SUPPLEMENTAL MATERIAL

A Markerless Gene Deletion System for Sphingomonads

Andreas Kaczmarczyk, Julia A. Vorholt, Anne Francez-Charlot*

Institute of Microbiology, ETH Zurich, 8093 Zurich, Switzerland

* Corresponding author. Anne Francez-Charlot, Institute of Microbiology, ETH Zurich, Wolfgang-Pauli-Strasse 10, 8093 Zurich, Switzerland. Phone: +41-44-632-3830. Fax: +41-44-632-1378. E-mail: fanne@micro.biol.ethz.ch.

Supplemental Methods:

- Cloning and plasmids
- Generation of gene deletions
- Plasmid delivery into sphingomonads

Supplemental Results:

- Sucrose and p-chloro-phenylalanine sensitivity of wild-type sphingomonads
- Sucrose susceptibility of strains carrying *sacB*

Supplemental Tables:

- Table S1. Sucrose and pCl-Phe sensitivity of wild-type sphingomonads

Supplemental Figures:

- Fig. S1. *rpsL* and *rpsL1* alignment
- Fig. S2. *ecfG*, *phyR*, *crtI* and *crtY* mutant phenotypes

SUPPLEMENTAL METHODS

1. Cloning and plasmids

All DNA manipulations were performed following standard molecular biology protocols (4). For polymerase chain reaction (PCR), Phusion polymerase in GC-buffer (Finnzymes) was used. Restriction enzymes were from Fermentas. T4 DNA ligase was from New England Biolabs.

Wild-type *rpsL* including 274 nucleotides upstream of the open reading frame (ORF) was amplified using primers oJVZ854 (5'-ATT TTG GTA CCA TGG GCG CGT TCT GCA CC-3') and oJVZ856 (5'-ATT TTG AAT TCG ACC GGC TTA CTT CGG ACG-3') and *Sphingomonas* sp. Fr1 genomic DNA as a template. The PCR product was digested with Acc65I and EcoRI and cloned into the same sites of pCM62, yielding pCM62-*rpsL*. The *rpsL*$_{R88K}$ allele was generated using a previously described PCR overlap extension technique (1) with primer pairs oJVZ853 (5'-GGG AAG ATC CTT CAC ACG GCC-3') and oJVZ854 (upstream region) and oJVZ852 (5'-GGC CGT GTG AAG GAT CTT CCC-3') and oJVZ856 (downstream region) and cloned via Acc65I and EcoRI sites into pCM62, yielding pCM62-*rpsL*$_{R88K}$. *sacB* including 492 nucleotides upstream of the *sacB* start codon was amplified from pK18*mobsacB* (5) using primers oJVZ1065 (5'-ATT TTG GAT CCA GCG GGA CTC TGG GGT TCG-3') and oJVZ1066 (5'-ATT TGA ATT CTC GCT CGG TAC CCA TCG GC-3') and cloned between the EcoRI and BamHI sites of pCM62, resulting in pCM62-*sacB*.

In a genetic screen for strong synthetic housekeeping promoters (A. Kaczmarczyk, unpublished data) we identified the sequence 5'-GGC CCA TGG TTG ACG GAT CGC CGC GAA GCC GCT AAC TGC GCC GGC AGA TC-3' (termed P_{syn2}) harboring putative -35 and -10 boxes (underlined) separated by 17 nucleotides, resembling the consensus sequence of *Escherichia coli* sigma70-dependent promoters (TTGACA-N_{17}-TATAAT). This sequence was cloned between the PscI and HindIII sites of pCM62 via oligonucleotide annealing of oligos oJVZ949 (5'-CAT GGG GCC CAT GGT TGA CGG ATC GCC GCG AAG CCG CTA ACY GCG CCG GCA GAT CTA-3') and oJVZ950 (5'-AGC TTA GAT CTG CCG GCG CRG TTA GCG GCT TCG CGG CGA TCC GTC AAC CAT GGG CCC-3'), generating pAK126a. *rpsL*$_{R88K}$ was amplified with primers oJVZ850 (5'-ATT TGG ATC CAT ATA CGA GGA GGA GGC TTC ATG C-3') and oJVZ856 from pCM62-*rpsL*$_{R88K}$, digested

with BamHI and EcoRI, and cloned between the BglII and EcoRI sites of pAK126a, yielding pAK126a-*rpsL*$_{R88K}$; oJVZ850 replaced the native ribosome binding site (RBS) with a stronger one (underlined). *rpsL1* was synthesized by GeneArt (Invitrogen), and PCR-amplified using primers oJVZ850 and oJVZ851 (5'-ATT TGA ATT C<u>AC GCG T</u>TA TCA CTT CGG GC-3'), digested with BamHI and EcoRI, and cloned between the BglII and EcoRI sites of pAK126a, resulting in pAK126a-*rpsL1*; oJVZ851 introduced an ApaI site downstream of *rpsL1* that was used for subsequent subcloning (underlined).

A fragment containing the *nptII* gene was amplified by PCR using primers oJVZ574 (5'-ATT TTG CTA GCA CGT AGA AAG CCA GTC CG-3') and oJVZ575 (5'-ATT TTA CCG GT<u>A CGC GT</u>T T<u>GG GCC C</u>TA GCG AAC CCC AGA GTC C-3') and pK18*mobsacB* as a template. The PCR product was digested with BglII and AgeI, and inserted between the SgrAI and BglII sites of pK18*mobsacB*. The resulting plasmid, pAK401, lacks the *sacB* gene of the original pK18*mobsacB* plasmid and harbors two unique restriction sites downstream of *nptII*, introduced by primer oJVZ575 (MluI and ApaI; underlined in the primer sequence). A fragment containing P_{syn2}, the strong RBS and the *rpsL1* ORF was excised from pAK126a-*rpsL1* by MluI and ApaI and the fragment was cloned into the same sites of pAK401, yielding pAK405. The full sequence of pAK405 is available in GenBank (accession no. JQ432562).

2. Generation of gene deletions

Upstream and downstream regions of approximately 750 bps flanking the respective gene were amplified, joint by PCR overlap extension, and cloned into the multiple cloning site of pAK405 (primer sequences are available upon request). Candidate plasmids were identified by blue/white screening on X-Gal and plasmid inserts were verified by sequencing. The suicide plasmid derivatives of pAK405 were introduced into *Sphingomonas* sp. Fr1 by electroporation or via conjugal transfer.

2.A. Electroporation

20-ml-cultures of *Sphingomonas* sp. Fr1 grown overnight in LB at 28°C, 220 rpm orbital shaking, were made electro-competent by washing 3 times with 25 ml of cold ddH_2O, followed by resuspension in 2 ml of 10% (vol/vol) glycerol. Aliquots of 50 ul were stored at -80°C. pAK405 derivatives (200-400 ng) were introduced into *Sphingomonas*

sp. Fr1 by electroporation (25 μF, 200 Ω, 2 kV) using a BIORAD GenePulser. Immediately after the pulse, cells were resuspended in 700 μl of LB and incubated at 28°C without shaking for 1-2 h. Cells were collected by centrifugation and resuspended in 100 μl of LB, and 50 μl were plated on LB containing 50 μg kanamycin/ml. After 2 days of incubation at 28°C, single colonies were restreaked on the same medium and incubated for 1 day at 28°C. Subsequently, a small loop of bacteria was dilution-restreaked on LB containing 100 μg streptomycin/ml to select for a second recombination event; alternatively, bacteria were resuspended in LB and 10-fold dilution series were plated on LB containing 100 μg streptomycin/ml. Resulting colonies were restreaked on both LB supplemented with 100 μg streptomycin/ml and LB containing 50 μg kanamycin/ml, and kanamycin-sensitive clones were analyzed further for wild-type or mutant genotypes.

2.B. Conjugal transfer

pAK405 derivatives were transformed into *E. coli* S17-1(λ*pir*) (6). 5 ml of *E. coli* donor culture was grown overnight at 37°C in LB supplemented with kanamycin (50 μg/ml). *Sphingomonas* sp. Fr1 was grown as described above. Cells were harvested by centrifugation, washed once in 10 mM $MgSO_4$ and resuspended in $MgSO_4$ at an optical density at 600 nm of 10. 50 μl of both suspensions were mixed, spotted onto minimal medium (3) plates without carbon source and incubated for 6-24 h at 28°C. Bacteria were scratched off the plates and resuspended in 200 μl of $MgSO_4$, and 50 μl aliquots were plated on LB supplemented with 50 μg kanamycin/ml (for selection of *Sphingomonas* sp. Fr1 merodiploids) and 50 μg carbenicillin/ml (for *E. coli* counterselection). Individual colonies were once restreaked on the same medium and then resuspended in LB and plated on LB supplemented with 100 μg streptomycin/ml and 50 μg carbenicillin/ml, or dilution-restreaked on the same medium, to select for a second recombination event. Resulting colonies were restreaked on both LB supplemented with 100 μg streptomycin/ml and LB containing 50 μg kanamycin/ml, and kanamycin-sensitive clones were analyzed further for wild-type or mutant genotypes.

3. Plasmid delivery into sphingomonads

All strains were grown on NB or LB at 28°C. For *Sphingomonas* sp. Fr1, *Sphingomonas* sp. C3, *Novosphingobium rosa*, *Sphingomonas mali*, *Sphingomonas aerolata*, and *Sphingomonas roseiflava*, replicative plasmids were delivered by electroporation according to the procedure described above. For *Sphingobium indicum*, *Sphingobium rhizovivinum*, and *Sphingomonas wittichii*, conjugal transfer as described above was used for plasmid delivery.

SUPPLEMENTAL RESULTS

1. Sucrose and p-chloro-phenylalanine sensitivity of wild-type sphingomonads

In order to test whether sucrose and p-chloro-phenylalanine (pCl-Phe) could be used in counterselection with their respective markers (*sacB* and *pheS*), we first analyzed susceptibility of the wild-type strain without markers to those substances. To this end, single bacterial colonies were restreaked on NB containing 5 or 10% (wt/vol) sucrose, or 1 or 0.1 mM pCl-Phe, and growth of bacteria was assessed. As illustrated in Table S1, wheareas most species could tolerate 5 and 10% sucrose, the majority of them was susceptible to 0.1 mM pCl-Phe, a concentration ten times lower than the pCl-Phe concentration usually used for counterselection in *E. coli* (2). This indicates that *pheS* cannot be used as a counterselection marker for most sphingomonads.

2. Sucrose susceptibility of strains carrying *sacB*

To test the efficiency of the *sacB* counterselectable markers, a single colony of *Sphingomonas* sp. Fr1, *Sphingomonas wittichii* or *Novosphingobium rosa* harboring pCM62-*sacB* was restreaked on NB plates containing tetracycline (10 μg/ml) and sucrose (5% [wt/vol] for *Sphingomonas wittichii*, 10% [wt/vol] for *Sphingomonas* sp. Fr1 and *Novosphingobium rosa*). After 2 to 3 days of incubation, a substantial number of colonies appeared for all strains tested, indicating that *sacB* is a leaky counterselectable marker for those strains. In contrast, as shown in Fig. 2B of the main text, no colonies appeared for *Sphingomonas* sp. Fr1 carrying the *rpsL1* counterselection marker at concentrations of streptomycin as little as 1 μg/ml. To quantitatively assess the

frequency of spontaneous sucrose resistance, cultures of strains carrying pCM62-*sacB* were grown in NB supplemented with tetracycline (10 μg/ml) and 10-fold serial dilutions were plated on NB supplemented with tetracycline (10 μg/ml) with or without sucrose (5 or 10% [wt/vol], see above). The frequency of spontaneous sucrose resistance (i.e. the ratio of the number of colonies on plates with and without sucrose) was 1.6×10^{-4} for *Sphingomonas* sp. Fr1, 3.3×10^{-4} for *Sphingomonas wittichii* and 2.1×10^{-5} for *Novosphingobium rosa*. These frequencies of spontaneous sucrose resistance are in the upper range of the frequency we typically observe for the second homologous recombination event in *Sphingomonas* sp. Fr1 (5×10^{-6} to 10^{-3}), indicating that spontaneous sucrose resistance is a major concern when using *sacB* as a counterselection marker in several sphingomonads.

Table S1. Sucrose and pCl-Phe sensitivity of wild-type sphingomonads

Strain	**Sucrose 5%**	**Sucrose 10%**	**pCl-Phe (0.1 mM)**
E. coli TOP10 (control)	+	ND	+
Sphingomonas sp. Fr1 DSM 24958	+	+	-
Sphingomonas sp. C3 DSM 24957	+	+	-
Novosphingobium rosa DSM 7285	+	+	+
Sphingomonas mali DSM 10565	+	-	-
Sphingomonas aerolata DSM 14746	+	+	-
Sphingobium indicum DSM 16412	+/-	-	-
Sphingobium rhizovicinum DSM 19845	+	+	+
Sphingomonas roseiflava CIP 106847	+	+	-
Sphingomonas wittichii DSM 6014	+	+/-	-

After incubation for 2-5 days: +, growth; -, no growth; +/-, weak growth; ND, not determined

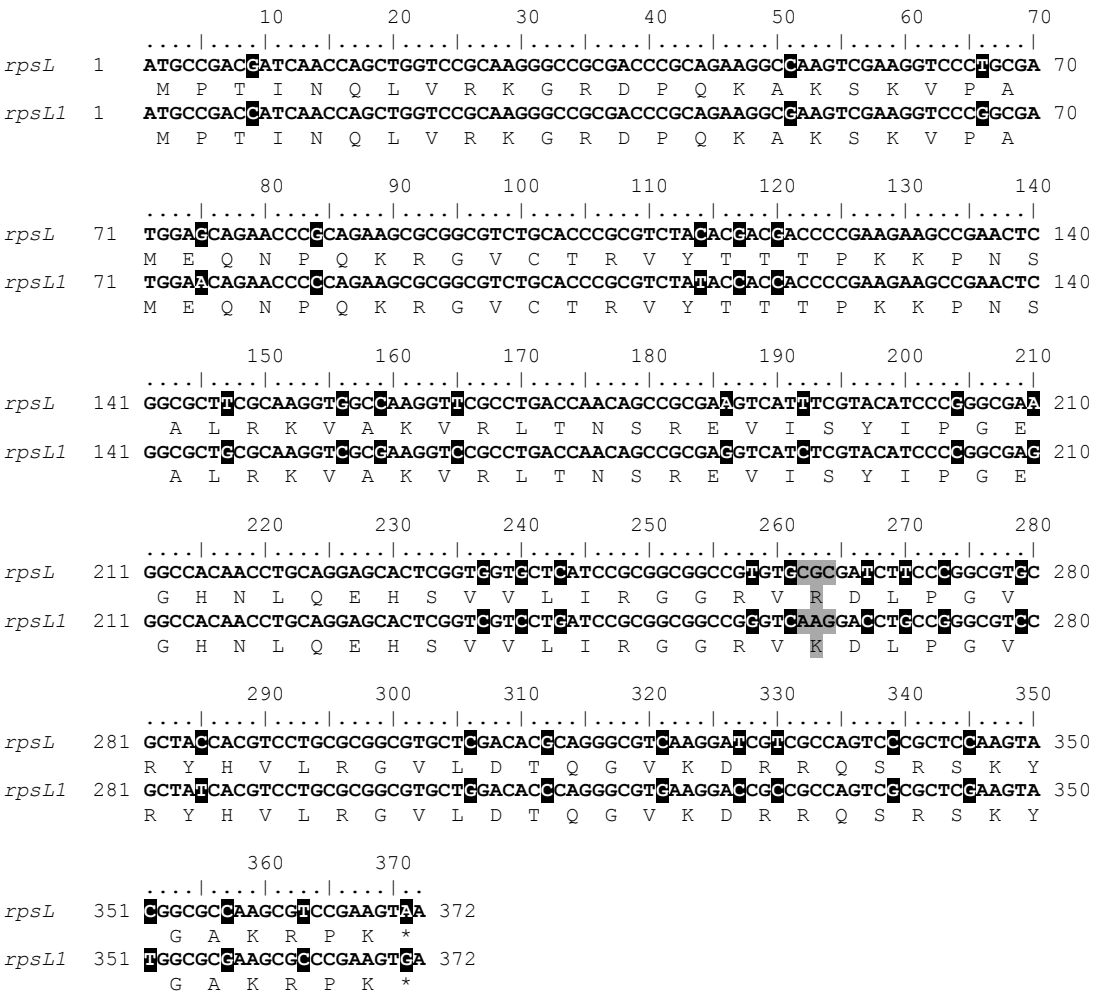

Fig. S1. Alignment of the *Sphingomonas* sp. Fr1 wild-type *rpsL* allele and the synthetic *rpsL1* allele carrying synonymous codons and encoding the Arg-88-Lys substitution (highlighted in gray). Substituted nucleotides in synonymous codons of *rpsL1* are highlighted in black.

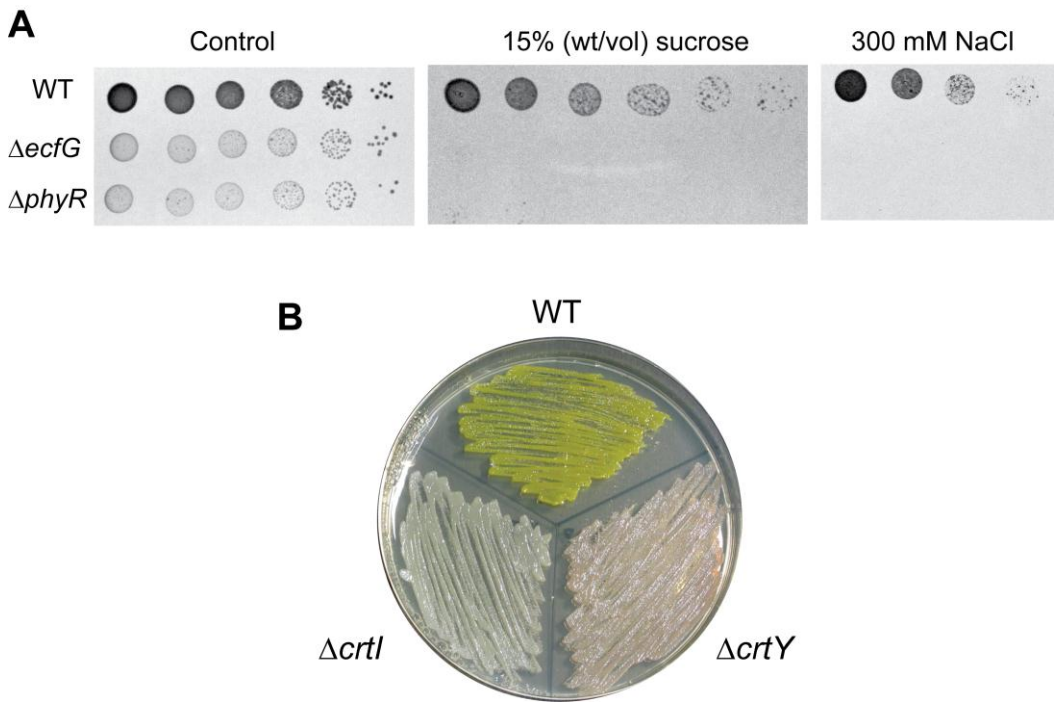

Fig. S2. (A) Osmotic stress phenotypes of *Sphingomonas* sp. Fr1 Δ*phyR* and Δ*ecfG*. Bacteria were grown and plated on NB containing 15% (wt/vol) sucrose or 300 mM NaCl as described before (1). (B) *Sphingomonas* sp. Fr1 Δ*crtI* and Δ*crtY* show defects in carotenoid biosynthesis.

1. **Kaczmarczyk A, Campagne S, Danza F, Metzger LC, Vorholt JA, and Francez-Charlot A.** 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:**6629-38.
2. **Kast P.** 1994. pKSS--a second-generation general purpose cloning vector for efficient positive selection of recombinant clones. Gene **138:**109-14.
3. **Peyraud R, Kiefer P, Christen P, Massou S, Portais JC, and Vorholt JA.** 2009. Demonstration of the ethylmalonyl-CoA pathway by using 13C metabolomics. Proc Natl Acad Sci U S A **106:**4846-51.
4. **Sambrook J, and Russel D.** 2001. Molecular Cloning: A Laboratory Manual, third ed. Cold Spring Harbor Laboratory Press.
5. **Schäfer A, Tauch A, Jäger W, Kalinowski J, Thierbach G, and Pühler A.** 1994. Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. Gene **145:**69-73.
6. **Simon R, Priefer U, and Pühler A.** 1983. A broad-host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology **1:**784-791.