2504	JVZ857 Δ*phyR* Δ*sdrG*	This study
JVZ3235	JVZ857 Δ*sdrG* $phyR^{D194A}$	This study
JVZ3216	JVZ857 Δ*pakB* Δ*pakF*	This study
JVZ3215	JVZ857 Δ*pakC* Δ*pakF*	This study
JVZ2259	JVZ857 Δ*pakC* Δ*pakB*	This study
JVZ2360	JVZ857 Δ*pakC* Δ*pakB* Δ*pakA*	This study
JVZ2385	JVZ857 Δ*pakC* Δ*pakB* Δ*pakA* Δ*pakF*	This study
JVZ2399	JVZ857 Δ*pakC* Δ*pakB* Δ*pakA* Δ*pakE* Δ*pakF*	This study
JVZ2453	JVZ857 Δ*pakC* Δ*pakB* Δ*pakA* Δ*pakE* Δ*pakF* Δ*pakG*	This study
JVZ3242	JVZ857 Δ*pakC* Δ*pakB* Δ*pakA* Δ*pakE* Δ*pakF* Δ*pakG* Δ*phyP*	This study
JVZ2507	JVZ857 Δ*pakC* Δ*pakB* Δ*pakA* Δ*pakE* Δ*pakF* Δ*pakG* ΔSphme2DRAFT_2368	This study
JVZ2546	JVZ857 Δ*pakC* Δ*pakB* Δ*pakA* Δ*pakE* Δ*pakF* Δ*pakG* ΔSphme2DRAFT_2368 Δ*phyP*	This study
JVZ1952	JVZ857 Δ*pakB* Δ*pkrB*	This study
JVZ1713	JVZ857 Δ*pakC* Δ*pkrC*	This study
JVZ3191	JVZ857 Δ*pakF* Δ*pkrF*	This study
JVZ2522	JVZ857 Δ*pkrC* Δ*pkrF*	This study
JVZ2541	JVZ857 Δ*pkrC* Δ*pkrF* Δ*pkrB*	This study
JVZ3188	JVZ857 Δ*pkrC* Δ*pkrF* Δ*pkrB* Δ*phyR*	This study
JVZ3189	JVZ857 Δ*pkrC* Δ*pkrF* Δ*pkrB* Δ*sdrG*	This study
JVZ1919	JVZ857 *att*Tn7::*nepRp-syfp2*/pAK145	This study

1. Innerebner G, Knief C, Vorholt JA (2011) Protection of *Arabidopsis thaliana* against leaf-pathogenic *Pseudomonas syringae* by *Sphingomonas* strains in a controlled model system. *Appl Environ Microbiol* 77(10):3202–3210.
2. Kaczmarczyk A, Vorholt JA, Francez-Charlot A (2012) Markerless gene deletion system for sphingomonads. *Appl Environ Microbiol* 78(10):3774–3777.

Table S3. Primers

Primer name	Primer sequence (5′ to 3′)	Purpose
Mutant strain construction		
PhyR194chrom_R	ATTTCTAGAATGCCTTCAGCAGCGTTTCC	Construction of phyRD194A strain
PhyR194chrom_F	ATTTTGGTACCACCAAAGTCGCGTTCGTTGG	Construction of phyRD194A strain
PhyR_FR1_D194A F	GGTCTGGTGCTGGCGGCGATCCAGCTGGCCGACGAT	Construction of phyRD194A strain
PhyR_FR1_D194A R	ATCGTCGGCCAGCTGGATCGCCGCCAGCACCAGACC	Construction of phyRD194A strain
419up_F	ATTTTGGTACCATGACCACCAAGACCATCG	pakA mutant construction
419up_R	GACGAATGGCGTGTGCGGC	pakA mutant construction
419dw_F	GCCGCACACGCCATTCGTC ATCGAGGCCCCGGTCTGAGC	pakA mutant construction
419dw_R	ATTTTCTAGATGCGCCGAACTCTATTACG	pakA mutant construction
885N_up_F	ATTTTCTAGAACCGCAGTGCATCAGCACG	pakB mutant construction
885N_up_R	GCGTCGCGATCGGCACGCGTTCACAGACGGGTGTCATC	pakB mutant construction
885N_dw_F	CGCGTGCCGATCGCGACGC	pakB mutant construction
885N_dw_R	ATTTGGTACCAATGGCGCATCCGCCTGTC	pakB mutant construction
1359N_up_F	ATTTTCTAGAAGATCGGGTCCGAACAGCC	pakC mutant construction
1359N_up_R	GACGGCGGCGATGGTCACG	pakC mutant construction
1359N_dw_F	CGTGACCATCGCCGCCGTCTATCAGGTTCAAGAGGCG	pakC mutant construction
1359N_dw_R	ATTTGGTACCTGTCCGTTCGGTGGTTTGG	pakC mutant construction
2513up_F	ATTTTGGTACCTCTCGCCCAGCGGATGGAGG	pakE mutant construction
2513up_R	GTGGGAAATCGCCATTATCAC	pakE mutant construction
2513dw_F	GTGATAATGGCGATTTCCCACACCGCCCCGATTGCGGAAG	pakE mutant construction
2513dw_R	ATTTTCTAGAATCACGATCCAGACCGTCC	pakE mutant construction
1301up_F	ATTTTGGTACCATCGGGCCAGCAGGACCAGC	pakF mutant construction
1301up_R	CGCCGCGCGCAGCGTTGC CGGCTGGCCCGCCGCCAGC	pakF mutant construction
1301dw_F	GCAACGCTGCGCGCGGCG	pakF mutant construction
1301dw_R	ATTTTCTAGATAGGCGGCGATATGTCGTCG	pakF mutant construction
3817up_R	GGCTCGCCATCGGCACCCTCTCGGAATCGTTCATGGC	pakG mutant construction
3817dw_F	AGGGTGCCGATGGCGAGCC	pakG mutant construction
3817dw_R	ATTTCTAGATTGTTCCTGCCGATCGCG	pakG mutant construction
3817up_F	ATTTGGTACCTGACAAGTTTCGGTCCAGC	pakG mutant construction
BK51_up_F	ATTTGGTACCTTACCGCGGCCGACGTGC	sdrG mutant construction
BK51_dw_R	ATTTTCTAGATTGCCGGCGCGCCCCCAGG	sdrG mutant construction
BK51_up_R	GGGGCGTTACAGCGCGGCGGTGAGCGCAGACATGCC	sdrG mutant construction
BK51_dw_F	GCCGCGCTGTAACGCCCC	sdrG mutant construction
1662up_R	GTGTTCCTCGACCTCGGGTTGATGGGCGTTCATGACG	pkrF mutant construction
1662dw_F	CCCGAGGTCGAGGAACAC	pkrF mutant construction
1662up_F	ATTTCTAGAACGAGGAAATCCAGCGC	pkrF mutant construction
1662dw_R	ATTTGGTACCATCGATTCGGCGACAAGG	pkrF mutant construction
1661-2_up_F	ATTTCTAGAAACGAGGAAATCCAGCGC	pkrF pakF double-mutant construction
1661-2_up_R	GCTTATGCCATCGCCGCGCGTTGATGGGCGTTCATGACG	pkrF pakF double-mutant construction
1661-2_dw_F	CGCGCGGCGATGGCATAAGC	pkrF pakF double-mutant construction
1661-2_dw_R	ATTTGGTACCATAGGCGGCGATATGTCG	pkrF pakF double-mutant construction
2368dw_F	CATCATGTCCGTCGAAGCAAGCGGGCTAGTGTACAGC	Sphme2DRAFT_2368 mutant construction
2368up_R	TGCTTCGACGGACATGATG	Sphme2DRAFT_2368 mutant construction
2368up_F	ATTTGGTACCACCGTGCCCGATGTCTGC	Sphme2DRAFT_2368 mutant construction
2368dw_R	ATTTCTAGAATCTGCCGCAGCATCGTGG	Sphme2DRAFT_2368 mutant construction
1360dw_F	CGACATGGGACAGTCCGAAAACCGCTCAGCTGCTTGAG	pkrC mutant construction
1360up_R	TTCGGACTGTCCCATGTCG	pkrC mutant construction
1360up_F	ATTTCTAGATATACGCCGGATCGCGACG	pkrC mutant construction
1360dw_R	ATTTGGTACCATCGATCTCGCCCGATGC	pkrC mutant construction
1359.1360dw_F	CGTGACCATCGCCGCCGTCCTGGTAAACCGCTCAGCTGC	pkrC pakC double-mutant construction
1359.1360up_R	GACGGCGGCGATGGTCACG	pkrC pakC double-mutant construction
1359.1360dw_R	ATTTTCTAGAGATCTCGCCCGATGCCAGC	pkrC pakC double-mutant construction
1359.1360up_F	ATTTTGGTACCCCTGACGCGCGTCTCATGG	pkrC pakC double-mutant construction
884dw_F	GGATGGATGATGACCAAAAGCCGTCAGGCCTAGGCG	pkrB mutant construction
884up R2	TTTGGTCATCATCCATCCTGG	pkrB mutant construction
884dw_R	ATTTGGTACCATCGAATATCGCCTGCGC	pkrB mutant construction
884 upF2	ATTTTCTAGAACTTCATGGAGCGTCTGTCG	pkrB mutant construction
884.885dw_F	GGTTTCTCGGCTCCAAGCCAGAACGACGGCTCAGAACGG	pkrB pakB double-mutant construction
884.885up_R	TGGCTTGGAGCCGAGAAACC	pkrB pakB double-mutant construction
884.885up_F	ATTTTGGTACCACGCGCTGCTCCGCCCCG	pkrB pakB double-mutant construction
884.885dw_R	ATTTTCTAGAAGCACGATCGCCGTGACC	pkrB pakB double-mutant construction
pENTR and expression plasmids		
pENTR4_linker_F	GCGGTAGCGGCAGCCATATGACCATGGGAACCAATTCAG	Construction of pENTR4b

Table S3. Cont.

Primer name	Primer sequence (5′ to 3′)	Purpose
pENTR4_linker_R	TACCGCTGCCGCTACCGGAGCCTGCTTTTTTGTACAAAG	Construction of pENTR4b
419FL_ENTR_F	ATTTGGTACCGAGTGCCGCACACGCCATTCG	pakA in pENTR4a
419_ENTR_R	ATTTTCTCGAGTCAGACCGGGGCCTCGATCG	pakA in pENTR4a; truncated pakA in pENTR4a (for purification)
885FL_ENTR_F	ATTTGGTACCGAATGACACCCGTCTGTGAAC	pakB in pENTR4a
885ENTR_XbaI_R	ATTTCTAGACTAGGCGCGGTTCAGCGTCG	pakB in pENTR4a
1359ENTR_F3	ATTTGGTACCGAGTGACCATCGCCGCCGTCC	pakC in pENTR4b
1359ENTR_R3	ATTTCTAGATGTCCCATGTCGTGTGCTG	pakC in pENTR4b
2513FL_ENTR_F	ATTTGGTACCGAATGGCGATTTCCCACCCG	pakE in pENTR4a
2513ENTR_R	ATTTTCTAGATTACTCTTCCGCAATCGGGGCG	pakE in pENTR4a; truncated pakE in pENTR4b (for purification)
1301FL_ENTR_F	ATTTGGTACCGACTGATCCTGACCCAGGTTC	pakF in pENTR4a
1301ENTR_R	ATTTTCTAGATTATGCCATCGCCGCGCGCAGC	pakF in pENTR4a
3817ENTR_F	ATTTGGTACCGAATGAACGATTCCGAGATGG	pakG in pENTR4a
3817ENTR_R	ATTTCTAGATGACGCCTAGTGGCTCGCC	pakG in pENTR4a
phyR_ENTR_F	ATTTTGGTACCGAATGTCGCTTGGACAGCAACTC	phyR in pENTR4a
phyR_ENTR_R	ATTTTCTCGAGTCACGCAACCGCCGTCGG	phyR in pENTR4a
BK51comp_F	ATTTTCTAGAAAGGGCTGGGGCATGTCTGC	sdrG complementation
BK51comp_R	ATTTGGTACCTTACAGCGCGGCGAGAGC	sdrG complementation
2886ENTR_F2	ATTTGGTACCGAATGTCTGCGCTCACCCAG	sdrG in pENTR4b
2886ENTR_R2	ATTTCTAGATTACAGCGCGGCGAGAGC	sdrG in pENTR4b
2886_pET_F	ATTTCATATGTCTGCGCTCACCCAGATC	sdrG in pET26bII
2886_pET_R	ATTTGGTACCAGCAGCGCGGCGAGAGCCTTGG	sdrG in pET26bII
2368ENTR_R	ATTTCTAGAATCACTCGCGCCACTCGTCG	Sphme2DRAFT_2368 in pENTR4a
2368ENTR_F2	ATTTGGTACCGAATGCAGAACTCTGTCCGG	Sphme2DRAFT_2368 in pENTR4a
3203ENTR_F	ATTTGGTACCGAGTGACGGACCGTCCGGGC	Sphme2DRAFT_3203 in pENTR4a
3203ENTR_R	ATTTCTAGAATCACTGGACGGCCCGTGG	Sphme2DRAFT_3203 in pENTR4a
419s_ENTR_F	ATTTTGGTACCGAACGGCGCGCAAATTCGCC	Truncated pakA in pENTR4a (for purification)
885s_ENTR_F	ATTTTGGTACCGACACGAACAGAAACTGGCGC	Truncated pakB in pENTR4a (for purification)
885_ENTR_R	ATTTTCTCGAGCTAGGCGCGGTTCAGCGTCG	Truncated pakB in pENTR4a (for purification)
2513ENTR_F	ATTTGGTACCGAGAGGTCAATCACCGCGTGC	Truncated pakE in pENTR4a (for purification)
Mutant alleles		
419H-A_F	CGCGAGCTGACGGCCCGGGTGAAGAAC	PakA(H305A)
419H-A_R	GTTCTTCACCCGGGCCGTCAGCTCGCG	PakA(H305A)
885H-A_F	CCAGGAGCTGTCAGCCCGGATCAAGAAC	PakB(H315A)
885H-A_R	GTTCTTGATCCGGGCTGACAGCTCCTGG	PakB(H315A)
1359H-A_F	GAATGAGGAACTGGCGGCTCGGATCAAGAACAC	PakC(H184A)
1359H-A_R	GTGTTCTTGATCCGAGCCGCCAGTTCCTCATTC	PakC(H184A)
3381H-A_F	CTCAATGGCGAATTGAGCGCTCGATTGAAAAACGTCTTG	PakD(H728A)
3381H-A_R	CAAGACGTTTTTCAATCGAGCGCTCAATTCGCCATTGAG	PakD(H728A)
2513H-A_F	GAAGGAGGTCAATGCCCGCGTGCAGAAC	PakE(H538A)
2513H-A_R	GTTCTGCACGCGGGCATTGACCTCCTTC	PakE(H538A)
1301H-A_F	CTGTCCGAAGTGAACGCCCGCGTCGCCAATTC	PakF(H162A)
1301H-A_R	GAATTGGCGACGCGGGCGTTCACTTCGGACAG	PakF(H162A)
3817H-A_F	GCGCGAAACCAATGCTCGGTGCAGCAAC	PakG(H33A)
3817H-A_R	GTTGCTGCACCGAGCATTGGTTTCGCGC	PakG(H33A)
2886(D56A)_R	CGCCGCGCAGGTTGACGGCGAGGATCGCAGCGTCG	SdrG(D56A)
2886(D56A)_F	CGACGCTGCGATCCTCGCCGTCAACCTGCGCGGCG	SdrG(D56A)
2886(D56E)_F	CGACGCTGCGATCCTCGAGGTCAACCTGCGCGGCG	SdrG(D56E)
2886(D56E)_R	CGCCGCGCAGGTTGACCTCGAGGATCGCAGCGTCG	SdrG(D56E)
Bacterial two-hybrid assay		
419BTH_F	ATTTTCTAGAGGTGCCGCACACGCCATTCG	pakA for BacTH
419BTH_R	ATTTTGGTACCGCGACCGGGGCCTCGATCGC	pakA for BacTH
885BTH_F	ATTTTCTAGAGATGACACCCGTCTGTGAAC	pakB for BacTH
885BTH_R	ATTTTGGTACCGCGGCGCGGTTCAGCGTCGC	pakB for BacTH
1359BTH_F	ATTTTCTAGAGGTGACCATCGCCGCCGTCC	pakC for BacTH
1359BTH_R	ATTTTGGTACCGCCGCCTCTTGAACCTGATAG	pakC for BacTH
3381BTH_F	ATTTTCTAGAGATGACTGATCTTGGAGCG	pakD for BacTH
3381BTH_R	ATTTTGGTACCGCCGCGGTTACCAATTCGCCTGC	pakD for BacTH
2513BTH_F	ATTTTCTAGAGATGGCGATTTCCCACCCG	pakE for BacTH
2513BTH_R	ATTTTGGTACCGCCTCTTCCGCAATCGGGGC	pakE for BacTH
1301BTH_F	ATTTTCTAGAGCTGATCCTGACCCAGGTTC	pakF for BacTH

Table S3. Cont.

Primer name	Primer sequence (5′ to 3′)	Purpose
1301BTH_R	ATTTTGGTACCGCTGCCATCGCCGCGCGCAGC	pakF for BacTH
3817BTH_F	ATTTTCTAGAGATGAACGATTCCGAGATGG	pakG for BacTH
3817BTH_R	ATTTTGGTACCGCGTGGCTCGCCATCGGCAC	pakG for BacTH
2368BTH_F2	ATTTTCTAGAGATGCAGAACTCTGTCCGG	Sphme2DRAFT_2368 for BacTH
2368BTH_R	ATTTTGGTACCGCCTCGCGCCACTCGTCGCG	Sphme2DRAFT_2368 for BacTH
Construction of transposons		
VanR_F	ATTTACATGTTTTCAGTCGGCGCGAATGC	
VanR_R	ATTTAAGCTTCACTCGAGGAATTAATCCTCCTCGCATCGTGGTTCG	
Pvan2_F	GTTTGATTACGATCTAACTGCATTGGATCCAATAG	
Pvan2_R	CTATTGGATCCAATGCAGTTAGATCGTAATCAAAC	
Pvan_R2	ATTTTAAGCTTCTCACTCGAGGAATTAATCGTTTCCTCGCATCGTGGTTCGG	
VanR_RBS2_s	CATGACCTCCTCTAGCCGACCGACTGAGACGCTCACAAGCGTTC	
VanR_RBS2_as	AATTGAACGCTTGTGAGCGTCTCAGTCGGTCGGCTAGAGGAGGT	
tnp_Oend_F	ATTTTATTAATGGGACTTGTGTATAAGAGTCAGATGATAACTTCTGCTCTTC	
tnp_R	ATTTTCGATCGAAAACTAGTCAGATCTTGATCCCCTGC	
CAT_F_SpeI	ATTTTACTAGTGCTGATGTCCGGCGGTGC	
CAT_R_IendPvuI	ATTTCGATCGAGATCTGATCAAGAGACAGAGCACCAGGCGTTTAAGG	
Himar1W119A_F	GCTCTGTGCGAAAGCGGTGCCGCGCGAGCTCAC	
Himar1W119A_R	GTGAGCTCGCGCGGCACCGCTTTCGCACAGAGC	
Himar1_F	ATTTATTAATGACTCTAGAGACCGGGGACTTATCAGCCAACCTGTTACTAG AATAGCAGGAGGAATTCACC	
Himar1_R	ATTTCGATCGATTACTAGTTATTATTCAACATAGTTCC	
CAT_himer1ITR_R	ATTTCGATCGCTCTAGAGACCGGGGACTTATCAGCCAACCTGTTACTAGAA GCACCAGGCGTTTAAGG	
PhyR cascade down selection and reporters fusions		
nepRp-CAS_F	ATTTTCTCGAGTTTTGCTCACATGTGATGG	
nepRp-CAS_R	ATTTTGGGCCCACGGCCAGTGAATTCTCTGG	
nepRpcore_as	GATCGGCGATGAAACCAAAGTCGCGTTCGTTGGTTCCATTGCAGC	
nepRpcore_s	AGCTGCTGCAATGGAACCAACGAACGCGACTTTGGTTTCATCGCC	
ecfG2p_F2	ATTTTAAGCTTACCGCGCGTTTGTTTCG	
ecfG2p_R2	ATTTTCTAGACGATCGAACCAGGCAGC	
rpsL88_RBS_F	ATTTGGATCCATATACGAGGAGGAGGCTTCATGC	
rpsL88_R2	ATTTGAATTCACGCGTTATCACTTCGGGC	
rpmB_R	ATTTGGTACCCTGAATTCGGTTGCCCGG	
rpmB_F	ATTTAAGCTTTGGCGTAACAACGCCGCG	
FLP_F	ATTTACGCGTTACTAAGGAGGTTGTATGC	
FLP_R	ATTTGAATTCTAAATGCGTACTTATATGC	
Arb-P1	GGCCACGCGTGCACTAGTCANNNNNNNNNNGCTCG	
Arb-P2	GGCCACGCGTGCACTAGTCANNNNNNNNNNGACTC	
Arb-P3	GGCCACGCGTGCACTAGTCANNNNNNNNNNGATAC	
Anchor-P	GGCCACGCGTGCACTAGTCA	
pAK411-nested1	CTTTATGCGCGGCATGTCC	
pAK411-nested2	GACTGAGACGCTCACAAGCG	

Table S4. Locus tags of genes described in this study

Name	Locus_tag (IMG annotation)
phyP	Sphme2DRAFT_1443
nepR	Sphme2DRAFT_1444
phyR	Sphme2DRAFT_1445
ecfG	Sphme2DRAFT_1447
sdrG	Sphme2DRAFT_3354
pakA	Sphme2DRAFT_2181
pakB	Sphme2DRAFT_0513
pakC	Sphme2DRAFT_2824
pakD	Sphme2DRAFT_3654
pakE	Sphme2DRAFT_2488
pakF	Sphme2DRAFT_1661
pakG	Sphme2DRAFT_3817
pkrB	Sphme2DRAFT_0512
pkrC	Sphme2DRAFT_2825
pkrD	Sphme2DRAFT_3653
pkrF	Sphme2DRAFT_1662

According to Integrated Microbial Genomes (IMG; img.jgi.doe.gov/cgi-bin/w/main.cgi) as of September 24, 2014.