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Development of a Gene Knockout System for the Halophilic Archaeon *Haloferax volcanii* by Use of the *pyrE* Gene

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So far, the extremely halophilic archaeon *Haloferax volcanii* has the best genetic tools among the archaea. However, the lack of an efficient gene knockout system for this organism has hampered further genetic studies. In this paper we describe the development of *pyrE*-based positive selection and counterselection systems to generate an efficient gene knockout system. The *H. volacanii pyrE1* and *pyrE2* genes were isolated, and the *pyrE2* gene was shown to code for the physiological enzyme orotate phosphoribosyl transferase. A Δ*pyrE2* strain was constructed and used to isolate deletion mutants by the following two steps: (i) integration of a nonreplicative plasmid carrying both the *pyrE2* wild-type gene, as a selectable marker, and a cloned chromosomal DNA fragment containing a deletion in the desired gene; and (ii) excision of the integrated plasmid after selection with 5-fluoroorotic acid. Application of this gene knockout system is described.

The archaea represent one of the three fundamental divisions of life (24). Archaea have features that are present in both the eukaryotic and prokaryotic kingdoms, and this fact has been very important in understanding the evolution of cellular processes. Thus, archaeal transcription and translation can be envisaged as a mosaic of eukaryotic and bacterial elements (1). While the archaeal basal transcription machinery resembles that of RNA polymerase II of eukaryotes, the regulation of gene expression has many of the characteristics of bacterial systems (2). Particularly interesting is the fact that most of the known archaea are extremophiles, and studies of the structure-function relationships in archaeal proteins have provided valuable insights into the mechanisms that enable biochemical systems to adapt and function in extreme physiological conditions (9).

The extremely halophilic archaeon *Haloferax volcanii* is an obligate halophile that was first isolated from the Dead Sea (13). *H. volcanii* is a genetically stable prototroph that has become a model organism for molecular genetic studies of the archaea (6, 21, 25). The presence in *H. volcanii* of an efficient transformation system (5), several shuttle vectors (7, 10), and selectable markers (8, 15) has made a wide variety of molecular genetic studies possible. However, a key tool for genetic analysis, namely, the availability of an efficient gene knockout system, has been lacking. One important tool for the creation of gene knockouts is a counterselectable genetic marker. The counterselectable markers commonly used in bacteria include the *Bacillus subtilis sacB* gene, which encodes levan sucrase and confers sensitivity of many bacteria to sucrose (19), and the *glkA* gene, which encodes glucose kinase and confers sensitivity of *Streptomyces* to 2-deoxyglucose (23). In *Saccharomyces cerevisiae,* genes involved in uracil biosynthesis serve as effective counterselectable markers (3). The selection takes advantage of the fact that *S. cerevisiae* that can synthesize uracil de novo is sensitive to the toxic analogue 5-fluoroorotic acid (5-FOA), whereas mutations in the *ura5* gene, which encodes orotate phosphoribosyl transferase (OPRTase), or the *ura3* gene, which encodes orotidine-5′-phosphate decarboxylase, are resistant to 5-FOA. In bacteria the genes corresponding to *ura5* and *ura3* are *pyrE* and *pyrF*, respectively.

In this report we describe the isolation of two *H. volcanii* genes whose products exhibit homology to OPRTases and demonstrate that the *pyrE2* gene codes for the physiological enzyme. Previously (16), other workers have employed the *pyrF* gene of *Halobacterium salinarum* as a counterselectable genetic marker for creating gene knockouts. Here we show that the *H. volcanii pyrE2* gene can conveniently serve both as a selectable genetic marker and as a counterselectable genetic marker for efficiently creating gene knockouts in *H. volcanii*.

MATERIALS AND METHODS

Strains and culture conditions. The properties of the various *H. volcanii* strains used in this work are given in Table 1. *H. volcanii* was routinely grown in rich (HY) medium containing (per liter) 206 g of NaCl, 36.9 g of $MgSO_4 \cdot 7H_2O$, 5 ml of a 1 M KCl solution, 1.8 ml of a 75-mg/liter $MnCl_2$ solution, and 50 mM Tris HCl (pH 7.2). After autoclaving and cooling, 5 ml of 10% (wt/vol) $CaCl_2$ and 25 ml of filter-sterilized 20% (wt/vol) yeast extract (Difco) were added. Agar plates contained 18 g of Bacto Agar (Difco) per liter. For uracil-minus medium (CA medium), the yeast extract was replaced by Casamino Acids (Difco) at the same final concentration (0.5%, wt/vol). When needed, 5-FOA (US Biological) was added to HY medium to a final concentration of 150 μg/ml.

Escherichia coli strains DH5α, XLI, and DH12S were grown in Luria-Bertani medium. When needed, ampicillin (Sigma) was added to the medium to a final concentration of 100 μg/ml.

Transformation procedures. Transformation of halobacteria was carried out as previously described (5). *E. coli* was transformed by using the $CaCl_2$ protocol (11) or a standard electroporation protocol.

Molecular genetic methods. Restriction endonuclease digestion, agarose gel electrophoresis, and molecular cloning techniques were performed by using standard procedures (11). Isolation of total halobacterial DNA was performed as previously described (20). The oligodeoxynucleotide primers used in this study are shown in Table 2.

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TABLE 1. *H. volcanii* strains and plasmids used in this investigation

Strain or plasmid	Relevant properties	Source or reference
Strains		
WR340	His^-	—[a]
WR341	Cys^-	—
WR445	WR341/Δ*hdrA* Δ*hdrB*	15
WR472	WR445 containing pGB53 (pop-in)	This study
WR473	WR445/Δ*pyrE1*	This study
WR479	WR445/Δ*pyrE1* Δ*pyrE2*	This study
WR480	WR340/Δ*pyrE2*	This study
WR501	WR480 containing pGB72 (pop-in)	This study
WR504	WR480/Δ*cmi4*	This study
Plasmids		
pGB53	pBR-Nov containing the flanking sequences of *pyrE1*	This study
pGB68	pBR-Nov containing the flanking sequences of *pyrE2*	This study
pGB70	pUC19 containing the *pyrE2* coding region under the ferredoxin promoter	This study
pGB72	pGB70 containing the flanking sequences of *cmi4*	This study
pBR-Nov	pBR containing the halobacterial novobiocin resistance gene *gyrB*	15

[a] —, Strain obtained in our laboratory by chemical mutagenesis of strain WFD11 (4) as described by Mevarech and Werczberger (12).

(i) Southern blot analysis. The nucleotide sequence of *Halobacterium* sp. strain NRC-1 *pyrE2* was obtained from the complete genome sequence (14) (accession no. NC 002607). The *H. salinarum* S9 *pyrE2* gene was obtained by PCR amplification of the chromosomal DNA of this archaeon (obtained from Felicitas Pfeifer, Technical University, Darmstadt, Germany) by using the N-*salpyrE2* and C-*salpyrE2* primers (Table 2). Hybridization probes were labeled with a digoxigenin (DIG) DNA labeling kit (Roche Diagnostics GmbH, Mannheim, Germany). The standard hybridization buffer contained 0.6 M NaCl, 0.06 M sodium citrate, 0.1% *N*-laurylsarcosine, 0.02% sodium dodecyl sulfate, 2% blocking reagent (Roche Diagnostics GmbH), and 50% (vol/vol) formamide. Hybridization was performed at 42°C. Chemiluminescent detection of the labeled fragments was performed as described in the instructions to a DIG luminescence detection kit (Roche Diagnostics GmbH).

(ii) DNA sequence analysis. Nucleotide sequences of cloned fragments were determined by using an ABI373 automated sequencer (Perkin-Elmer ABI) as described by the supplier. DNA database searches were performed by using the National Center for Biotechnology Information Blast web site (http://www.ncbi.nlm.nih.gov/BLAST/). Multiple-sequence alignments were constructed by using the ClustalW program (22) on a web server (http://www.ch.embnet.org/software/ClustalW.html).

Plasmid construction. The plasmids used in this work are shown in Table 1, and the oligodeoxynucleotide PCR primers used are shown in Table 2.

(i) pGB53, used for creating a deletion in the *pyrE1* gene. A 1.4-kb DNA fragment containing the upstream flanking sequence of the *H. volcanii pyrE1* gene was amplified by PCR by using primers *pyrE1*-Rev and 1.4 us *pyrE1*, which contain *Bam*HI and *Cla*I restriction sites, respectively. A 1.4-kb DNA fragment containing 300 bp of the 3′ coding region of *H. volcanii pyrE1* and 1.1 kb of the downstream sequence was amplified by PCR by using primers *pyrE1*-M and ds *pyrE1*, which contain *Bam*HI and *Asp*718 restriction sites, respectively. The two PCR products were cloned by triple ligation into pBR-Nov digested with *Cla*I and *Asp*718.

(ii) pGB68, used for *pyrE2* gene deletion. An 850-bp fragment upstream from the first codon of the *H. volcanii pyrE2* gene was amplified by PCR with primers 850 us *pyrE2* and us *pyrE2*-Rev, which contain *Asp*718 and *Bam*HI restriction sites, respectively. Similarly, an 850-bp fragment containing the downstream flanking sequence of this gene was amplified by PCR with primers 850 ds *pyrE2*-Rev and ds *pyrE2*, which contain *Hin*dIII and *Bam*HI restriction sites. The two fragments were cloned by triple ligation into pBR-Nov digested with *Hin*dIII and *Asp*718.

(iii) pGB70. The coding region of *pyrE2* was amplified by PCR by using primers N-*pyrE2* and C-*pyrE2*, which contain *Nco*I and *Xba*I restriction sites, respectively. The amplified coding region was fused to the promoter region of the *Halobacterium halobium* ferredoxin gene (17) and then cloned into pUC19 digested with *Bam*HI and *Xba*I. A schematic diagram of pGB70 is shown in Fig. 1.

(iv) pGB72, used for creating a deletion in the *cmi4* gene. A 300-bp fragment upstream from the first codon of the *H. volcanii cmi4* gene was amplified by PCR with primers 300 us *cmi4* and us *cmi4*-Rev, which contain *Eco*RI and *Nco*I restriction sites, respectively. Similarly, a fragment containing the last 92 bp of

TABLE 2. Oligodeoxynucleotide primers used in this study

Primer	Sequence	Location
pyrE1-Rev	5′-CGGGATCCGTTCTTCATACAGTACC	5′ end of *pyrE1*; reverse primer
1.4 us *pyrE1*	5′-CCATCGATGTCGACGACATCGAG	1.4-kb upstream to *pyrE1*; forward primer
pyrE1-M	5′-CGGGATCCCCCGCTTGCGACCTCCGTCTC	Middle of the *pyrE1* ORF; forward primer
ds *pyrE1*	5′-GCGGTACCGTTGTCGCGCCAGTTGATTCCG	1.1-kb downstream to *pyrE1*; reverse primer
850 us *pyrE2*	5′-GGGGTACCGGGGCCTCTAATCGACGTAGGC	850 bp upstream to *pyrE2*; forward primer
us *pyrE2*-Rev	5′-CGGGATCCGCGTGGATTACCACGGCTCG	5′ end of *pyrE2*; reverse primer
ds *pyrE2*	5′-CGGGATCCCGCCGACGGCTAATACACGC	3′ end of *pyrE2*; forward primer
850 ds *pyrE2* Rev	5′-GCCAAGCTTGTGCCTATTTCTACGTCACC	850-bp downstream to *pyrE2*; reverse primer
N-*pyrE2* (*H. volcanii*)	5′-CGGCCATGGCGAACGCAGCACTCATCG	5′ end of *pyrE2* from *H. volcanii*; forward primer
C-*pyrE2* (*H. volcanii*)	5′-GCTCTAGATTAGCCGTCGGCGTCGGCCAGC	3′ end of *pyrE2* from *H. volcanii*; reverse primer
N-*salpyrE2*	5′-ATGAGTCCAACTGACGACCT	5′ end of *pyrE2* from *H. salinarum*; forward primer
C-*salpyrE2*	5′-TTACTCGGCGTCCAAGAGGTC	3′ end of *pyrE2* from *H. salinarum*; reverse primer
300 us *cmi4*	5′-CGGAATTCATCGTCACCGGAATCGGTGAG	300 bp upstream to *cmi4*; forward primer
us *cmi4*-Rev	5′-CGCCCATGGCCGGAACGTGGCACGCCC	5′ end of *cmi4*; reverse primer
ds *cmi4*	5′-CGCCCATGGACGACGACCTCTCGAACCGG	3′ end of *cmi4*; forward primer
ds *cmi4*-Rev	5′-CGCGGGGAGGTCGAACAGGC	Downstream to *cmi4*; reverse primer
415 us *cmi4*	5′-AACACCGGCGCGCCGAGACC	415-bp upstream to *cmi4*; forward primer

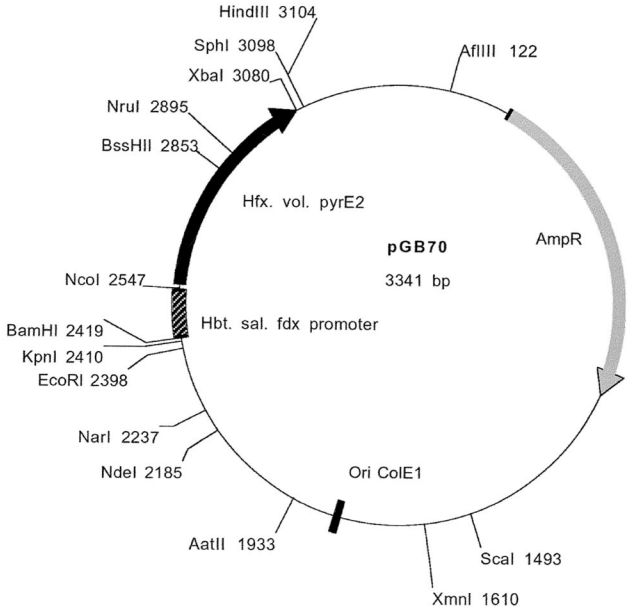

FIG. 1. Restriction map of *H. volcanii* gene knockout plasmid pGB70. Hbt. sal., *Halobacterium salinarum*.

cmi4 and 176 bp of the downstream flanking sequence of this gene was amplified by PCR with the ds *cmi4* (containing an *Nco*I restriction site) and pUC reverse primers. The template for the second PCR product was a subclone of a 1.4-kb *Bsi*WI-*Cla*I genomic fragment that contained the *cmi4* gene cloned in pUC19 digested with *Acc*I and *Asp*718. The two PCR fragments were cloned by triple ligation into pGB70 digested with *Eco*RI and *Asp*718.

Nucleotide sequence accession numbers. The nucleotide sequences of the *H. volcanii pyrE1* and *pyrE2* genes have been deposited in the EMBL nucleotide sequence database under accession numbers AJ492197 and AJ492198, respectively.

RESULTS

Cloning of *pyrE1* and its flanking sequences. When a collection of random *H. volcanii* genomic clones was sequenced, a sequence was identified in a BLAST search which exhibited homology to part of the *Halobacterium* sp. strain NRC-1 *pyrE1* gene. To clone the entire gene and the flanking sequences, *H. volcanii* chromosomal DNA was digested with the *Pst*I and *Bsi*WI restriction enzymes and analyzed by Southern blotting by using the incomplete *pyrE*-containing clone as a probe. A DNA fragment of about 3 kb was found to hybridize to the probe. A genomic DNA minilibrary of *Pst*I-*Bsi*WI 3-kb fragments was constructed and screened by using the incomplete *pyrE1* probe, and this resulted in isolation of a clone containing the entire coding region of the *H. volcanii pyrE1* homologue and its flanking sequences. As shown in Fig. 2, the deduced amino acid sequence of the protein encoded by the *pyrE1* gene of *H. volcanii* exhibits low but significant homology with the sequences of the *E. coli* and *S. cerevisiae* OPRTases. The *H. volcanii pyrE1* structural gene contains 630 bp and encodes a putative protein, PyrE1,consisting of 210 amino acids. Like other halophilic proteins, PyrE1 is acidic and contains 20% negatively charged amino acid residues and 8% positively charged residues. BLAST analysis of the sequence immediately upstream of the *pyrE1* gene revealed an open reading frame (ORF) homologous to the *gcd* gene coding for glucose dehydrogenase (18). The TGA termination codon of *gcd* overlaps the translation initiation ATG codon of the *pyrE1* gene. A similar arrangement occurs in the corresponding *Halobacterium* sp. strain NRC-1 (*H. salinarum*) (14) and *Haloferax mediterranei* DNA regions (unpublished results).

Construction of a deletion in *pyrE1* and phenotypic analysis. To determine whether the *pyrE1* gene encodes a functional OPRTase, deletion of the first 330 bp of the gene was performed by using the pop-in–pop-out method shown in Fig. 3. A 2.8-kb DNA fragment containing a deletion of the first 330 bp of the *pyrE1* gene was cloned into an *E. coli* plasmid that carries a halobacterial novobiocin resistance gene (*gyrB*) (8) to create plasmid pGB53. pGB53 was transformed into *H. volcanii* WR445 (pop in). Following transformation, novobiocin-resistant colonies were obtained, and the chromosomal DNAs of several colonies were screened by using Southern blot hybridization. One clone, in which pGB53 had integrated into the *pyrE1* flanking region, was designated WR472. Excision of pGB53 in WR472 was performed by propagating WR472 for approximately 30 generations in rich liquid medium containing uracil and lacking novobiocin, and the cultures were spread on agar plates containing the same medium. Colonies were screened by replica plating on media with and without novobiocin; novobiocin-sensitive colonies were picked, and their DNAs were analyzed by Southern blot hybridization. Excision of pGB53 by a homologous recombination event may result in either reconstitution of the wild-type allele or deletion of 330 bp from the chromosomal *pyrE1* gene (Fig. 3A). A strain in which excision of pGB53 resulted in deletion of 330 bp from the *pyrE1* gene was designated WR473 (Fig. 3B). This excision did not affect the adjacent coding region of the *gcd* gene. *H. volcanii* WR473 was found to be partially resistant to 5-FOA (it grew in HY medium containing 150 μg 5-FOA per ml but did not grow in HY medium containing 450 μg of 5-FOA per ml), and surprisingly, it did not require uracil for growth.

Cloning of *pyrE2*. Because WR473 is prototrophic for uracil and *Halobacterium* sp. strain NRC-1 possesses two *pyrE* genes (14), we supposed that *H. volcanii* might also have two *pyrE* genes. *H. volcanii* genomic DNA was digested with various combinations of restriction enzymes, and the DNA was resolved by electrophoresis and blotted onto nylon filters. The filters were hybridized to a PCR product of *H. salinarum pyrE2* labeled with DIG. A 5.7-kb *Mlu*I-*Asp*718 fragment was found to hybridize to *H. salinarum pyrE2* DNA and was subsequently cloned. The sequence of this fragment revealed that it contains the *H. volcanii pyrE2* homologue and its flanking sequence. The *H. volcanii pyrE2* structural gene is 531 bp long and encodes protein PyrE2, which has 176 amino acid residues (Fig. 2). Similar to PyrE1, PyrE2 contains 20% acidic amino acid residues and 9% basic residues. A comparison of the deduced amino acid sequences of PyrE2 and PyrE1 showed that these proteins have limited sequence similarity (Fig. 2).

Construction of a Δ*pyrE2* strain and phenotypic analysis. Deletion of the *H. volcanii pyrE2* gene was performed by using the strategy that was used to create a deletion in the *pyrE1* gene (Fig. 3). Plasmid pGB68 contains the 850-bp upstream and 850-bp downstream flanking sequences of *pyrE2* and the gene coding for novobiocin resistance as a selectable marker. The deletion was created in *H. volcanii* WR340, and the strain

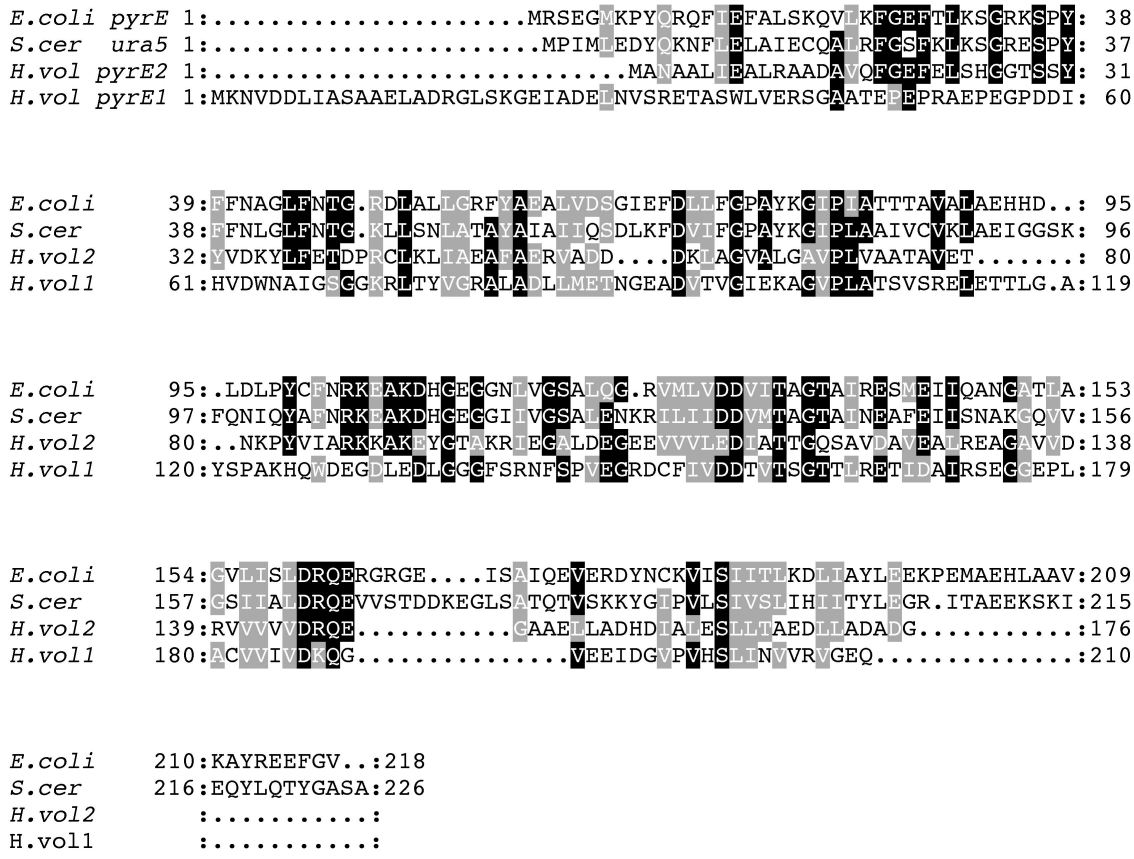

FIG. 2. Multiple alignment of the *E. coli* PyrE, *S. cerevisiae* Ura5 (*S.cer*), *H. volcanii* PyrE1 (*H.vol1*), and *H. volcanii* PyrE2 (*H.vol2*) proteins.

with *pyrE2* deleted, designated WR480, was found to be 5-FOA resistant and to require uracil for growth.

Construction of a Δ*pyrE1* Δ*pyrE2* strain and phenotypic analysis. By using the pop-in–pop-out approach, an *H. volcanii* strain containing deletions in both the *pyrE1* and *pyrE2* genes was constructed. Strain WR473 (Δ*pyrE1*) was transformed with pGB68, and an isolate containing the Δ*pyrE2* deletion was obtained. As expected, the phenotype of the Δ*pyrE1* Δ*pyrE2* strain (WR479) was similar to that of WR480 (Δ*pyrE2*) (namely, 5-FOA resistance and uracil auxotrophy).

Construction of a Δ*cmi4* mutation by using the Δ*pyrE2* strain for positive and negative selection. We used WR480 (Δ*pyrE2*), which is unable to grow without uracil and is 5-FOA resistant, and the *pyrE2* gene to develop an effective tool for creating gene knockouts in *H. volcanii*. The coding region of *pyrE2* was fused to the constitutive ferredoxin promoter, and the construct was inserted into pUC19 to obtain plasmid pGB70 (Fig. 1). pGB70 served as the basic knockout vector into which the flanking sequences of the desired gene to be deleted were cloned. To determine the efficiency of this system, we performed a knockout of the *cmi4* gene, an *H. volcanii* gene whose expression we have shown to be conditioned medium induced. Two 300-bp fragments containing the 5′ and 3′ flanking sequences of the *cmi4* gene were fused and cloned into pGB70 to create plasmid pGB72 (see Materials and Methods). Plasmid pGB72 was transformed into *H. volcanii* WR480 with selection for transformants that grew without added uracil (pop in). A single colony of one of the pop-in isolates (WR501) was inoculated into liquid medium lacking uracil and grown for 48 h. The culture was then spread onto HY medium plates containing 5-FOA. Since only Δ*pyrE2* cells can grow on 5-FOA-containing media, it was expected that only cells in which pGB72 had been excised from the chromosome could grow. Excision of pGB72 was expected to leave in the chromosome either the wild-type *cmi4* gene or a deletion of the *cmi4* gene. PCR analysis was used to analyze the genotypes of 16 isolates. As shown in Fig. 4B, in six isolates excision of pGB72 resulted in deletion of the *cmi4* gene, and in the other 10 isolates excision of pGB72 resulted in reversion to the parental *cmi4* gene. These results were confirmed by a Southern blot analysis of one of the clones (Fig. 4C). Δ*cmi4* mutants did not show any phenotype when they were grown in rich or minimal medium.

DISCUSSION

Genome analysis depends largely on the ability to assign functions to the various putative ORFs identified by DNA sequencing. For a given gene, the most straightforward approach is to create a knockout in that gene and to characterize the phenotype of the mutant. In the pop-in–pop-out approach, the first step results in integration of the vector into the chro-

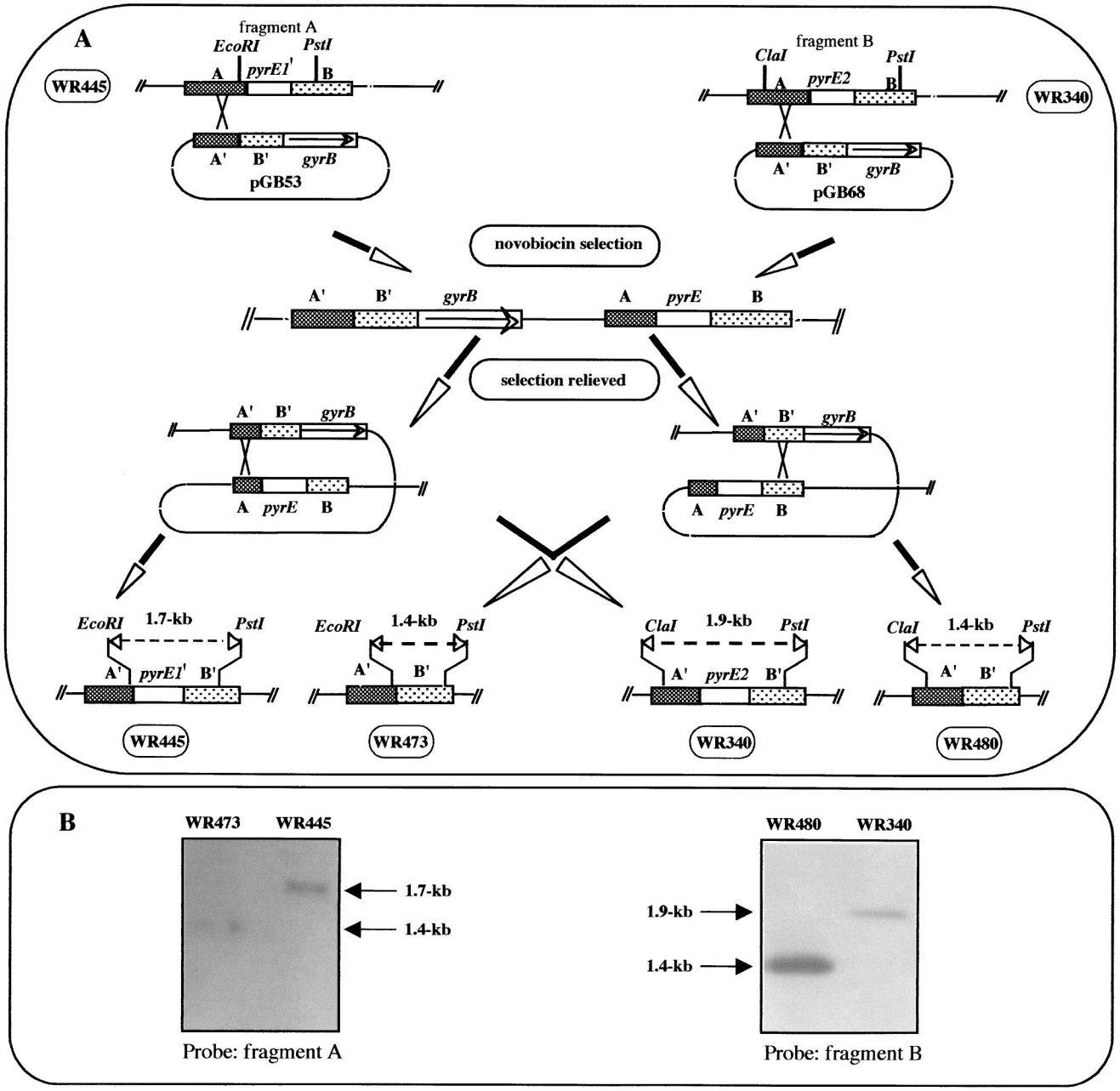

FIG. 3. Schematic diagram of disruption of the *pyrE1* gene in strain WR445 and disruption of the *pyrE2* gene in strain WR340 and Southern blot analyses of the mutant strains. (A) Plasmids pGB53 and pGB68 were constructed as described in Materials and Methods. The plasmids were integrated into the chromosome by homologous recombination between the chromosomal sequence A and the plasmid sequence A′, and novobiocin-resistant recombinants were selected. Following relief of selection, recombination events could result in either reconstitution of the wild-type allele or deletion of the chromosomal *pyrE* gene. *pyrE1′* is the first 330 bp of the *pyrE1* gene. (B) Analysis of *pyrE1* mutant. Total DNA was prepared from WR445 (parental strain) and WR473 (Δ*pyrE1*), digested with the *Eco*RI and *Pst*I restriction enzymes, and analyzed by Southern blotting by using fragment A as a probe. For analysis of the Δ*pyrE2* mutant, total DNA was prepared from WR430 (parental strain) and WR480 (Δ*pyrE2*), digested with the *Cla*I and *Pst*I restriction enzymes, and analyzed by Southern blotting by using fragment B as a probe.

mosome, creating a tandem arrangement of the wild-type and mutant gene copies; in the second step, the two alleles are resolved, resulting either in the desired knockout strain or in the parental strain. Two advantages of this method are (i) that isolation of the initial integrant provides a means to assess successful transformation and insertion of the vector into the chromosome and (ii) that the selectable marker used for construction is removed at the end of the process and thus can be used again.

We previously employed the pop-in–pop-out method to create Δ*hdr* mutations in *H. volcanii* (15), and in this work we created Δ*pyrE1* and Δ*pyrE2* strains by using a plasmid vector

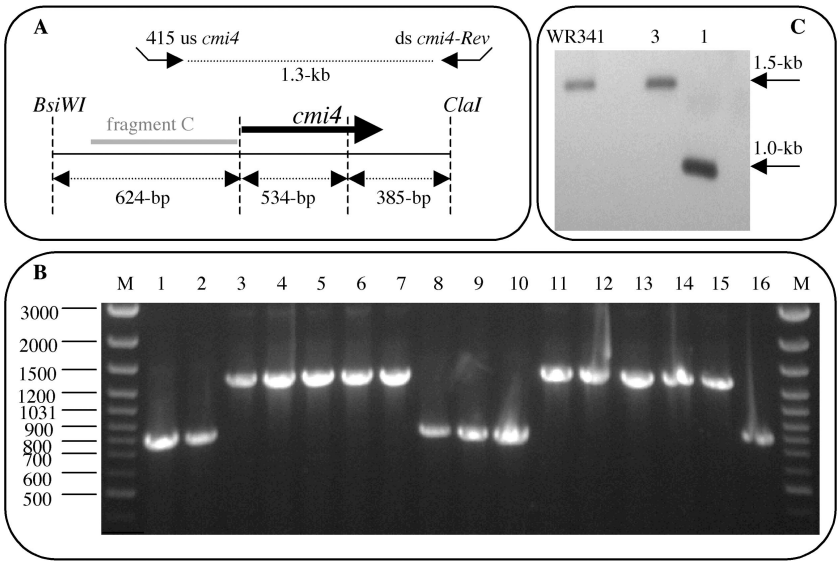

FIG. 4. Partial restriction map of the *cmi4* gene and its flanking sequences (A) and PCR analysis (B) and Southern blot analysis (C) of cells that underwent excision of the integrated plasmid and could grow on media containing 5-FOA. (A) The long solid arrow represents the *cmi4* gene. The locations of PCR primers 415 us *cmi4* and ds *cmi4*-Rev are indicated by short arrows, and fragment C was used as a probe for Southern blot analysis. (B) PCR were performed with DNA isolated from 16 colonies that grew on media containing 5-FOA by using primers 415 us *cmi4* and ds *cmi4*-Rev. Lanes M contained molecular weight markers. (C) Total DNA was prepared from WR341 (parental strain), one *cmi4* deletion strain (as determined by PCR) (lane 1), and a *cmi4* reconstituted strain (as determined by PCR) (lane 3). The DNA was digested with the *Bsi*WI *and Cla*I restriction enzymes and analyzed by Southern blotting by using fragment C as a probe.

carrying the novobiocin resistance gene for selection. A disadvantage of the novobiocin marker is that it is not possible to positively select for excision of the plasmid when this marker is used. The low frequency of spontaneous excision necessitates tedious screening of cells for mutants in which the selectable marker has been lost. In contrast, the uracil biosynthetic pathway for selection and counterselection is widely used in *S. cerevisiae* (3), and use of this pathway for counterselection was recently introduced for *H. salinarum* by employing the *pyrF* gene (16).

In this paper we describe construction of a selection-counterselection system based on the *H. volcanii pyrE* gene. *pyrE* encodes the enzyme OPRTase, which participates in uracil biosynthesis. We identified in the *H. volcanii* genome two genes that encode proteins that exhibit low but significant (~30%) homology with *E. coli* PyrE and *S. cerevisiae* Ura5 (Fig. 2). Previously, genome sequence analysis showed that the phylogenetically distinct halophilic archaeon *Halobacterium* sp. strain NRC-1 (14) also contains two ORFs (designated *pyrE1* and *pyrE2*) that exhibit homology with *pyrE* genes of many organisms. Interestingly, in a search of the databases of complete microbial genomes we noticed that some archaea have two ORFs that are homologous to *pyrE1* and *pyrE2,* while other archaea have only one *pyrE* ORF that is more similar to *pyrE2*.

In order to demonstrate the functionality of the two *pyrE* genes, deletion mutations of both genes were created. It was found that the Δ*pyrE1* mutant has partial resistance to 5-FOA and is a uracil prototroph, whereas the Δ*pyrE2* mutant shows the expected phenotype, 5-FOA resistance and uracil auxotrophy. Plausibly, *pyrE2* encodes the physiological OPRTase of the cell.

It is not clear what the cellular function of *pyrE1* is. On the one hand, the Δ*pyrE1* strain is prototrophic, and the deletion could not be complemented by an intact copy of *pyrE1* (a Δ*pyrE2* mutant is auxotrophic to uracil). On the other hand, the partial resistance of the Δ*pyrE1* strain to 5-FOA indicates that *pyrE1* is involved in uracil biosynthesis. It seems likely that the level of OPRTase activity encoded by *pyrE1* is, by itself, insufficient to enable growth without uracil but is sufficient to increase sensitivity to 5-FOA. In the three halophilic archaea known to contain *pyrE1* (*H. salinarum, H. volcanii*, and *H. mediterranei*) (unpublished data), *pyrE1* occurs in an operon with the gene encoding for glucose dehydrogenase (18). This is not the case in other archaea containing two *pyrE* genes.

In this study we developed for *H. volcanii* a new genetic tool that enables efficient creation of gene knockouts. The *pyrE2* gene provides a valuable genetic marker that enables both positive and negative selection. We demonstrated the efficiency of this system for creating deletions by employing the *cmi4* gene as a target for gene knockouts. Our previous attempts to delete *cmi4* failed mainly due to the very low level of spontaneous plasmid excision even after long exposure to nonselective conditions (unpublished data). When the *pyrE2*-based system was used, the desired Δ*cmi4* mutants were obtained at a high frequency (6 of 16 isolates) (Fig. 4B).

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