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Development of a Method for Markerless Gene Deletion in *Pseudomonas putida*▽†

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We developed a negative counterselection system for *Pseudomonas putida* based on uracil phosphoribosyltransferase (UPRTase) and sensitivity against the antimetabolite 5-fluorouracil (5-FU). We constructed a *P. putida* strain that is resistant to 5-FU and constructed vectors for the deletion of the surface adhesion protein gene, the flagellum biosynthesis operon, and two endonuclease genes. The genes were efficiently disrupted and left a markerless chromosomal in-frame deletion.

Since the genome of the rod-shaped Gram-negative bacterium *Pseudomonas putida* KT2440 has been completely sequenced (GenBank accession number AE015451) (10), the study of gene functions is more and more focused. Usually genes are simply replaced by antibiotic resistance markers (15) to see what phenotypic effects result. The generation of a strain with multiple deletions, however, cannot be achieved with this method, because the availability of resistance markers is limited. Therefore, other gene disruption methods based on homologous recombination have been developed. By flanking the resistance cassette with recognition sites for site-specific recombinases, such as Flp/Flp recombination target (FRT) and Cre/*loxP* (5, 8), the cassette can precisely be removed and used again for the next deletion step. However, scars are left in the chromosome after each deletion step in the form of FRT or *loxP* sites, respectively. This may cause severe problems because the recognition sites in the chromosome can become recombined, which would then lead to large chromosomal deletions or inversions. Another disadvantage is the elaborate selection for positive clones that have dropped out the resistance cassette. To solve this problem, counterselectable marker systems have been developed. The most powerful are the fusaric acid (*tetAR*), streptomycin (*rpsL*), and sucrose (*sacB*) sensitivity systems (13). Recently, a novel counterselection system has been approved first for *Bacillus subtilis* (3) and subsequently for several other microorganisms (4, 6, 7). This system is based on *upp*, encoding the uracil phosphoribosyltransferase (UPRTase). This enzyme (EC 2.4.2.9) belongs to the pyrimidine salvage pathway and creates UMP from uracil and phosphoribosylpyrophosphate (11). The toxic antimetabolite 5-fluorouracil (5-FU) also becomes converted by UPRTase into 5-fluoro-UMP. After metabolization into 5-fluoro-dUMP, it acts as a suicide inhibitor of the thymidylate synthase, subsequently resulting in cell death. Therefore, microorganisms with UPRTase activities are sensitive to 5-FU. In *P. putida* KT2440, an *upp* gene (PP_0746) has been identified as well (10).

In this study, we describe a gene deletion procedure based on the *upp* gene as a new counterselectable marker in *P. putida*. We deleted the *upp* gene to get a 5-FU-resistant strain. With that strain, we were able to delete further genes efficiently with regard to creating a strain applicable in industrial production.

Plasmids, bacterial strains, and growth conditions. The bacterial strains and plasmids used in this study are shown in Table 1. *P. putida* strains and *Escherichia coli* JM109 (16) were grown at 30°C in LB medium (2), supplemented with kanamycin (Kan; 50 μg ml^{-1}) (LB_{Kan}) or 5-FU (20 μg ml^{-1}). 5-FU was purchased from Sigma-Aldrich Corporation (Taufkrichen, Germany) and prepared as a stock solution of 100 mg ml^{-1} in dimethyl sulfoxide (DMSO). *P. putida* strains were also grown in M9 minimal medium (48 mM $Na_2HPO_4 \cdot 7H_2O$, 22 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl), supplemented with 0.2% glucose, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 6 μM thiamine hydrochloride.

Plasmid and strain construction for *upp*-based counterselection. Standard recombinant DNA techniques and transformation methods were used as described by Sambrook et al. (14). The oligonucleotide primers used in this study are shown in Table S1 in the supplemental material. The usage of the *upp* counterselection system (Fig. 1) for *P. putida* requires a strain resistant to 5-FU. The MIC was determined as 10 μg ml^{-1} 5-FU for *P. putida* KT2440, but 20 μg ml^{-1} was chosen for selection. That reduced the appearance of false-positive clones without affecting the growth rate. The plasmid used to delete the *upp* gene in *P. putida* KT2440 was constructed by starting with plasmid pIC20HE (1). That vector was cut with BamHI/ClaI, and the kanamycin resistance gene (*neo*), which was cut out from the plasmid pNEO (GenBank accession no. U13862), was inserted by ligation, resulting in pJOE6186.1.

For chromosomal deletion of *upp*, the up- and downstream regions were PCR amplified, using oligonucleotides s5380/s5381 and s5382/s5383, respectively. Chromosomal DNA from *P. putida* KT2440 was used as a template. The fragments, which included homologous regions of 792 bp and 883 bp, respectively, were cut with BamHI and HindIII. Integration occurred via 3-fragment ligation into BamHI-cut pJOE6168.1, creating pJOE6227.1.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or relevant characteristics	Reference or source
Strains		
E. coli JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	16
P. putida		
KT2440	Wild type	ATCC 47054
ΔUPP4	Δ*upp*	This study
GN24	Δ*upp* ΔPP_0168	This study
GN109	Δ*upp* ΔPP_0168 ΔPP_2451	This study
GN112	Δ*upp* ΔPP_0168 Δ PP_4333-4396	This study
GN125	Δ*upp* ΔPP_0168 ΔPP_2451 ΔPP_3375	This study
Plasmids		
pIC20HE	Cloning vector for blue-white screening	1
pNEO	Cloning vector with kanamycin resistance gene	GenBank accession no. U13862
pJOE6186.1	pIC20HE with a kanamycin resistance gene cloned into BamHI/ClaI sites	This study
pJOE6227.1	pJOE6186.1 with the up- and downstream regions of *upp* cloned into BamHI site	This study
pJOE6261.2	pJOE6186.1 with a copy of *upp* from *P. putida* KT2440 cloned into NdeI/NheI sites	This study
pJOE6348.1	pJOE6261.2 with the up- and downstream regions of PP_0168 cloned into BamHI site	This study
pNG90.7	pJOE6261.2 with the upstream region of PP_4333 and the downstream region of PP_4396 cloned into BamHI site	This study
pNG100.1	pJOE6261.2 with the up- and downstream regions of PP_3375 cloned into BamHI site	This study
pNG105.2	pJOE6261.2 with the up- and downstream regions of PP_2451 cloned into SalI site	This study

P. putida KT2440 was transformed with pJOE6227.1 by electroporation. Since the plasmid cannot be autonomously replicated, it has to integrate via single crossover into the chromosome. Selection occurred on LB_{Kan}. Positive clones should be Kan^r and 5-FU^s. Therefore, clones were checked on M9 minimal plates containing 20 μg ml^{-1} 5-FU and 0.2% glucose ($M9_{Glc + 5\text{-}FU}$). One of the Kan^r 5-FU^s clones was incubated in LB for 24 h at 30°C. During this time the plasmid should have been excised again through homologous recombination and been lost. By this means, either the original genotype would be reestablished or the *upp* gene would be lost. Afterwards, different dilutions (10^{-3} and 10^{-5}) were plated on $M9_{Glc + 5\text{-}FU}$. After incubation for 48 h at 30°C, up to 250 CFU was gained. Fifty clones thereof were checked on LB_{Kan} and on $M9_{Glc + 5\text{-}FU}$. Of the clones, which were 5-FU^r and Kan^s, 3 were checked by colony PCR: cells were picked from M9 minimal plates and

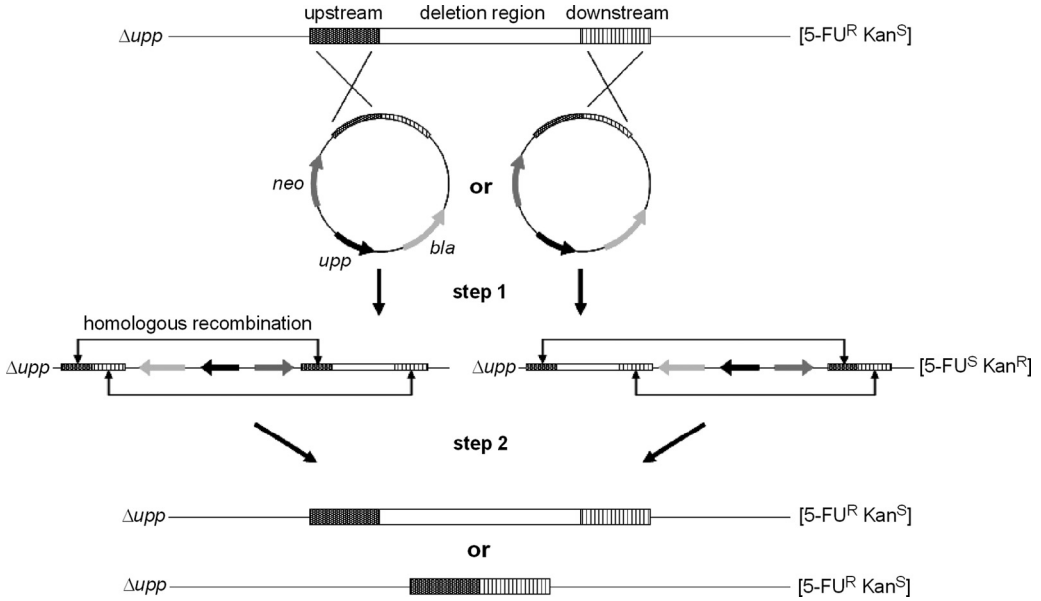

FIG. 1. Schematic presentation of the *upp* counterselection system for *P. putida*. The integration vectors for deletion of a chromosomal region (white box) contain the up- and downstream regions (each about 1 kb; shaded boxes), kanamycin and ampicillin resistance genes (*neo* and *bla*), and the *upp* gene. In the first step, integration occurs via a single-crossover recombination event in either the up- or the downstream region. The resulting strain is sensitive to 5-FU and resistant to kanamycin. In the second step, another recombination event takes place, in which either the original state is restored or the region of interest is deleted. These strains are now resistant to 5-FU and sensitive to kanamycin. When other genes are inserted between the up- and downstream regions of the integration vectors, this method can also be used for gene integration or gene replacement in the *P. putida* chromosome.

TABLE 2. Overview of the strain constructions

Parameter	Deleted gene/region			
	PP_0168 (*lapA*)	PP_4333-4396 (flagellum biosynthesis)	PP_2451 (*endA-1*)	PP_3375 (*endA-2*)
Region length (bp)	26,049	64,722	693	966
Upstream primers (length of homology, bp)	s5278/s5279 (976)	s5840/s5841 (944)	s5943/s5944 (864)	s5947/s5948 (950)
Downstream primers (length of homology, bp)	s5280/s5281 (1,031)	s5842/s5843 (1,047)	s5945/s5946 (1,051)	s5949/s5950 (1,052)
Restriction sites by which cloned	BamHI/HindIII	BamHI/EcoRI	SalI/HindIII	BamHI/HindIII
Integration vector	pJOE6348.1	pNG90.7	pNG105.2	pNG100.1
Target strain	ΔUPP4	GN24	GN24	GN109
Colony PCR primers	s5761/s5762, s5764/s5762	s6257/s6258, s6368/s6258	s6261/s6262, s6370/s6262	s6263/s6264, s6371/s6264
Deletion ratio (%)	40	20	10	30
Resulting strain	GN24	GN112	GN109	GN125

resuspended in 100 μl of double-distilled water. After treatment for 10 min at 99°C, cells were pelletized (5 min, 16.1 g, room temperature). From the supernatant, 10 μl was used as template for the PCR, using s5381/s5383 as primers. The resulting positive strain was designated ΔUPP4. The counterselection system can be used only in *P. putida*, if 5-FU sensitivity can be restored by reintroducing the *upp* gene. Therefore, *upp* was PCR amplified using oligonucleotides s5378/s5379 and chromosomal DNA from *P. putida* KT2440 as template. The fragment was integrated between the restriction sites NdeI and NheI of vector pJOE6168.1, creating pJOE6261.2. In the BamHI restriction site of that vector, the flanking regions of target genes can now be easily integrated.

Deletion of selected chromosomal genes. To demonstrate the potential of the new developed *upp* counterselection system for *P. putida* (Fig. 1) and with regard to constructing an eligible production strain, different genes were selected and deleted: PP_0168 (*lapA*), coding for the surface adhesion protein, which is an important stress response element and responsible for the formation of biofilms (12); PP_4333–4396, coding for flagellum biosynthesis; and PP_2451 and PP_3375, coding for endonucleases. Table 2 summarizes the constructions of the different strains. Generally, construction began with PCR amplification of the up- and downstream regions (each about 1 kb), including the start and stop codons of the target gene. The PCR fragments were cut and cloned into pJOE6261.2 according to the data shown in Table 2. Then, *P. putida* with deleted *upp* was transformed with the constructed integration vectors by electroporation. Selection steps occurred as described above. The transformation efficiencies were about 25 CFU μg^{-1} DNA using 5×10^9 electroporated cells. In each case, 10 putative positive clones were checked by colony PCR, whereas the reverse primers were located in the downstream region and the forward primers once in the upstream region (positive control) and once in the region to be deleted (negative control). As further controls, the initial strain and the strain with the integrated vector were used. Theoretically, half of the clones should have lost the gene, while the other half should have restored the original sequence. For reasons already discussed by Fabret et al. (3), the maximum ratio of 50% could not be achieved (Table 2). Deletion of PP_0168 resulted in strains no longer capable of forming biofilms, which was verified by a modified version of a biofilm assay described by Merritt et al. (9) as detailed in the supplemental material. Deletion of the flagellum biosynthesis operon led to strains with a lack of motility, which was observed microscopically and on motility agar plates (see Fig. S2 in the supplemental material). Deletion of the endonucleases, however, had no significant effect on transformation efficiencies.

Conclusion. The *upp* gene has been approved for *B. subtilis*, *Enterococcus faecalis*, *Lactobacillus acidophilus*, and *Desulfovibrio vulgaris* already as an efficient negative selection marker for gene deletion, integration, and replacement (3, 4, 6, 7). Here we demonstrate that it can also be an efficient genetic tool for *P. putida*.

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REFERENCES

1. **Altenbuchner, J., P. Viell, and I. Pelletier.** 1992. Positive selection vectors based on palindromic DNA sequences. Methods Enzymol. **216:**457–466.
2. **Bertani, G.** 1951. Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli.* J. Bacteriol. **62:**293–300.
3. **Fabret, C., S. D. Ehrlich, and P. Noirot.** 2002. A new mutation delivery system for genome-scale approaches in *Bacillus subtilis*. Mol. Microbiol. **46:**25–36.
4. **Goh, Y. J., et al.** 2009. Development and application of a *upp*-based counterselective gene replacement system for the study of the S-layer protein SlpX of *Lactobacillus acidophilus* NCFM. Appl. Environ. Microbiol. **75:** 3093–3105.
5. **Hoang, T. T., R. R. Karkhoff-Schweizer, A. J. Kutchma, and H. P. Schweizer.** 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. Gene **212:**77–86.
6. **Keller, K. L., K. S. Bender, and J. D. Wall.** 2009. Development of a markerless genetic exchange system for *Desulfovibrio vulgaris* Hildenborough and its use in generating a strain with increased transformation efficiency. Appl. Environ. Microbiol. **75:**7682–7691.
7. **Kristich, C. J., D. A. Manias, and G. M. Dunny.** 2005. Development of a method for markerless genetic exchange in *Enterococcus faecalis* and its use in construction of a *srtA* mutant. Appl. Environ. Microbiol. **71:**5837–5849.
8. **Marx, C. J., and M. E. Lidstrom.** 2002. Broad-host-range *cre-lox* system for antibiotic marker recycling in Gram-negative bacteria. Biotechniques **33:** 1062–1067.
9. **Merritt, J. H., D. E. Kadouri, and G. A. O'Toole.** 2005. Growing and analyzing static biofilms. Curr. Protoc. Microbiol. **2005:**1B.1.1–1.B.1.17.
10. **Nelson, K. E., et al.** 2002. Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. Environ. Microbiol. **4:**799–808.

11. **Neuhard, J.** 1983. Utilization of preformed pyrimidine bases and nucleosides, p. 95–148. *In* A. Munch-Petersen (ed.), Metabolism of nucleotides, nucleosides and nucleobases in microorganisms. Academic Press, New York, NY.
12. **Reva, O. N., et al.** 2006. Functional genomics of stress response in *Pseudomonas putida* KT2440. J. Bacteriol. **188:**4079–4092.
13. **Reyrat, J. M., V. Pelicic, B. Gicquel, and R. Rappuoli.** 1998. Counterselectable markers: untapped tools for bacterial genetics and pathogenesis. Infect. Immun. **66:**4011–4017.
14. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
15. **Schweizer, H. P., and T. T. Hoang.** 1995. An improved system for gene replacement and *xylE* fusion analysis in *Pseudomonas aeruginosa*. Gene **158:** 15–22.
16. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.