

Adaptation of the Yeast *URA3* Selection System to Gram-Negative Bacteria and Generation of a $\Delta betCDE$ *Pseudomonas putida* Strain

Teca Calcagno Galvão and Víctor de Lorenzo*

Department of Microbial Biotechnology, Centro Nacional de Biotecnología-CSIC,
Campus UAM–Cantoblanco, Madrid, Spain

Received 3 August 2004/Accepted 20 September 2004

A general procedure for efficient generation of gene knockouts in gram-negative bacteria by the adaptation of the *Saccharomyces cerevisiae* *URA3* selection system is described. A *Pseudomonas putida* strain lacking the *URA3* homolog *pyrF* (encoding orotidine-5'-phosphate decarboxylase) was constructed, allowing the use of a plasmid-borne copy of the gene as the target of selection. The delivery vector pTEC contains the *pyrF* gene and promoter, a conditional origin of replication (*oriR6K*), an origin of transfer (*mobRK2*), and an antibiotic selection marker flanked by multiple sites for cloning appropriate DNA segments. The versatility of *pyrF* as a selection system, allowing both positive and negative selection of the marker, and the robustness of the selection, where *pyrF* is associated with uracil prototrophy and fluoroarotic acid sensitivity, make this setup a powerful tool for efficient homologous gene replacement in gram-negative bacteria. The system has been instrumental for complete deletion of the *P. putida* choline-*O*-sulfate utilization operon *betCDE*, a mutant which could not be produced by any of the other genetic strategies available.

Homologous gene replacement is a basic tool for the study of biological systems, and as such the efficiency of methods for gene knockout generation contributes to the ease with which a system can be characterized by permitting genetic manipulation. The diversity of bacterial species under study has harbored the development of a plethora of cloning, expression, and mutagenesis tools, but those for the generation of gene knockouts are still in need of improvement (39). PCR-based one-step techniques for gene replacement and disruption (6, 46, 47) greatly facilitate gene knockout construction but thus far have only been shown to work in a limited number of species (i.e., *Escherichia coli* and *Salmonella* spp.). For other bacteria, including a large number of genera of considerable environmental and biotechnological interest, exchange of DNA segments to and from the chromosome depends on the use of counterselection markers, but these are few and of limited efficiency.

The dearth of suitable tools to effect such exchanges limits the possibilities of genetic analysis and chromosomal engineering of most non-*E. coli* strains. The most widely used counterselection marker in gram-negative bacteria is the *sacB* gene of *Bacillus subtilis*, whose product, levan sucrose, confers sucrose sensitivity on a large number of gram-negative species (11, 40). Also, in *Streptomyces* spp., the *glkA* gene product, glucose kinase, confers sensitivity to 2-deoxyglucose (44). Use of the *glkA* system in other bacteria has not been reported. In *Bacillus subtilis*, uracil phosphoribosyltransferase has been used successfully as a counterselection marker in one-step generation of chromosomal point mutations, in-frame deletions, and gene knockouts (9). Uracil phosphoribosyltransferase con-

verts 5-fluorouracil into a compound, 5-fluoro-UMP, that is metabolized into 5-fluoro-dUMP, which strongly inhibits thymidylate synthetase (28).

Except for *sacB*-based vectors, none of the counterselection markers have been employed in *Pseudomonas putida* to any significant extent. Yet, although useful in many cases, *sacB* is by no means an optimal gene for this end, as its function depends on the transport of sucrose. This often has to be forced by adding high extracellular concentrations (5% or more) of the sugar, causing undue stress and favoring the rise of mutations that bypass the stringency of the selection. Having a reliable system for allelic exchange and chromosomal engineering of *P. putida* is a prerequisite to realize the full potential of this species in biocatalysis and bioremediation. The same applies to many other gram-negative bacteria which are not yet amenable to genetic analysis (34).

In our pursuit of improved genetic tools for *P. putida*, we came across the uracil biosynthesis gene *URA3*, which has been used widely in positive and negative selection in *Saccharomyces cerevisiae*. *URA3* encodes an enzyme, orotidine-5'-phosphate decarboxylase (ODCase), that can catalyze the transformation of 5-fluoroarotic acid (FOA, a uracil analogue) into a highly toxic compound (3). Thus, counterselection works on the basis that the presence of *URA3* confers sensitivity to FOA, while *URA3*-negative cells are FOA resistant. Alternatively, *URA3* as a positive selection marker works based on the ability of an exogenous or plasmid-borne *URA3* gene complementing uracil auxotrophy of a *URA3*-negative strain.

Assuming that *URA3*/FOA counterselection could work so long as an ODCase activity exists in an organism, successful adaptation of the URA counterselection system has allowed homologous gene replacement in the archaea *Halobacterium salinarum* (33), *Thermococcus kodakaraensis* KOD1 (38), and *Halorerox volcanii* (2). Also, *URA3*-based counterselection has

* Corresponding author. Mailing address: Centro Nacional de Biotecnología-CSIC, Campus de Cantoblanco, Madrid 28049, Spain. Phone: 34 915854536. Fax: 34 91 5854506. E-mail: vdlorenzo@cnb.uam.es.

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Reference
Strains		
<i>P. putida</i> KT2442	Rif ^r ; derived from <i>P. putida</i> KT2440	15
<i>P. putida</i> TEC1	Rif ^r ; <i>P. putida</i> KT2442 with a nearly complete internal deletion of <i>pyrF</i>	This work
<i>P. putida</i> TEC1 $\Delta betCDE$	Rif ^r Gm ^r ; TEC1 whose <i>betCDE</i> putative operon was deleted by use of pTGLB	This work
<i>E. coli</i> CC118 λ pir	$\Delta(ara-leu) araD \Delta lacX74 galE galK phoA20 thi-1 rpsE rpoB argE recA1 \lambda$ pir lysogen	15
<i>E. coli</i> HB101	Sm ^r ; <i>rpsL recA thi pro leu hsdR⁻ M⁺</i> (<i>E. coli</i> K-12/ <i>E. coli</i> B. hybrid)	37
<i>E. coli</i> X82	<i>lacZ53(Am) λ^- trpC50 pyrF287 hisG1(Fs) rpsL8</i> ; uracil auxotroph	<i>E. coli</i> Stock Center CGSC 5889 ID 6952
Plasmids		
pUC19NotI	Ap ^r ; pUC-type plasmid with MCS of pUC19 flanked by NotI sites	15
pUP	Ap ^r ; pUC19NotI inserted with a 2-kb EcoRI-HindIII PCR fragment containing the <i>P. putida pyrF</i> chromosome region	This work
pUPX	Ap ^r ; pUP with an <i>xylE</i> SmaI fragment from pXYLE10 cloned into the Eco47III and SmaI sites of pUP	This work
pKNG101	Sm ^r ; <i>sacRB⁺</i> , broad-host-range plasmid of conditional replication	17
pKPX	Sm ^r ; pKNG1 with the pUPX $\Delta pyrF::xylE$ NotI fragment	This work
pKT231	Km ^r , Sm ^r , broad-host-range plasmid derived from RSF1010	10
pKP	Km ^r ; pKT231 with the <i>P. putida pyrF</i> gene and its promoter region	This work
pGAPN	Ap ^r ; pGEM-T Easy carrying a PCR fragment of <i>pyrF</i> (with endogenous promoter)	This work
pGP704	Ap ^r ; <i>oriR6K, mobRK2</i> , MCS of M13 tg131	25
pGEM-T Easy	Ap ^r ; PCR fragment cloning plasmid	Promega
pGGLB	Ap ^r ; pGEM-T Easy with the <i>betCDE</i> deletion cassette cloned into the NotI site	Galvão et al., unpublished
pTEC	Km ^r FOA ^s Ura ⁺ ; ligation of MCS-Km-MCS, <i>oriR6K</i> /origin of transfer <i>mobRK2</i> , and <i>pyrF</i> PCR fragments	This work
pTGLB	Km ^r Gm ^r FOA ^s Ura ⁺ ; pTEC with the <i>betCDE</i> deletion cassette cloned into the NotI site	This work
pRK600	Cm ^r ; <i>oriColE1, mobRK2, traRK2</i>	19

been used in *Mycobacterium smegmatis* for obtaining unmarked gene integration into the chromosome (20) and in shuttle vectors for the hyperthermophilic archaeon *Pyrococcus abyssi* (21). The *pyr* genes are *URA* homologs, such that adaptation of the *URA3* system to bacteria is based on identification of the ODCase-encoding *pyr* gene and isolation of mutants that are FOA resistant and uracil auxotrophs, followed by the use of allelic exchange to isolate the gene replacement.

Using the complete genome sequence of *P. putida* KT2440 (27), we identified a single *pyrF/URA3* ortholog in the chromosome. This opened the possibility of recreating in *P. putida* the many advantages and utilities of the *URA3* system of *S. cerevisiae*. In this work, we present a complete genetic system for generation of knockouts in gram-negative bacteria that uses the *pyrF* gene of *Pseudomonas putida* as a positive and negative marker. We prove that *pyrF*-based selection is highly efficient by creating a *P. putida* strain entirely deleted of the osmotic stress-related operon *betCDE*, which could not have been generated by any other method.

MATERIALS AND METHODS

Biological materials, culture conditions, and general procedures. The bacteria and plasmids used in this work are listed in Table 1. *Pseudomonas putida* KT2442 (15) and *Escherichia coli* strains were grown at 30°C and 37°C, respectively, in LB or 2YT broth (37). *P. putida* strains were also grown in M9 minimal medium (37) with citrate (2 g/liter) as the carbon source. Antibiotics were used at the following concentrations: rifampin, 50 µg/ml; ampicillin, 150 µg/ml; kanamycin, 50 µg/ml; streptomycin, 50 µg/ml (*E. coli*) or 100 µg/ml (*P. putida*); gentamicin, 10 µg/ml; and chloramphenicol, 30 µg/ml.

For the *P. putida* $\Delta pyrF$ TEC1 strain, uracil (Sigma Aldrich, Madrid, Spain)

was used at 20 µg/ml. 5-Fluoroorotic acid (FOA; Zymo Research) was used at 250 µg/ml. Plasmids were transferred from *E. coli* to *P. putida* by tripartite conjugation on membrane filters (0.45 µm, Millipore), including helper strain *E. coli* HB101(pRK600). After 6 to 8 h of incubation at 30°C on LB agar, the conjugation mixture was plated on minimal selective medium as indicated in each case. All oligonucleotides were synthesized by Sigma-Genosys. DNA constructs were sequenced with the dideoxy method (ABI-Prism automated DNA sequencer, Perkin Elmer). Endurance to osmotic stress was assayed by plating serial dilutions of cultures onto solid M9 minimal medium supplemented with citrate and sodium chloride to 0.6 or 0.7 M.

Plasmid and strain construction. The *P. putida* $\Delta pyrF$ TEC1 strain was created as follows. A 1.8-kb region of the *P. putida* genome spanning the *pyrF* gene (identified with sequence information from the *Pseudomonas putida* genome, <http://www.tigr.org>) was amplified by PCR with primers 37pyr5 (5'-CCGTAAAGCTTGGATGCACTTAGCCTGCAAAAAGGAGC-3') and 37pyr3 (5'-TGCA GAATTCCTGAAGCGGAATGCCCAAAGGCGTG-3'), which added EcoRI and HindIII sites at the ends of the targeted DNA segment. The resulting product was then cloned into pUC19NotI (Table 1), yielding plasmid pUP. This plasmid was cut with Eco47III and SmaI, releasing an internal 404-bp segment of the 702-bp-long *pyrF* gene, and treated with T4 DNA polymerase (Roche Applied Science) to produce blunt ends at the sequences left attached to the plasmid. This was ligated with the SmaI fragment from pXYLE10 (42) containing the *xylE* gene (for catechol 2,3-dioxygenase), yielding pUPX. Plasmid pKPX, used for chromosomal delivery of the resulting assembly $\Delta pyrF::xylE$, was generated by excising the 2,418-bp NotI fragment of pUPX (containing the deleted *pyrF* genomic region inserted with *xylE*) into the NotI site of the *sacB⁺* suicide vector pKNG101 (17).

To create a *pyrF⁺* broad-host-range plasmid, the *P. putida pyrF* gene and its promoter (890 bp) was amplified with primers NotIpyrProm (5'-GATATACA GCGGC CGCATCGGGCCGTGGGCAACCGGT-3') and Asc pyrF (5'-GAAT GGCGCGCCCTTACCCACGGATCTC-3'), introducing NotI and AscI sites to the 5' and 3' ends of the amplified segment, respectively. The resulting 882-bp product was ligated to pGEM-T Easy (Promega), resulting in pGAPN. Digestion of pGAPN with EcoRI (vector sites flanking the *pyrF* insert) released a 920-bp fragment containing *pyrF*, which was ligated with EcoRI-digested plasmid

pKT231 (10) and electroporated into *E. coli* X82, a uracil auxotroph (from S. Brenner, through the *E. coli* Stock Center, CGSC 5889 ID 6952), and plated on M9 minimal medium (37) supplemented with glucose (2 g/liter), tryptophan (0.1 mM), histidine (1 mg/liter), thymidine (0.5 mg/liter), and kanamycin. The plasmid borne by transformants growing without uracil was named pKP and consisted of pKT231 with a functional *pyrF* insertion.

pTEC, the *pyrF*⁺ vector for allelic exchange, was generated as follows. The same primers (NotIpyrFprom and Asc pyrF) employed for construction of pKP were used to amplify the 870-bp region of the *P. putida pyrF* gene and its promoter. A 1.0-kb kanamycin resistance gene was amplified separately from pUT mini-Tn5 Km1 (7) with primers 5'MCS Km (5'-ATAAGAATCGCGCCGCTCTAGAAGTACTAGGCCTATCGATGAGCTCCGCCACGTTGTGTCTC AAAATCTCTGATGTTACATTGCACAAGATAA-3') and 3'MCS Km (5'-TGCAGTTTCATTGATGCTCGATGAGTTTTTCTAAG GTACCAGATCT GATATCACGCGTGAATTCAGAC-3') which flanked the kanamycin resistance determinant with multiple restriction sites. Finally, a 2.0-kbp fragment from plasmid pGP704 (25) containing the origin of transfer (*oriT*) of RK2 (12, 45) and the vegetative origin of replication (*oriV*) of R6K (which functions only in concert with the replication protein II encoded by gene *pir*) (16) was amplified with primers 5'ORI EcoNotAvr (5'-TACGGAATTCGCGGCCGCCCTAGGC CATGTCAGCCGTTAAGTGTCTGT-3') and 3'ORI EagApaAsc (5'-CATGTCGGCGCGCCGGCCCGCGCCGCTGGCTCCGGCGTCTT-3'), which add EcoRI and AscI sites to the corresponding DNA. The three PCR products (one bearing *pyrF*, other kanamycin resistance, and the last the *oriV/oriT* origins) were digested with the cognate enzymes required to produce cohesive ends, ligated, and transformed into *E. coli* CC118 λ *pir* (15). Transformants growing on LB-kanamycin agar plates were found to bear the plasmid shown in Fig. 3, which was subsequently named pTEC (EMBL accession number BN000674).

Δ *pyrF P. putida* strain TEC1 was constructed by mobilizing pKPX from *E. coli* CC118 λ *pir* into *P. putida* KT2442 by tripartite mating with *E. coli* HB101(pRK600) as the helper and selecting exconjugants in minimal medium plates with citrate and streptomycin. Putative cointegrates were grown in LB without selection, and resolution of the cointegrate resulting in chromosomal acquisition of the deleted *pyrF* gene was selected by plating on minimal medium plates with citrate, uracil, and FOA (counterselection of *sacB* with sucrose never worked in this case).

The plasmid used for deletion of the *P. putida* putative *betCDE* operon, pTGLB, was constructed as follows. A PCR product with a gentamicin resistance cassette and a β -galactosidase gene flanked by *betCDE* upstream (ORF PP0074) and downstream (*betR*) sequences, forming a transcriptional fusion of the *betCD* promoter with the β -galactosidase gene (Galvão et al., unpublished data) was cloned into plasmid pGEM-T Easy (Promega) to generate pGGLB. pGGLB was cut with NotI, and the deletion cassette was cloned into the NotI site of pTEC to generate pTGLB.

RESULTS AND DISCUSSION

***P. putida pyrF* gene and FOA sensitivity.** A BLAST search on the *P. putida* genome with the *P. aeruginosa pyrF* gene sequence yielded an open reading frame (ORF) with 90% identity (locus PP1815 in the *P. putida* genome). The *P. aeruginosa pyrF* gene has been shown to encode orotidine-5'-monophosphate decarboxylase (ODCase) by enzymatic assays of the gene product (43). In *P. putida*, *pyrF* is not part of an operon (Fig. 1A), being located between a conserved hypothetical protein and a predicted alcohol dehydrogenase.

In a first step to develop a counterselection system based on *pyrF*, we tested the sensitivity of *P. putida* KT2442 to FOA. Plating of up to 10⁹ CFU on solid minimal medium containing uracil and FOA revealed sensitivity to the compound, the spontaneous mutation frequency to FOA resistance being 0.8 \times 10⁻⁶. In *T. kodakaraensis* and *H. salinarum*, the frequency of FOA resistance arising is 1 \times 10⁻⁵ to 5 \times 10⁻⁵ and 6.9 \times 10⁻⁶, respectively (33, 38). Growth of FOA-resistant colonies on solid minimal medium showed that, contrary to what happens in these two microorganisms, mutation to FOA resistance was not associated with uracil auxotrophy. Instead, of 50 FOA-

resistant *P. putida* colonies tested, all grew in the absence of uracil, such that resistance is likely to be associated either with FOA uptake or with the specific reaction catalyzed by the ODCase on FOA but not on uracil. The particularity in FOA resistance of *P. putida* is further noted by the fact that the plating of 10⁹ CFU on solid medium with FOA but lacking uracil did not yield any resistant colonies. In any case, these figures were well within the range of what is useful for selection and counterselection of chromosome segments.

As a prerequisite to set up a counterselection system based on *pyrF*, we pursued the generation of a Δ *pyrF P. putida* strain so that *pyrF* in a plasmid could serve as a marker. To this end, a 2.0-kb region containing the *pyrF* gene was PCR cloned as described in the Materials and Methods section, and the subsequent cloning of an *xylE* cassette was carried out to allow *pyrF* deletion (Fig. 1B). The deletion construct (Fig. 1B) was cloned in pKNG101 (17), yielding pKPX, and introduced into *P. putida* KT2442. Chromosomal integration of the plasmid was selected by streptomycin resistance. Following growth without selection, a second recombination event that maintained the Δ *pyrF* copy was pursued. Efforts to use the *sacB* gene of pKPX as a counterselection marker in the presence of 5% or 8% sucrose to force resolution of the cointegrates repeatedly generated sucrose-resistant spontaneous mutants. On the contrary, plating the streptomycin-resistant exconjugants on minimal medium containing uracil and FOA resulted in the rise of FOA-resistant colonies, of which two of eight tested were uracil auxotrophs. These *ura* FOA-resistant colonies were also sensitive to streptomycin and turned yellow when sprayed with catechol (10 g/liter), the substrate of the *xylE* gene product, catechol-2,3-dioxygenase (not shown). These properties were indicative of the successful double recombination of the Δ *pyrF::xylE* segment into the chromosome of *P. putida*.

To confirm this, one of the *ura* clones that was FOA resistant, streptomycin-sensitive, and *xylE*⁺ (hereafter referred to as *P. putida* TEC1) was subjected to PCR analysis of the relevant chromosomal sequences. Figure 1C shows that the uracil auxotrophy and FOA resistance of *P. putida* TEC1 are associated with a band that corresponds in size to the deletion cassette with *xylE* (lanes 2 and 5). Note that when *P. putida* TEC1 carried a plasmid with the wild-type *pyrF* gene, the *pyrF::xylE* deletion cassette was not amplified with the *pyrF* primers, possibly due to preferential annealing of the primers to the wild-type gene. Indeed, whenever a wild-type copy of *pyrF* was present, amplification of the *pyrF::xylE* deletion cassette was not observed (see also Fig. 5, lane 6). *P. putida* TEC1 was thus chosen as the *pyrF* mutant of reference. That *pyrF* turned out to be far more powerful than *sacB* to select resolution of the cointegrate of pKPX with the chromosome of *P. putida* provided a good indication of the potential of the *URA3* homologue for vector development.

To confirm that *pyrF* encodes the *P. putida* orotidine-5'-monophosphate decarboxylase, growth of the wild-type and mutant strains was compared. Clearly, while the Δ *pyrF P. putida* strain TEC1 failed to grow in standard minimal medium, uracil addition sufficed to make growth of *P. putida* TEC1 and its wild-type counterpart *P. putida* KT2442 indistinguishable in liquid medium (Fig. 2A). This suggests that so long as an adequate amount of uracil is present, deletion of *pyrF* does not alter the growth rate. The uracil auxotrophy of *P.*

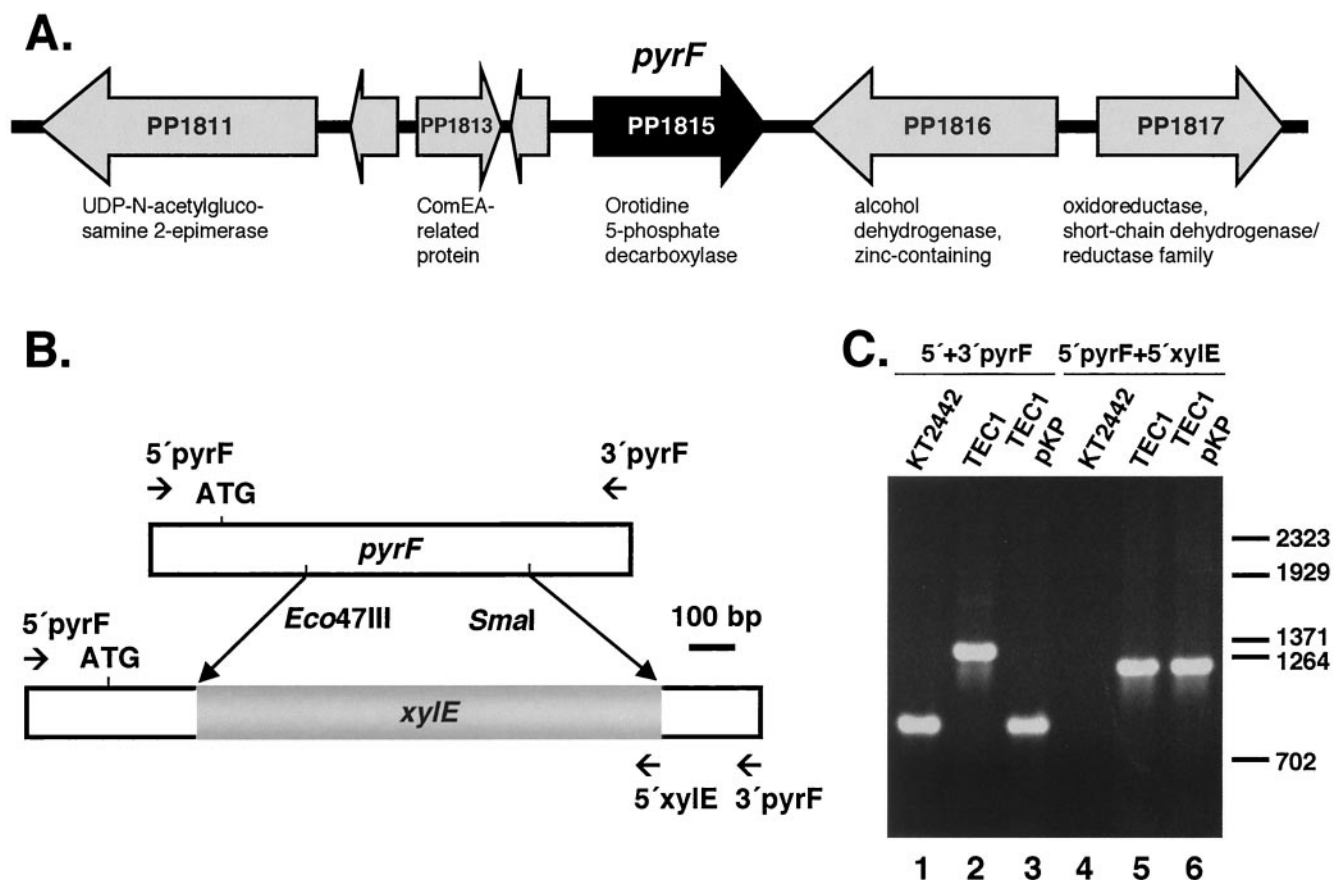


FIG. 1. Generation of $\Delta pyrF$ *P. putida* TEC1. (A) Schematic diagram of the *P. putida* *pyrF* gene (ORF PP1815) chromosomal context. The locus name of each ORF with assigned or predicted function is shown; hypothetical ORFs are unmarked. (B) Schematic diagram of the *P. putida* *pyrF* gene and of the $\Delta pyrF$ in *P. putida* TEC1. The *xylE* gene was cloned in the *Eco47III* and *SmaI* sites of *pyrF*. Primers are shown as arrows, indicating primer combinations used to amplify the *pyrF* and *xylE* sequences. (C) PCR analysis of wild-type and $\Delta pyrF$ strains. Lanes 1 to 3: primers annealing to the *pyrF* promoter and to the 3' end of the gene were used to amplify the *pyrF* gene of *P. putida* KT2442, of the $\Delta pyrF$ strain *P. putida* TEC1, and of TEC1 with the *pyrF*-containing pKP plasmid. Lanes 4 to 6: the chromosomal copy of $\Delta pyrF$ in *P. putida* TEC1 carrying pKP was amplified with primers annealing at the 5' end of *pyrF* and *xylE*.

putida TEC1 can be complemented by the *pyrF*⁺ plasmid pKP, carrying *pyrF* under its own promoter (Fig. 2B). That *pyrF* deletion confers FOA resistance, as should the deletion of ODCase, was shown by a growth assay on solid medium (Fig. 2C). While *P. putida* TEC1 is FOA resistant, *P. putida* KT2442 is sensitive. Uracil prototrophy and FOA sensitivity in *P. putida* TEC1 is regained upon complementation with pKP. Thus, the *P. putida* *pyrF* gene is a bona fide orotidine-5'-monophosphate decarboxylase, and this activity is associated with FOA sensitivity. Reversion of the $\Delta pyrF$ strains back to the wild-type *pyrF* genotype is straightforward, as *P. putida* TEC1 and its derivatives can be electroporated with the *pyrF*⁺ pUP plasmid (which does not replicate in *P. putida*) (Table 1), and following a round of antibiotic selection and growth without selection, plated on minimal medium without uracil. Spraying colonies with catechol allows visualization of *xylE* marker loss (not shown).

Structure and qualities of pTEC, a suicide plasmid for homologous gene replacement. On the basis of the data above, we anticipated that the $\Delta pyrF$ background should permit the use of *pyrF* as a counterselection marker in a suicide vector. Thus, the essential elements of such a plasmid were put together by

PCR cloning to make pTEC (Fig. 3A). These elements were the *pyrF* gene under its own promoter, a broad-host-range kanamycin resistance cassette flanked by multiple cloning sites, facilitating cloning of the desired genomic regions through which recombination is to take place, a segment of plasmid pGP704 (25) carrying a conditional origin of replication (*oriV* R6K), and an origin of transfer (*oriT*) for RK2-mediated mobilization (see Materials and Methods for details). This design allows, in a vector of small size, the insert of any DNA segment of interest and its propagation in specialized *E. coli* hosts (such as *E. coli* CC118λ*pir*) engineered to express in *trans* the replication protein II of plasmid R6K. Finally, pTEC derivatives can be passed to *P. putida* $\Delta pyrF$ by bipartite or tripartite conjugation mediated by the *tra* genes of plasmid RK2 (which can be provided by a helper strain, such as HB101 carrying pRK600; see Table 1). Formation of a cointegrate between pTEC derivatives and the chromosome of *P. putida* TEC1 can be selected by plating in minimal medium without uracil (and, if desired, with kanamycin), while its resolution can be forced by replating in medium containing FOA.

Generation and characterization of a *P. putida* $\Delta betCDE$ strain. The performance and survival of microorganisms, in-

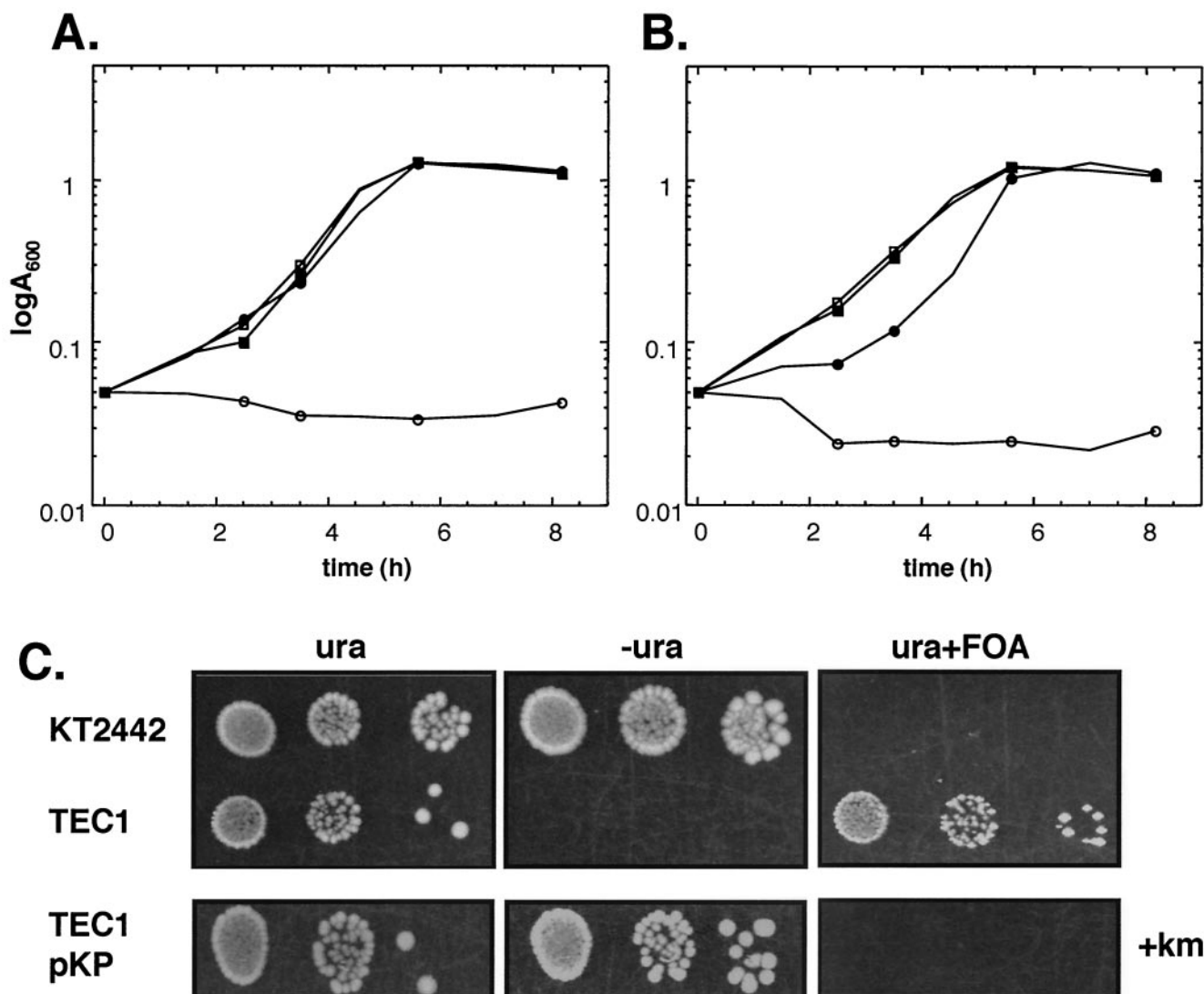


FIG. 2. Uracil auxotrophy and FOA resistance conferred by *pyrF* deletion is complemented by plasmid-borne *pyrF*. (A) Strains *P. putida* KT2442 (squares) and *P. putida* TEC1 (circles) were grown at 30°C in liquid M9 minimal medium supplemented with citrate in the presence (solid symbols) or absence (open symbols) of uracil at 20 $\mu\text{g/ml}$. Growth was monitored at 600 nm after inoculation to 0.05 at 0 h. (B) Strains *P. putida* TEC1 carrying pKT231 (circles) or pKP (pKT231 carrying *pyrF* under its own promoter) (squares) were grown as in A. (C) Upper panel: Deletion of *pyrF* confers uracil auxotrophy and FOA resistance. Serial dilutions of *P. putida* KT2442 or TEC1 were plated on solid M9 minimal medium in the presence or absence of uracil (20 $\mu\text{g/ml}$) or with uracil and 5-fluoroorotic acid (250 $\mu\text{g/ml}$). Lower panel: Serial dilutions of *P. putida* TEC1/pKP were plated as above but with kanamycin, the resistance marker of pKP.

cluding *P. putida*, in soil and the rhizosphere of plants relies to a large extent on their ability to endure desiccation and osmotic stress (18, 24). Choline-*O*-sulfate has been shown to be an efficient osmoprotectant in several plants (14, 36) and microbial species (4, 13, 14, 23, 26, 32). It is thought that choline-*O*-sulfate is found in soil, as plants (30), fungi (1, 22), and bacteria have transport systems for this ester (4, 26, 29). In bacteria, choline-*O*-sulfate can be transformed into choline (31, 35), which is subsequently transformed into glycine betaine (41), a potent osmoprotectant (5).

We have found that the genome of *P. putida* bears a putative choline-*O*-sulfate utilization operon, named *betCDE*, for choline-*O*-sulfate uptake (ORFs PP0075 and PP0076) and conversion to choline (ORF PP0077; Galvão et al., unpublished data).

To examine the role of choline-*O*-sulfate in osmoprotection of *P. putida* in a rhizosphere microcosm system, we set out to generate a *lacZ*⁺-traceable $\Delta betCDE$ strain, the expectation being that such a deletion would affect *P. putida*'s response to desiccation and osmotic stress. Production of such a strain with a standard *sacB*-based vector system such as pKNG101 (17) failed repeatedly. Besides the problems inherent in the use of *sacB* mentioned above, mutations involved in osmotic stress may be particularly difficult to produce with a counterselection system which involves the addition of $\geq 5\%$ solute, which causes considerable osmotic pressure. This scenario provided the most appropriate case to test the performance of the *pyrF*-based allelic exchange system presented above.

To this end, a DNA segment carrying a gentamicin resis-

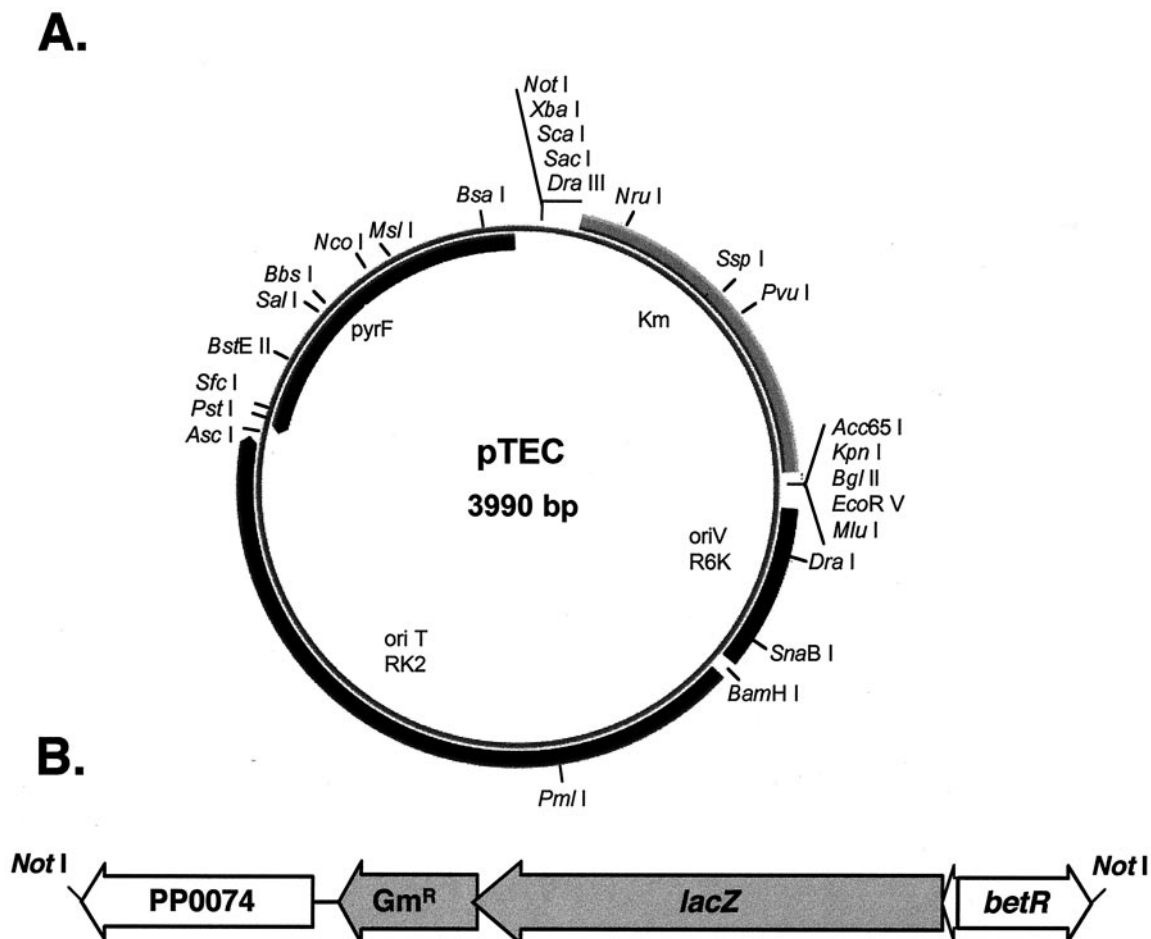


FIG. 3. Map of pTEC. (A) The plasmid contains the *P. putida pyrF* gene and its promoter, a kanamycin resistance cassette flanked by multiple cloning sites, the broad-host-range origin of replication *oriR6K*, and the origin of transfer *mobRK2*. Only unique restriction sites are shown. The diagram was drawn with PlasMapper (<http://wishart.biology.ualberta.ca/PlasMapper/index.html>) (8). (B) Schematic diagram of the cassette cloned into the pTEC NotI site for generating a *betCDE* deletion mutant. In the cassette, the *betCDE* genes have been replaced by the gentamicin resistance cassette and *lacZ* genes while maintaining the flanking sequences, ORF PP0074 and *betR*.

tance cassette and the β -galactosidase (*lacZ*) gene flanked by the genomic sequences upstream and downstream of *betCDE* (ORF PP0074 and *betR*) was assembled (Fig. 3B). The β -galactosidase gene is cloned as a transcriptional fusion to the *betCD* promoter, as indicated by a small arrow ahead of the *lacZ* gene (Galvão et al., unpublished data). This segment was cloned into the NotI site of pTEC to yield plasmid pTGLB. This construct was transferred to *P. putida* TEC1 by tripartite conjugation. Recombination of pTGLB into the chromosome was selected on minimal medium with kanamycin and gentamicin but lacking uracil, thus using positive selection for uracil prototrophy upon plasmid integration. Following growth in liquid medium supplemented with uracil but without antibiotic selection, cells in which a second recombination event had taken place (resulting in the *betCDE* deletion) were selected by plating on minimal medium with gentamicin, uracil, and FOA (Fig. 4).

A total of 810 colonies of two morphologies grew when 10^8 CFU were plated in these conditions. The major morphotype (760, or 88%) represented $\Delta betCDE$ deletion mutants, as judged by the uracil auxotrophy and kanamycin sensitivity of 25

colonies analyzed. This represents a respectable double-recombination frequency of nearly 1.3×10^{-5} . PCR analysis showed that the FOA-resistant, kanamycin-sensitive colonies no longer had pTGLB in the chromosome, as judged by loss of the wild-type *pyrF* copy, the *betC* gene, and the kanamycin cassette while maintaining the β -galactosidase gene in the deletion cassette (Fig. 5, lanes 1 to 5). The second morphotype consisted of faster-growing colonies, which were found to be FOA resistant uracil prototrophs as well as resistant to kanamycin. That these colonies carry mutation to FOA resistance containing integrated pTGLB was confirmed by PCR analysis, which showed the presence of both wild-type and $\Delta pyrF$ copies as well as of the *betC* gene and the kanamycin cassette (Fig. 5, lanes 6 to 10). The frequency at which these FOA-resistant colonies arose is 2×10^{-6} (50 FOA-resistant colonies per 10^8 CFU plated), near that of *P. putida* KT2442 spontaneous mutation to FOA resistance (see above). That efficient homologous gene replacement is possible through use of *pyrF*-based selection is made clear by the fact that the frequency of double-recombination events generating knockout strains (1.3×10^{-5})

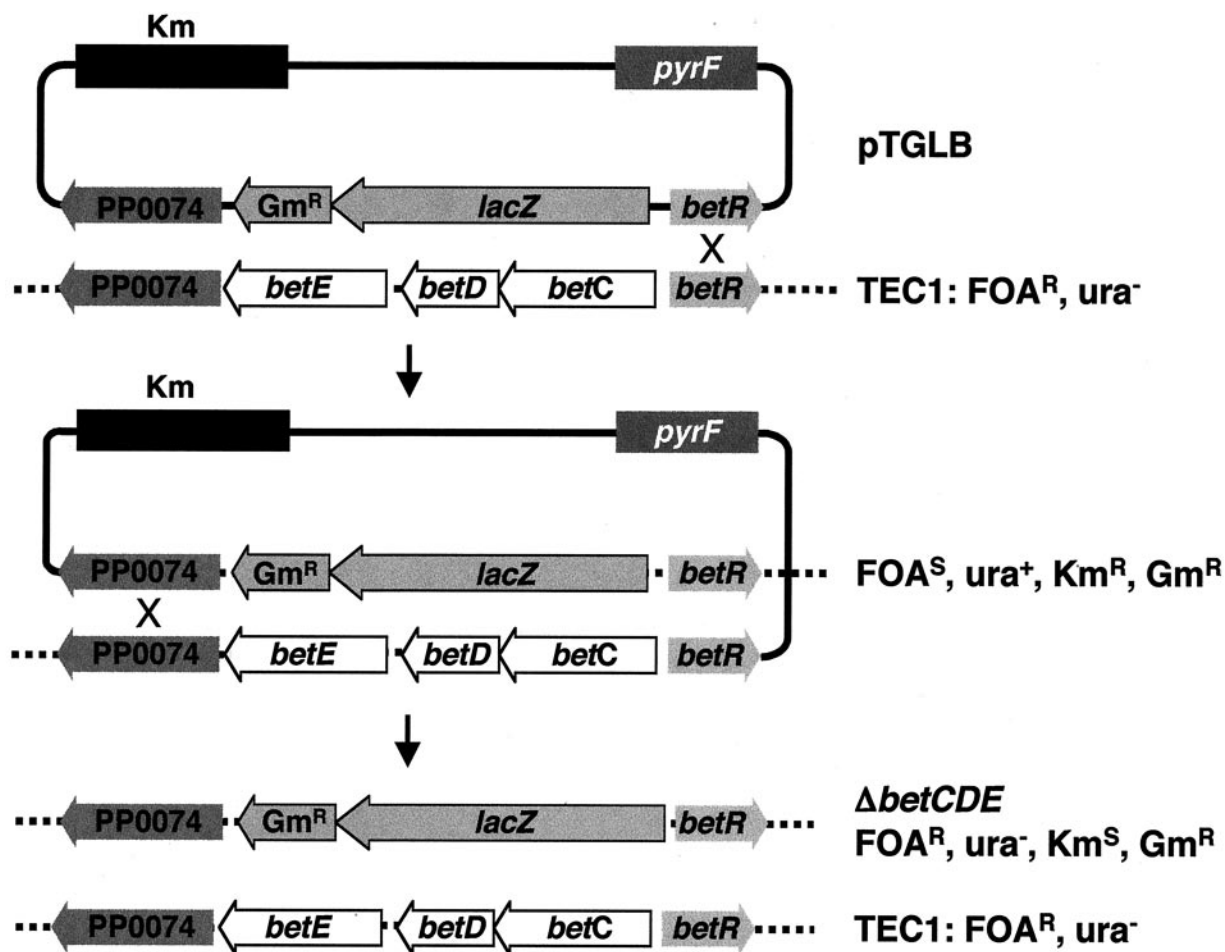


FIG. 4. Schematic map for *pyrF*-based counterselection for generation of homologous gene recombination with pTEC. Chromosomal integration events by homologous recombination between PP0074 or *betR* chromosomal and plasmid (pTGLB) sequences are selected by plating on medium with kanamycin (Km) or gentamicin (Gm) or both but lacking uracil. Cells with the plasmid integrated in the chromosome are grown without selection to allow a second recombination event that maintains either the wild-type or the deleted *betCDE* operon. The $\Delta betCDE$ mutant is isolated by selecting cells that grow in the presence of FOA, gentamicin, and uracil but are kanamycin sensitive and uracil auxotrophs.

is at least 6.5 times higher than that of spontaneous FOA resistance (2×10^{-6}).

To first characterize the *P. putida* TEC1 $\Delta betCDE$ strain, its sensitivity to osmotic stress was tested by looking at growth in solid medium containing high salt. Figure 6 shows that the $\Delta betCDE$ mutant was more sensitive to high salt than *P. putida* TEC1, as in the presence of 0.6 M NaCl the mutant grows much more slowly than *P. putida* TEC1 and is not viable in 0.7 M NaCl. Thus, deletion of *betCDE* renders *P. putida* more sensitive to osmotic stress. Sensitivity to osmotic stress in the absence of protectant compounds is interesting despite the fact that the putative role of the *betCDE* gene products is related to osmoprotectant uptake and transformation. Because the *betCDE* mutant lacks a putative transmembrane protein, BetE (Galvão et al., unpublished data), it is possible that the internal membrane is altered, becoming more susceptible to osmotic stress. The *betCDE* mutant is the basis of further studies under laboratory conditions as well as in the field.

Use of pTEC in other gram-negative bacteria. Employing pTEC for homologous gene replacement in other gram-nega-

tive bacteria requires generating a *pyrF* background. This requirement is compensated for by the possibility of obtaining such mutants by plating on FOA, as was done with *T. kodakaraensis* KOD1 (38) and *H. salinarum* (33). While full deletion of *pyrF* has the advantage of avoiding pTEC integration in the *pyrF* locus, in practice the use of *pyrF*-negative, FOA-resistant strains generated by spontaneous mutations implies that $\leq 50\%$ of cointegrates are inadequate and thus require an additional screening step, easily achieved by PCR. Alternatively, *pyrF* deletion mutants may be generated with pKPX, the pKNG-based suicide plasmid carrying the *P. putida* gene (see above; Table 1), if there is a high sequence similarity between the *pyrF* genes of the organism being used and *P. putida*.

A BLAST search with the *P. putida pyrF* sequence against the EMBL database shows it to be highly homologous to sequences of several gram-negative bacteria (*Pseudomonas syringae*, 83%; *P. aeruginosa*, 82%; *Chromobacterium violaceum*, 71%; *Neisseria meningitidis*, 65%; *E. coli*, 64%; *Vibrio vulnificus*, 63%; *Salmonella enterica* serovar Typhimurium, 64%; *Shigella flexneri*, 63%; *Photobacterium luminescens*, 61%; *Yersinia*

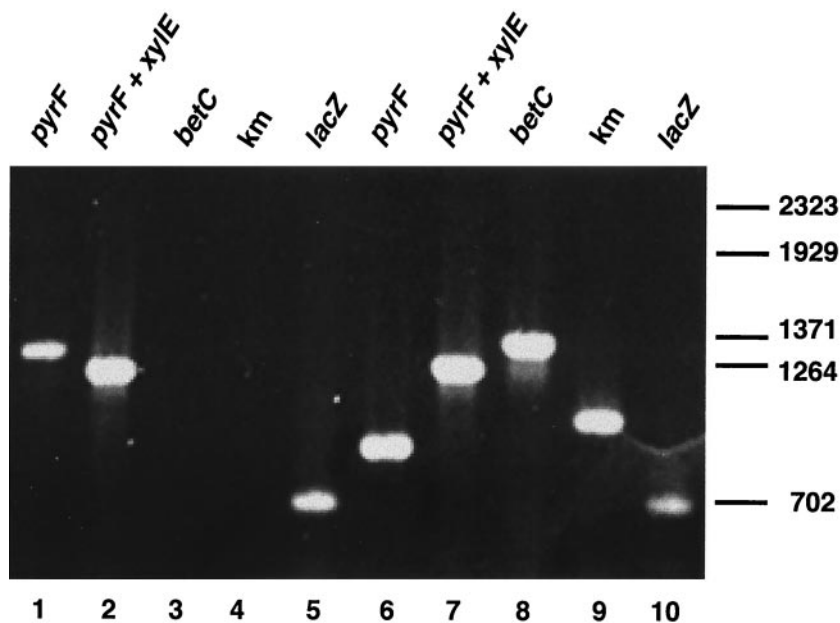


FIG. 5. PCR analysis of *P. putida* TEC1 $\Delta betCDE$ and FOA-resistant cointegrate colonies. Lanes 1 to 5, *P. putida* TEC1 $\Delta betCDE$ colonies. Lanes 6 to 10, FOA-resistant colonies carrying a chromosomal insertion of pTGLB. Primers: lanes 1 and 6, anneal at *pyrF* gene promoter and 3' end (as shown in Fig. 1B); lanes 2 and 7, anneal at *pyrF* gene 5' end and *xylE* gene 5' end (as shown in Fig. 1B); lanes 3 and 8, *betC* internal primers; lanes 4 and 9, kanamycin internal primers; lanes 5 and 10, *lacZ* internal primers.

pestis, 61%; and *Shewanella oneidensis*, 60%). Whether these degrees of homology are sufficient to allow homologous recombination will have to be checked for each organism individually with the simple test provided by the *xylE*⁺ marker of the $\Delta pyrF$ deletion.

Conclusion. We have developed a vector system that employs the *pyrF* gene as a selection marker to allow efficient homologous gene replacement. We tested the plasmid in *Pseudomonas putida* and found that *pyrF* is a reliable marker in positive and negative selection. At least three strategies can be

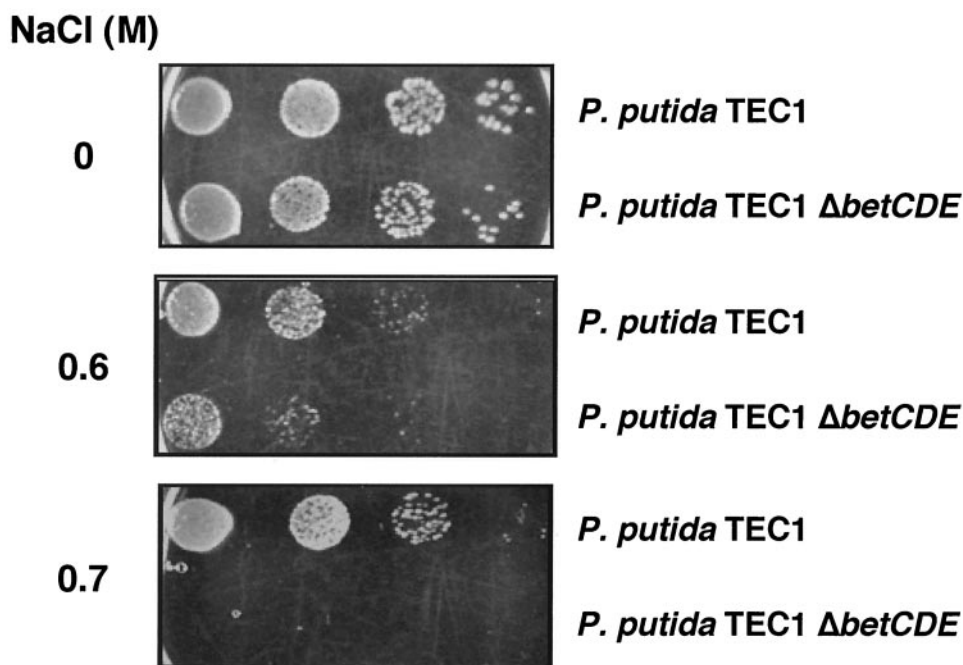


FIG. 6. Deletion of *betCDE* renders *P. putida* TEC1 susceptible to osmotic stress. Serial dilutions of *P. putida* TEC1 and TEC1 $\Delta betCDE$ were plated on M9 minimal medium supplemented with uracil in the presence or absence of 0.6 or 0.7 M NaCl and grown at 30°C for 24 h (0 M NaCl) or 72 h (0.6 and 0.7 M NaCl).

applied for generating gene knockouts with pTEC. First is the one presented here, where a deletion cassette containing an antibiotic resistance marker is cloned directly into pTEC, yielding a knockout strain in which pTEC can be employed again. Second, PCR products of genomic regions adjacent to the gene of interest may be cloned in the multiple cloning sites flanking the kanamycin resistance cassette in pTEC. Third, a deletion cassette that contains only the genomic regions adjacent to the gene of interest is cloned into pTEC, such that the resulting knockout is antibiotic marker free and additional deletions can be generated with the same vector system. In all cases, the essential features of the system are *pyrF*-based selection, where the initial recombination event is selected by uracil prototrophy and the double-recombination event is selected by resistance to FOA and by uracil auxotrophy.

ACKNOWLEDGMENTS

David Canovas is acknowledged for providing the pGGLB plasmid and for useful discussions. The help of the *E. coli* Stock Center with strain X82 is greatly appreciated.

This work was supported by the Biocarte and Lindane Contracts of the European Union and by Comunidad Autónoma de Madrid grant 07 M/0075/2002.

REFERENCES

- Bellenger, N., P. Nissen, T. C. Wood, and I. H. Segel. 1968. Specificity and control of choline-*O*-sulfate transport in filamentous fungi. *J. Bacteriol.* **96**:1574–1585.
- Bitan-Banin, G., R. Ortenberg, and M. Mevarech. 2003. Development of a gene knockout system for the halophilic archaeon *Haloferax volcanii* by use of the *pyrE* gene. *J. Bacteriol.* **185**:772–778.
- Boeke, J. D., F. LaCroute, and G. R. Fink. 1984. A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol. Gen. Genet.* **197**:345–346.
- Canovas, D., C. Vargas, L. N. Csonka, A. Ventosa, and J. J. Nieto. 1996. Osmoprotectants in *Halomonas elongata*: high-affinity betaine transport system and choline-betaine pathway. *J. Bacteriol.* **178**:7221–7226.
- Csonka, L. N., and A. D. Hanson. 1991. Prokaryotic osmoregulation: genetics and physiology. *Annu. Rev. Microbiol.* **45**:569–606.
- Datsenko, K. A., and B. L. Wanner. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* **97**:6640–6645.
- de Lorenzo, V., M. Herrero, U. Jakubzik, and K. N. Timmis. 1990. Mini-Tn5 transposon derivatives for insertion mutagenesis, promoter probing, and chromosomal insertion of cloned DNA in gram-negative eubacteria. *J. Bacteriol.* **172**:6568–6572.
- Dong, X., P. Stothard, I. J. Forsythe, and D. S. Wishart. 2004. PlasMapper: a web server for drawing and auto-annotating plasmid maps. *Nucleic Acids Res.* **32**:660–664.
- 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.
- Franklin, F. 1985. Broad host range cloning vectors for Gram-negative bacteria. DNA cloning: a practical approach. IRL Press, Oxford, England.
- Gay, P., D. Le Coq, M. Steinmetz, T. Berkelman, and C. I. Kado. 1985. Positive selection procedure for entrapment of insertion sequence elements in gram-negative bacteria. *J. Bacteriol.* **164**:918–921.
- Guiney, D. G., and E. Jakobson. 1983. Location and nucleotide sequence of the transfer origin of the broad host range plasmid RK2. *Proc. Natl. Acad. Sci. USA* **80**:3595–3598.
- Gutierrez, J. A., and L. N. Csonka. 1995. Isolation and characterization of adenylate kinase (*adk*) mutations in *Salmonella typhimurium* which block the ability of glycine betaine to function as an osmoprotectant. *J. Bacteriol.* **177**:390–400.
- Hanson, A. D., B. Rathinasabapathi, B. Chamberlin, and D. A. Gage. 1991. Comparative physiological evidence that β -alanine betaine and choline-*O*-sulfate act as compatible osmolytes in halophytic *Limonium* species. *Plant Physiol.* **97**:1199–1205.
- Herrero, M., V. de Lorenzo, and K. N. Timmis. 1990. Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J. Bacteriol.* **172**:6557–6567.
- Inuzuka, M., and D. R. Helinski. 1978. Requirement of a plasmid-encoded protein for replication in vitro of plasmid R6K. *Proc. Natl. Acad. Sci. USA* **75**:5381–5385.
- Kaniga, K., I. Delor, and G. R. Cornelis. 1991. A wide-host-range suicide vector for improving reverse genetics in Gram-negative bacteria: inactivation of the *blaA* gene of *Yersinia enterocolitica*. *Gene* **109**:137–141.
- Kempf, B., and E. Bremer. 1998. Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. *Arch. Microbiol.* **170**:319–330.
- Kessler, B., V. de Lorenzo, and K. N. Timmis. 1992. A general system to integrate *lacZ* fusions into the chromosomes of gram-negative eubacteria: regulation of the *Pm* promoter of the TOL plasmid studied with all controlling elements in monocopy. *Mol. Gen. Genet.* **233**:293–301.
- Knipfer, N., A. Seth, and T. E. Shrader. 1997. Unmarked gene integration into the chromosome of *Mycobacterium smegmatis* via precise replacement of the *pyrF* gene. *Plasmid* **37**:129–140.
- Lucas, S., L. Toffin, Y. Zivanovic, D. Charlier, H. Moussard, P. Forterre, D. Prieur, and G. Erauso. 2002. Construction of a shuttle vector for, and spheroplast transformation of, the hyperthermophilic archaeon *Pyrococcus abyssi*. *Appl. Environ. Microbiol.* **68**:5528–5536.
- Marzluf, G. A. 1972. Genetic and metabolic control of sulfate metabolism in *Neurospora crassa*: a specific permease for choline-*O*-sulfate. *Biochem. Genet.* **7**:219–233.
- Mason, T., and G. Blunden. 1989. Quaternary ammonium and tertiary sulfonium compounds of algal origin as alleviators of osmotic stress. *Bot. Mar.* **32**:313–316.
- Miller, K. J., and J. M. Wood. 1996. Osmoadaptation by rhizosphere bacteria. *Annu. Rev. Microbiol.* **50**:101–136.
- Miller, V. L., and J. J. Mekalanos. 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. *J. Bacteriol.* **170**:2575–2583.
- Nau-Wagner, G., J. Boch, J. A. Le Good, and E. Bremer. 1999. High-affinity transport of choline-*O*-sulfate and its use as a compatible solute in *Bacillus subtilis*. *Appl. Environ. Microbiol.* **65**:560–568.
- Nelson, K. E., C. Weinel, I. T. Paulsen, R. J. Dodson, H. Hilbert, V. A. Martins dos Santos, D. E. Fouts, S. R. Gill, M. Pop, M. Holmes, L. Brinkac, M. Beanan, R. T. DeBoy, S. Daugherty, J. Kolonay, R. Madupu, W. Nelson, O. White, J. Peterson, H. Khouri, I. Hance, P. Chris Lee, E. Holtzapple, D. Scanlan, K. Tran, A. Moazzes, T. Utterback, M. Rizzo, K. Lee, D. Kosack, D. Moestl, H. Wedler, J. Lauber, D. Stjepandic, J. Hoheisel, M. Straetz, S. Heim, C. Kiewitz, J. A. Eisen, K. N. Timmis, A. Dusterhoft, B. Tummler, and C. M. Fraser. 2002. Complete genome sequence and comparative analysis of the metabolically versatile *Pseudomonas putida* KT2440. *Environ. Microbiol.* **4**:799–808.
- Neuhard, J. 1983. Utilization of preformed pyrimidine bases and nucleosides. p. 95–148. In O. Munch-Petersen (ed.), *Metabolism of nucleotides, nucleosides and nucleobases in microorganisms*. Academic Press, New York, N.Y.
- Nissen, P. 1968. Choline sulfate permease: transfer of information from bacteria to higher plants? *Biochem. Biophys. Res. Commun.* **32**:696–703.
- Nissen, P., and A. A. Benson. 1964. Active transport of choline sulfate by barley roots. *Plant Physiol.* **39**:586–589.
- Osteras, M., E. Boncompagni, N. Vincent, M. C. Poggi, and D. Le Rudulier. 1998. Presence of a gene encoding choline sulfatase in *Sinorhizobium meliloti* bet operon: choline-*O*-sulfate is metabolized into glycine betaine. *Proc. Natl. Acad. Sci. USA* **95**:11394–11399.
- Park, Y., and J. E. Gander. 1998. Choline derivatives involved in osmotolerance of *Penicillium fellutanum*. *Appl. Environ. Microbiol.* **64**: 273–278.
- Peck, R. F., S. Dassarma, and M. P. Krebs. 2000. Homologous gene knockout in the archaeon *Halobacterium salinarum* with *ura3* as a counterselectable marker. *Mol. Microbiol.* **35**:667–676.
- Pieper, D. H., V. A. Martins dos Santos, and P. N. Golyshin. 2004. Genomic and mechanistic insights into the biodegradation of organic pollutants. *Curr. Opin. Biotechnol.* **15**:215–224.
- Rathinasabapathi, B., M. Burnet, B. L. Russell, D. A. Gage, P. C. Liao, G. J. Nye, P. Scott, J. H. Golbeck, and A. D. Hanson. 1997. Choline monooxygenase, an unusual iron-sulfur enzyme catalyzing the first step of glycine betaine synthesis in plants: prosthetic group characterization and cDNA cloning. *Proc. Natl. Acad. Sci. USA* **94**:3454–3458.
- Rivoal, J., and A. D. Hanson. 1994. Choline-*O*-Sulfate Biosynthesis in Plants (Identification and Partial Characterization of a Salinity-Inducible Choline Sulfotransferase from Species of *Limonium* (Plumbaginaceae). *Plant Physiol.* **106**:1187–1193.
- Sambrook, J., T. Maniatis, and T. Fritsch. 1989. *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Sato, T., T. Fukui, H. Atomi, and T. Imanaka. 2003. Targeted gene disruption by homologous recombination in the hyperthermophilic archaeon *Thermococcus kodakaraensis* KOD1. *J. Bacteriol.* **185**:210–220.
- Schweizer, H. P., and V. de Lorenzo. 2004. Molecular Tools for Genetic Analysis of Pseudomonads, p. 317–350. In J. L. Ramos (ed.), *Pseudomonas*, 1st ed., vol. 1. Kluwer Academic/Plenum Publishers, New York, N.Y.

40. **Simon, R., B. Hotte, B. Klauke, and B. Kosier.** 1991. Isolation and characterization of insertion sequence elements from gram-negative bacteria by using new broad-host-range, positive selection vectors. *J. Bacteriol.* **173**: 1502–1508.
41. **Smith, L. T., J. A. Pocard, T. Bernard, and D. Le Rudulier.** 1988. Osmotic control of glycine betaine biosynthesis and degradation in *Rhizobium meliloti*. *J. Bacteriol.* **170**:3142–3149.
42. **Stein, D. C.** 1992. Plasmids with easily excisable *xylE* cassettes. *Gene* **117**: 157–158.
43. **Strych, U., S. Wohlfarth, and U. K. Winkler.** 1994. Orotidine-5'-monophosphate decarboxylase from *Pseudomonas aeruginosa* PAO1: cloning, overexpression, and enzyme characterization. *Curr. Microbiol.* **29**:353–359.
44. **van Wezel, G. P., and M. J. Bibb.** 1996. A novel plasmid vector that uses the glucose kinase gene (*glkA*) for the positive selection of stable gene disruptants in *Streptomyces*. *Gene* **182**:229–230.
45. **Yakobson, E. A., and D. G. Guiney, Jr.** 1984. Conjugal transfer of bacterial chromosomes mediated by the RK2 plasmid transfer origin cloned into transposon Tn5. *J. Bacteriol.* **160**:451–453.
46. **Yu, D., H. M. Ellis, E. C. Lee, N. A. Jenkins, N. G. Copeland, and D. L. Court.** 2000. An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc. Natl. Acad. Sci. USA* **97**:5978–5983.
47. **Zhang, Y., F. Buchholz, J. P. Muyrers, and A. F. Stewart.** 1998. A new logic for DNA engineering using recombination in *Escherichia coli*. *Nat. Genet.* **20**:123–128.