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Development of Additional Selectable Markers for the Halophilic Archaeon *Haloferax volcanii* Based on the *leuB* and *trpA* Genes

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Since most archaea are extremophilic and difficult to cultivate, our current knowledge of their biology is confined largely to comparative genomics and biochemistry. *Haloferax volcanii* offers great promise as a model organism for archaeal genetics, but until now there has been a lack of a wide variety of selectable markers for this organism. We describe here isolation of *H. volcanii leuB* and *trpA* genes encoding 3-isopropylmalate dehydrogenase and tryptophan synthase, respectively, and development of these genes as a positive selection system. Δ*leuB* and Δ*trpA* mutants were constructed in a variety of genetic backgrounds and were shown to be auxotrophic for leucine and tryptophan, respectively. We constructed both integrative and replicative plasmids carrying the *leuB* or *trpA* gene under control of a constitutive promoter. The use of these selectable markers in deletion of the *lhr* gene of *H. volcanii* is described.

Less than 25 years ago, the archaea were virtually unknown. We now recognize that these organisms represent one of the fundamental domains of life (32) and constitute a significant fraction of the total biomass (9). Acceptance of the distinct status of the archaea has been largely due to genome sequencing projects (6). The data from the sequenced genomes have revealed that in spite of their prokaryotic morphology, the archaea have numerous similarities with eukaryotes (5), particularly in enzymes involved in core processes such as transcription (2), translation (10), and DNA replication (23). As the archaeal transcription and replication systems are considerably less complex than those found in eukaryotes, they are more amenable to analysis. Notwithstanding this sequence similarity, the archaea have a unique identity (12), which is best exemplified by novel enzymes such as the Holliday junction resolvase Hjc (19).

In order to realize the true potential of the archaea, both as stripped-down models to dissect more complex eukaryotic systems and as a source of novel enzymes, it is essential to harness the power of genetics to underpin advances in biochemistry and genomics. *Haloferax volcanii* is an obligate halophile (25) that is genetically stable and grows aerobically in both complex and minimal media (24). It has some of the best genetic tools among the archaea, including a transformation system (8), reporter genes (17), shuttle vectors with antibiotic resistance (14, 16, 22), auxotrophic markers (26), and a recently developed gene knockout system based on the *pyrE2* gene (3) (Fig. 1A), which encodes orotate phosphoribosyl transferase and is involved in uracil biosynthesis.

Since the number of selectable genetic markers in *H. volcanii* is still limited, we wished to supplement it with additional markers. We describe here the isolation of *H. volcanii leuB* and *trpA* genes encoding 3-isopropylmalate dehydrogenase and tryptophan synthase, respectively. Deletion of *leuB*, a homologue of the widely used *Saccharomyces cerevisiae LEU2* gene (1), confers leucine auxotrophy in minimal medium. Deletion of the *trpA* gene (20) confers tryptophan auxotrophy in minimal or Casamino Acids medium. The *trpA* marker is of particular value when it is used with the *pyrE2* gene knockout system, as it allows direct selection for deleterious mutations that are otherwise difficult to recover (Fig. 1B). Here we describe the use of this system for deletion of the *H. volcanii* homologue of the *Escherichia coli lhr* gene (28). The *leuB* and *trpA* markers were combined with a deletion of the *hdrB* gene (26), conferring thymidine auxotrophy in rich (yeast extract) medium, and both integrating and shuttle plasmid vectors in which *pyrE2*, *leuB*, *trpA*, and *hdrB* were used as selectable markers were generated.

MATERIALS AND METHODS

Unless stated otherwise, chemicals were obtained from Sigma and restriction endonucleases were obtained from New England Biolabs.

Strains and culture conditions. The *H. volcanii* strains used are shown in Table 1 and were routinely grown in rich medium (Hv-YPC) containing (per liter) 144 g of NaCl, 21 g of $MgSO_4 \cdot 7H_2O$, 18 g of $MgCl_2 \cdot 6H_2O$, 4.2 g of KCl, and 12 mM Tris HCl (pH 7.5). For solid media, agar (Difco) was added at a concentration of 15 g per liter and was dissolved by heating the medium in a microwave oven. Yeast extract (0.5%, wt/vol; Difco), 0.1% (wt/vol) peptone (Oxoid), and 0.1% (wt/vol) Casamino Acids (Difco) were added, and the medium was autoclaved. After cooling, $CaCl_2$ was added to a final concentration of 3 mM. When required, novobiocin was added to a concentration of 2 μg/ml, mevinolin was added to a concentration of 4 μg/ml, and thymidine was added to a concentration of 40 μg/ml. Casamino Acids medium (Hv-Ca) was made in a similar manner, except that yeast extract and peptone were omitted and Casamino Acids was added to a final concentration of 0.5% (wt/vol). When required, thymidine or hypoxanthine was added at a concentration of 40 μg/ml, and tryptophan or uracil was added at a concentration of 50 μg/ml; for pop-out selection medium, 5-fluoroorotic acid (5-FOA) was added to a concentration of 50 μg/ml and uracil was added to a concentration of 10 μg/ml. Minimal medium (Hv-Min) contained the same concentration of salts as Hv-YPC, except that Tris HCl (pH 7.5) was added to a concentration of 42 mM. After autoclaving and cooling, 4.25 ml of a sodium DL-lactate solution (60%, wt/vol), 3.83 g of disodium succinic acid $\cdot 6H_2O$, 0.25 ml of glycerol, 5 ml of a 1 M NH_4Cl solution, 6 ml of a 0.5 M $CaCl_2$ solution, 2 ml

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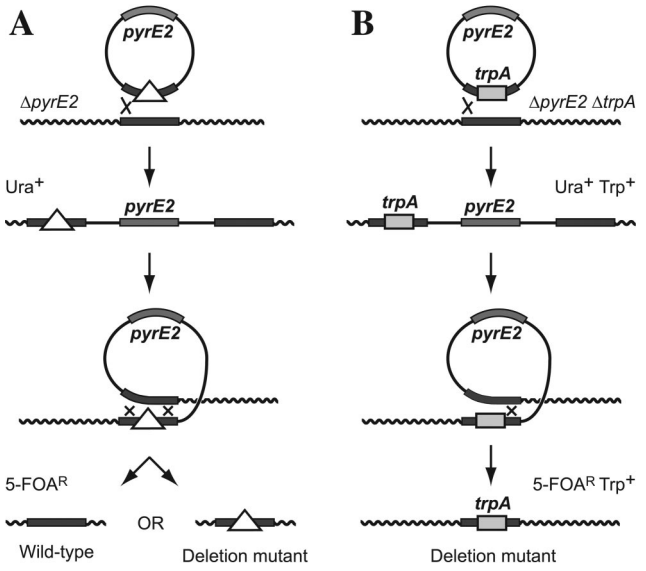

FIG. 1. Gene knockout system based on the *pyrE2* gene. (A) A plasmid carrying the *pyrE2* marker and flanking sequences of the gene to be deleted is used to transform a Δ*pyrE2 H. volcanii* strain to uracil prototrophy. Here, the crossover used to integrate the plasmid (pop-in) has occurred to the left of the deletion. Subsequent loss of the plasmid by intrachromosomal crossing over can occur on the left of the deletion, restoring the gene to wild type, or on the right of the deletion, resulting in the desired mutant. In either case the cell is rendered auxotrophic for uracil and is therefore resistant to 5-FOA by virtue of its inability to convert this compound to the toxic analog 5-fluorouracil. (B) The gene is replaced with the *trpA* marker, and the plasmid is used to transform a Δ*pyrE2* Δ*trpA H. volcanii* strain to prototrophy for uracil and tryptophan. Loss of the plasmid by crossing over on the right of the deletion, resulting in a *trpA*-marked mutant, can be selected in one step.

of 0.5 M potassium phosphate buffer (pH 7.5), 1 ml of a trace element solution (24), 0.8 mg of thiamine, and 0.1 mg of biotin were added per liter of Hv-Min. When required, thymidine or hypoxanthine was added to a concentration of 40 μg/ml, and leucine, tryptophan, uracil, methionine, glycine, or pantothenic acid was added to a concentration of 50 μg/ml.

E. coli strains XL1-Blue MRF′ (Δ*mcrA183* Δ*mcrCB-hsdSMR-mrr173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB lacI*^{q}*Z*ΔM15 Tn*10*]) and GM121 (F$^-$ *dam-3 dcm-6 ara-14 fhuA31 galK2 galT22 hdsR3 lacY1 leu-6 thi-1 thr-1 tsx-78*) were grown in Luria-Bertani medium containing 100 μg of ampicillin per ml. The latter strain was used to prepare unmethylated plasmid DNA for efficient transformation of *H. volcanii* (18).

Transformation procedures. Transformation of *H. volcanii* was carried out by using polyethylene glycol 600 as described previously (8). The media used for transformation were prepared as described above, except that sucrose was added to a concentration of 15% (wt/vol). *E. coli* was transformed by using a standard electroporation protocol (29).

Molecular genetic methods. Restriction endonuclease digestion, agarose gel electrophoresis, Southern blot analysis, and molecular cloning techniques were performed by using standard procedures (29). Probes were generated by PCR by using the provisional *H. volcanii* genome sequence (University of Scranton, Scanton, Pa.) and the primers listed in Table 2.

Isolation of *H. volcanii* total genomic DNA. One milliliter of a saturated culture (grown in Hv-YPC broth) was centrifuged at 3,300 × *g* for 8 min and resuspended in 200 μl of 1 M NaCl–20 mM Tris HCl (pH 7.5). Then 200 μl of 100 mM EDTA (pH 8.0)–0.2% (wt/vol) SDS was added to lyse the cells, followed by 1 ml of ethanol. The DNA was spooled out of solution onto a capillary, washed twice with ethanol, and resuspended in 500 μl of TE (10 mM Tris HCl, 1 mM EDTA; pH 7.5). The DNA was precipitated with isopropanol, washed thoroughly with 70% ethanol, and resuspended in 100 μl of TE containing 0.1 mg of RNase A per ml.

Plasmid construction. The plasmids used are shown in Table 3. Apart from the plasmids shown in Fig. 5 (see below), plasmids were constructed as described in the Results. The PCR primers used for generation of deletion constructs are listed in Table 2. Sequence files are available on request.

(i) Integrative plasmids. To generate pTA131, a *Bam*HI-*Xba*I fragment from pGB70 (3) containing the *pyrE2* gene under control of the constitutive ferredoxin promoter was inserted into the *Psi*I site of pBluescript II. The *Psi*I site lies outside the polylinker in the *lacZ* promoter; therefore, the blue-white screening capability is retained. To generate pTA133, pTA132, and pTA192, the coding regions of *leuB*, *trpA*, and *hdrB* were amplified from pTA44, pTA49, and pD4 (26), respectively. The primers used (Table 2) incorporated *Pci*I or *Bsp*HI sites at the ATG start codon, which were compatible with the *Nco*I site at the 3′ end of the ferredoxin promoter. The ensuing fusion constructs were inserted into the *Psi*I site of pBluescript II.

(ii) Shuttle vectors. An *Nco*I-*Hin*dIII fragment of pWL102 containing the pHV2 replication origin (7, 22) was inserted into the *Pci*I sites of pTA131, pTA132, pTA133, and pTA192 to generate pTA230, pTA231, pTA232, and pTA233, respectively.

DNA sequence analysis. DNA database searches of the *H. volcanii* genome were performed by using NCBI BLAST for Mac OS X (ftp://ftp.ncbi.nih.gov/blast/executables/), and sequence files were downloaded from the University of Scranton (http://wit-scranton.mbi.scranton.edu/Haloferax/). Genomic clones were sequenced by using an in-house service and were used to amend the University of Scranton data. Sequences of *pyrE2* (3), *hdrB* (26), and the *trpCBA* operon (20) have been published previously.

Nucleotide sequence accession number. The corrected nucleotide sequence of the *H. volcanii leuCDB* operon has been deposited in the EMBL nucleotide sequence database under accession number AJ571689.

RESULTS

Construction of *H. volcanii* DS70 Δ*pyrE2* strain. A Δ*pyrE2* mutant was constructed previously in the *H. volcanii* WFD11 background (3). Since WFD11 strains (22) have been reported to suffer from growth reduction and plasmid instability (31), we implemented the *pyrE2* gene knockout system in the improved *H. volcanii* strain DS70 (31) using the method described by Bitan-Banin et al. (3). DS70 cells were transformed to novobiocin resistance with pGB68, which contains the 850-bp upstream and 850-bp downstream flanking sequences of *pyrE2* (3). Transformants (pop-in) were screened by Southern blot hybridization, and a clone (H18) was selected which had integrated at the *pyrE2* locus and not *gyrB*; since novobiocin resistance is encoded by a mutant allele of the DNA gyrase gene *gyrB* (15), plasmids containing this marker can integrate at the chromosomal *gyrB* locus (which was the case in two of the five transformants examined). Excision of pGB68 (pop-out) was performed by propagating H18 for ~30 generations in rich medium (Hv-YPC) in the absence of novobiocin and plating on Casamino Acids (Hv-Ca) agar containing either uracil alone or uracil and 5-FOA. Approximately 2% of the cells were 5-FOA resistant and were subsequently determined to be auxotrophic for uracil and sensitive to novobiocin. These clones were analyzed by Southern blot hybridization, and a strain in which pGB68 had been excised, resulting in deletion of *pyrE2*, was designated H26.

Cloning of *leuB* and flanking sequences. An operon containing the leucine biosynthesis genes *leuB*, *leuC*, and *leuD* was identified in the *H. volcanii* genome sequence. The *leuB* gene, encoding 3-isopropylmalate dehydrogenase, was chosen for development as a genetic marker, since it is the terminal gene in the operon and a deletion would not have a polar effect on transcription of *leuC* and *leuD*. To clone the gene and its flanking sequences, a fragment of *leuB* was amplified by PCR and used to probe a Southern blot of *H. volcanii* chromosomal DNA digested with *Bss*HII. A 4.2-kb DNA fragment was found

TABLE 1. *H. volcanii* strains used

Strain	Background	Derivation or reference[a]	Genotype and/or phenotype
DS70	(DS70)	31	
WR340	WFD11	3	His$^-$
WR480	WFD11	3	His$^-$ Δ*pyrE2*
H18	DS70	DS70, pGB68 pop-in	*pyrE2*$^+$::[Δ*pyrE2* NovR]
H23	DS70	DS70, pTA70 pop-in	*leuB*$^+$::[Δ*leuB* MevR]
H26	DS70	H18 pop-out	Δ*pyrE2*
H30	WFD11	WR480, pTA73 pop-in	His$^-$ Δ*pyrE2 leuB*$^+$::[Δ*leuB pyrE2*$^+$]
H37	DS70	H23 pop-out	Δ*leuB*
H40	WFD11	H30 pop-out	His$^-$ Δ*pyrE2* Δ*leuB*
H42	WFD11	WR340, pTA93 pop-in	His$^-$ *trpA*$^+$::[Δ*trpA* MevR]
H43	DS70	DS70, pTA93 pop-in	*trpA*$^+$::[Δ*trpA* MevR]
H45	WFD11	WR480, pTA95 pop-in	His$^-$ Δ*pyrE2 trpA*$^+$::[Δ*trpA pyrE2*$^+$]
H47	DS70	H26, pTA95 pop-in	Δ*pyrE2 trpA*$^+$::[Δ*trpA pyrE2*$^+$]
H52	WFD11	H45 pop-out	His$^-$ Δ*pyrE2* Δ*trpA*
H53	DS70	H47 pop-out	Δ*pyrE2* Δ*trpA*
H60	DS70	H26, pTA73 pop-in	Δ*pyrE2 leuB*$^+$::[Δ*leuB pyrE2*$^+$]
H66	DS70	H60 pop-out	Δ*pyrE2* Δ*leuB*
H76	WFD11	H42 pop-out	His$^-$ Δ*trpA*
H77	DS70	H43 pop-out	Δ*trpA*
H90	DS70	H26, pTA155 pop-in	Δ*pyrE2 hdrB*$^+$::[Δ*hdrB pyrE2*$^+$]
H91	DS70	H53, pTA155 pop-in	Δ*pyrE2* Δ*trpA hdrB*$^+$::[Δ*hdrB pyrE2*$^+$]
H92	DS70	H66, pTA155 pop-in	Δ*pyrE2* Δ*leuB hdrB*$^+$::[Δ*hdrB pyrE2*$^+$]
H98	DS70	H90 pop-out	Δ*pyrE2* Δ*hdrB*
H99	DS70	H91 pop-out	Δ*pyrE2* Δ*trpA* Δ*hdrB*
H100	DS70	H92 pop-out	Δ*pyrE2* Δ*leuB* Δ*hdrB*
H107	DS70	H26, pTA166 pop-in	Δ*pyrE2 lhr*$^+$::[Δ*lhr pyrE2*$^+$]
H108	DS70	H53, pTA166 pop-in	Δ*pyrE2* Δ*trpA lhr*$^+$::[Δ*lhr pyrE2*$^+$]
H109	DS70	H53, pTA172	Δ*pyrE2* Δ*trpA lhr*$^+$::[Δ*lhr*::*trpA*$^+$ *pyrE2*$^+$]
H111	DS70	H53, pTA73 pop-in	Δ*pyrE2* Δ*trpA leuB*$^+$::[Δ*leuB pyrE2*$^+$]
H119	DS70	H111 pop-out	Δ*pyrE2* Δ*trpA* Δ*leuB*
H120	DS70	H107 pop-out	Δ*pyrE2* Δ*lhr*
H121	DS70	H108 pop-out	Δ*pyrE2* Δ*trpA* Δ*lhr*
H122	DS70	H109 pop-out	Δ*pyrE2* Δ*trpA* Δ*lhr*::*trpA*$^+$
H126	DS70	H119, pTA155 pop-in	Δ*pyrE2* Δ*trpA* Δ*leuB hdrB*$^+$::[Δ*hdrB pyrE2*$^+$]
H133	DS70	H126 pop-out	Δ*pyrE2* Δ*trpA* Δ*leuB* Δ*hdrB*

[a] Unless indicated otherwise, the source of strains was this study.

to hybridize with the probe. A genomic DNA mini-library of 4.2-kb *Bss*HII fragments was constructed in pBluescript II and screened by colony hybridization by using the *leuB* fragment probe. A clone (pTA44) was sequenced and found to contain the 972-bp *leuB* gene, as well as 1,604 bp of upstream flanking sequences and 1,584 bp of downstream flanking sequences (Fig. 2).

Deletion of *leuB* and phenotypic analysis. To delete *leuB*, a 1,268-bp fragment upstream of *leuB* and a 1,395-bp downstream fragment were amplified by PCR by using pTA44 as a template. The internal primers contained *Eco*RI sites used to ligate the PCR products, and the external primers contained *Bam*HI sites used to clone the Δ*leuB* fragment in pBluescript II SK+, generating pTA65 (Fig. 2). A *Not*I fragment from pMDS99 (31) containing the mevinolin resistance gene (MevR) was inserted at the *Not*I site of pTA65 to generate pTA70. Alternatively, an *Xba*I-*Hin*dIII fragment of pTA65 containing the Δ*leuB* construct was inserted at the *Xba*I and *Hin*dIII sites of the *pyrE2*-marked plasmid pGB70 (3), generating pTA73 (Fig. 2).

H. volcanii DS70 was transformed to mevinolin resistance with pTA70, and transformants were screened for integration at the *leuB* locus by Southern blot hybridization (Fig. 3); this was observed in all eight transformants analyzed. One clone was chosen (H23), and excision of pTA70 was performed by propagating H23 in the absence of mevinolin. Colonies were screened by replica plating on rich agar with and without mevinolin, as well as minimal agar (Hv-Min). Mevinolin-sensitive clones that failed to grow on minimal agar without added leucine were analyzed by Southern blot hybridization. A strain in which pTA70 had been excised, resulting in deletion of 942 bp, including *leuB*, was designated H37 (Fig. 3).

To construct Δ*pyrE2* Δ*leuB* strains, the *H. volcanii* DS70 Δ*pyrE2* strain H26 was transformed to uracil prototrophy with pTA73, and transformants were screened for integration at the *leuB* locus (Fig. 3). One clone was chosen (H60), and excision of pTA73 was performed by propagating H60 in rich medium and plating the culture on Casamino Acids (Hv-Ca) agar containing either uracil alone or uracil and 5-FOA. Approximately 2% of the cells were 5-FOA resistant (Ura$^-$) and were screened by replica plating on minimal agar with and without added leucine. Five of 30 Ura$^-$ clones tested were auxotrophic for leucine and were analyzed by Southern blotting (Fig. 3B). A strain in which pTA73 had been excised, resulting in deletion of *leuB*, was designated H66. A Δ*pyrE2* Δ*leuB* strain was made in a similar manner in the WFD11 background by transforming WR480 (3) with pTA73. The pop-in strain was designated H30, and the Δ*leuB* pop-out strain was designated H40.

Cloning of *trpA* and flanking sequences. To clone the operon containing the tryptophan biosynthesis genes *trpC*, *trpB*, and

TABLE 2. Oligonucleotide primers used

Primer	Sequence (5′-3′)[a]	Relevant properties[b]
HvLeuF	CGCCGGCGACCACGTCAAAGAAGA	*leuB* probe, forward primer
HvLeuR	AGCAGCATCGCCGCGGACAGAATC	*leuB* probe, reverse primer
TrpAF2	CGCCGAGGGGCCGACCATCC	*trpA* probe, forward primer
TrpAR	CGTTGCGACGCGCCCGCTACC	*trpA* probe, reverse primer
dLeu5F	GCGTTCAGCACGAATTCCGCCGCCGGGATGACCT	*leuB* deletion, upstream internal primer, *Eco*RI deletion site
dLeu5R	CGCGGGATCCGTCAACCCCGACGAGACCACCTACGA	*leuB* deletion, upstream external primer, *Bam*HI cloning site
dLeu3F	GCACGGATCCGCGGGCCGTTGTGATTGAGT	*leuB* deletion, downstream external primer, *Bam*HI cloning site
dLeu3R	GGCGGAATTCGTTTCGAACGCGCCCGTTTTCGTTTCTGAT	*leuB* deletion, downstream internal primer, *Eco*RI deletion site
dTrp5F	GCTCTAGAACGCGCTCGGGCAGGTCTTACTGG	*trpA* deletion, upstream external primer, *Xba*I cloning site
dTrp5R	GGACGAATTCCGGGCCGTCGGAGAAGG	*trpA* deletion, upstream internal primer, *Eco*RI deletion site
dTrp3F2	CGAACTCGAATTCGGTGCGGTAGCG	*trpA* deletion, downstream internal primer, *Eco*RI deletion site
dTrp3R	CCGGTGAGTCTCTAGACGTTTTCGTCCG	*trpA* deletion, downstream external primer, *Xba*I cloning site
TrpPci	GCCTGACATGTCGCTCGAAGACGCC	*trpA* coding sequence, forward primer, *Pci*I site
TrpXba	GGGTTCTAGAGCAGTTATGTGCGTTCC	*trpA* coding sequence, reverse primer, *Xba*I site
LeuBsp	GCCCTACGTTCATGACTGAGGAAATCG	*leuB* coding sequence, forward primer, *Bsp*HI site
LeuXba	CGGGTCGCTCTAGATCAGAGTCGGTCG	*leuB* coding sequence, reverse primer, *Xba*I site
LhrF2	GAAGCTGAAGGCGGGCGAGTTACG	*lhr* probe, forward primer
LhrR2	ATGGCGGCGAGGTTCAGTTTGTCT	*lhr* probe, reverse primer
dHdrBF2	CCCGATCTAGAGCCGGCTGGTCATC	*hdrB* deletion, upstream external primer, *Xba*I cloning site
dHdrB5R	CCCAGAAAGCTGCTAGCCGCTCATTCG	*hdrB* deletion, upstream internal primer, *Nhe*I deletion site
dHdrB3F	CTCGGGCTAGCGGGAGTACAAAATCGTC	*hdrB* deletion, downstream internal primer, *Nhe*I deletion site
dHdrB3R	GCCAAGCTCGAAATTAACCCTCAC	*hdrB* deletion, downstream external primer (*Xba*I site used)
dLhr5F	GAGCGCGCGTAATACGACTCACT	*lhr* deletion, upstream external primer (*Xho*I site used)
dLhr5R	GCGCGTCGCGGCCGCAATCAACGACG	*lhr* deletion, upstream internal primer, *Not*I deletion site
dLhr3F	GCGAGCGGCCGCGCCGGGTCATTACC	*lhr* deletion, downstream internal primer, *Not*I deletion site
dLhr3R	CGCGCAATTAACCCTCACTAAAGGG	*lhr* deletion, downstream external primer (*Xho*I site used)
HdrBsp	TGGCCTCATGAGCGGCGAGGAGC	*hdrB* coding sequence, forward primer, *Bsp*HI site
HdrXba	CTCCCACTCTCTAGAGTTACTCATCGG	*hdrB* coding sequence, reverse primer, *Xba*I site

[a] Restriction endonuclease sites used in cloning are underlined.
[b] Deletion sites were used to ligate flanking sequences. Cloning sites were used to clone deletion constructs in plasmid vectors. Cloning sites in parentheses were located in the amplified sequence (not the primer).

trpA and their flanking sequences (20), a fragment of *trpA* was amplified and used to probe a Southern blot of *H. volcanii* DNA digested with *Sau*3AI. As predicted (20), a 3.7-kb DNA fragment hybridized with the probe. A genomic DNA mini-library of 3.7-kb *Sau*3AI fragments was constructed in pBluescript II and screened by colony hybridization. A clone (pTA49) (Fig. 4A) was isolated that contained the 834-bp *trpA* gene, as well as 2,531 bp of upstream flanking sequences and

TABLE 3. Plasmids used

Plasmid	Relevant properties	Source or reference(s)
pBluescript II SK+	Standard cloning vector	Stratagene
pD4	pBluescript KS with *H. volcanii* 3,566-bp *Mbo*I-*Hin*dIII fragment containing *hdrB* gene	26
pGB68	pBR322 with NovR and flanking sequences of *pyrE2*	3
pGB70	pUC19 with *pyrE2* under ferredoxin promoter	3
pMDS99	Shuttle vector based on pOK12 with pHV2 replication origin and MevR from *Haloarcula hispanica*	31
pWL102	Shuttle vector based on pAT153 with pHV2 replication origin and MevR from *H. volcanii*	7, 22
pTA44	pBluescript II with *H. volcanii* 4,162-bp *Bss*HII fragment containing *leuB* gene	This study
pTA49	pBluescript II with *H. volcanii* 3,676-bp *Sau*3AI fragment containing *trpA* gene	This study
pTA65	pBluescript II with *Bam*HI PCR fragment containing flanking regions of *leuB*	This study
pTA70	pTA65 with *Not*I fragment from pMDS99 containing MevR	This study
pTA73	pGB70 with *Xba*I-*Hin*dIII Δ*leuB* fragment from pTA65	This study
pTA92	pBluescript II with *Xba*I PCR fragment containing flanking regions of *trpA*	This study
pTA93	pTA92 with *Not*I fragment from pMDS99 containing MevR	This study
pTA95	pGB70 with *Xba*I Δ*trpA* fragment from pTA92	This study
pTA131	pBluescript II with *Bam*HI-*Xba*I fragment from pGB70 containing *pyrE2* under ferredoxin promoter	This study
pTA132	pBluescript II with PCR fragment containing *trpA* under ferredoxin promoter	This study
pTA133	pBluescript II with PCR fragment containing *leuB* under ferredoxin promoter	This study
pTA150	pBluescript II with *H. volcanii* 3,974-bp *Xho*I-*Nru*I fragment containing *lhr* gene	This study
pTA155	pTA131 with *Hin*dIII-*Xba*I PCR fragment containing flanking regions of *hdrB*	This study
pTA166	pTA131 with *Xho*I-*Spe*I PCR fragment containing flanking regions of *lhr*	This study
pTA172	pTA166 with PCR fragment containing *trpA* under ferredoxin promoter, inserted at site of *lhr* deletion	This study
pTA192	pBluescript II with PCR fragment containing *hdrB* under ferredoxin promoter	This study
pTA230	pTA131 with *Nco*I-*Hin*dIII fragment from pWL102 containing pHV2 replication origin	This study
pTA231	pTA132 with *Nco*I-*Hin*dIII fragment from pWL102 containing pHV2 replication origin	This study
pTA232	pTA133 with *Nco*I-*Hin*dIII fragment from pWL102 containing pHV2 replication origin	This study
pTA233	pTA192 with *Nco*I-*Hin*dIII fragment from pWL102 containing pHV2 replication origin	This study

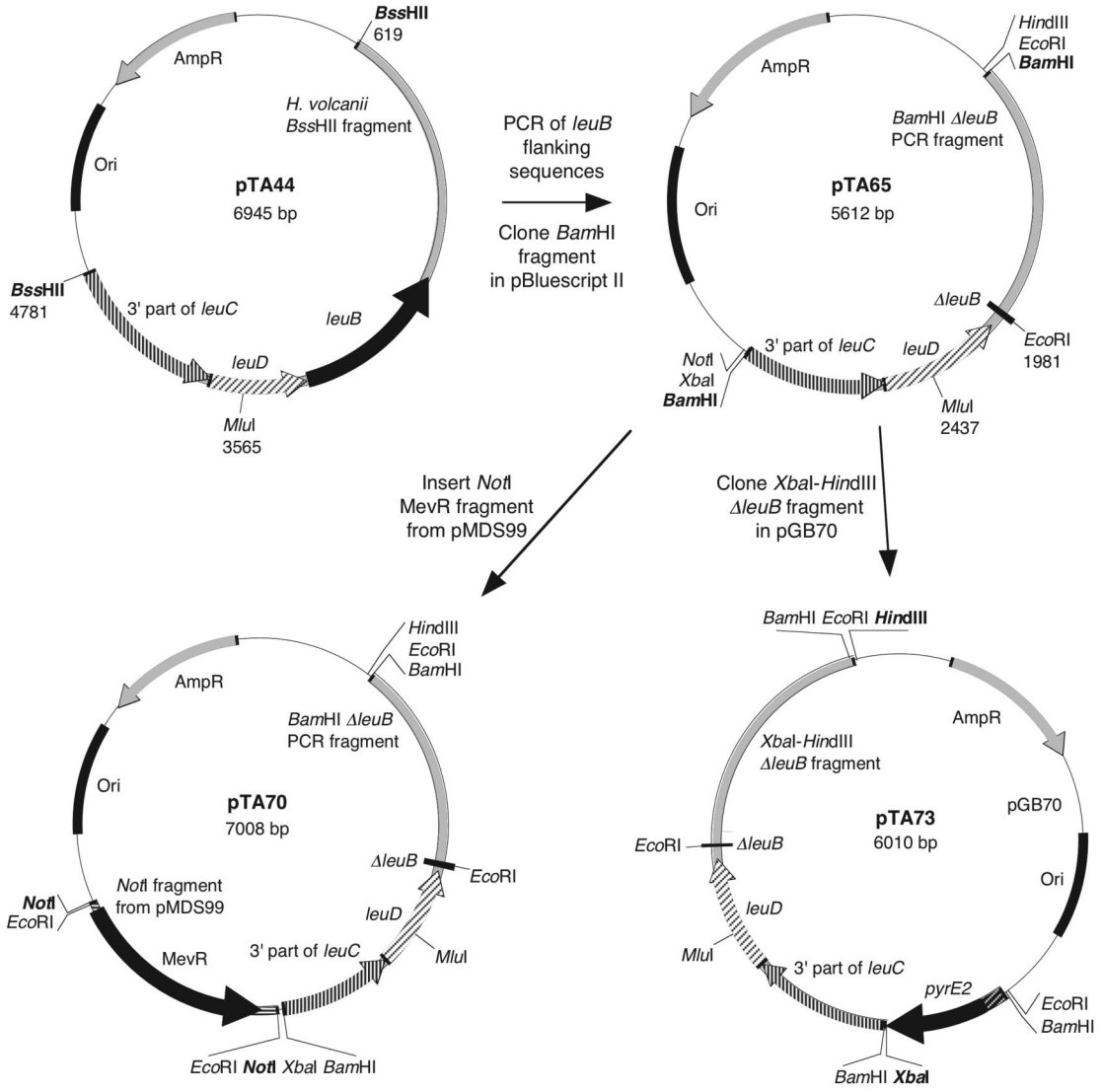

FIG. 2. Construction of *leuB* deletion plasmids. The genomic *leuB* clone pTA44 contains a 4,162-bp genomic DNA *Bss*HII fragment cloned in pBluescript II. Sequences flanking *leuB* were amplified from pTA44 and cloned in pBluescript II to generate the Δ*leuB* construct pTA65. The mevinolin resistance fragment from pMDS99 (31) was inserted into pTA65 to generate pTA70. Alternatively, the Δ*leuB* construct from pTA65 was cloned in the *pyrE2*-marked gene knockout plasmid pGB70 (3), generating pTA73. Only relevant sites are shown; full plasmid maps are available on request.

311 bp of downstream flanking sequences. The *trpA* gene, encoding the 277-amino-acid tryptophan synthase alpha subunit, was chosen for development as a genetic marker since it is the terminal gene in the operon.

Deletion of *trpA* and phenotypic analysis. To delete *trpA*, a 1,048-bp fragment upstream of *trpA* and a 355-bp downstream fragment were amplified by PCR by using pTA49. The internal primers contained *Eco*RI sites used to ligate the PCR products, and the external primers contained *Xba*I sites used to clone the Δ*trpA* fragment in pBluescript II, generating pTA92 (Fig. 4A). The *Not*I MevR fragment from pMDS99 (31) was inserted at the *Not*I site of pTA92 to generate pTA93 (data not shown; similar to pTA70 in Fig. 2). Alternatively, an *Xba*I fragment of pTA92 containing the Δ*trpA* construct was inserted at the *Xba*I site of pGB70 (3), generating pTA95 (data not shown; similar to pTA73 in Fig. 2).

Plasmid pTA93 was used to transform *H. volcanii* DS70. Transformants were screened for integration at *trpA* by Southern blotting (Fig. 4B). One integrant was chosen (H43), and excision of pTA93 was performed as described above. Colonies were also screened by replica plating on Casamino Acids (Hv-Ca) agar, which contained no detectable tryptophan. Mevinolin-sensitive clones exhibiting tryptophan auxotrophy on Casamino Acids agar were analyzed by Southern blotting. A strain in which pTA93 had been excised to obtain a 744-bp deletion of *trpA* was designated H77 (Fig. 4). A Δ*trpA* strain was made in a similar manner in the WFD11 background, by transforming WR340 (3) with pTA93. The pop-in strain was designated H42, and the Δ*trpA* pop-out strain was designated H76.

To construct a Δ*pyrE2* Δ*trpA* strain, *H. volcanii* H26 was transformed with pTA95, and transformants were screened for

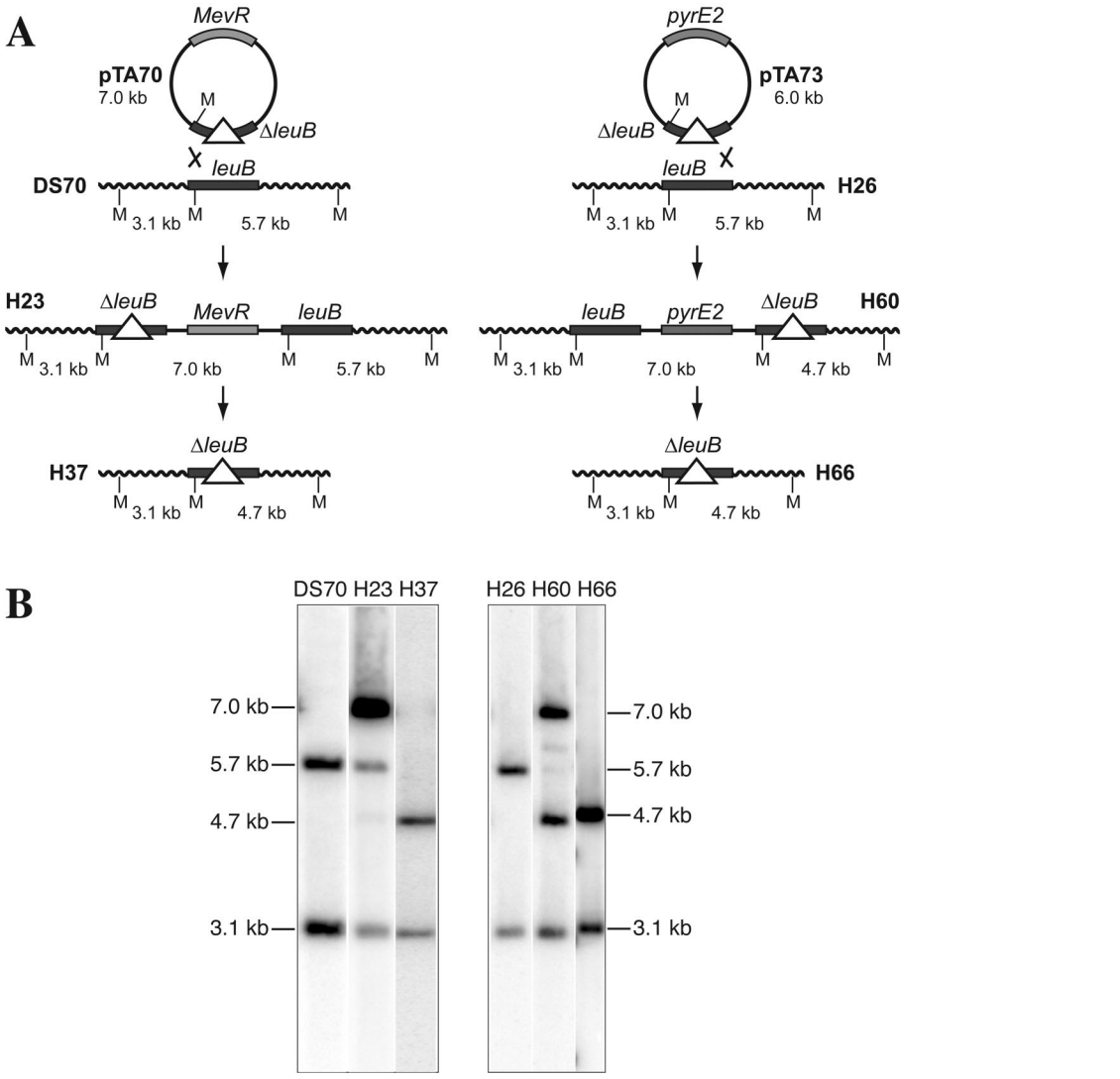

FIG. 3. Deletion of *leuB* gene. (A) Plasmids pTA70 and pTA73 were constructed as described in the legend to Fig. 2. Integration of pTA70 into the chromosome of DS70 by homologous recombination upstream of *leuB* resulted in strain H23. Loss of the plasmid by intrachromosomal recombination (Fig. 1) resulted in the Δ*leuB* strain H37. Integration of pTA73 (in H26) by recombination downstream of *leuB* yielded strain H60, and loss of the plasmid resulted in the Δ*leuB* strain H66. Integration and deletion events were monitored by digestion with *Mlu*I (M), resulting in the fragments indicated. (B) Southern blot analysis of Δ*leuB* strain construction. Genomic DNA were prepared from the strains indicated, digested with *Mlu*I, and probed with the flanking regions of *leuB*.

integration at *trpA*. One integrant was chosen (H47) (Fig. 4B), and excision of pTA95 was performed as described above. 5-FOA-resistant (Ura$^-$) cells were screened for tryptophan auxotrophy by replica plating on Casamino Acids agar and were analyzed by Southern blot hybridization (Fig. 4B). A strain in which pTA95 had been excised to delete *trpA* was designated H53. A Δ*pyrE2* Δ*trpA* strain was made in a similar manner in the WFD11 background, by transforming WR480 (3) with pTA95. The pop-in strain was designated H45, and the Δ*trpA* pop-out strain was designated H52.

We examined the potential of Δ*trpA* strains in a counterselectable system similar to that based on *pyrE2*. In *S. cerevisiae*, 5-fluoroanthranilic acid has been used for counterselection of tryptophan biosynthesis genes (30). This compound is converted to toxic 5-fluorotryptophan in *trp*$^+$ cells, which is analogous to the action on 5-FOA in uracil biosynthesis (4). We tested a number of anthranilic acid derivatives, including 4-, 5-, and 6-fluoroanthranilic acids and 5- and 6-methylanthranilic acids, as well as 5-fluoroindole, for the ability to select against *trpA*$^+$ strains but not Δ*trpA* strains. Strains H26 and H53 were tested by plating on Casamino Acids agar containing between 0.05 and 1 mg of the anthranilic acid derivatives per ml; tryptophan was added at a relative concentration of 10 to 40% to support growth of Δ*trpA* cells. None of these compounds discriminated between H26 (*trpA*$^+$) and H53 (Δ*trpA*) cells; the anthranilic acid derivatives prevented growth of both strains at concentrations above 0.25 mg/ml, and 5-fluoroindole was toxic at all concentrations tested. This was most likely due to feedback inhibition of tryptophan biosynthesis, leading to insufficient conversion of anthranilic acid derivatives to toxic fluoro-

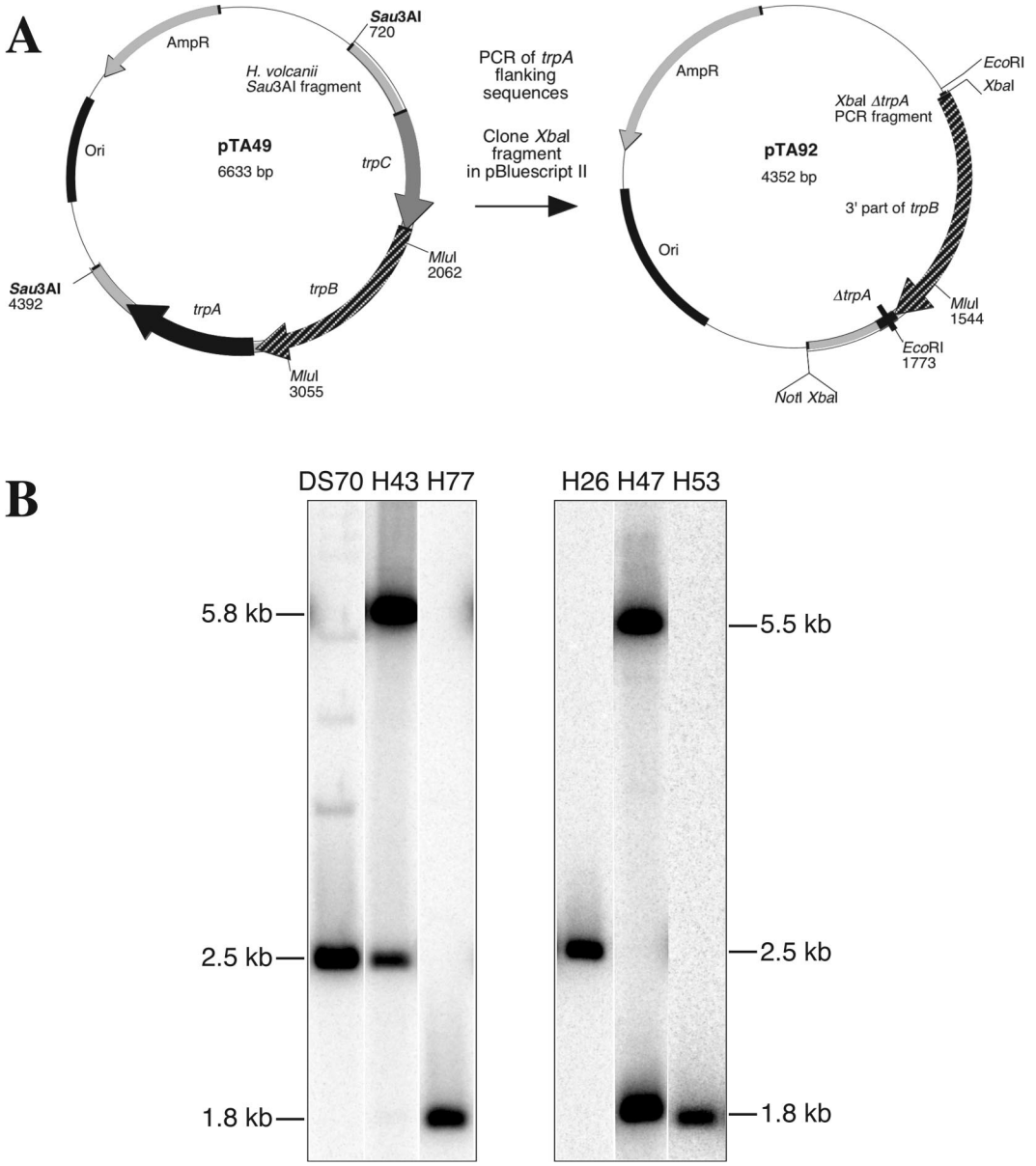

FIG. 4. Deletion of *trpA* gene. (A) Construction of *trpA* deletion plasmids. The genomic *trpA* clone pTA49 contains a 3,676-bp genomic DNA *Sau*3AI fragment cloned in pBluescript II. Sequences flanking *trpA* were amplified from pTA49 and cloned in pBluescript II to generate the Δ*trpA* plasmid pTA92. The mevinolin resistance fragment from pMDS99 (31) was inserted into pTA92 to generate pTA93 (data not shown; similar to pTA70 in Fig. 2). Alternatively, the Δ*trpA* construct from pTA92 was cloned in pGB70 (3), generating pTA95 (data not shown; similar to pTA73 in Fig. 2). Plasmid maps are available on request. (B) Deletion of the *trpA* gene in strains H77 and H53 was analyzed by *Mlu*I digestion and Southern blot hybridization by using flanking regions of *trpA* as a probe (similar to the *leuB* deletion in Fig. 3). Integration of pTA93 into the chromosome of DS70 by recombination upstream of *trpA* gave a novel 5.8-kb *Mlu*I fragment, producing strain H43. Loss of the plasmid, which deleted *trpA* (1.8-kb *Mlu*I fragment instead of 2.5-kb *Mlu*I fragment) resulted in Δ*trpA* strain H77. Integration of pTA95 into H26 by recombination downstream of *trpA* gave a novel 5.5-kb *Mlu*I fragment, producing strain H47, and loss of the plasmid resulted in Δ*trpA* strain H53.

or methyltryptophan, and a failure to discriminate between *trpA*$^+$ and Δ*trpA* strains. In addition, the anthranilic acid derivatives most probably exhibited nonspecific toxicity at higher concentrations, affecting both *trpA*$^+$ and Δ*trpA* cells.

Construction of strains with deletions in *pyrE2*, *leuB*, *trpA*, and *hdrB*. The *hdrB* marker is a useful addition to the current genetic repertoire, as deletion of this gene confers thymidine auxotrophy in rich medium (Hv-YPC) (26). We therefore constructed a Δ*hdrB* mutant in the *H. volcanii* DS70 Δ*pyrE2* strain H26. Sequences flanking *hdrB* (577 bp upstream and 292 bp downstream) were amplified by PCR by using the genomic clone pD4 as a template (26) and were cloned in the *pyrE2*-marked gene knockout plasmid pTA131 (see below) (Fig. 5A) to generate the Δ*hdrB* construct pTA155. *H. volcanii* H26 was transformed with pTA155, and transformants were screened for integration at *hdrB* (data not shown). One integrant was chosen (H90), and excision of pTA155 was performed as described above. 5-FOA-resistant (Ura$^-$) cells were screened for

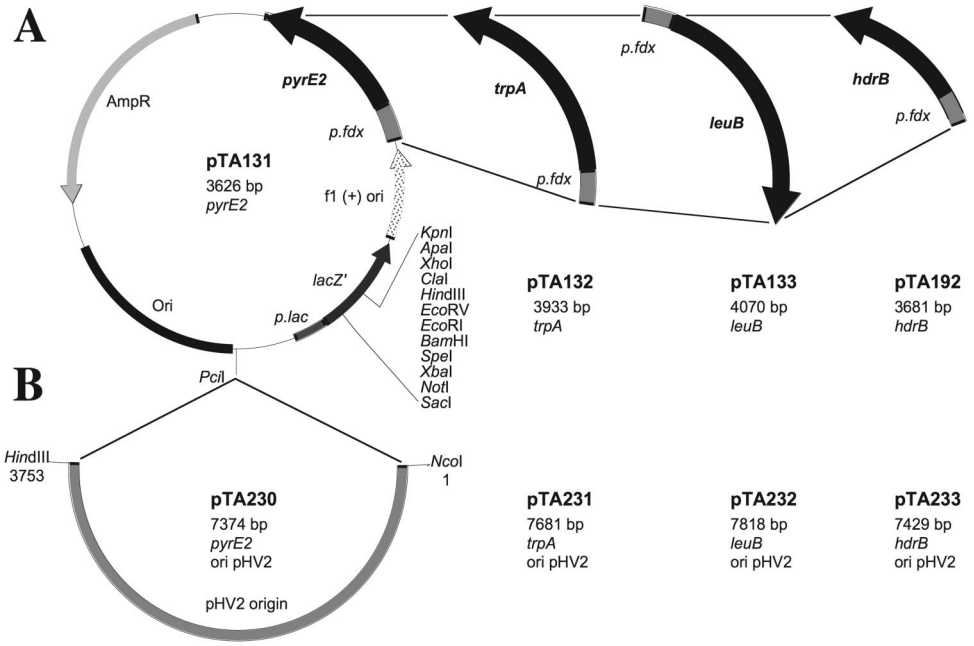

FIG. 5. Plasmid vectors marked with *pyrE2*, *leuB*, *trpA*, and *hdrB*. (A) Gene knockout plasmids. The *pyrE2*, *leuB*, *trpA*, and *hdrB* genes were placed under control of the ferredoxin promoter and cloned in pBluescript II to generate pTA131, pTA132, pTA133, and pTA192, respectively. (B) Shuttle vectors. The pHV2 replication origin from pWL102 (7, 22) was cloned in pTA131, pTA132, pTA133, and pTA192 to generate pTA230, pTA231, pTA232, and pTA233, respectively. Some sites in the polylinker are not unique in the shuttle vectors; full plasmid maps are available on request.

thymidine auxotrophy by replica plating on rich agar. A strain in which pTA155 had been excised to delete *hdrB* was designated H98.

We constructed strains with combinations of the deletions described above. A Δ*pyrE2* Δ*leuB* Δ*trpA* strain was made by transforming H53 with pTA73; the pop-in strain was designated H111, and the Δ*leuB* pop-out strain was designated H119. Derivatives of H53, H66, and H119 with an additional deletion of *hdrB* were constructed by transforming these strains with pTA155; the pop-in strains were designated H91, H92, and H126, respectively, and the Δ*hdrB* pop-out strains were designated H99, H100, and H133, respectively. All of the strains described here are listed in Table 1.

Construction of plasmid vectors by using *pyrE2*, *leuB*, *trpA*, and *hdrB* as selectable markers. To construct an improved *pyrE2*-marked gene knockout plasmid, a fragment from pGB70 containing the *pyrE2* gene under control of the constitutive ferredoxin promoter of *Halobacterium salinarum* (27) was inserted into pBluescript II. This plasmid (pTA131) (Fig. 5A) retained the blue-white screening facility of pBluescript. Similar plasmids were made by using *leuB*, *trpA*, and *hdrB* as selectable markers. The coding regions of these genes were amplified from pTA44, pTA49, and pD4, fused to the ferredoxin promoter, and inserted into pBluescript II to generate pTA133, pTA132, and pTA192, respectively (Fig. 5A).

Shuttle vectors were derived from these plasmids by inserting the replication origin of the *H. volcanii* episome pHV2 (Fig. 5B); both strain WFD11 and strain DS70 have been cured of this indigenous plasmid (7, 22, 31). The shuttle vectors were able to transform the corresponding *H. volcanii* deletion strains to prototrophy for the appropriate markers. The transformation efficiencies were $\sim 10^5$ CFU/μg of DNA, as expected. Dual-resistance vectors based on pMDS20 (14), which also contained the novobiocin resistance gene, were also constructed (details are available on request).

Construction of a Δ*lhr* strain by using the Δ*pyrE2* Δ*trpA* strain. In the pop-in–pop-out gene knockout system, spontaneous excision of the integrated plasmid can occur in one of two ways, either restoring the gene to wild-type information or resulting in a deletion (Fig. 1A). Even if both outcomes are equally likely, the mutant rapidly becomes underrepresented (in the population of 5-FOA-resistant cells) if the gene deletion leads to a slow-growth phenotype. By replacing the gene to be deleted with the *trpA* marker and plating the culture on Casamino Acids medium with 5-FOA (but no added tryptophan), it is possible to select directly for pop-out events that lead to the mutation (Fig. 1B).

To demonstrate that the *trpA pyrE2* system allows direct selection for gene knockouts that might otherwise be difficult to recover, we deleted the *lhr* gene, which was identified in the genome sequence on the basis of the homology of its product to the Lhr protein of *E. coli*. Lhr is a member of helicase superfamily II (28) and is well conserved among prokaryotes, although the C-terminal ~650 amino acids of the *E. coli* protein are found only in bacteria. A 3,974-bp *Xho*I-*Nru*I fragment of *H. volcanii* genomic DNA, containing the 2,757-bp *lhr* gene, 677 bp of upstream flanking sequences, and 540 bp of downstream flanking sequences, was cloned in pBluescript II to generate pTA150 (Fig. 6A). To delete *lhr*, a 653-bp upstream fragment and a 551-bp downstream fragment were amplified by PCR by using pTA150. The internal primers contained *Nco*I sites used to ligate the PCR products, and the external primers contained *Xho*I and *Spe*I sites used to clone the Δ*lhr* fragment in pTA131, generating pTA166 (Fig. 6A). A 972-bp fragment

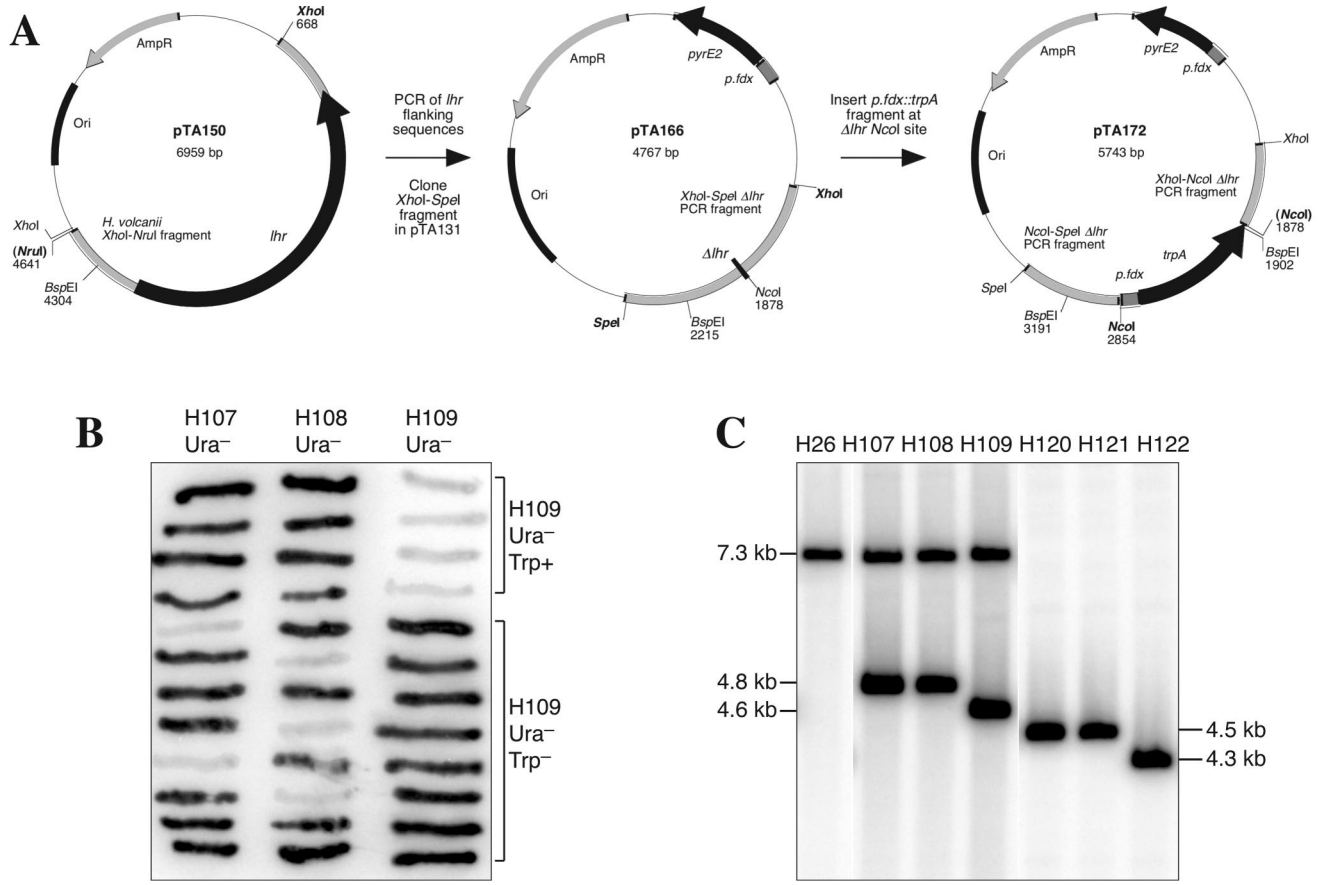

FIG. 6. Deletion of *lhr* gene. (A) Construction of *lhr* deletion plasmid. The genomic *lhr* clone pTA150 was used to amplify the flanking regions of *lhr*, which were inserted into pTA131 to generate the Δ*lhr* construct pTA166. A fragment containing the *trpA* gene under control of the ferredoxin promoter (*p.fdx*) was inserted at the site of the *lhr* deletion (*Nco*I), generating the Δ*lhr*::*trpA* plasmid pTA172. (B) Twelve 5-FOA-resistant (Ura$^-$) derivatives of pop-in strains H107 to H109 were grown on rich agar, transferred to a nylon filter, and probed with the *lhr* coding sequence. In the case of H109, the four Ura$^-$ clones with *lhr* deleted had previously been shown to be prototrophic for tryptophan (Trp$^+$), whereas the remaining eight clones in which *lhr* was not deleted were Trp$^-$. (C) Deletion of the *lhr* gene was analyzed by *Bsp*EI digestion and Southern blot hybridization by using the downstream flanking region of *lhr* as a probe. Integration of pTA166 into the chromosomes of H26 and H53 gave a novel 4.8-kb *Bsp*EI fragment, producing strains H107 and H108, respectively, and loss of pTA166, which deleted *lhr* (4.5-kb *Bsp*EI fragment instead of 7.3-kb *Bsp*EI fragment), resulted in Δ*lhr* strains H120 and H121, respectively. Integration of pTA172 in the chromosome of H53 gave a novel 4.6-kb *Bsp*EI fragment, producing strain H109, and loss of pTA172, which deleted *lhr* (4.3-kb *Bsp*EI fragment), resulted in Δ*lhr*::*trpA* strain H122.

containing the *trpA* gene under control of the ferredoxin promoter (see pTA132 in Fig. 5A) was inserted into the *Nco*I site at the *lhr* deletion to generate pTA172 (Fig. 6A).

H26 was transformed with pTA166, and H53 was transformed with pTA166 or pTA172. Integrants at *lhr* were verified (H107 to H109, respectively) (Fig. 6C), and excision of pTA166 and pTA172 was performed as usual. Cultures were plated on Casamino Acids agar containing uracil and 5-FOA with or without added tryptophan. Approximately 2% of the cells were 5-FOA resistant (Ura$^-$), and in the case of H109 (transformed with Δ*lhr*::*trpA* plasmid pTA172), 4 of the 30 Ura$^-$ clones tested were prototrophic for tryptophan. Ura$^-$ clones were screened for deletion of *lhr* by colony hybridization by using the *lhr* coding sequence as a probe (Fig. 6B). All four of the Trp$^+$ derivatives of H109 were Δ*lhr*::*trpA*, while in the remaining Trp$^-$ derivatives *lhr* was not deleted. Among derivatives of H107 and H108 (transformed with Δ*lhr* plasmid pTA172) only 2 of 12 and 3 of 12 of the Ura$^-$ clones, respectively, proved to be Δ*lhr* (Fig. 6B). Deletions were confirmed by Southern blot hybridization (Fig. 6C), and Δ*lhr* derivatives of H107 to H109 were designated H120 to H122, respectively. Δ*lhr* mutants did not show any detectable growth deficiency in rich or minimal medium and were no more sensitive to UV or γ radiation than isogenic *lhr*$^+$ strains were.

DISCUSSION

A cornerstone of genetic analysis is the ability to manipulate the genome. Traditionally, this has been done by chemical mutagenesis, followed by laborious screening for the appropriate phenotype. In the postgenomic era, when homologues are readily identifiable in model organisms, it is more expedient to perform targeted gene knockout and characterize the phenotype of the mutant. Selectable markers provide the means to accomplish this end.

The development of antibiotic resistance markers for the

TABLE 4. Growth characteristics of selected *H. volcanii* mutants

Strain	Genotype	Growth on:		
		Hv-YPC	Hv-Ca	Hv-Min
H26	Δ*pyrE2*	+	Ura$^-$	Ura$^-$
H37	Δ*leuB*	+	+	Leu$^-$
H77	Δ*trpA*	+	Trp$^-$	Trp$^-$
H66	Δ*pyrE2* Δ*leuB*	+	Ura$^-$	Ura$^-$ Leu$^-$
H53	Δ*pyrE2* Δ*trpA*	+	Ura$^-$ Trp$^-$	Ura$^-$ Trp$^-$
H98[a]	Δ*pyrE2* Δ*hdrB*	Thy$^-$	Ura$^-$ Thy$^-$	Ura$^-$ Thy$^-$

[a] In addition to thymidine, Δ*hdrB* strain cultures should be supplemented with hypoxanthine in Hv- Ca and with hypoxanthine, methionine, glycine, and pantothenic acid in Hv-Min (26).

archaea has been hampered by the lack of drug targets. Bacterial antibiotics are safe for medical use as their targets are generally not found in eukaryotic cells. Due to the greater similarity of the archaea to eukaryotes, it is hardly surprising that most commonly used antibiotics are ineffective against archaea (13). The few antibiotics currently available for *H. volcanii* have shortcomings. For example, the mevinolin resistance marker is an up-promoter mutation of the chromosomal gene *hmgA* (21). Recombination between the chromosomal gene and the resistance marker can therefore lead to constitutive antibiotic resistance. This problem was recently alleviated by development of a mevinolin resistance marker from *Haloarcula hispanica*, which is stably maintained in *H. volcanii* (31). However, spontaneous resistance to mevinolin can still arise (at an inconveniently high frequency) by promoter point mutation or amplification of the *hmg* gene (T.A., unpublished observations). Stable auxotrophic mutants provide a solution to these problems.

We have developed *leuB* and *trpA* as selectable markers, in conjunction with existing systems based on *pyrE2* and *hdrB*, since this suite of genes takes full advantage of the media available for *H. volcanii* (Table 4). In order to ensure that the mutants are stable, complete gene deletions were constructed. The coding sequence of the deleted gene can then be used as a selectable marker on a replicative (shuttle) vector without the risk of integration by homologous recombination. We have generated shuttle vectors and integrative plasmids for gene knockouts that complement these deletions (Fig. 5). Implementation of the *leuB*, *trpA*, and *hdrB* deletions in the Δ*pyrE2* background provides the widest variety of genetic markers available in any archaeal species.

The genetic tools described here, particularly the *trpA pyrE2* system, should be useful for isolation of mutants that are deleterious and therefore difficult to recover. By using a construct in which the gene of interest is replaced with the *trpA* marker, it is possible to select directly for deletion events (Fig. 1B). We demonstrated the utility of this system with a deletion of the *lhr* gene of *H. volcanii* (Fig. 6). Complete failure to recover 5-FOA-resistant Trp$^+$ cells from such a pop-in strain would be a strong indication that the gene deletion is lethal. This facility should in turn permit development of more sophisticated genetic tools, such as synthetic lethal screening methods (11).

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