

## *sacB*–5-Fluoroorotic Acid–*pyrE*-Based Bidirectional Selection for Integration of Unmarked Alleles into the Chromosome of *Rhodobacter capsulatus*

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**The gram-negative, purple nonsulfur, facultative photosynthetic bacterium *Rhodobacter capsulatus* is a widely used model organism and has well-developed molecular genetics. In particular, interposon mutagenesis using selectable gene cartridges is frequently employed for construction of a variety of chromosomal knockout mutants. However, as the gene cartridges are often derived from antibiotic resistance-conferring genes, their numbers are limited, which restricts the construction of multiple knockout mutants. In this report, *sacB*–5-fluoroorotic acid (5FOA)—*pyrE*-based bidirectional selection that facilitates construction of unmarked chromosomal knockout mutations is described. The *R. capsulatus pyrE* gene encoding orotate phosphoribosyl transferase, a key enzyme of the de novo pyrimidine nucleotide biosynthesis pathway, was used as an interposon in a genetic background that is auxotrophic for uracil (Ura<sup>−</sup>) and hence resistant to 5FOA (5FOA<sup>r</sup>). Although Ura<sup>+</sup> selection readily yielded chromosomal allele replacements via homologous recombination, selection for 5FOA<sup>r</sup> to replace *pyrE* with unmarked alleles was inefficient. To improve the latter step, 5FOA<sup>r</sup> selection was combined with sucrose tolerance selection using a suicide plasmid carrying the *Bacillus subtilis sacB* gene encoding levansucrase that induces lethality upon exposure to 5% (wt/vol) sucrose in the growth medium. Sucrose-tolerant, 5FOA<sup>r</sup> colonies that were obtained carried chromosomal unmarked mutant alleles of the target gene via double crossovers between the resident *pyrE*-marked and incoming unmarked alleles. The effectiveness of this double selection was proven by seeking insertion and deletion alleles of *helC* involved in *R. capsulatus* cytochrome *c* biogenesis, which illustrated the usefulness of this system as a genetic means for facile construction of *R. capsulatus* unmarked chromosomal mutants.**

The gram-negative facultative photosynthetic bacterium *Rhodobacter capsulatus* belongs to the group of  $\alpha$ -proteobacteria and is evolutionarily closely related to mitochondria (43). This bacterium grows under a variety of conditions and has well-developed genetics (17, 34). It has been used as a suitable model organism for a multitude of studies, including studies of respiration and photosynthesis, biosynthesis, and metabolism, as well as regulation of gene expression in response to various environmental stimuli (9). In *R. capsulatus*, isolation of chromosomal knockout mutations can be carried out by using gene transfer agent (GTA)-mediated interposon mutagenesis (34), and a large number of genetic and biochemical studies have benefited from this technique. However, as antibiotic resistance gene cassettes (e.g., cassettes for resistance to kanamycin [Km<sup>r</sup>], gentamicin [Gm<sup>r</sup>], spectinomycin [Sp<sup>r</sup>], or tetracycline [Tet<sup>r</sup>] in *R. capsulatus*) are used as selectable markers, the number of chromosomal knockout mutations that can be generated simultaneously in a given strain is limited. This could be a restrictive factor for generation of multiple-knockout mutations in a given strain. In addition, some interposons might be polar on the expression of the downstream genes, which might

cause undesired complications. Thus, a method to alleviate these limitations would be a valuable addition to the arsenal of genetic tools available for *R. capsulatus*.

In various organisms, genes of the pyrimidine biosynthesis pathway provide an interesting opportunity for genetic studies due to the mode of action of 5-fluoroorotic acid (5FOA), which is a bactericidal pyrimidine analogue. The key enzymes of this pathway, orotate phosphoribosyl transferase (OPRTase) (encoded by *pyrE*) and orotidine 5'-monophosphate decarboxylase (OMPdecase) (encoded by *pyrF*), are at the basis of this toxicity. 5FOA is converted to 5-fluorouridine monophosphate and can be incorporated into RNA, as well as further metabolized to 5-fluoro-2'-dUMP to act as a potent inhibitor of thymidylate synthase, causing cessation of DNA synthesis (Fig. 1). Mutants that lack either of these enzymes are unable to metabolize 5FOA and hence are resistant to 5FOA (5FOA<sup>r</sup>), but they are uracil auxotrophs (Ura<sup>−</sup>) for growth. These properties can be used for selection in both directions (i.e., Ura<sup>+</sup> and 5FOA<sup>r</sup>) and have been exploited successfully in *Saccharomyces cerevisiae* genetics (5). More recently, similar approaches have been extended to other organisms, including the thermophilic gram-negative bacterium *Thermus thermophilus* (39), the halophilic archaeon *Haloferax volcanii* (4), and the hyperthermophilic archaea *Pyrococcus abyssi* (20) and *Thermococcus kodakaraensis* (31).

We thought that the development in *R. capsulatus* of a similar 5FOA-*pyrE*-based genetic selection system might be useful for facilitating construction of unmarked chromosomal knock-

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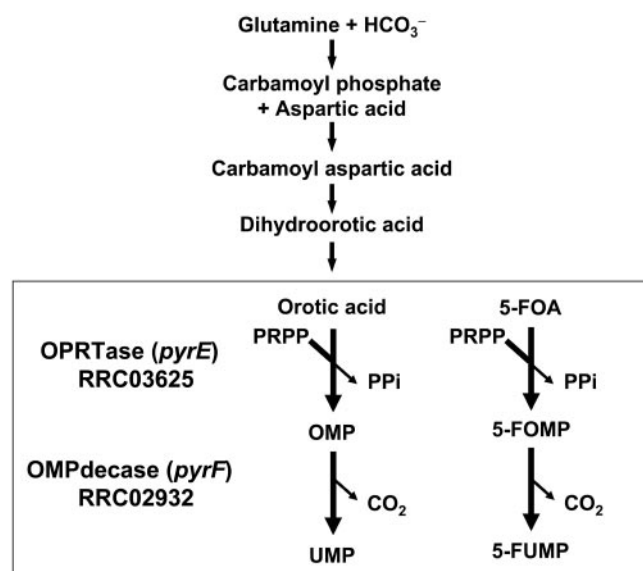


FIG. 1. Late steps of the de novo pyrimidine biosynthesis pathway of *R. capsulatus*. The last two reaction steps of the de novo pyrimidine biosynthesis pathway deduced from the *R. capsulatus* genome sequence that involve *pyrE* and 5FOA are enclosed in a box. The toxicity of 5FOA is considered to appear via its conversion to 5-fluoroorotidine monophosphate (5FOMP) and 5-fluorouridine monophosphate (5FUMP) by OPRtase and OMPdecase, respectively. OMP, orotidine 5'-monophosphate; PRPP, 5-phosphoribosylpyrophosphate; UMP, uridine monophosphate; PPI, pyrophosphate.

out mutants. Therefore, we cloned the *R. capsulatus pyrE* gene and constructed a  $\text{Ura}^-$  5FOA<sup>r</sup> mutant carrying a chromosomal insertion-deletion [ $\Delta(\text{pyrE}::\text{kan})$ ] allele. By using *pyrE* as an interposon, we then isolated a *pyrE*-marked chromosomal knockout mutation in *helC* [ $\Delta(\text{helC}::\text{pyrE})$ ], a gene that is involved in cytochrome *c* biogenesis in *R. capsulatus* (1). To facilitate the isolation of a mutant with unmarked chromosomal knockout mutations in *helC* (*helC* or  $\Delta\text{helC}$ ), we combined 5FOA<sup>r</sup> selection with sucrose lethality provided by the *Bacillus subtilis sacB* gene encoding levansucrase (7, 37). Selection for sucrose-tolerant and 5FOA<sup>r</sup> colonies in the presence of uracil readily yielded chromosomal unmarked *helC* or  $\Delta\text{helC}$  alleles, illustrating the efficiency of *sacB*-5FOA-*pyrE* selection as a useful genetic tool for facile isolation of unmarked chromosomal knockout mutants of *R. capsulatus*.

#### MATERIALS AND METHODS

**Bacterial strains and growth conditions.** The strains and plasmids used are listed in Table 1. *Escherichia coli* strains and their plasmid-harboring derivatives were grown in Luria broth (LB) containing appropriate antibiotics (ampicillin, 100  $\mu\text{g}/\text{ml}$ ; kanamycin, 50  $\mu\text{g}/\text{ml}$ ; tetracycline, 12.5  $\mu\text{g}/\text{ml}$ ; streptomycin, 25  $\mu\text{g}/\text{ml}$ ; gentamicin, 10  $\mu\text{g}/\text{ml}$ ) at 37°C. *R. capsulatus* strains were grown at 35°C in Siström's minimal medium A (MedA) (36) or enriched medium (MPYE) (17) supplemented as needed with 40  $\mu\text{g}/\text{ml}$  rifampin, 10  $\mu\text{g}/\text{ml}$  kanamycin, 2.5  $\mu\text{g}/\text{ml}$  tetracycline, or 1.25  $\mu\text{g}/\text{ml}$  gentamicin, either chemoheterotrophically in the dark (Res conditions) or photoheterotrophically in the light (Ps conditions) using anaerobic jars and BBL GasPaks. When grown in MedA, *pyrE* mutants were supplemented with 0.1 mg/ml uracil and 0.1 mg/ml Casamino Acids.

**Bacterial genetic techniques.** Plasmid pRK415 derivatives were conjugated from *E. coli* to *R. capsulatus*, and GTA-mediated crosses were performed as described previously (17). The transferable suicide plasmid pZJP29a, which carries a Gm<sup>r</sup> cassette and the *B. subtilis sacB* gene encoding levansucrase expressed from the *R. capsulatus pucAB* promoter, was a gift from C. Bauer

(Indiana University). As pZJP29a is an R6K derivative, it cannot replicate in *R. capsulatus* and becomes toxic in the presence of 5% (wt/vol) sucrose in the growth medium. This toxicity is due to the expression of *sacB* (21, 38) and the production of branched-chain fructose-derived compounds (levans) (7, 37). All pZJP29a derivatives were maintained in *E. coli* JM109( $\lambda\text{pir}$ ) and spot mated from *E. coli* S17-1( $\lambda\text{pir}$ ) into *R. capsulatus* strains on nonselective MPYE plates, using a recipient-to-donor cell ratio of 10:1. Gm<sup>r</sup> transconjugants were subsequently selected on MPYE plates containing 1.25  $\mu\text{g}/\text{ml}$  gentamicin and 40  $\mu\text{g}/\text{ml}$  rifampin, which yielded presumably single-crossover chromosomal integrants. The colonies were then subcultured in nonselective MPYE at 35°C so that there was a second homologous recombination event to excise the plasmid DNA. The culture was serially diluted in MPYE and spread onto MPYE agar plates containing either 5% (wt/vol) sucrose or 5% (wt/vol) sucrose and 0.1 mg/ml 5FOA. Colonies that appeared on these plates were then characterized in detail.

**Molecular cloning techniques.** All DNA manipulations were carried out according to standard protocols described by Sambrook et al. (29). Extraction of DNA fragments from agarose gels was performed according to the manufacturer's protocol using QIAGEN gel extraction kits. DNA sequences were determined by automated DNA sequencing with a Big-Dye terminator cycle sequencing kit (Applied Biosystems Inc., Foster City, CA) used according to the supplier's specifications by using the T7, M13 forward, and M13 reverse primers, as well as custom-designed primers, as needed.

**Southern hybridization analyses of *R. capsulatus* mutants.** *R. capsulatus* chromosomal DNA was isolated from fully grown cultures by using a QIAGEN DNeasy kit and was digested with appropriate restriction enzymes (New England Biolabs Inc., Beverly, MA). DNA fragments were separated by electrophoresis on a 0.75% (wt/vol) agarose gel and blotted onto a MAGNA nylon membrane (MSI, Westborough, MA). DNA probes were purified from appropriate plasmids after restriction enzyme digestions and agarose gel electrophoresis and were labeled with digoxigenin (DIG)-dUTP with a DIG High Prime DNA labeling kit (Roche Inc., Indianapolis, IN). DNA hybridizations were performed at 55°C. The membranes were washed twice with 2 $\times$  SSC buffer containing 0.1% sodium dodecyl sulfate (SDS) at room temperature for 10 min and twice with 0.5 $\times$  SSC buffer containing 0.1% SDS at 65°C for 15 min (1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Detection of hybridized probes was carried out by using anti-digoxigenin-alkaline phosphatase with a ready-to-use substrate (CPSD) according to the manufacturer's recommendations (Roche Inc., Indianapolis, IN).

**PCR analysis of *R. capsulatus* mutants.** Chromosomal DNA isolated using a QIAGEN DNeasy kit from appropriate *R. capsulatus* mutants was used as a template, and the *helC* locus of the chromosome was amplified using primers *helC*2 (5'-CTT ATT CTA GAT TGA TGC GAG T-3') and *helC*3 (5'-GAC GCA AGG TAC CTG ACG GCG TAT TTT C-3') (the underlining indicates newly introduced XbaI and KpnI sites, respectively) and Platinum *Taq* DNA polymerase enzyme (Invitrogen Inc., Carlsbad, CA). The PCR mixture contained 1 $\times$  reaction buffer (without MgCl<sub>2</sub>), each deoxynucleoside triphosphate at a concentration of 0.2 mM, 1.5 mM MgCl<sub>2</sub>, each PCR primer at a concentration of 1.0  $\mu\text{M}$ , 0.8 ng/ $\mu\text{l}$  chromosomal DNA, 35% (vol/vol) glycerol, and 0.05 U/ $\mu\text{l}$  *Taq* DNA polymerase. The reaction conditions were as follows: denaturation at 98°C for 4 min, 30 cycles of amplification (98°C for 0.5 min, 55°C for 1 min, and 72°C for 1.5 min), and one final elongation step at 72°C for 4 min. PCR products were analyzed by 1.0% (wt/vol) agarose gel electrophoresis, extracted from gels, and cloned into the pCR 2.1-TOPO vector using a TOPO TA cloning kit (Invitrogen Inc., Carlsbad, CA).

**Cloning and sequencing of the *pyrE* locus of *R. capsulatus*.** An approximately 2.0-kb DNA fragment encompassing *R. capsulatus pyrE* was PCR amplified using either *Pfu* Turbo DNA polymerase (Stratagene Inc., La Jolla, CA) or Platinum *Pfx* DNA polymerase (Invitrogen Inc., Carlsbad, CA) according to the manufacturer's protocols. As appropriate, primers PYR1FKPN (5'-GCG GTA CCC CGC CGC GCG ACG CCC GGG C-3'), PYR1RXHO (5'-GCC TCG AGG CAT CCG CGC GGT CAA CCG G-3'), PYR2FCLA (5'-CGA TCG ATG GCA CCA CGG TCG AAA AGA G-3'), PYR2RBAM (5'-GCG GAT CCC CAC AGG CGT ATC CCC GGG G-3'), PYR3FXHO (5'-GCC TCG AGG AGC GCC CCG CGC GCC TGG C-3'), and PYR3RXBA (5'-GCT CTA GAG CGC GCC CTC GGT CTT GAC G-3') (the underlining indicates newly introduced KpnI, XhoI, ClaI, BamHI, XhoI, and XbaI sites, respectively) were employed as follows. Amplification using PYR1FKPN and PYR1RXHO yielded a 726-bp DNA fragment, which contained 560 bp of the 3' end of *pyrC*, the 106-bp intergenic region between *pyrC* and *pyrE*, and the 54-bp 5' end of *pyrE*. Using PYR2FCLA and PYR2RBAM, a 782-bp DNA fragment that contained the entire *pyrE* gene and its 60-bp upstream region was obtained. With PYR3FXHO and PYR3RXBA, a 756-bp DNA fragment which consisted of 43 bp of the *pyrE* 3' end, the 245-bp intergenic region between *pyrE* and *dnaB*, and the 462-bp 5'

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Relevant phenotype	Reference or source
<i>E. coli</i> strains			
HB101	$F^-$ <i>mcrB mrr hsdS20</i> ( $r_B^-$ $m_B^-$ ) <i>recA13 leu ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20</i> ( $Sm^r$ ) <i>supE44</i> $\lambda$		29
DH5 $\alpha$	<i>hsdR17</i> ( $r_K^-$ $m_K^+$ ) <i>deoR thi-1 phoA supE44 <math>\lambda^-</math> <i>gyrA96 relA1</i></i>	Tet <sup>r</sup>	Invitrogen Stratagene
XL1-Blue	$F^-::Tn10$ (Tet <sup>r</sup> ) <i>proA^- B^+</i> <i>lacI<sup>s</sup></i> ( <i>lacZ</i> )M15/ <i>recA1 endA1 gyrA96(Nal<sup>r</sup>) thi-1 hsdR17</i> ( $r_K^-$ $m_K^+$ ) <i>glnV44 relA1 lac</i>		22
JM109( $\lambda$ pir)	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 <math>\Delta</math>(<i>lac-proAB</i>)F' [<i>traD36 proAB^+ laqI<sup>s</sup></i> <i>lacZAM15</i>] <math>\lambda</math>pirR6K</i>		27
S17-1 ( $\lambda$ pir)	<i>thi pro hsdR hsdM^+ recA::</i> (RP4-2-Tc::Mu-Km::Tn7) $\lambda$ pir	Sm <sup>r</sup> Tp <sup>r</sup> St <sup>r</sup>	
<i>R. capsulatus</i> strains			
MT1131	<i>crtD121</i> Rif <sup>r</sup>	Wild type	35
Y262	GTA overproducer		44
TJK11	<i>crtD121</i> Rif <sup>r</sup> $\Delta$ ( <i>pyrE::kan</i> )	Ps <sup>+</sup> Nadi <sup>+</sup> PyrE <sup>-</sup> Km <sup>r</sup>	This study
TJK11R	<i>crtD121</i> Rif <sup>r</sup> $\Delta$ ( <i>pyrE::kan</i> )	Ps <sup>+</sup> Nadi <sup>+</sup> PyrE <sup>-</sup> Km <sup>r</sup>	This study
TCK1	<i>crtD121</i> Rif <sup>r</sup> $\Delta$ ( <i>pyrE::kan</i> ) <i>helC::pyrE</i>	Ps <sup>-</sup> Nadi <sup>-</sup> , all cytochrome <i>c^-</i> , Km <sup>r</sup>	This study
TCK2	<i>crtD121</i> Rif <sup>r</sup> $\Delta$ ( <i>pyrE::kan</i> ) <i>helC::pyrE</i>	Ps <sup>-</sup> Nadi <sup>-</sup> , all cytochrome <i>c^-</i> , Km <sup>r</sup>	This study
TCK4	<i>crtD121</i> Rif <sup>r</sup> $\Delta$ ( <i>pyrE::kan</i> ) <i>helC</i>	Ps <sup>-</sup> Nadi <sup>-</sup> , all cytochrome <i>c^-</i> , Km <sup>r</sup>	This study
TCK5	<i>crtD121</i> Rif <sup>r</sup> $\Delta$ ( <i>pyrE::kan</i> ) $\Delta$ <i>helC</i>	Ps <sup>-</sup> Nadi <sup>-</sup> , all cytochrome <i>c^-</i> , Km <sup>r</sup>	This study
TRK1	<i>crtD121</i> Rif <sup>r</sup> $\Delta$ ( <i>pyrE::kan</i> ) <i>helC::pyrE</i> , pZJDhelABCDX integrated	Ps <sup>+</sup> Nadi <sup>+</sup> Km <sup>r</sup> Gm <sup>r</sup>	This study
TRK2	<i>crtD121</i> Rif <sup>r</sup> $\Delta$ ( <i>pyrE::kan</i> ) <i>helC::pyrE</i> , pZJD $\Delta$ helC integrated	Ps <sup>-</sup> Nadi <sup>-</sup> , all cytochrome <i>c^-</i> , Km <sup>r</sup> Gm <sup>r</sup>	This study
Plasmids			
pRK415	Broad-host-range plasmid	Tet <sup>r</sup>	8
pCHB500	pRK415 with a <i>cycA</i> promoter	Tet <sup>r</sup>	3
pRK2013	Helper	Km <sup>r</sup>	8
pUC4K	Kanamycin cassette	Amp <sup>r</sup> Km <sup>r</sup>	Amersham
pCR-Blunt	pCR-Blunt II-TOPO	Amp <sup>r</sup>	Invitrogen
pBluescript	pBluescript II SK(+)	Amp <sup>r</sup>	Stratagene
pTZ18U	Mobilizable suicide vector	Gm <sup>r</sup>	BioRad Laboratories
pZJD29a	pCR-Blunt with 782-bp PCR product of <i>R. capsulatus pyrE</i> including 60 bp of its upstream sequence	Km <sup>r</sup>	38
pCR-PyrE	pCR-Blunt with 737-bp PCR product of <i>R. capsulatus pyrE</i> with Shine-Dalgarno sequence	Km <sup>r</sup>	This study
pCR-PyrESD	pCR-Blunt with 718-bp PCR product of <i>R. capsulatus pyrE</i> without Shine-Dalgarno sequence	Km <sup>r</sup>	This study
pCR-PyrC	pCR-Blunt with 726-bp KpnI-XhoI PCR product of <i>R. capsulatus pyrC</i>	Km <sup>r</sup>	This study
pCR-DnaB	pCR-Blunt with 756-bp XhoI-XbaI PCR product of <i>R. capsulatus dnaB</i>	Km <sup>r</sup>	This study
pPyrE01	pBluescript with the ClaI-BamHI fragment of pCR-PyrE	Amp <sup>r</sup>	This study
pPyrE02	pBluescript with the EcoRI fragment of pCR-PyrE	Amp <sup>r</sup>	This study
pRK-PyrE-PB	pRK415 with the PstI-BamHI fragment of pPyrE02	Tet <sup>r</sup>	This study
pRK-PyrE-PH	pRK415 with the PstI-HindIII fragment of pPyrE02	Tet <sup>r</sup>	This study
pRK-PyrENde	pRK415 with the BamHI-PstI fragment of pCR-PyrENde	Tet <sup>r</sup>	This study
pRK-PyrESD	pRK415 with the BamHI-PstI fragment of pCR-PyrESD	Tet <sup>r</sup>	This study
pAPyrE::Km	pBluescript with the <i>pyrC-Km-dnaB</i> fragment	Amp <sup>r</sup>	This study
pRK- $\Delta$ PyrE::Km	pRK415 with the KpnI-XbaI fragment of pAPyrE::Km	Tet <sup>r</sup>	This study
p2helABCDX	pUC118 with <i>R. capsulatus helABCDX</i> cluster	Amp <sup>r</sup>	This study
pCS1530	pRK415 with 830-bp XbaI-KpnI PCR product containing <i>R. capsulatus helC</i>	Tet <sup>r</sup>	R. Kranz
pCS1650#3	pCS1530 with the <i>pyrE</i> cassette in the BamHI site of <i>helC</i> gene (polar orientation)	Tet <sup>r</sup>	30
pCS1650#4	pCS1530 with the <i>pyrE</i> cassette in the BamHI site of <i>helC</i> gene (nonpolar orientation)	Tet <sup>r</sup>	This study
pZJD1652	pCS1530 with a frameshift at the BamHI site of <i>helC</i> gene	Tet <sup>r</sup>	This study
pZJDhelABCDX	pZJD29a with the SacI-XbaI 3.5-kb fragment of pUChelABCDX	Gm <sup>r</sup>	This study
pZJD $\Delta$ helC	pZJD29a with the SacI-XbaI 830-bp fragment of pCS1652	Gm <sup>r</sup>	This study
pZJDhelABACDX	pZJDhelABCDX with 250-bp in-frame deletion in <i>helC</i>	Gm <sup>r</sup>	This study

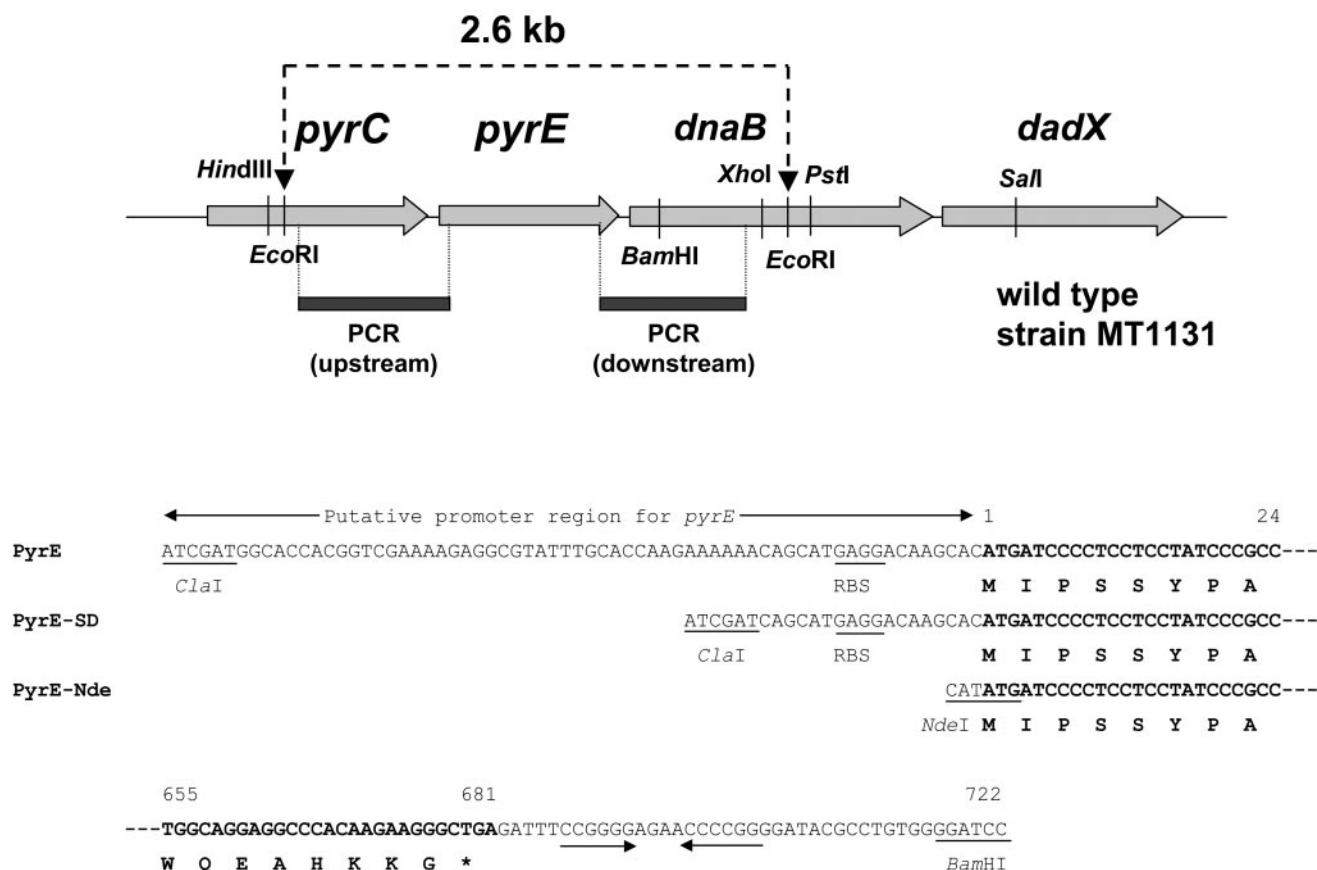


FIG. 2. *R. capsulatus pyrE* locus and *pyrE* gene cassettes. (Top panel) Physical map of the *pyrE* locus of the wild-type *R. capsulatus* chromosome. *pyrC* (RRC03624) encoding a second copy of dihydroorotic acid synthase and *dnaB* (RRC03625) encoding a probable replicative DNA helicase plus *dadX* (RRC03627) encoding alanine racemase are present upstream and downstream of *pyrE*, respectively. Two flanking DNA segments encompassing *pyrC* and *dnaB*, as indicated by solid bars, were amplified by PCR and used to construct the knockout plasmid pRK-Δ*PyrE*::Km. Restriction enzyme recognition sites for BamHI, EcoRI, HindIII, PstI, SalI, and XhoI are indicated. Digestion of the wild-type chromosomal DNA with EcoRI generated a 2.6-kb fragment containing the 3' half of *pyrC*, all of *pyrE*, and the 5' half of *dnaB*, as indicated. (Bottom panel) Molecular structure of *R. capsulatus pyrE* gene cassettes. The 5'- and 3'-terminal DNA sequences of the three *pyrE* gene cassettes are shown. The coding region is indicated by boldface type, and the putative ribosome binding sequences (RBS) are underlined. The ClaI, NdeI, and BamHI restriction enzyme recognition sites are also indicated. The inverted arrows indicate a hairpin structure found immediately after the translation stop codon of *pyrE*, which might form a stem-loop structure with a predicted Δ*G*<sup>o</sup> of -14.9 kcal/mol.

end of *dnaB*, was amplified. These three PCR products overlapped each other, yielding a total of 2,050 bp of DNA sequence surrounding *pyrE*. Appropriate PCR products were cloned into the pCR-Blunt II TOPO vector using a Zero Blunt TOPO PCR cloning kit (Invitrogen Inc., Carlsbad, CA) (Table 1), and their DNA sequences were determined and compared with that of the *R. capsulatus* genome to avoid any possible genome sequencing errors.

**Construction of a Δ(*pyrE*::*kan*) knockout allele and various *pyrE* cassettes.** First, the 726-bp fragment of pCR-PyrC was restricted with KpnI and XhoI and ligated into the respective sites of pBluescript II, yielding pBluescript(*pyrC*), into which the 756-bp fragment of pCR-DnaB restricted with XhoI and XbaI was ligated at the corresponding sites to obtain pBluescript(*pyrC*-DnaB). A Km<sup>r</sup> cassette was then digested from vector pUC4K as a SalI fragment and inserted at the compatible XhoI site of pBluescript(*pyrC*-DnaB). A plasmid containing the Km<sup>r</sup> cassette in the same orientation as the *pyrC*, *pyrE*, and *dnaB* genes was selected and designated pΔ*PyrE*::Km, from which a DNA fragment containing *pyrC*, *pyrE*::*kan*, and *dnaB* was obtained after digestion with KpnI and XbaI and transferred into the corresponding sites of pRK415 (Table 1) to obtain plasmid pRK-Δ*PyrE*::Km.

Aside from the *pyrE* cassette, which contains 60 bp of the 5' upstream sequence of the *pyrE* start codon and hence encompasses its own expression region as described above, two additional variants, *pyrE*-SD and *pyrE*-Nde, were also constructed. *pyrE*-SD contained only 20 bp of the 5' upstream sequence of the *pyrE* start codon, including a putative ribosome binding sequence, which was

amplified by PCR using primers PYR2FSD (5'-GCA TCG ATA CAG CAT GAG GAC AAG CAC-3') and PYR2RBAM (5'-GCG GAT CCC CAC AGG CGT ATC CCC GGG G-3') (the underlining indicates newly introduced ClaI and BamHI sites, respectively). *pyrE*-Nde contained only the coding sequence of *pyrE* starting at the first ATG, which was amplified by PCR using primers PYR2FNDE (5'-GTC ATA TGA TCC CCT CCT CCT ATC-3'; the underlining indicates a newly introduced NdeI site) and PYR2RBAM. *pyrE*-SD and *pyrE*-Nde were both cloned into the pCR-Blunt II TOPO cloning vector, as shown in Table 1.

**Construction of *helC*::*pyrE* and Δ*helC* knockout alleles.** A *pyrE*-marked knockout allele of *R. capsulatus helC* was constructed by inserting the 790-bp *pyrE* cassette, retrieved from p*PyrE*02 by BamHI digestion, into the unique BamHI site in a 850-bp *helC* gene fragment of the pRK415 derivative pCS1530 (30) (Table 1). This yielded two marked knockout alleles of *helC* (*helC*::*pyrE* in pCS1650#3 and pCS1650#4), in which *pyrE* was inserted in different orientations (Table 1). The unmarked allele of *helC* was constructed by self-ligation of pCS1530 after its unique BamHI site was blunted with T4 DNA polymerase, yielding pCS1652, which contained a frameshift mutation in *helC*, as verified by DNA sequencing. Finally, the suicide plasmids pZJD*helABC*DX and pZJDΔ*helC* were obtained by transferring the SacI and XbaI fragments of p2*helABC*DX (Table 1), containing the entire *R. capsulatus helABC*DX genes, and pCS1652, respectively, into the corresponding sites of pZJD29a.

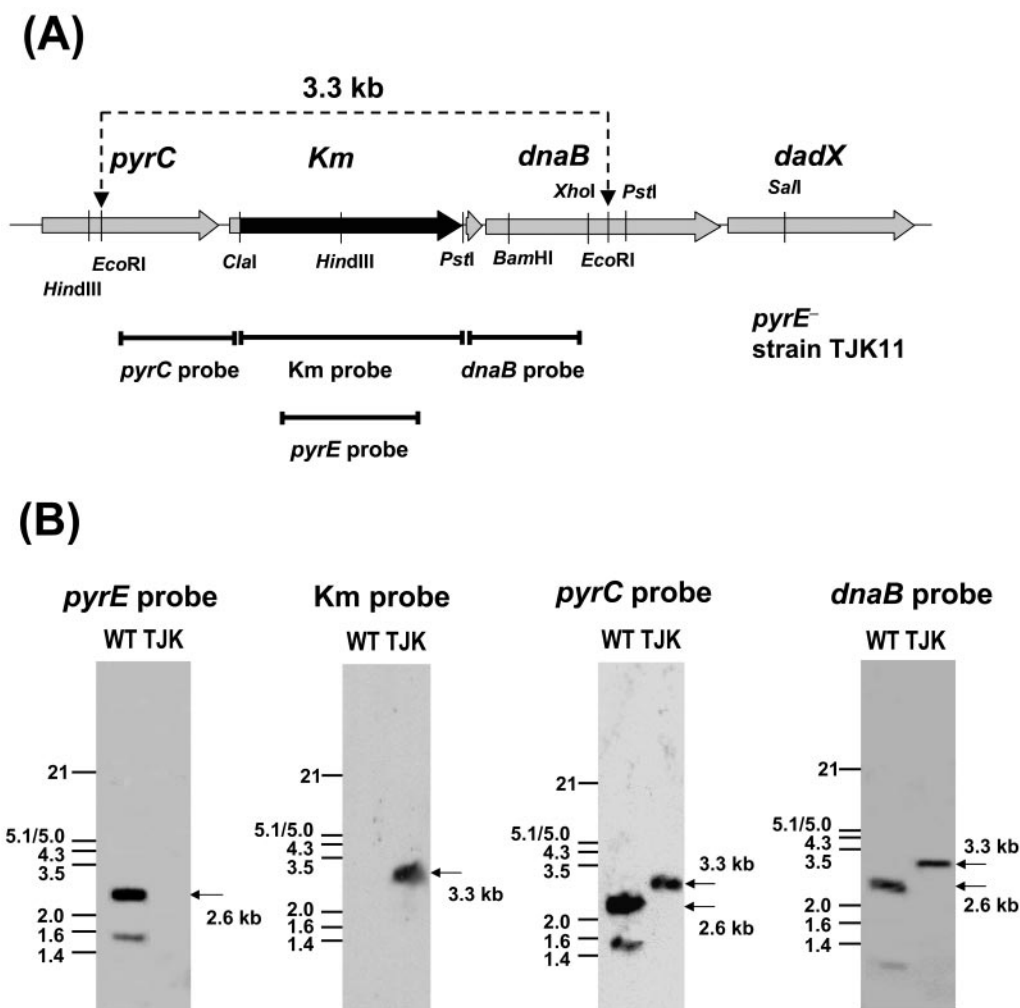


FIG. 3. Southern hybridization analyses of TJK11 [ $\Delta(pyrE::kan)$ ]. (A) Physical map of the *pyrE* locus in TJK11. A 2.6-kb wild-type *pyrE* allele of *R. capsulatus* MT1131 was replaced in TJK11 by a  $\Delta(pyrE::kan)$  allele via GTA crosses. Hybridization probes used for the Southern hybridization analyses are indicated by solid bars. In TJK11, EcoRI digestion generated a 3.3-kb DNA fragment containing the 3' half of *pyrC*, the entire  $Km^r$  gene cassette, and the 5' half of *dnaB*, which was 0.7 kb longer than its counterpart in the wild-type strain (see Fig. 2). (B) Southern hybridization analyses of *R. capsulatus* wild-type strain MT1131 (WT) and PyrE null mutant TJK11 (TJK). Genomic DNA was isolated from MT1131 and TJK11, digested with EcoRI, and analyzed by using DIG-labeled *pyrE*, kanamycin ( $Km$ ), *pyrC*, and *dnaB* gene probes. The molecular sizes of the positive blots are indicated by arrows.

**Biochemical techniques.** Cytoplasmic membrane vesicles or chromatophores were prepared in 20 mM morpholinepropanesulfonic acid (MOPS)-KOH buffer (pH 7.0) containing 1 mM KCl, 5 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride using a French pressure cell as described previously (15). Protein concentrations were determined by the method of Bradford (6) using the Bio-Safe Coomassie blue stain from Bio-Rad Laboratories (Hercules, CA) or a BCA kit from Pierce Biotechnology Inc. (Rockford, IL). Membrane proteins were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (16.5% T, 6% C) as described by Schägger and von Jagow (32), and the *c*-type cytochromes were revealed by their endogenous peroxidase activities using tetramethylbenzidine and  $H_2O_2$  (41). The cytochrome *c* oxidase activity of the colonies was detected colorimetrically using the Nadi stain, as described by Keilin (16).

For determination of the OPRase activity, cytoplasmic fractions prepared from *R. capsulatus* cells grown at 35°C to the late exponential phase in 5 ml of MedA were used. Cells were harvested in microtubes by centrifugation at 14,000 rpm for 15 min, washed once in 50 mM MOPS-KOH buffer (pH 7.0) containing 100 mM KCl and 1.0 mM phenylmethylsulfonyl fluoride, resuspended in 1 ml of the same buffer, and disrupted by ultrasonication with a Sonifier (Misonix, New York) in the 1-s pulse mode at 15% power output for a total of 1 min on ice. The treated suspensions were then ultracentrifuged in a 75Ti rotor at  $184,000 \times g$  for 60 min, and the cleared supernatant was used for enzyme assays. OPRase

activity was measured as described by Beckwith et al. (2) with a reaction mixture containing 20 mM Tris-HCl (pH 8.0), 2 mM  $MgCl_2$ , 30 mM sodium orotidylic acid, 100  $\mu M$  5-phosphoribosylpyrophosphate, and 0.6 U/ml yeast OMPdecase (Sigma, St. Louis, MO). The reaction was initiated by adding cytoplasmic fractions prepared from appropriate *R. capsulatus* strains, and changes in absorbance at 295 nm were monitored spectrophotometrically at 25°C. The OPRase activity was determined using the initial reaction rate and an extinction coefficient of  $3.67 \mu M^{-1} cm^{-1}$ . One unit of enzyme was defined as the amount that removed 1  $\mu mol$  of orotate per h, and the specific activity was expressed in units of enzyme per mg of cytoplasmic proteins.

## RESULTS

**De novo pyrimidine biosynthesis pathway of *R. capsulatus* and *pyrE*.** Examination of the *R. capsulatus* genome (<http://ergo.integratedgenomics.com>) revealed that nine open reading frames have been annotated to encode the six essential enzymes of the de novo pyrimidine nucleotide biosynthesis pathway. These genes are not contiguous on the chromosome

TABLE 2. Phenotypes of various *R. capsulatus* *pyrE* and *helC* mutant strains and their derivatives

Strain <sup>a</sup>	Plasmid <sup>a</sup>	MedA <sup>b</sup>		MedA + uracil <sup>b</sup>		MedA + uracil + 5FOA (Res) <sup>b</sup>	Nadi <sup>d</sup>
		Res <sup>c</sup>	Ps <sup>c</sup>	Res	Ps		
MT1131 (wild type)	None	+	+	+	+	–	+
TJK11 [ $\Delta$ ( <i>pyrE::kan</i> )]	None	–	–	+	+	+	+
	pRK-PyrE-PB	+	+	+	+	–	ND <sup>f</sup>
	pRK-PyrE-PH	+	+	+	+	–	ND
	pRK-PyrESD	–	–	+	+	+	ND
	pRK-PyrENde	–	–	+	+	+	ND
	pCS1650#3	+	+	+	+	–	ND
	pCS1650#4	+	+	+	+	–	ND
TCK1 or TCK2 [ $\Delta$ ( <i>pyrE::kan</i> ) <i>helC::pyrE</i> ] <sup>e</sup>	None	+	–	+	–	–	–
	pCS1650#3	+	–	+	–	ND	–
	pCS1650#4	+	–	+	–	ND	–
	pCS1530	+	+	+	+	ND	+
TCK4 [ $\Delta$ ( <i>pyrE::kan</i> ) <i>helC</i> ] <sup>e</sup>	None	–	–	+	–	+	–
	pCS1650#3	+	–	+	–	–	–
	pCS1650#4	+	–	+	–	–	–
	pCS1530	–	–	+	+	ND	+
TCK5 [ $\Delta$ ( <i>pyrE::kan</i> ) $\Delta$ <i>helC</i> ] <sup>e</sup>	None	–	–	+	–	+	–

<sup>a</sup> The strains and the plasmids that they carry are described in Table 1.

<sup>b</sup> MedA was the minimal growth medium. MedA + uracil contained 0.1 mg/ml uracil. MedA + uracil + 5FOA contained 0.1 mg/ml uracil and 0.1 mg/ml 5FOA.

<sup>c</sup> Res and Ps indicate respiratory and photosynthetic growth conditions, respectively. +, strain was able to grow; –, strain was not able to grow.

<sup>d</sup> The Nadi reaction was examined for the strains grown on MedA containing uracil under Res conditions. +, Nadi reaction; –, no Nadi reaction.

<sup>e</sup> Strains TCK1 and TCK2 are phenotypically identical, although they differ genotypically. The same is true for TCK4 and TCK5 (for details see Materials and Methods).

<sup>f</sup> ND, not determined.

with the exception of *pyrC* (RRC03624), which encodes a second copy of dihydroorotic acid synthase, and *pyrE* (RRC03625), which encodes OPRTase (Fig. 2). Inactivation of *pyrE* or *pyrF* (RRC02932), which encodes orotic acid monophosphate decarboxylase and catalyzes the last step of the de novo pyrimidine biosynthesis pathway, is known to result in 5FOA<sup>r</sup> and uracil auxotrophy (Ura<sup>–</sup>) in some organisms (4, 5, 20, 31, 39) (Fig. 1). Whether this is also the case in *R. capsulatus* was tested by determining the inhibitory effect of 5FOA on aerobic growth of wild-type strain MT1131 in the dark on MedA plates containing 0, 0.1, 0.2, 0.3, 0.5, and 1.0 mg/ml 5FOA. In the presence of 0.2 mg/ml 5FOA or a higher concentration, growth of wild-type *R. capsulatus* was significantly inhibited for 3 to 4 days, after which 5FOA<sup>r</sup> colonies appeared, especially in the presence of 0.1 mg/ml of uracil and 0.1 mg/ml of Casamino Acids, at a frequency of  $\sim 10^{-7}$ . In any event, the sensitivity to 5FOA suggested that *R. capsulatus* contains a de novo pyrimidine biosynthesis pathway similar to that of *E. coli* (24, 25).

**$\Delta$ (*pyrE::kan*) knockout mutants of *R. capsulatus* are Ura<sup>–</sup> and 5FOA<sup>r</sup>.** In order to examine whether an *R. capsulatus* *pyrE* knockout mutant is indeed Ura<sup>–</sup> and 5FOA<sup>r</sup>, two DNA fragments flanking *pyrE* located 103 bp downstream from *pyrC* and 250 bp upstream of *dnaB* (RRC03625), which has been annotated as the replicative DNA helicase gene (Fig. 2), were amplified by PCR using MT1131 chromosomal DNA as a template. The PCR products were assembled with a kanamycin gene cassette to construct a *pyrE* knockout plasmid, pRK- $\Delta$ *pyrE::Km*, carrying a  $\Delta$ (*pyrE::kan*) allele, which was then integrated into the chromosome of MT1131 via GTA crosses with selection for Km<sup>r</sup> colonies on MedA plates supplemented with 0.1 mg/ml uracil. One Km<sup>r</sup> and Ura<sup>–</sup> colony, TJK11, was

retained (Table 1) and examined by Southern hybridization analysis (Fig. 3). The data indicated that the 2.6-kb EcoRI DNA fragment carrying the wild-type *pyrE* allele in MT1131 was replaced in TJK11 with a 3.3-kb DNA fragment corresponding to the expected  $\Delta$ (*pyrE::kan*) allele, whereas the upstream and downstream regions containing *pyrC* and *dnaB*, respectively, remained intact.

As expected, TJK11 was 5FOA<sup>r</sup> (up to a concentration of 1 mg/ml) and exhibited virtually no OPRTase activity. In addition to uracil, uridine (0.5 mg/ml) could also support the growth of TJK11, whereas 5'-UMP, adenosine, guanosine, cytosine, thymine, and 5'-GMP could not support growth (data not shown). In liquid MedA, the growth of TJK11 was significantly poorer than it was on agar plates supplemented with uracil alone. Addition of Casamino Acids (1.0 mg/ml) or yeast extract (1.0 mg/ml) improved the growth, which still depended on supplementation with uracil. The additional requirement of an *R. capsulatus* *pyrE* mutant for Casamino Acids or yeast extract is intriguing, but this issue was outside the scope of this work and was not pursued further.

**Complementation of TJK11 [ $\Delta$ (*pyrE::kan*)] to Ura<sup>+</sup> and 5FOA<sup>s</sup> phenotypes by *pyrE*.** Three different constructs, designated PyrE, PyrE-SD, and PyrE-Nde, as described in Materials and Methods, were used to complement the Ura<sup>–</sup> and 5FOA<sup>r</sup> mutant TJK11. One of these, PyrE-Nde, contained only the coding sequence of *pyrE* from its putative initiating codon ATG at position 1, while PyrE-SD and PyrE carried an additional 18 bp (encompassing a putative ribosome binding site) and 60 bp (including a putative promoter sequence) 5' upstream of this ATG, respectively (Fig. 2). Only plasmids containing PyrE but not PyrE-SD and PyrE-Nde were able to render TJK11 Ura<sup>+</sup> and 5FOA<sup>s</sup> (Table 2), and only the strains

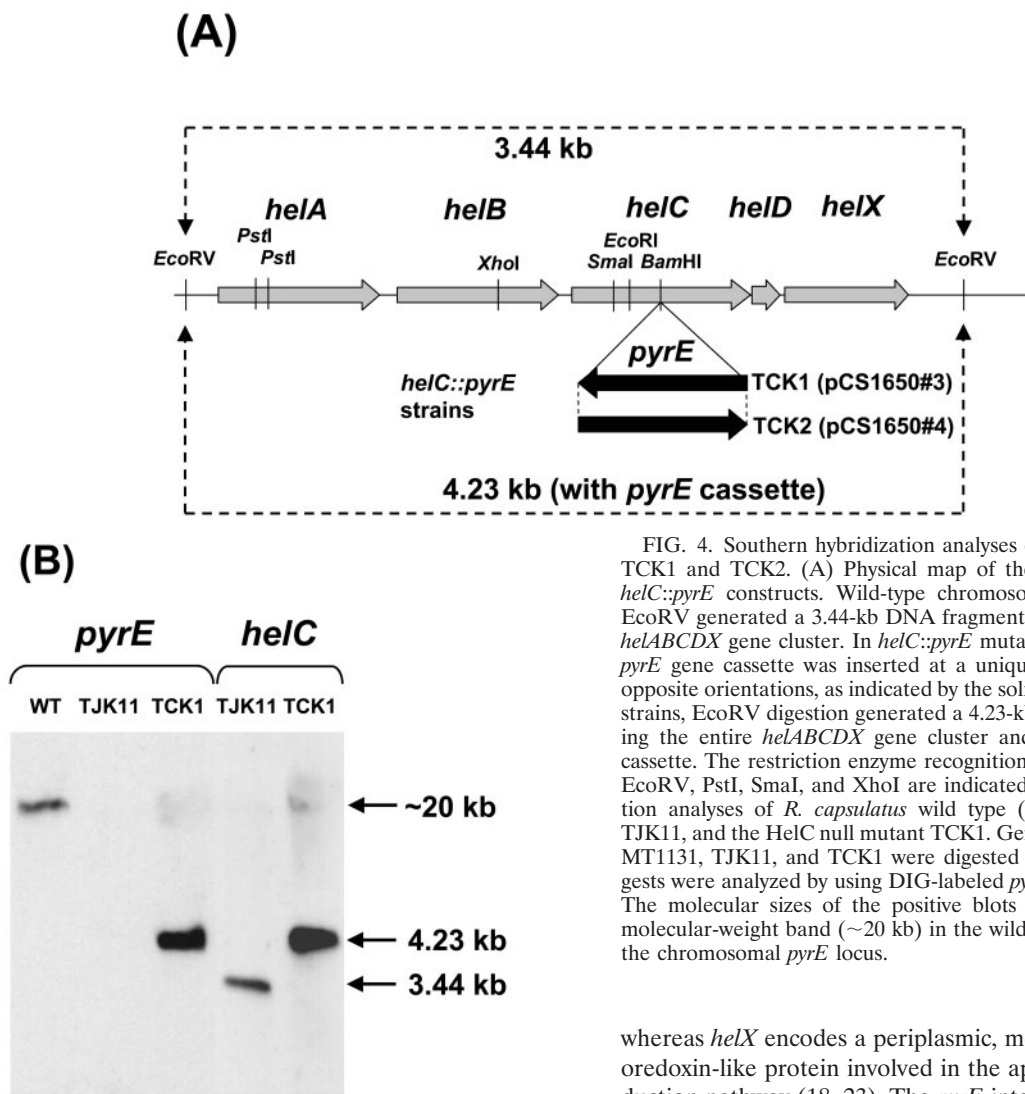


FIG. 4. Southern hybridization analyses of the *helC::pyrE* mutants TCK1 and TCK2. (A) Physical map of the *hel* gene cluster of the *helC::pyrE* constructs. Wild-type chromosomal DNA digested with *EcoRV* generated a 3.44-kb DNA fragment that contained the entire *helABCDX* gene cluster. In *helC::pyrE* mutants TCK1 and TCK2, the *pyrE* gene cassette was inserted at a unique *Bam*HI site of *helC* in opposite orientations, as indicated by the solid arrows. In these mutant strains, *EcoRV* digestion generated a 4.23-kb DNA fragment containing the entire *helABCDX* gene cluster and the inserted *pyrE* gene cassette. The restriction enzyme recognition sites for *Bam*HI, *Eco*RI, *Eco*RV, *Pst*I, *Sma*I, and *Xho*I are indicated. (B) Southern hybridization analyses of *R. capsulatus* wild type (WT), the *PyrE*<sup>-</sup> mutant TJK11, and the *HelC* null mutant TCK1. Genomic DNA isolated from MT1131, TJK11, and TCK1 were digested with *EcoRV*, and the digests were analyzed by using DIG-labeled *pyrE* and *helC* gene probes. The molecular sizes of the positive blots are indicated. The high-molecular-weight band (~20 kb) in the wild-type lane corresponds to the chromosomal *pyrE* locus.

harboring *PyrE* exhibited levels of OPRTase activity (0.26 to 0.31  $\mu\text{mol/h/mg}$  of proteins) similar to those found in MT1131 (0.39  $\mu\text{mol/h/mg}$  of proteins). Moreover, TJK11 was complemented with both plasmid pRK-PyrE-PB and plasmid pRK-PyrE-PH, which harbored *pyrE* in opposite orientations. Therefore, the data indicated that the *Ura*<sup>-</sup> and 5FOA<sup>+</sup> phenotypes of TJK11 were due to inactivation of *pyrE* and that the DNA fragment carrying the 60-bp 5' upstream sequence was necessary and sufficient to mediate *pyrE* expression.

**Isolation of *helC::pyrE* chromosomal knockout derivatives of TJK11.** We chose to isolate a chromosomal knockout allele of *helC* of *R. capsulatus* in order to assess the usefulness of *pyrE* as an interposon. The *helC* gene is located in the *orf124-helABCDX* cluster encoding several components required for cytochrome *c* biogenesis (1). These genes are tightly packed together, and recent work has indicated that their transcriptional organization is more complex than a simple operon structure (1, 30). The *helABCD* cluster encodes an ATP-binding cassette containing a transporter complex believed to export heme across the cytoplasmic membrane (12–14, 33),

whereas *helX* encodes a periplasmic, membrane-anchored thioredoxin-like protein involved in the apocytochrome *c* thioreduction pathway (18, 23). The *pyrE* interposon was inserted at a unique *Bam*HI site of *helC* in both orientations (pCS1650#3 and pCS1650#4) (Table 1) to obtain two *helC::pyrE* insertion alleles, which were then integrated into the chromosome of TJK11 via GTA crosses with selection for *Ura*<sup>+</sup> colonies on kanamycin-containing *MedA* plates in the absence of uracil. Two such colonies, TCK1 with pCS1650#3 and TCK2 with pCS1650#4, were retained for further analyses.

Both TCK1 and TCK2 were 5FOA<sup>s</sup> and grew slowly on enriched or appropriately supplemented minimal media under respiratory conditions; however, they were unable to grow under Ps conditions. Furthermore, they were *Nad*<sup>-</sup> and excreted intermediates of heme biosynthesis into the growth media, unless they carried a wild-type copy of *helC* harbored by pCS1530 (Table 2). These phenotypes were consistent with both TCK1 and TCK2 being *HelC*<sup>-</sup> and unable to produce *c*-type cytochromes. Indeed, Southern hybridization analyses confirmed that in these mutants, a 4.23-kb *EcoRV* fragment carrying *helC::pyrE*, which was 0.79 kb longer than the wild-type *helC* (3.44 kb) of parental strain TJK11, replaced the wild-type chromosomal *helC* (Fig. 4). Similarly, TMBZ-SDS-PAGE data revealed that TCK1 and TCK2 derivatives carrying the cloning vector pRK415 as a control were unable to produce

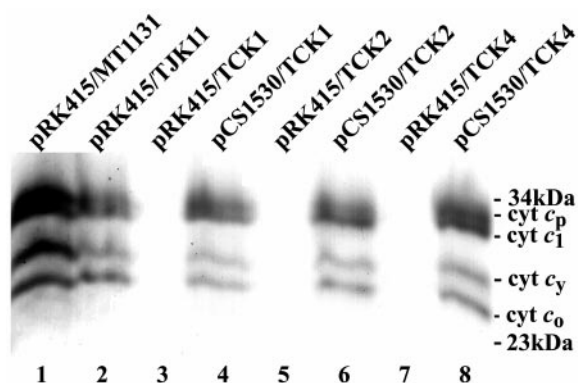


FIG. 5. TMBZ-SDS-PAGE analyses of various *R. capsulatus* strains. Chromatophore membranes prepared from *R. capsulatus* wild-type strain MT1131, the *pyrE* mutant TJK11, and the *helC* mutants TCK1, TCK2, and TCK4 (harboring either pRK415 or pCS1530 carrying an expressed *helC* gene) grown in MPYE under respiratory conditions were subjected to TMBZ-SDS-PAGE analyses as described in Materials and Methods. Approximately 75  $\mu$ g of proteins was loaded in each lane, and the positions of the molecular mass markers (in kDa) are indicated on the right. The four *c*-type cytochromes detected in this gel system correspond to cytochrome  $c_1$  (cyt  $c_1$ ) of the cytochrome  $bc_1$  complex, the membrane-anchored cytochrome  $c_y$  (cyt  $c_y$ ), and cytochromes  $c_p$  (cyt  $c_p$ ) and  $c_o$  (cyt  $c_o$ ) of the *ccb\_3*-type cytochrome *c* oxidase.

various *c*-type cytochromes (cytochromes  $c_p$ ,  $c_1$ ,  $c_o$ , and  $c_y$  in the chromatophore membranes), but the derivatives that carried pCS1530 were able to produce these cytochromes (Fig. 5). Therefore, a *pyrE* cassette can be used as an interposon to knock out a desired gene, provided that the parental strain is auxotrophic for uracil, so that Ura<sup>+</sup> selection could be applied.

**Isolation of a chromosomal unmarked  $\Delta$ *helC* knockout mutant.** The availability of a strain like TCK1 (*helC::pyrE pyrE::kan*), which is HelC<sup>-</sup> and 5FOA<sup>s</sup>, allowed us to probe whether homologous recombination between an unmarked  $\Delta$ *helC* allele and *helC::pyrE* could yield an unmarked HelC<sup>-</sup> chromosomal mutant simply by selection for 5FOA<sup>r</sup>. For this purpose, strong selection for the loss of *pyrE* was applied by combining 5FOA<sup>r</sup> with sucrose lethality conferred by the *B. subtilis* *sacB* gene. An 850-bp *helC* gene containing a frameshift mutation at its unique BamHI site (Table 1) was cloned into the suicide plasmid pZJD29a harboring *sacB* expressed via an *R. capsulatus* *pucAB* promoter. The *sacB* gene is known to be toxic to this species in the presence of 5% (wt/vol) sucrose in the growth medium (7, 21, 37, 38). As a control, pZJD*helC* ABCDX that contained a 3.46-kb DNA fragment encompassing the entire wild-type *helC* ABCDX cluster was also constructed (Fig. 6A and Table 1). These nonreplicative plasmids were conjugated into the *R. capsulatus* HelC<sup>-</sup> mutant TCK1, as described in Materials and Methods, and Rif<sup>r</sup> and Gm<sup>r</sup> colonies were obtained on appropriate MPYE plates under Res growth conditions at a frequency of about 10<sup>-3</sup>. Transconjugants TRK1 and TRK2 with pZJD*helC* ABCD and pZJD*helC*, respectively, corresponding to integrants of the nonreplicative plasmids into the chromosome, were retained for further analysis.

TRK1 was Ps<sup>+</sup> Nadi<sup>+</sup> and did not excrete porphyrin, indicating that it became HelC<sup>+</sup>, whereas TRK2 was Ps<sup>-</sup> Nadi<sup>-</sup>

and excreted porphyrin derivatives, remaining HelC<sup>-</sup> like its parent, TCK1 (data not shown). TRK1 and TRK2 were subcultured under Res growth conditions in MPYE in the absence of gentamicin and plated on enriched MPYE plates containing either 5% (wt/vol) sucrose or 5% (wt/vol) sucrose and 0.1 mg/ml 5FOA. In the case of TRK1, approximately 1,200 sucrose-tolerant and 350 sucrose-tolerant and 5FOA<sup>r</sup> colonies were obtained on the respective plates. Of the 46 sucrose-tolerant colonies tested, 39 were Gm<sup>s</sup> and 5FOA<sup>r</sup> (~85%), and 7 were Gm<sup>r</sup> and 5FOA<sup>s</sup>, and of the 52 sucrose-tolerant and 5FOA<sup>r</sup> colonies tested, 50 were Gm<sup>s</sup> and 5FOA<sup>r</sup> (~96%) and 2 were Gm<sup>r</sup> and 5FOA<sup>s</sup>. Therefore, the data indicated that single selection and double selection yielded similar results. One of the Gm<sup>s</sup> and 5FOA<sup>r</sup> colonies from the double-selection plates, which exhibited wild-type phenotypes (HelC<sup>+</sup>, Ps<sup>+</sup> Nadi<sup>+</sup>), was designated TJK11R and retained for further analysis. In the case of TRK2, 122 sucrose-tolerant colonies and only two sucrose-tolerant and 5FOA<sup>r</sup> colonies appeared on the respective plates. Of the 52 sucrose-tolerant colonies tested, only 1 was Gm<sup>s</sup> and 5FOA<sup>r</sup>. Both of the colonies that appeared on sucrose- and 5FOA-containing plates were Gm<sup>s</sup> and 5FOA<sup>r</sup>, suggesting that double selection was more efficient for identifying the clones that had evicted the integrated plasmid (i.e., Gm<sup>s</sup>) and inactivated *pyrE* (i.e., 5FOA<sup>r</sup>). One of the three Gm<sup>s</sup> and 5FOA<sup>r</sup> colonies, which were also Ps<sup>-</sup> Nadi<sup>-</sup> and excreted porphyrin derivatives (Table 2), was designated TCK4 and analyzed further.

The *helC* locus on the chromosome of TJK11R (HelC<sup>+</sup>) and TCK4 (HelC<sup>-</sup>) was compared to that of TJK11 (HelC<sup>+</sup>) by using PCR with the *helC2* and *helC3* primers (see Materials and Methods) to establish the molecular nature of the events leading to the sucrose-tolerant, Gm<sup>s</sup>, and 5FOA<sup>r</sup> phenotypes. Although an approximately 850-bp DNA fragment encompassing *helC* was amplified by using chromosomal DNA of all three strains, only the fragments from TJK11 and TJK11R contained a unique BamHI site, like the wild-type *helC* (Fig. 6B). The absence of this enzyme recognition site was further confirmed by DNA sequencing of the PCR product, which was cloned into the pCR2.1-TOPO vector (pTOPO-*helC* $\Delta$ BamHI) (data not shown). Strain TCK4 was also examined by Southern hybridization, which revealed that the 3.44-kb EcoRV DNA fragment containing *helC* ABCDX was devoid of *pyrE* (data not shown). Furthermore, TMBZ-SDS-PAGE data indicated that TCK4 was unable to produce *c*-type cytochromes, like TCK1 and TCK2, unless it was complemented with pCS1530, which carried a wild-type allele of *helC* (Fig. 5). Taken together, the overall data demonstrated that the *helC::pyrE* allele present in TCK1 was replaced in TCK4 with an unmarked  $\Delta$ *helC* allele devoid of the BamHI site. Therefore, the combined *sacB*-5FOA selection was a very efficient way to integrate unmarked alleles into the *R. capsulatus* chromosome.

## DISCUSSION

**De novo pyrimidine biosynthesis pathway of *R. capsulatus* and *pyrE*.** The *R. capsulatus* genome data indicated that the genes encoding the enzymes of the de novo pyrimidine biosynthetic pathway are scattered throughout the chromosome, except that *pyrC* and *pyrE* are adjacent to each other. Cloning and subsequent inactivation of RRC03625, which is annotated as *pyrE*, using a *kan* interposon (26, 40) yielded *R. capsulatus*



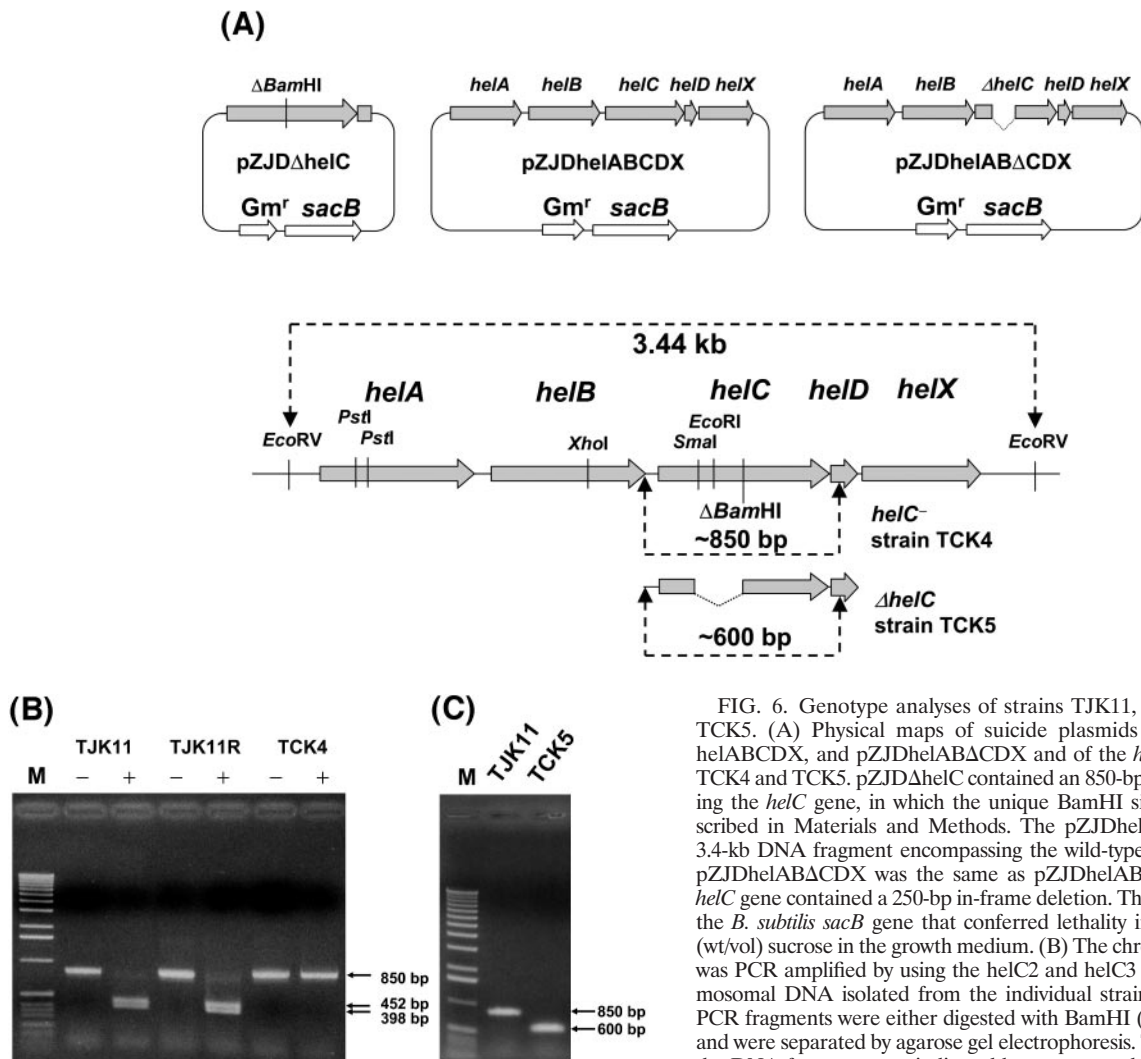


FIG. 6. Genotype analyses of strains TJK11, TJK11R, TCK4, and TCK5. (A) Physical maps of suicide plasmids pZJDΔ*helC*, pZJD*helABCDX*, and pZJD*helABΔCDX* and of the *hel* gene cluster loci of TCK4 and TCK5. pZJDΔ*helC* contained an 850-bp fragment encompassing the *helC* gene, in which the unique BamHI site was deleted as described in Materials and Methods. The pZJD*helABCDX* contained a 3.4-kb DNA fragment encompassing the wild-type *hel* gene cluster, and pZJD*helABΔCDX* was the same as pZJD*helABCDX* except that the *helC* gene contained a 250-bp in-frame deletion. These plasmids harbored the *B. subtilis sacB* gene that conferred lethality in the presence of 5% (wt/vol) sucrose in the growth medium. (B) The chromosomal *helC* region was PCR amplified by using the *helC2* and *helC3* primers and the chromosomal DNA isolated from the individual strains as a template. The PCR fragments were either digested with BamHI (+) or not treated (-) and were separated by agarose gel electrophoresis. The molecular sizes of the DNA fragments are indicated by arrows on the right. (C) The chromosomal *helC* region of TJK11 and TCK5 was amplified by PCR as described above for panel B, and the PCR products were analyzed by agarose gel electrophoresis without restriction enzyme treatment. For a description of the strains see Table 1. Lane M, DNA molecular weight marker.

strain TJK11, which is *Ura*<sup>-</sup>, *5FOA*<sup>r</sup>, and devoid of OPRase activity. As expected, TJK11 was fully complemented by an active copy of *pyrE* in *trans*, which conferred both the *Ura*<sup>+</sup> and *5FOA*<sup>s</sup> phenotypes. Therefore, RRC03625 is the only active copy of *pyrE* that is required for de novo pyrimidine biosynthesis in this species, and this metabolic pathway appears to be similar to that in *E. coli* (24, 25) and *S. cerevisiae* (19).

***pyrE* as a novel interposon in *R. capsulatus*.** The experiments reported here indicated that *R. capsulatus pyrE* is expressed autonomously and could itself be used as an interposon to inactivate any gene of interest, provided that the parental strain is *Ura*<sup>-</sup>. As interposon mutagenesis is a common tool used for construction of chromosomal knockout mutants, *pyrE* is a welcome addition to the antibiotic resistance gene cassettes that are available for *R. capsulatus* (17). Some of the antibiotic resistance-based interposons are available as transcriptionally

polar or nonpolar forms (11, 28) and can be preferentially used depending on the expected outcomes of the gene knockout experiments. The *pyrE* cassette isolated here has a hairpin structure at its 3' end, which might act as a rho-independent transcription termination signal (10, 42). Although we isolated *helC::pyrE* insertions in two opposite orientations, due to the complex operon structure of the *helABCDX* cluster with its multiple internal promoters (30), we could not deduce rigorously whether *pyrE* is a polar or nonpolar interposon with respect to transcription of the genes located downstream from its insertion site. Additional experiments and construction of variants of the *pyrE* interposon, which might be more desirable for various purposes, are required to settle this issue.

***sacB-5FOA-pyrE* as powerful bidirectional selection markers.** In knockout experiments with conventional antibiotic resistance-conferring interposons, such as *kan* and *spe*, removal

of the inserted antibiotic marker from the chromosome is often a laborious process, if it is possible at all. A clear advantage of an interposon like *pyrE* is the ease with which one can excise it from the chromosome by selecting for 5FOA<sup>r</sup> derivatives. However, it should be kept in mind that this selection does not yield exclusively *pyrE* excisions via homologous recombination, as mutations inactivating *pyrE* also confer the 5FOA<sup>r</sup> phenotype. Although in the case of *S. cerevisiae* this does not appear to be a major drawback (5), in *R. capsulatus*, in which the frequency of the 5FOA<sup>r</sup> colonies is apparently high, it becomes a potential restricting factor when the loss of *pyrE* is selected for. However, combining 5FOA<sup>r</sup> and sucrose toxicity mediated by *sacB* readily overcomes this hurdle and yields chromosomal allele replacements even when the incoming unmarked allele is carried by a homologous flanking DNA fragment as short as ~400 bp, as in the case of the  $\Delta helC$  construct used here. Conceivably, when larger DNA fragments with longer flanking homologous regions are used, even single 5FOA<sup>r</sup> might be sufficient to obtain the desired outcome without the need for *sacB*-mediated selection. Indeed, we observed that when a derivative of the suicide plasmid pZJD29a harboring the entire *helABCDX* gene cluster with a 250-bp in-frame deletion in *helC* (pZJD $\Delta$ helAB $\Delta$ CDX) was used, the desired mutants were obtained at comparable frequencies (approximately 500 colonies per plate) by selecting for either only 5FOA<sup>r</sup> colonies or 5FOA<sup>r</sup> and sucrose-tolerant colonies.

Another advantage of delivering a homologous DNA fragment via a nonreplicating plasmid instead of using GTA is the possibility that the double-crossover homologous recombination events can be divided into two parts. A first homologous recombination yields a chromosomal integrant selected using a marker carried by the nonreplicating plasmid. The chromosomal duplication generated in this way is then resolved via a second homologous recombination that can be selected for sucrose tolerance via the loss of *sacB*. Moreover, when this second step is further reinforced for the loss of *pyrE* by 5FOA<sup>r</sup> selection, then homologous replacement of a *pyrE*-marked allele of a chromosomal gene of interest by its unmarked allele becomes easy, as documented here by the construction of  $\Delta helC$ .

Finally, once the *pyrE*-marked allele of a chromosomal gene of interest is replaced by an unmarked allele, then the strain can be subjected to additional similar rounds of gene knockout experiments with the *pyrE* interposon as a selectable marker. The procedure described here could be used as many times as it is needed for introduction of various type of mutations (frameshifts, in-frame or out-of-frame deletions, site-specific mutations or tag insertions, or other mutations) into a chromosomal gene of interest. The *sacB*-5FOA-*pyrE* bidirectional selection system is, therefore, applicable to a variety of experiments, including structure-function studies of large multisubunit enzymes and elucidation of complex multicomponent pathways, and thus it is a useful addition to the well-developed arsenal of *R. capsulatus* genetic tools.

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