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Markerless Gene Deletion System for Sphingomonads

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Here, we suggest that natural streptomycin resistance of many sphingomonads resides within *rpsL*. We constructed a dominant, streptomycin-sensitive *rpsL* allele and demonstrated its use as a counterselection marker in several sphingomonads. An *rpsL*-based markerless gene deletion system was developed and validated by deleting four genes in *Sphingomonas* sp. strain Fr1.

Bacteria of the genus *Sphingomonas* and the closely related genera *Novosphingobium*, *Sphingopyxis*, *and Sphingobium* (commonly referred to as sphingomonads) (1, 29) are well known for their potential in bioremediation and use in industrial applications (2–4, 16, 18, 22) and have recently also been described as potential agents for biocontrol (7). Genetic studies of sphingomonads have suffered from a limited number of genetic tools, e.g., the lack of a markerless gene deletion and allelic exchange system. Such a system is advantageous over existing methods for insertional gene inactivation or gene replacement in that it avoids polar effects on the expression of downstream genes and allows recycling of the antibiotic resistance marker. Markerless gene deletion systems follow a two-step homologous recombination strategy that involves successive selection and counterselection (28). Whereas selection markers are usually antibiotic resistance cassettes, most counterselectable markers render the host sensitive to a specific substance, such as sucrose (*sacB*) or *p*-chlorophenylalanine (*pheS*) (9, 10, 23). However, several wild-type sphingomonads are impaired in growth in the presence of p-chlorophenylalanine (see Table S1 in the supplemental material), making *pheS* an unsuitable counterselection marker. Moreover, although *sacB* has been reported as a counterselection marker in *Sphingomonas* sp. strain SYK-6 (15), we failed to reproducibly obtain mutants using existing *sacB*-based markerless gene deletion systems (13, 25) in *Sphingomonas* sp. strain Fr1 (data not shown), probably because of the high frequency of spontaneous sucrose resistance (see Results in the supplemental material), a common drawback of *sacB*-based counterselection systems (20, 25, 32). Thus, a generally applicable counterselectable marker is needed for sphingomonads.

Another commonly used counterselection marker is based on *rpsL*, which encodes the ribosomal protein S12, a target of streptomycin (19). In the classical approach based on *rpsL*, naturally streptomycin-sensitive bacteria are first selected for spontaneous streptomycin-resistant mutants with mutations usually found within *rpsL* and the dominant, streptomycin-sensitive wild-type *rpsL* allele (12) is subsequently used in the streptomycin-resistant background as a counterselectable marker (23, 24). One disadvantage of this method is that it requires prior manipulation of the wild type to make it streptomycin resistant. Most sphingomonads are naturally streptomycin resistant (31), and we therefore questioned (i) whether this property resided within the *rpsL* gene and, if so, (ii) whether it could be exploited to construct a dominant, streptomycin-sensitive allele for use as a counterselectable marker in wild-type sphingomonads. A comparison of *rpsL* alleles of several sphingomonads revealed that all encoded arginine at position 88 (Fig. 1), which is precisely the amino acid known to render naturally streptomycin-sensitive *rpsL* alleles resistant when replacing the original Lys-88 residue in diverse species (6, 17, 30). To test whether this residue was responsible for resistance, Arg-88,

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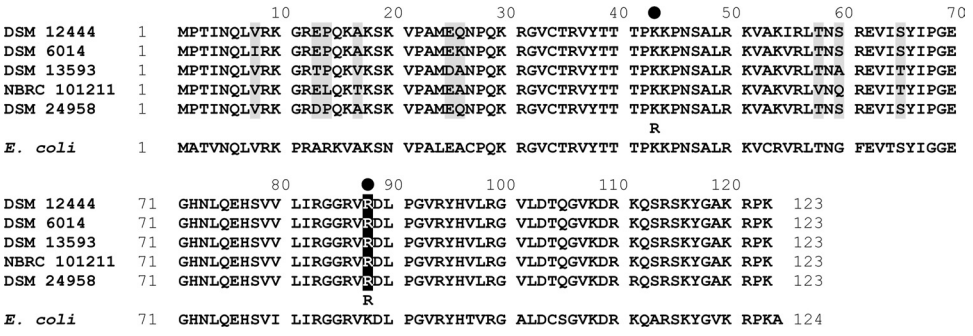

FIG 1 Alignment of wild-type ribosomal protein S12 sequences of selected sphingomonads and *E. coli*. DSM 12444, *Novosphingobium aromaticivorans* F199; DSM 6014, *Sphingomonas wittichii* RW1; DSM 13593, *Sphingopyxis alaskensis* RB2256; NBRC 101211, *Sphingobium japonicum* UT26; DSM 24958, *Sphingomonas* sp. Fr1; *E. coli*, *Escherichia coli* K-12. Amino acids that differ among the selected sphingomonads are highlighted in gray. Arg-88 is highlighted in black. Amino acid substitutions known to render naturally streptomycin-sensitive S12 proteins resistant are shown above the *E. coli* S12 sequence and are highlighted by a black dot on top.

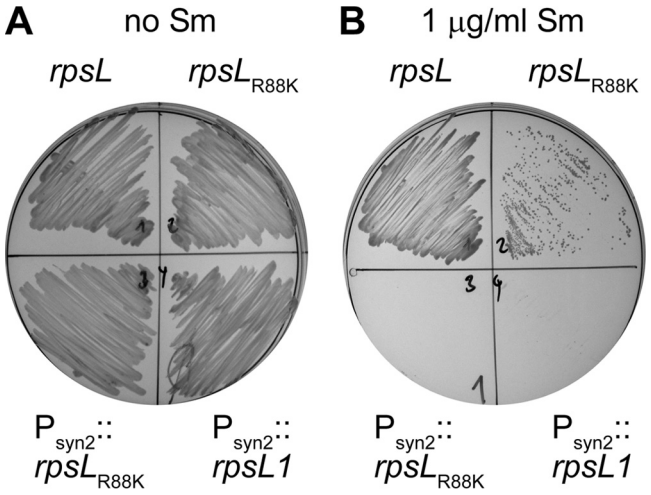

FIG 2 Growth of *Sphingomonas* sp. Fr1 expressing different *rpsL* alleles on nutrient broth without (A) or with (B) 1 μg streptomycin/ml. *rpsL*, wild-type *rpsL* expressed from pCM62 under the control of its native promoter; $rpsL_{R88K}$, $rpsL_{R88K}$ allele expressed from pCM62 under the control of its native promoter; P_{syn2}::$rpsL_{R88K}$, $rpsL_{R88K}$ with an improved ribosome-binding site expressed from pAK126a under the control of the strong synthetic promoter P_{syn2}; P_{syn2}::*rpsL1*, *rpsL1* allele with an improved ribosome-binding site expressed from pAK126a under the control of the strong synthetic promoter P_{syn2}.

encoded by *Sphingomonas* sp. Fr1 *rpsL*, was replaced by lysine, and the mutant allele, $rpsL_{R88K}$, was cloned in a multicopy plasmid, pCM62 (14), under the control of its native promoter. Whereas expression of wild-type *rpsL* from pCM62 did not affect growth on Luria broth (LB; Lennox) supplemented with 200 μg streptomycin/ml, expression of $rpsL_{R88K}$ from pCM62 abolished growth of *Sphingomonas* sp. Fr1 at a streptomycin concentration of 20 μg/ml or higher but did not impair growth on LB without streptomycin. Given that streptomycin-sensitive *rpsL* alleles are dominant over streptomycin-resistant *rpsL* alleles (12), our results suggest that Arg-88 in wild-type *rpsL* confers natural streptomycin resistance to *Sphingomonas* sp. Fr1 and show that the $rpsL_{R88K}$ allele is a suitable counterselection marker for the *Sphingomonas* sp. Fr1 wild-type strain.

In order to use the $rpsL_{R88K}$ allele in a markerless gene deletion system, homologous recombination between plasmid-borne and chromosomally encoded *rpsL* alleles should be avoided. To this end, an $rpsL_{R88K}$ allele in which several codons were replaced by synonymous ones, so that no more than 41 successive nucleotides were identical to the wild-type sequence while maintaining the original GC bias, was synthesized (GeneArt; Invitrogen) (see Fig. S1 in the supplemental material). This allele, *rpsL1*, together with an optimized ribosome-binding site, was then cloned under the control of a strong synthetic promoter in pAK126a, a pCM62 derivative, to yield plasmid pAK126a-*rpsL1*. As a control, $rpsL_{R88K}$ was cloned in the same plasmid, resulting in pAK126a-$rpsL_{R88K}$. *Sphingomonas* sp. Fr1 strains carrying pAK126a-*rpsL1* or pAK126a-$rpsL_{R88K}$ were sensitive to as little as 1 μg streptomycin/ml, indicating expression and functionality of *rpsL1* (Fig. 2). To construct a plasmid suitable for generating gene deletions, a fragment containing the synthetic promoter and *rpsL1* was subcloned into a derivative of pK18*mobsacB* (25) lacking the *sacB* gene, yielding pAK405 (Fig. 3A). Cloning details are given in the supplemental material.

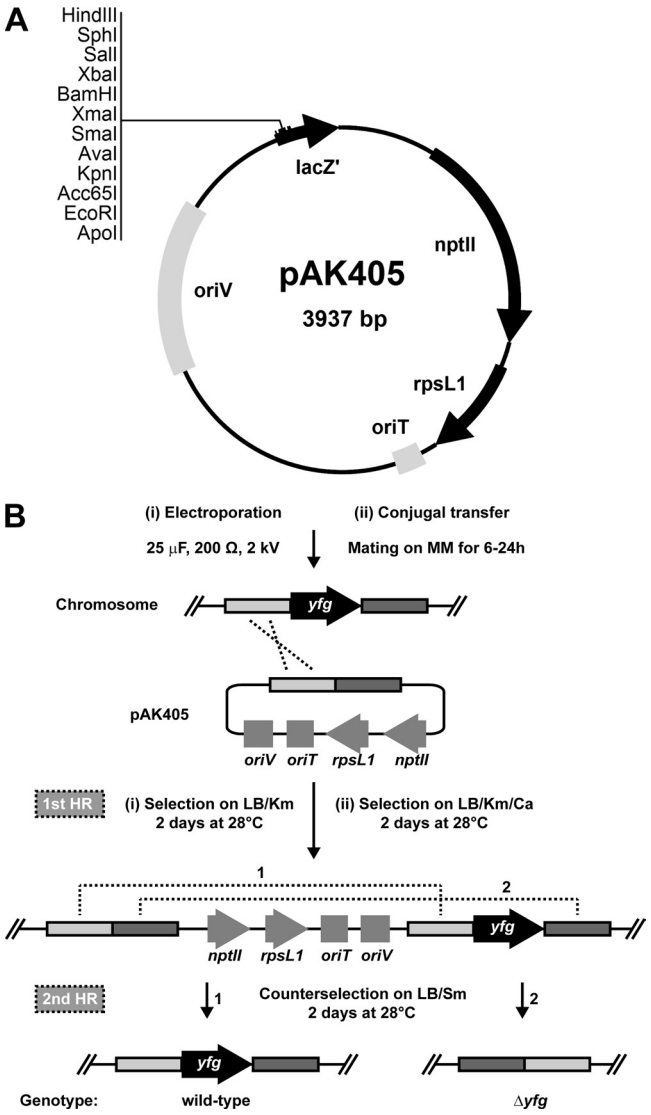

FIG 3 pAK405 plasmid map and schematic of the gene deletion strategy. (A) Plasmid pAK405 has the following features: a pBR322 *oriV* (suicide plasmid for *Sphingomonas*), the RP4 *oriT* (for conjugal transfer between *E. coli* and *Sphingomonas*), the pUC18 multiple cloning site in *lacZ'* (allowing blue/white screening), *nptII* (for selection on kanamycin), and *rpsL1* (for streptomycin counterselection). Unique restriction sites in the multiple cloning site are shown. (B) Outline of the gene deletion strategy with delivery of pAK405 through electroporation (i) or conjugal transfer (ii). The gene of interest (*yfg*, "your favorite gene") to be deleted is shown as a black arrow, and up- and downstream regions are depicted in light and dark gray boxes, respectively. Homologous recombination (HR) events are represented by dashed lines. For simplicity, only one of two possibilities for the first HR event is shown (HR via the upstream region). For the second HR event, the two possibilities leading to the wild-type (1) and mutant (2) genotypes are depicted. Antibiotics are indicated as follows: Km, 50 μg kanamycin/ml; Ca, 50 μg carbenicillin/ml; Sm, 100 μg streptomycin/ml.

To test the pAK405-based gene deletion system, four genes—*ecfG*, *phyR*, *crtI*, and *crtY*—with easily traceable mutant phenotypes (see below) were deleted in *Sphingomonas* sp. Fr1 following the general methodology outlined in Fig. 3B (see the supplemental material for the detailed methods). Briefly, upstream and downstream regions of approximately 750 bp that flanked each gene were PCR amplified, joined by PCR overlap extension, and cloned

into pAK405. pAK405 derivatives were subsequently transformed into *Sphingomonas* sp. Fr1 by electroporation or delivered via conjugal transfer from *Escherichia coli* S17-1(λ*pir*) (27). For conjugal transfer, mating was performed on minimal medium (MM) without a carbon source (21) and bacteria were subsequently 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 restreaked once on the same medium and then plated on LB supplemented with 100 μg streptomycin/ml to select for the second homologous 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 by colony PCR using primers flanking the respective loci. Of 200 colonies tested, 8.5% (17/200) of streptomycin-resistant clones were kanamycin resistant, indicating that spontaneous streptomycin resistance is a minor concern in the counterselection step. Overall, 62.7% (94/150), 64.3% (30/84), 30.6% (22/72), and 42.7% (50/117) of kanamycin-sensitive, streptomycin-resistant clones carried the *ecfG*, *phyR*, *crtI*, and *crtY* deletions, respectively, while the remaining had reverted to the wild type. These values are close to the theoretical 1:1 ratio of the wild type to the mutant genotype and thus prove the efficiency of the system.

All mutants displayed the expected phenotypes. *phyR* and *ecfG* encode the response regulator PhyR and the alternative sigma factor σ^{EcfG}, respectively, two master regulators of the general stress response in *Alphaproteobacteria* (5, 8), and the mutants showed the osmotic stress-sensitive phenotypes (see Fig. S2A in the supplemental material) described previously (8). *crtY* encodes a putative lycopene cyclase and *crtI* encodes a putative phytoene desaturase/dehydrogenase that are predicted to be involved in carotenoid biosynthesis (26). The *crtI* mutant was white, which is in line with the predicted role of phytoene desaturase/dehydrogenase in converting colorless phytoene into lycopene (see Fig. S2B in the supplemental material). The *crtY* mutant was reddish, which is also consistent with a defect in carotenoid biosynthesis and the accumulation of the red intermediate lycopene due to the lack of lycopene cyclase (see Fig. S2B in the supplemental material).

The above-described results show that *rpsL1* is an efficient tool for counterselection and targeted gene deletion in *Sphingomonas* sp. Fr1. To evaluate whether *rpsL1* could also be used for other sphingomonads, *rpsL1* was expressed from pAK126a in several phylogenetically diverse representatives of this class and the resulting strains were assessed for their capacity to grow on streptomycin. pAK126a-*rpsL1* carries a tetracycline resistance marker and thus could be selected for and maintained in all tested sphingomonads due to their sensitivity to tetracycline (Table 1). Whereas wild-type strains grew at streptomycin concentrations of 50 μg/ml or higher, expression of *rpsL1* rendered all tested species streptomycin sensitive (Table 1). In addition, most strains were naturally sensitive to kanamycin and resistant to carbenicillin (Table 1), indicating that pAK405 and the gene deletion strategy via conjugal transfer as outlined above can also be used for these organisms. Finally, pAK405 or the general strategy of generating streptomycin-sensitive *rpsL* alleles as described here might also be applicable to closely related bacteria not belonging to the sphingomonads, such as *Erythrobacter*, *Citromicrobium*, or *Zymomonas* species, which are naturally streptomycin resistant and whose *rpsL* alleles encode Arg-88 (11, 33, 34).

TABLE 1 Antibiotic susceptibility of selected sphingomonads and effect of *rpsL1*

Strain	Growth with each antibiotic[a]				
	Wild type				+ pAK126a-*rpsL1*
	Tc10	Km50	Ca50	Sm200	Sm50 and Tc10[b]
Sphingomonas sp. Fr1 (DSM 24958)	−	−	+	+	−
Sphingomonas sp. C3 (DSM 24957)	−	−	+	+	−
Novosphingobium rosa (DSM 7285)	−	−	+	+	−
Sphingomonas mali (DSM 10565)	−	−	+	+	−
Sphingomonas aerolata (DSM 14746)	−	−	−	+[c]	−
Sphingobium indicum (DSM 16412)	−	−	+	+[c]	−
Sphingobium rhizovicinum (DSM 19845)	−	−	+	+	−
Sphingomonas roseiflava (CIP 106847)	−	−	+	+	−
Sphingomonas wittichii (DSM 6014)	−	−	+	+	−

[a] Growth of wild-type strains (wild type) or otherwise-wild-type strains expressing *rpsL1* from pAK126a (+ pAK126a-*rpsL1*) was assessed on nutrient broth (NB) supplemented with different antibiotics. Tc10, 10 μg tetracycline/ml; Km50, 50 μg kanamycin/ml; Ca50, 50 μg carbenicillin/ml; Sm200, 200 μg streptomycin/ml. +, growth after 2 days of incubation; −, no growth after 5 days of incubation.
[b] A total of 10 μg tetracycline/ml was added for maintenance of pAK126a-*rpsL1*.
[c] Growth was observed at a concentration of 50 μg streptomycin/ml but not at 200 μg streptomycin/ml.

In conclusion, we showed that a mutant *rpsL* allele encoding the R88K substitution (*rpsL1*) behaves as a dominant, streptomycin-sensitive allele in the wild-type background of several sphingomonads, suggesting that Arg-88, encoded by the endogenous *rpsL* allele, confers natural streptomycin resistance to these species. A plasmid based on *rpsL1*, pAK405, that can be used for markerless gene deletions and allelic exchange in *Sphingomonas* sp. Fr1 and most likely in a number of other, naturally streptomycin-resistant sphingomonads was constructed. The simple and efficient method presented in this work expands the genetic toolbox for sphingomonads.

Nucleotide sequence accession number. The sequence of pAK405 is available in GenBank (http://www.ncbi.nlm.nih.gov/) under accession number JQ432562.

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