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Improved Strains and Plasmid Vectors for Conditional Overexpression of His-Tagged Proteins in *Haloferax volcanii*▽

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Research into archaea will not achieve its full potential until systems are in place to carry out genetics and biochemistry in the same species. *Haloferax volcanii* is widely regarded as the best-equipped organism for archaeal genetics, but the development of tools for the expression and purification of *H. volcanii* proteins has been neglected. We have developed a series of plasmid vectors and host strains for conditional overexpression of halophilic proteins in *H. volcanii*. The plasmids feature the tryptophan-inducible p*.tnaA* promoter and a 6×His tag for protein purification by metal affinity chromatography. Purification is facilitated by host strains, where *pitA* is replaced by the ortholog from *Natronomonas pharaonis*. The latter lacks the histidine-rich linker region found in *H. volcanii* PitA and does not copurify with His-tagged recombinant proteins. We also deleted the *mrr* restriction endonuclease gene, thereby allowing direct transformation without the need to passage DNA through an *Escherichia coli dam* mutant.

Over the past century, our understanding of fundamental biological processes has grown exponentially, and this would have been impossible without the use of organisms that are amenable to experimental manipulation. Model species, such as *Escherichia coli*, *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Mus musculus*, and *Arabidopsis thaliana*, have become a byword for scientific progress (15). The rational choice of a model organism is critically important, and certain features are taken for granted, such as ease of cultivation, a short generation time, and systems for genetic manipulation. This list has now grown to include a genome sequence and methods for biochemical analysis of purified proteins *in vitro*.

Research into archaea has lagged behind work on bacteria and eukaryotes but has nonetheless yielded profound insights (2). One hurdle has been the paucity of archaeal organisms suitable for both biochemistry and genetics. For example, *Methanothermobacter thermautotrophicus* is a stalwart of archaeal biochemistry but has proved resistant to even the most rudimentary genetic manipulation (2). Progress has recently been made with another biochemical workhorse, *Sulfolobus* spp., and a few genetic tools are now available (6, 13, 37). *Methanosarcina* spp. and *Thermococcus kodakaraensis* offer alternative systems with an increasing array of techniques (16, 35, 36), but sophisticated genetics has traditionally been the preserve of haloarchaea, of which *Haloferax volcanii* is the organism of choice (39). It is easy to culture, the genome has been sequenced (19), and there are several selectable markers and plasmids for transformation and gene knockout (3, 7, 31), including a Gateway system (14), as well as reporter genes (20, 33) and a tightly controlled inducible promoter (26).

The genetic prowess of *H. volcanii* is not yet fully matched by corresponding systems for protein overexpression and purification. Like other haloarchaea, *H. volcanii* grows in high salt concentrations (2 to 5 M NaCl), and to cope with the osmotic potential of such environments, it accumulates high intracellular concentrations of potassium ions (12). Consequently, halophilic proteins are adapted to function at high salt concentrations and commonly feature a large excess of acidic amino acids; the negative surface charge is thought to be critical to solubility (28). This can pose problems for expression in heterologous hosts, such as *E. coli*, since halophilic proteins can misfold and aggregate under conditions of low ionic strength. The purification of misfolded halophilic enzymes from *E. coli* has relied on the recovery of insoluble protein from inclusion bodies, followed by denaturation and refolding in hypersaline solutions (8, 11). This approach is feasible only where the protein is well characterized and reconstitution of the active form can be monitored (for example, by an enzymatic assay). Furthermore, archaeal proteins expressed in heterologous bacterial hosts lack posttranslational modifications, such as acetylation or ubiquitination (4, 22), which are critical to understanding their biological function.

Systems for expression of halophilic proteins in a native haloarchaeal host are therefore required. A number of studies have successfully purified recombinant proteins with a variety of affinity tags after overexpression in *H. volcanii*. For example, Humbard et al. employed tandem affinity tagging to purify 20S proteasomal core particles from the native host (23). However, the protein expression constructs used in these studies were custom made and somewhat tailored to the application in question. We report here the development of "generic" plasmid vectors and host strains for conditional overexpression of halophilic proteins in *H. volcanii*. The plasmids feature a tryp-

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TABLE 1. *H. volcanii* strains used

Strain	Genotype[a]	Source or reference[b]
H26	Δ*pyrE2*	3
H98	Δ*pyrE2* Δ*hdrB*	3
H133	Δ*pyrE2* Δ*trpA* Δ*leuB* Δ*hdrB*	3
H989	Δ*pyrE2* Δ*hdrB* {p.*tnaA*::6×His tag *pyrE2*$^+$ *hdrB*$^+$}	H98 pTA963
H1045	Δ*pyrE2* Δ*hdrB* {p.*tnaA*::6×His tag::*radA*$^+$ *pyrE2*$^+$ *hdrB*$^+$}	H98 pTA1041
H1154	Δ*pyrE2-pitA*$_{Nph}$	H26 (pTA1106)
H1155	Δ*pyrE2* Δ*hdrB pitA*$_{Nph}$	H98 (pTA1106)
H1172	Δ*pyrE2* Δ*hdrB pitA*$_{Nph}$ {p.*tnaA*::6×His tag *pyrE2*$^+$ *hdrB*$^+$}	H1155 pTA963
H1173	Δ*pyrE2* Δ*hdrB pitA*$_{Nph}$ {p.*tnaA*::6×His tag::*radA*$^+$ *pyrE2*$^+$ *hdrB*$^+$}	H1155 pTA1041
H1174	Δ*pyrE2* Δ*hdrB pitA*$_{Nph}$ {p.*tnaA*::6×His tag::*radB*$^+$ *pyrE2*$^+$ *hdrB*$^+$}	H1155 pTA1043
H1206	Δ*pyrE2* Δ*mrr*	H26 (pTA1150)
H1207	Δ*pyrE2 pitA*$_{Nph}$ Δ*mrr*	H1154 (pTA1150)
H1208	Δ*pyrE2* Δ*hdrB* Δ*mrr*	H98 (pTA1150)
H1209	Δ*pyrE2* Δ*hdrB pitA*$_{Nph}$ Δ*mrr*	H1155 (pTA1150)
H1227	Δ*pyrE2* Δ*hdrB pitA*$_{Nph}$ Δ*mrr* {p.*tnaA*::6×His tag::*radA*(*A196V*) *pyrE2*$^+$ *hdrB*$^+$}	H1209 pTA1182
H1228	Δ*pyrE2* Δ*hdrB pitA*$_{Nph}$ Δ*mrr cdc48d*$^+$::[Δ*cdc48d pyrE2*$^+$]	H1209 pTA1180[c]
WR755	Δ*pyrE2* Δ*trpA* Δ*leuB* Δ*hdrB pitA*$^+$::[Δ*pitA pyrE2*$^+$]	H133 pMM1231[c]
WR756	Δ*pyrE2* Δ*trpA* Δ*leuB* Δ*hdrB pitA*$^+$::[Δ*pitA*::*hdrB*$^+$ *pyrE2*$^+$]	H133 pMM1232[c]

[a] Episomal plasmids are indicated by braces ({}); plasmids integrated on the chromosome are indicated by brackets ([]).
[b] Deletion constructs used successfully are indicated by parentheses.
[c] Pop-in strain from which a pop-out strain could not be obtained.

tophan-inducible promoter derived from the *tnaA* gene of *H. volcanii* (26). We demonstrate the utility of these vectors by overexpressing a hexahistidine-tagged recombinant version of the *H. volcanii* RadA protein. Purification was greatly facilitated by a host strain in which the endogenous *pitA* gene was replaced by an ortholog from *Natronomonas pharaonis*. The latter protein lacks the histidine-rich linker region found in *H. volcanii* PitA (5) and therefore does not copurify with His-tagged recombinant proteins. Finally, we deleted the *mrr* gene of *H. volcanii*, which encodes a restriction enzyme that cleaves foreign DNA methylated at GATC residues. The *mrr* deletion strain allows direct transformation of *H. volcanii* without the need to passage plasmid DNA through an *E. coli dam* mutant (21).

MATERIALS AND METHODS

Unless stated otherwise, chemicals were from Sigma and restriction enzymes were from New England Biolabs. Standard molecular techniques were used (34).

Strains and plasmids. *H. volcanii* strains (Table 1) were grown at 45°C on complete (Hv-YPC) or Casamino Acids (Hv-Ca) agar or in Hv-YPC or Hv-Ca

TABLE 2. Plasmids used

Plasmid	Relevant properties	Source or reference[a]
pTA131	Integrative vector based on pBluescript, with *pyrE2* marker	3
pTA187	Integrative vector based on pUC19, with *hdrB* marker	3
pTA230	Shuttle vector derived from pTA131, with *pyrE2* marker and pHV2 replication origin	3
pTA354	Shuttle vector based on pBluescript, with *pyrE2* marker and pHV1/4 replication origin	31
pTA927	Overexpression vector with *pyrE2* marker and pHV2 origin, derived from pTA230 by insertion of 131-bp t.L11e terminator at KpnI site, 224-bp p.*tnaA* promoter at ApaI and ClaI sites, and 35-bp t.Syn terminator at NotI and BstXI sites	This study; FN645893
pTA929	Overexpression vector with 6×His tag, *pyrE2* marker, and pHV2 origin, derived from pTA927 by insertion of 26-bp fragment containinga His tag $(CAC)_6$ tract at the NdeI and ClaI sites	This study; FN645892
pTA949	Shuttle vector derived from pTA230, with *pyrE2* and *hdrB* markers and pHV2 replication origin	This study; FN645894
pTA962	Overexpression vector with *pyrE2* and *hdrB* markers and pHV2 origin, derived from pTA949 by insertion of t.L11e terminator, p.*tnaA* promoter and t.Syn terminator (see pTA927 for details)	This study; FN645891
pTA963	Overexpression vector with 6×His tag, *pyrE2* and *hdrB* markers, and pHV2 origin, derived from pTA962 by insertion of a His tag $(CAC)_6$ tract (see pTA929 for details)	This study; FN645890
pTA1041	pTA963 with insertion of 1.0-kb NcoI-BamHI *radA*$^+$ fragment at the PciI and BamHI sites; for overexpression of 6×His-tagged RadA	This study
pTA1043	pTA963 with insertion of 0.7-kb BspHI-BamHI *radB*$^+$ fragment at the PciI and BamHI sites; for overexpression of 6×His-tagged RadB	This study
pTA1106	pTA131 with 3.8-kb *pitA*$_{Nph}$ gene replacement construct, consisting of HindIII-EcoRI upstream Δ*pitA*$_{Hvo}$ fragment, EcoRI-XbaI *pitA*$_{Nph}$$^+$ fragment, and XbaI-NotI downstream Δ*pitA*$_{Hvo}$ fragment inserted at HindIII and NotI sites	This study
pTA1150	pTA131 with 1.3-kb Δ*mrr* construct consisting of a KpnI-BamHI upstream Δ*mrr* fragment and a BamHI-XbaI downstream Δ*mrr* fragment inserted at KpnI and XbaI sites	This study
pTA1180	pTA131 with 878-bp Δ*cdc48d* construct consisting of KpnI-BamHI upstream Δ*cdc48d* fragment and BamHI-EcoRI downstream Δ*cdc48d* fragment inserted at KpnI and EcoRI sites	This study
pTA1182	pTA963 with insertion of 1.0-kb NcoI-BamHI *radA*(*A196V*) fragment at PciI and BamHI sites; for overexpression of 6×His-tagged RadA(A196V)	This study
pMM1231	pTA131 with 2.0-kb Δ*pitA*$_{Hvo}$ construct consisting of a HindIII-EcoRI upstream Δ*pitA*$_{Hvo}$ fragment and an EcoRI-NotI downstream Δ*pitA*$_{Hvo}$ fragment; inserted at HindIII and NotI sites	This study
pMM1232	pTA131 with 2.7-kb Δ*pitA*$_{Hvo}$::*hdrB*$^+$ construct consisting of a HindIII-EcoRI upstream Δ*pitA*$_{Hvo}$ fragment, an EcoRI-XbaI fragment of pTA187 with an *hdrB* marker, and an XbaI-NotI downstream Δ*pitA*$_{Hvo}$ fragment; inserted at HindIII and NotI sites	This study

[a] The accession number is given where appropriate.

TABLE 3. Oligonucleotides used

Primer	Sequence (5′–3′)[a]	Relevant properties	Plasmid(s)
TERF	GACGGTACCGACTTCGACGACTACTTCGACG	t.L11e forward primer; KpnI site	pTA927 pTA962
TERR	GGCGGTACCGGGTCGAATCGGGTCGGTG	t.L11e reverse primer; KpnI site	pTA927 pTA962
PtnaApa2	CGAGTTCTGGGCCCGTTCTCGTCG	p.tnaA forward primer; ApaI site	pTA927 pTA962
PtnaNde3	CCTTGATCGATTT**CATATG**CGCAATAGGTCC	p.tnaA reverse primer; ClaI and NdeI sites	pTA927 pTA962
t.syn F	GGCCGCACCTCTGGACCATCGCATTTTTCGGCGCG	Top strand of t.Syn fragment; NotI and BstXI ends	pTA927 pTA962
t.syn R	CCGAAAAATGCGATGGTCCAGAGGTGC	Bottom strand of t.Syn fragment	pTA927 pTA962
HisndeI	TATGCACCACCACCACCACC**ACATGT**	Top strand of 6×His tag; NdeI end; *Pci*I site	pTA929 pTA963
HisclaI	CG**ACATGT**GGTGGTGGTGGTGGTGCA	Bottom strand of 6×His tag; ClaI end; PciI site	pTA929 pTA963
radANcoF	GAACGACTGGCCATGGCAGAAGACG	*radA* forward primer; NcoI site	pTA1041 pTA1182
radABamR	CCGACGGATCCACGGCTTACTCGG	*radA* reverse primer; BamHI site	pTA1041 pTA1182
radBBsF	CCTCCTGTCATGACAGAGTCAGTCTCC	*radB* forward primer; BspHI site	pTA1043
radBBamR2	CCGCGTGGATCCCTTTTCTACACG	*radB* reverse primer; BamHI site	pTA1043
mrrUSF	GGATGGTACCGCCGTAGAACAGCG	Δ*mrr* upstream external primer; KpnI site	pTA1150
mrrUSR	CCGGGGATCCCGTCATCGCTCGAC	Δ*mrr* upstream internal primer; BamHI site	pTA1150
mrrDSF	GGCGGATCCGTCGGCATTTGGCTC	Δ*mrr* downstream internal primer; BamHI site	pTA1150
mrrDSR	CCGCTCTAGAAGGCCGAGGAGGCC	Δ*mrr* downstream external primer; XbaI site	pTA1150
cdc48dUF	ACGGGTACCCACGTTGCTGG	Δ*cdc48d* upstream external primer; KpnI site	pTA1180
cdc48dUR	GGACGGATCCGTCGAACCGAG	Δ*cdc48d* upstream internal primer; BamHI site	pTA1180
cdc48dDF	CACGGATCCCCCAGAAATTGC	Δ*cdc48d* downstream internal primer; BamHI site	pTA1180
cdc48dDR	GCCGAATTCGAGCCGAGGTGG	Δ*cdc48d* downstream external primer; EcoRI site	pTA1180
5′Up HindIII	AAGCTTTCCGACCCGATTCGCGTGAC	Δ$pitA_{Hvo}$ upstream external primer; HindIII site	pMM1231 pMM1232 pTA1106
3′Up EcoRI	GAATTCACGAGGGCCTAGGGAGTCATCC	Δ$pitA_{Hvo}$ upstream internal primer; EcoRI site	pMM1231 pMM1232 pTA1106
5′Down EcoRI	GAATTCCTTCGCGACCATCGGGAGC	Δ$pitA_{Hvo}$ downstream internal primer; EcoRI site	pMM1231
3′Down NotI	GCGGCCGCCGGCATCGACCGCTTCGAC	Δ$pitA_{Hvo}$ downstream external primer; NotI site	pMM1231 pMM1232 pTA1106
5′Down XbaI	TCTAGACTTCGCGACCATCGGGAGC	Δ$pitA_{Hvo}$ downstream internal primer; XbaI site	pMM1232 pTA1106
5′NatroEcoRI	GAATTCATGCCACAACGCCAACCAC	$pitA_{Nph}$ forward primer;, EcoRI site	pTA1106
3′NatroXbaI	TCTAGATCAGGCGAGGAAGACGTGG	$pitA_{Nph}$ reverse primer; XbaI site	pTA1106
pitAF	GGAAAATCAAGCAGGTCATCGC	Forward primer specific to $pitA_{Hvo}$	NA
pitAR	GTAGAACATCCCCATCGTGCC	Reverse primer specific to $pitA_{Hvo}$	NA
NphPitAF	GCAGTATGCCGACAAGGTCTCC	Forward primer specific to $pitA_{Nph}$	NA
NphPitAR	CCCGCTCGTTTTTCCACAG	Reverse primer specific to $pitA_{Nph}$	NA
mrrF	TGGGCGTTCAGGCGAAGC	Forward primer for *mrr* probe	NA
mrrR	CGGGTGAGCGACCAGCGG	Reverse primer for *mrr* probe	NA

[a] Restriction endonuclease sites used in cloning are underlined; internal sites used subsequently are in boldface. NA, not applicable.

broth, as described previously (3, 17). Gene deletion/replacement mutants were constructed using a knockout system described previously (3, 7). The plasmids for gene deletion/replacement and protein overexpression are shown in Table 2 and were generated by PCR using the primers in Table 3. *E. coli* strains XL1-Blue MRF′ (Δ*mcrA183* Δ*mcrCB-hsdSMR-mrr173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15* 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 with 100 μg/ml ampicillin where appropriate. The latter strain was used to prepare unmethylated DNA for transformation of *H. volcanii*.

Protein overexpression and purification. A starter culture was grown overnight in 40 ml Hv-Ca broth to an optical density at 650 nm (OD_{650}) of ~0.6 and used to inoculate 360 ml Hv-YPC broth containing 1 mM tryptophan (Trp) to induce protein expression. The culture was incubated at 42°C with shaking (175 rpm) for 5 to 6 h to an OD_{650} of ~0.5, when protein expression was further induced by adding 36 ml prewarmed 25 mM Trp dissolved in 18% salt water (SW) (3), and the culture was incubated at 42°C with shaking for a further 1 h. The culture was then centrifuged at 3,300 × *g* for 10 min at 4°C, and the cells were resuspended in 7 ml ice-cold binding buffer (2 M NaCl, 20 mM HEPES, pH

7.5, 20 mM imidazole, 1 mM phenylmethanesulfonyl fluoride) and lysed by sonication on ice until the suspension was no longer turbid. The cell lysate was clarified by centrifugation at 16,000 × *g* for 15 min at 4°C and incubated overnight at 4°C with 0.5 ml of IMAC Sepharose 6 FastFlow beads (GE Healthcare) that had been charged with Ni^{2+} and equilibrated in binding buffer. The slurry was applied to a Poly-Prep column (Bio-Rad), and the flowthrough was collected and reloaded onto the column, followed by three washes with 4 ml of ice-cold binding buffer and one wash with 1 ml ice-cold binding buffer containing 50 mM imidazole. Bound protein was eluted with 1 ml binding buffer containing 500 mM imidazole. Samples were analyzed on 12.5% SDS-PAGE gels with PageRuler size marker (Fermentas), and quantification of protein bands stained with Coomassie blue was carried out using ImageGauge v4.22 (Fuji).

Mass spectrometry. Proteins in gel bands were reduced, carboxyamidomethylated, and digested with Trypsin Gold (Promega) on a robotic platform for protein digestion (MassPREP station; Waters). The resulting peptides were analyzed by electrospray ionization-tandem mass spectrometry (MS/MS) after on-line separation on a PepMap C_{18} reversed-phase, 75-μm-inner-diameter, 15-cm column (LC Packings) on a CapLC system attached to a Q-TOF2 mass spectrometer equipped with a nanolockspray source (Waters) and operated with MassLynx version 4.0 acquisition software. ProteinLynxGlobalSERVER software version 2.1 (Waters) was used to generate a peak list file of uninterpreted fragment mass data, which was used to search the Swissprot 57.1 and NCBInr databases using the MASCOT search engine (32). Only protein identifications with probability-based MOWSE scores above a threshold of $P < 0.05$ were accepted. The *H. volcanii* genome sequence (19) is not currently available for interrogation with uninterpreted mass spectral data; therefore, protein identifications relied on matches to publicly available sequences from related haloarchaea (see Table 4), which were used to find corresponding sequences in the *H. volcanii* genome at HaloLex (https://www.halolex.mpg.de/public/).

Protein sequence alignment. Alignment of the Mrr core domain was carried out in MacVector using ClustalW (BLOSUM; penalty for open gap = 10; extend gap = 2). A neighbor-joining tree was built using *Schizosaccharomyces pombe* SPAC824.03c as an outgroup.

Nucleotide sequence accession numbers. The sequences of the major plasmid vectors constructed in this study have been deposited in the EMBL nucleotide sequence database under accession numbers FN645893 (pTA927), FN645892 (pTA929), FN645894 (pTA949), FN645891 (pTA962), and FN645890 (pTA963).

RESULTS

Plasmid vectors for conditional overexpression of hexahistidine-tagged proteins. A series of plasmid shuttle vectors for conditional protein overexpression that utilized the p.*tnaA* tryptophanase promoter of *H. volcanii* were constructed (Fig. 1). Genes under the control of the p.*tnaA* promoter show rapid and strong induction of expression upon addition of ≥1 mM tryptophan (26). For expression of proteins with an N-terminal hexahistidine (6×His) tag, a $(CAC)_6$ tract was incorporated downstream of the p.*tnaA* promoter; plasmid variants without the 6×His tag were also generated (Fig. 1). To ensure that the gene was insulated from read-through transcription initiated elsewhere on the plasmid, the expression cassette was flanked by two transcriptional terminators, the L11e rRNA terminator (t.L11e) (38) and a synthetic terminator (t.Syn) comprising a T tract flanked by G/C-rich sequences. The vectors were based on pTA230 (3) and used the pHV2 replication origin, which maintained the plasmid at a copy number of ~6 per genome equivalent (10). Selection was based on the *pyrE2* and *hdrB* markers, which allowed growth on media lacking uracil and thymidine, respectively (3, 7). The former marker complemented the *pyrE2* deletion found in almost all laboratory strains of *H. volcanii* (3), while the latter marker allowed the plasmid to be maintained in rich medium (Hv-YPC); plasmid variants are available with the *pyrE2* marker only (Fig. 1).

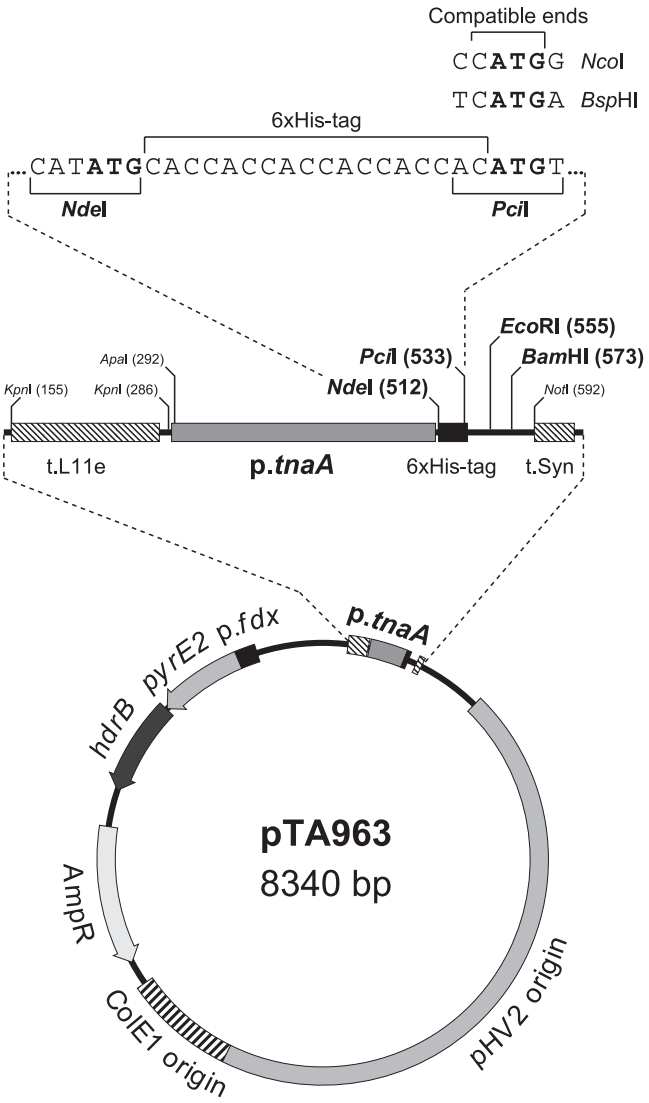


FIG. 1. Conditional overexpression vector pTA963. pTA963 features a tryptophan-inducible promoter from the *tnaA* gene (p.*tnaA*) (26) flanked by the L11e rRNA terminator (t.L11e) (38) and a synthetic terminator (t.Syn). For expression of native proteins, the coding sequence is inserted between the NdeI site downstream of the promoter and either the EcoRI or BamHI site. For N-terminal 6×His-tagged proteins, the 5′ end of the gene is ligated instead with the PciI site located downstream of a $(CAC)_6$ tract. PciI-compatible ends are generated by NcoI and BspHI and are used where the second codon starts with G and A, respectively. pTA963 uses a pHV2 replication origin and has *hdrB* and *pyrE2* selectable markers; variants are available with *pyrE2* only (pTA929) and without a $(CAC)_6$ tract (pTA962, or pTA927 for *pyrE2* only).

Replacement of the *H. volcanii pitA* gene with an ortholog from *Natronomonas pharaonis*. The *H. volcanii* PitA protein is a fusion of chlorite dismutase-like and antibiotic synthesis monooxygenase-like domains within a single open reading frame (5). PitA is unique to haloarchaea and in almost all species features a histidine-rich linker between the conserved N- and C-terminal domains (Fig. 2A). Owing to the numerous histidines in this region, PitA is a major contaminant of His-tagged recombinant proteins purified from *H. volcanii* by immobilized metal affinity chromatography (5). We attempted to delete *pitA* by the pop-in/pop-out technique based on the *pyrE2* counterselectable marker (7), using the deletion constructs pMM1231

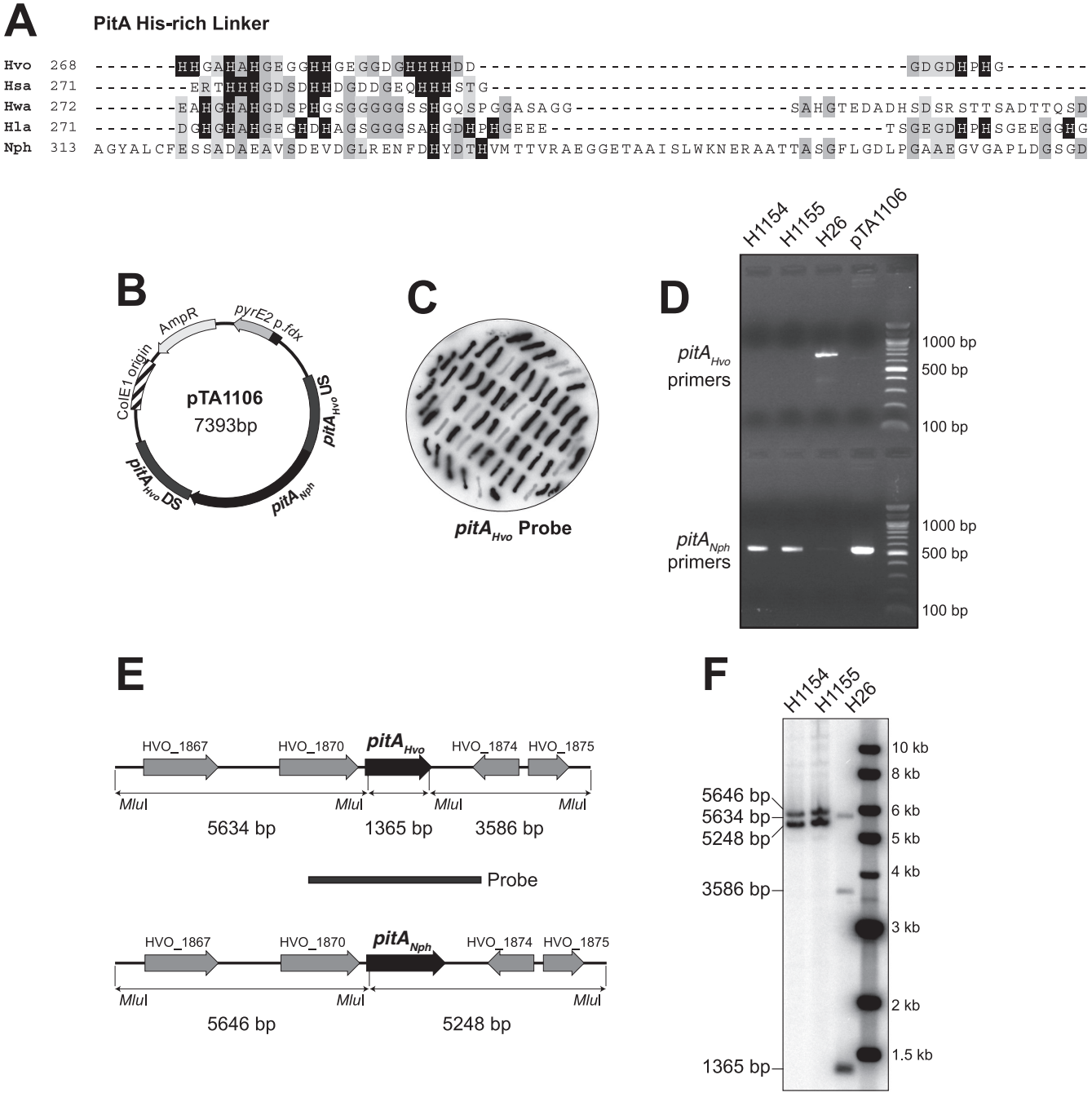
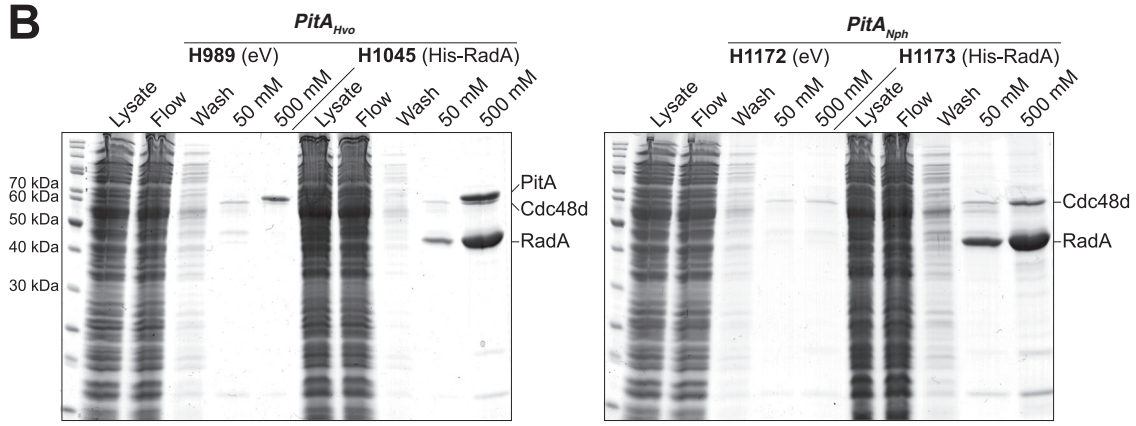
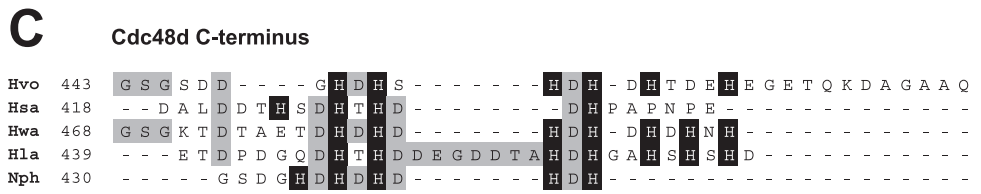


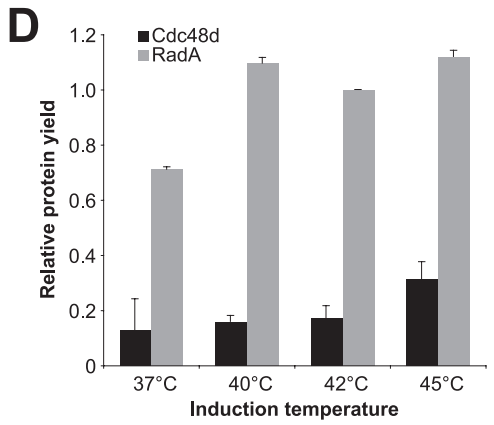
FIG. 2. Replacement of the *pitA* gene. (A) Protein sequence alignment of the central region of PitA (5), linking the N-terminal chlorite dismutase-like and C-terminal antibiotic biosynthesis monooxygenase-like domains, from selected species of haloarchaea. Histidine residues are indicated by a black background; conserved residues are indicated by gray shading. Hvo, *H. volcanii*; Hsa, *Halobacterium salinarum*; Hwa, *Haloquadratum walsbyi*; Hla, *Halorubrum lacusprofundi*; Nph, *N. pharaonis*. (B) Gene replacement construct pTA1106, containing *pitA* from *N. pharaonis* ($pitA_{Nph}$) flanked by upstream (US) and downstream (DS) regions of *H. volcanii pitA* ($pitA_{Hvo}$). (C) Colony hybridization of 5-fluoro-orotic acid (5-FOA)-resistant *H. volcanii* clones, after pop-in/pop-out gene replacement with pTA1106. *H. volcanii pitA* sequences (amplified with pitAF/pitAR primers) were used as a probe, and clones failing to hybridize therefore carried the *N. pharaonis pitA* gene. (D) Verification of *pitA* replacement in H1154 and H1155 using PCR with primers specific for either *H. volcanii* (pitAF/pitAR) or *N. pharaonis* (NphPitAF/NphPitAR) genes. H26 genomic DNA was used as a negative control and pTA1106 plasmid DNA as a positive control. (E) Map of the *H. volcanii pitA* region, indicating MluI sites and restriction fragment sizes of the native *pitA* gene ($pitA_{Hvo}$) and the replacement with *N. pharaonis pitA* ($pitA_{Nph}$). (F) Verification of *pitA* replacement in H1154 and H1155 by MluI digestion and Southern blotting. The probe used is indicated in panel E.

A

Induction regime	Relative Protein Yield
6 h Hv-Ca	0.02
6 h Hv-YPC	1.00
5 h YPC, 1 h YPC+1 mM Trp	4.60
6 h YPC+1 mM Trp	5.23
5 h YPC+1 mM Trp, 1 h YPC+3 mM Trp	14.5
6 h YPC+3 mM Trp	12.1







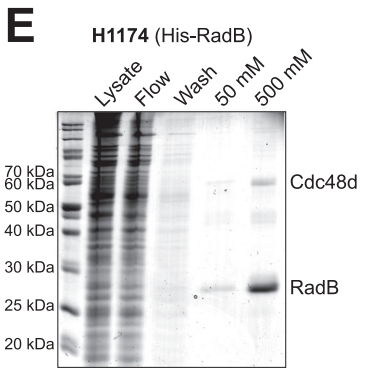


FIG. 3. Overexpression of RadA in a *pitA*$_{Nph}$ replacement strain. (A) Induction regime for protein overexpression using the tryptophan-inducible p.*tnaA* promoter. H1173 was grown for the times indicated in either Hv-Ca or Hv-YPC broth supplemented with tryptophan at the concentrations shown. 6×His-tagged RadA was purified, and the relative protein yield was determined by quantification of Coomassie blue-stained bands displayed on polyacrylamide gels as in panel B. (B) 6×His-tagged RadA was overexpressed in strains with either the native *pitA* gene (PitA $_{Hvo}$; H1045) or replacement with *N. pharaonis pitA* (PitA $_{Nph}$; H1173). The induction regime used was 5 h in Hv-YPC plus 1 mM tryptophan and 1 h in Hv-YPC plus 3 mM tryptophan at 42°C. The control strains H989 and H1172 contained the empty vector (eV) pTA963. 6×His-tagged RadA was purified from the soluble fraction (Lysate) by affinity chromatography on an Ni^{2+} chelating column, and samples were taken from the flowthrough (Flow) and after washing with 20 mM imidazole (Wash). Bound protein was eluted using 50 mM and 500 mM imidazole. Two additional bands were identified by mass spectrometry, PitA and Cdc48d. (C) Protein sequence alignment of the C termini of Cdc48d from selected species of haloarchaea. Histidine residues are indicated by a black background; conserved residues are indicated by gray shading. (D) Contamination by Cdc48d is reduced by growth at <45°C. 6×His-tagged RadA was overexpressed in H1173 and purified by affinity chromatography on a Ni^{2+} chelating column as in panel B. The relative yields of RadA and Cdc48d (standardized to the RadA yield at 42°C) were determined by quantification of Coomassie blue-stained bands displayed on polyacrylamide gels. The averages and standard errors of two experiments are shown. (E) Overexpression of 6×His-tagged RadB results in less contamination by Cdc48d. 6×His-tagged RadB was overexpressed in strain H1174 and purified by affinity chromatography on a Ni^{2+} chelating column as in panel B.

TABLE 4. Mass spectrometry identification of *H. volcanii* proteins obtained by immobilized metal affinity chromatography

Protein name	NCBI or Swiss-Prot entry	MASCOT score	Coverage (%)	No. of unique peptides	Peptide sequences	Predicted mass (kDa)	Observed mass (kDa)	HaloLex entry
PitA	gi‖224820302 (*Natrialba magadii* chlorite dismutase)	63	3	2	SIDWDAWR DVLADRPR	56.1	60.9	HVO_1871
Cdc48d	gi‖110668990 (*Haloquadratum walsbyi* AAA-type ATPase)	369	12	5	ILFVGPPGTGK EAVLEALTEER ILFIDEFDSVAK[a] LSMITSQYLGETAK RFDEIVNFPKPDR	53.4	55.7	HVO_1907
RadA	Q48328 (*Haloferax volcanii* DNA repair and recombination protein RadA)	546	32	9	ADIGSSTASDIINAA DAADVGGFETGSMVLER GLEDEALEATLDDR EMEGSIDDEETIK ALVDDFLDK VDDFLDKIHVAK[a] AGEHEDTEWPVR[a] LVDAPNLADGEAIMR VQDAGLKPE	38.3[b]	41.9	HVO_0104

[a] Semitryptic peptide.
[b] Including the hexahistidine tag.

and pMM1232. In the latter construct, the *pitA* coding sequence is replaced with an *hdrB* marker, allowing direct selection for gene deletion events (3). With either construct (in strains WR755 and WR756, respectively), we were unable to recover cells with a *pitA* deletion (data not shown), indicating that this gene is most likely essential.

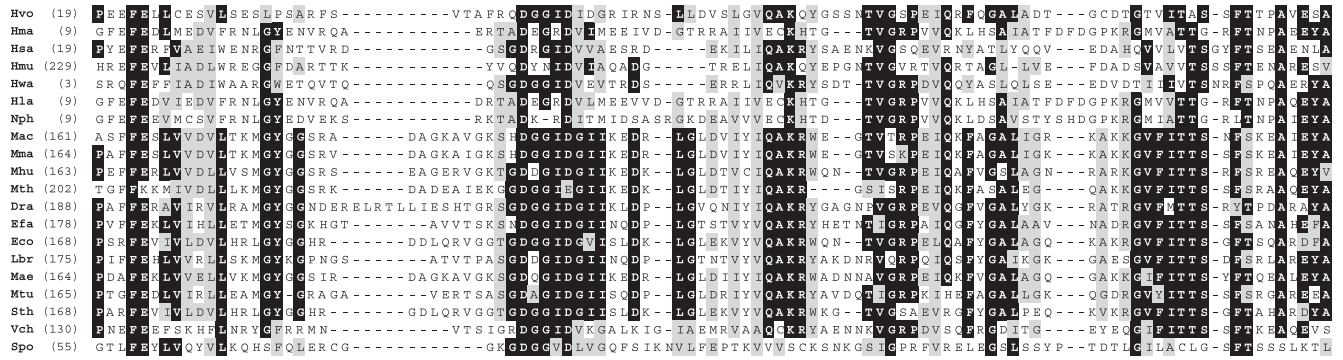
The PitA ortholog from the haloalkaliphile *Natronomonas pharaonis* is unique in that it does not feature a high number of histidines in the central linker region. We reasoned that replacing *H. volcanii pitA* ($pitA_{Hvo}$) with the *N. pharaonis* gene ($pitA_{Nph}$) would prevent copurification with His-tagged recombinant proteins. Gene replacement with the construct in pTA1106 (Fig. 2B) was carried out using the pop-in/pop-out technique (3, 7). Successful replacement of $pitA_{Hvo}$ with $pitA_{Nph}$ was established by colony lift and verified by both PCR and Southern blotting of a restriction digest (Fig. 2C to F). The resulting *H. volcanii* strains, H1154 and H1155, with the $pitA_{Nph}$ gene replacement showed no obvious growth defects in Hv-YPC and Hv-Ca broth and agar.

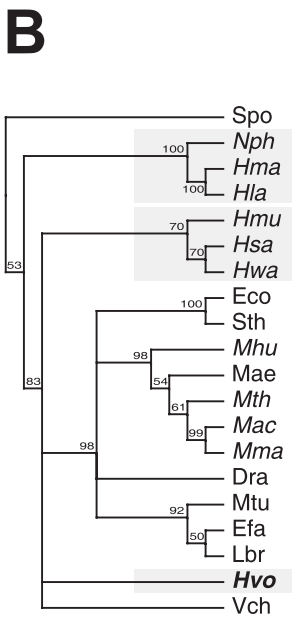
Overexpression of 6×His-tagged RadA in $pitA_{Hvo}$ and $pitA_{Nph}$ strains. 6xHis-tagged RadA was used to test the conditional protein overexpression system. RadA is the archaeal RecA family recombinase; it forms a nucleoprotein filament with single-stranded DNA and catalyzes strand exchange during homologous recombination (18, 40). Purification of recombinant 6×His-tagged RadA from *E. coli* has proved problematic. The protein copurifies with DNA, and removal of the latter requires harsh conditions (denaturation and benzonase treatment) that interfere with RadA activity (K. Bunting, personal communication).

The *radA* coding sequence was cloned in pTA963 and used to transform *H. volcanii* strains H98 and H1155. To determine the optimal induction regime for protein overexpression, the conditions shown in Fig. 3A were used. Since concentrations of >1 mM tryptophan affect the growth of *H. volcanii* (even without the RadA expression construct), it proved best to delay full induction (with 3 mM tryptophan) until 1 h before the cells were harvested. Metal affinity chromatography was used to purify 6×His-tagged RadA in the presence of 2 M NaCl, using an IMAC Sepharose column charged with Ni^{2+}. We had previously established that the yield of 6×His-tagged protein from columns charged with Co^{2+} was very low when the buffers contained >1 M NaCl (data not shown). The 6×His-tagged RadA purified from the $pitA_{Hvo}$ strain H1045 showed significant contamination with PitA, while this was not seen when 6×His-tagged RadA was purified from the $pitA_{Nph}$ strain, H1173 (Fig. 3B); the identities of both proteins were confirmed by mass spectrometry (Table 4).

The absence of $PitA_{Hvo}$ in cell lysates of H1173 revealed an additional contaminant, which was identified by mass spectrometry as Cdc48d (HVO_1907); Cdc48d is also present in H1045 but is difficult to distinguish in size from PitA (Fig. 3B). HVO_1907 is one of four *H. volcanii* isoforms of a putative AAA^+ ATPase that show homology to the yeast Ccd48 and *E. coli* FtsH proteins. In eukaryotes, Cdc48 is a ubiquitin-dependent chaperone that is involved in protein degradation (29), while bacterial FtsH is a Zn^{2+} metalloprotease that degrades a set of short-lived proteins (24); both are thought to regulate cell division at the level of protein stability. Notably, *H. volcanii* Cdc48d features a histidine-rich C terminus (Fig. 3C) that is almost certainly responsible for its copurification on metal affinity chromatography columns.

We attempted to delete *cdc48d* by the pop-in/pop-out technique (3, 7), using the deletion construct pTA1180 (in strain H1228). We were unable to recover cells with a *cdc48d* deletion (data not shown), indicating that the gene is essential. Unfortunately, all known haloarchaeal homologs of Cdc48d feature the conserved histidine-rich C terminus (Fig. 3C), ruling out the gene replacement strategy that had proved successful in eliminating PitA contamination. Increased concentrations of imidazole in the binding and wash buffers (>20 mM) were not helpful, and the yield of 6×His-tagged RadA was reduced significantly without eliminating Cdc48d contamination. However, growth at lower


A
Mrr Conserved Core
Hvo (19)
Hma (9)
Hsa (19)
Hmu (229)
Hwa (3)
Hla (9)
Nph (9)
Mac (161)
Mma (164)
Mhu (163)
Mth (202)
Dra (188)
Efa (178)
Eco (168)
Lbr (175)
Mae (164)
Mtu (165)
Sth (168)
Vch (130)
Spo (55)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Spo
Nph
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Hla
Hmu
Hsa
Hwa
Eco
Sth
Mhu
Mae
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92
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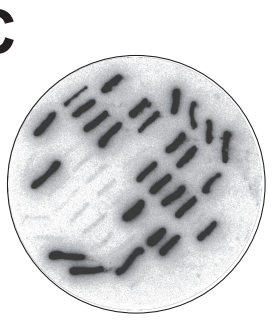
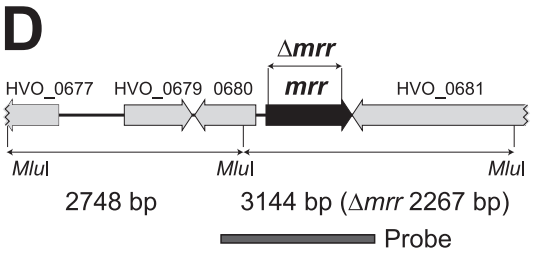
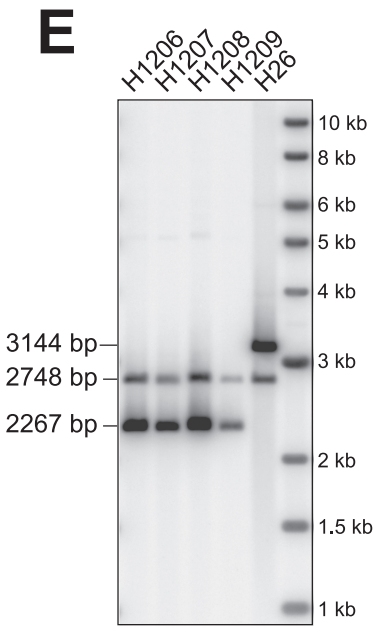

C
mrr Probe


D
Δmrr
mrr
HVO_0677
HVO_0679
0680
HVO_0681
MluI
MluI
MluI
2748 bp
3144 bp (Δmrr 2267 bp)
Probe


E
H1206
H1207
H1208
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H26
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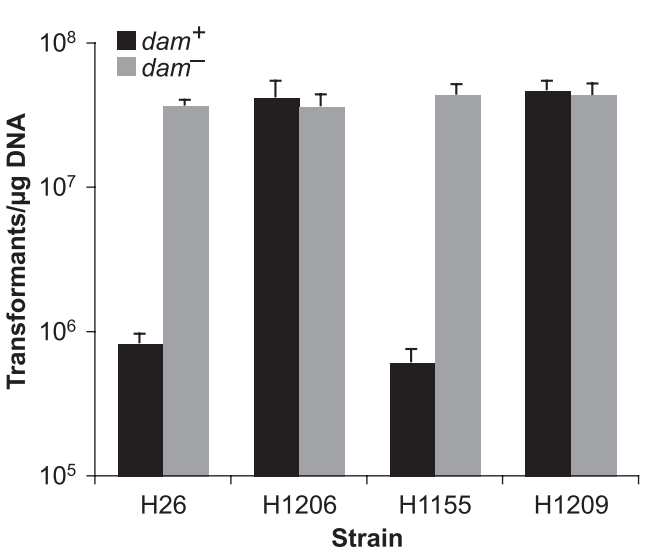
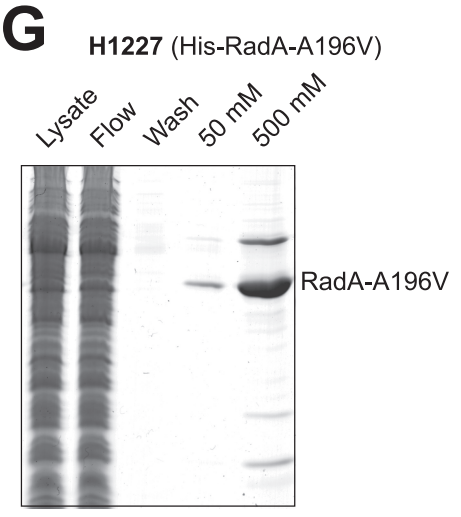
2748 bp
2267 bp


F
dam+
dam−
Transformants/μg DNA
10^8
10^7
10^6
10^5
H26
H1206
H1155
H1209
Strain


G
H1227 (His-RadA-A196V)
Lysate
Flow
Wash
50 mM
500 mM
RadA-A196V

temperatures was more successful; copurification of Cdc48d was reduced by 50% when cultures were grown at 40°C or 42°C, rather than the standard cultivation temperature of 45°C (Fig. 3D). Contamination by Cdc48d was also less pronounced during the purification of other recombinant proteins, such as 6×His-tagged RadB (Fig. 3E).

Deletion of the *Haloferax volcanii mrr* restriction endonuclease gene. It has long been suspected that *H. volcanii* encodes a methylation-sensitive restriction enzyme that targets 5′-GATC-3′ sequences. Transformation of *H. volcanii* is much more efficient when the DNA is purified from *E. coli dam* mutants, which are unable to methylate 5′-GATC-3′, than from *dam*$^+$ strains (21). Furthermore, transformation of *H. volcanii* with DNA methylated at 5′-GATC-3′ sequences leads to frequent plasmid loss, presumably due to cutting by restriction enzymes followed by recombination with chromosomal sequences (21). We hypothesized that the *H. volcanii* restriction endonuclease might be HVO_0682, which belongs to the Mrr family of enzymes that recognize and cleave N^6-methyladenine- and 5-methylcytosine-containing DNA (9). The conserved core of HVO_0682 shows homology to other Mrr family members from archaea, bacteria, and yeast (Fig. 4A). However, in a phylogenetic tree, the *H. volcanii* Mrr protein does not group with homologs from other haloarchaea (Fig. 4B).

We successfully deleted the *mrr* gene (from both $pitA_{Hvo}$ and $pitA_{Nph}$ strains) by the pop-in/pop-out technique (3, 7), using the construct pTA1150. Successful deletion of *mrr* was established by colony lift and verified by Southern blotting of a restriction digest (Fig. 4C to E). The *H. volcanii* Δ*mrr* mutants H1206 to H1209 showed no obvious growth defects in Hv-YPC and Hv-Ca broth and agar. To test the methylation-dependent restriction barrier, *mrr*$^+$ and Δ*mrr* strains (and the $pitA_{Nph}$ equivalents) were transformed with the shuttle vector pTA354 (31), which had been purified from either an *E. coli dam* mutant or a *dam*$^+$ strain. The number of transformants obtained in an *mrr*$^+$ strain was ~50-fold higher with DNA from the *E. coli dam* mutant than with DNA from the *dam*$^+$ strain, while the Δ*mrr* mutants were transformed efficiently regardless of the source of the DNA (Fig. 4F). Therefore, deletion of the *mrr* gene removed the need to passage DNA through an *E. coli dam* mutant prior to transformation of *H. volcanii*. Furthermore, it allowed direct transformation of *H. volcanii* with a DNA ligation, which might be useful for cloning of protein overexpression constructs that are not tolerated by *E. coli*.

To test this possibility, we PCR amplified a mutant allele of *radA* [*radA*(*A196V*)], ligated the cut product with pTA963, and transformed the *H. volcanii* Δ*mrr* strain H1209. Although only ~200 transformants were obtained (from ~1 μg vector DNA), 3 out of the 6 tested contained the correct plasmid construct (pTA1182). In contrast, we had failed on six previous occasions to construct this plasmid in *E. coli*, even though the PCR primers and restriction digests used were identical to those that had been used successfully to construct the *radA*$^+$ overexpression plasmid pTA1041. This suggests that in *E. coli*, the p.*tnaA* promoter is leaky and RadA(A196V) is toxic. To confirm that 6×His-tagged RadA(A196V) could be purified from *H. volcanii*, we induced overexpression with tryptophan and carried out metal affinity chromatography as usual using strain H1227 (Fig. 4G). There was no significant difference in the level of expression of mutant RadA(A196V) compared to the wild-type protein.

DISCUSSION

Existing methods for expression of halophilic enzymes in *E. coli* are far from ideal; the protein is often insoluble and must be reactivated by denaturation and refolding, which is not always successful (11). Even when the protein is soluble, problems can arise. For example, *H. volcanii* RadA expressed in *E. coli* is bound to host DNA that cannot be removed easily. Purification from the native host is the obvious solution, but until now, systems for overexpression in *H. volcanii* have failed to capitalize on recent advances in haloarchaeal genetics (2, 39). For example, existing vectors feature constitutive gene promoters and rely on mevinolin resistance or novobiocin resistance markers for selection (30), which are far from ideal, since the use of these antibiotics impairs cell growth.

We have harnessed the conditional p.*tnaA* promoter to develop a series of plasmid vectors (Fig. 1) for rapid and strong induction of protein expression upon addition of tryptophan (26). Our constructs use *pyrE2* and *hdrB* markers that maintain plasmids in rich medium (Hv-YPC) without the use of antibiotics (3); they are available with and without an in-frame 6×His tag for protein purification by metal affinity chromatography. This technique is ideal for purification of halophilic proteins, since it is compatible with the high salt concentrations used. However, it has until now been problematic in *H. volcanii* due to contamination by PitA, a protein with a histidine-rich linker region. We have replaced *pitA* with the gene from *N. pharaonis*, which encodes an ortholog lacking the histidine-rich

FIG. 4. Deletion of the *mrr* gene improves transformation with *dam*-methylated DNA. (A) Protein sequence alignment of the conserved cores of Mrr homologs from selected archaea and bacteria and *Schizosaccharomyces pombe* SPAC824.03c (9). Conserved residues are indicated by gray shading, and identical residues are indicated by a black background. Hvo, *H. volcanii*; Hma, *Haloarcula marismortui*; Hsa, *H. salinarum*; Hmu, *Halomicrobium mukohataei*; Hwa, *H. walsbyi*; Hla, *H. lacusprofundi*; Nph, *N. pharaonis*; Mac, *Methanosarcina acetivorans*; Mhu, *Methanospirillum hungatei*; Mth, *Methanothermobacter thermautotrophicus*; Dra, *Deinococcus radiodurans*; Efa, *Enterococcus faecalis*; Eco, *E. coli*; Lbr, *Lactobacillus brevis*; Mae, *Microcystis aeruginosa*; Mtu, *Mycobacterium tuberculosis*; Sth, *Salmonella thyphimurium*; Vch, *Vibrio cholerae*; Spo, *S. pombe*. (B) Phylogenetic tree representing evolutionary relationships between Mrr homologs, constructed using neighbor joining and rooted using *S. pombe* SPAC824.03c. Support for individual branches is indicated by bootstrap values (1,000-fold resampling); values of <50% are not recorded. Pairwise distances between sequences are uncorrected. (C) Colony hybridization of 5-FOA-resistant *H. volcanii* clones after pop-in/pop-out gene deletion with pTA1150. *H. volcanii mrr* sequences (amplified with mrrF/mrrR primers) were used as a probe, and clones failing to hybridize therefore had the *mrr* gene deleted. (D) Map of the *H. volcanii mrr* region indicating MluI sites and restriction fragment sizes. (E) Verification of *mrr* deletion in H1206 to H1209 by MluI digestion and Southern blotting; the probe used is indicated in panel D. (F) Transformation efficiencies of Δ*mrr* strains H1206 and H1209 compared to the *mrr*$^+$ strains H26 and H1155 using a methylated pTA354 plasmid purified from an *E. coli dam*$^+$ strain or unmethylated pTA354 DNA from an *E. coli dam* mutant (31). The averages and standard errors of three experiments are shown. (G) 6×His-tagged RadA(A196V) was overexpressed in the Δ*mrr* strain H1209 and purified by affinity chromatography on a Ni^{2+} chelating column as in Fig. 3B.

region (Fig. 2). Proteins purified from the $pitA_{Nph}$ gene replacement strain are free of PitA contamination (Fig. 3). Interestingly, contamination by *H. volcanii* PitA is more pronounced in strains overexpressing 6×His-tagged RadA than in strains containing the empty vector (Fig. 3B). This is also true for Cdc48d, another histidine-rich contaminant that we identified. PitA and Cdc48d might be upregulated in response to cell stress, in this case due to overexpression of a recombinant protein, which is consistent with our observation that contamination by Cdc48d is reduced by growth at lower temperatures (Fig. 3D). Furthermore, proteins that are expressed at lower levels than 6×His-tagged RadA, such as 6×His-tagged RadB, exhibit less contamination by Cdc48d (Fig. 3E).

Finally, we have deleted the *mrr* gene, which encodes a homolog of the methylation-sensitive restriction enzyme Mrr (9). In contrast to the wild type, *H. volcanii* Δ*mrr* strains exhibit high transformation efficiencies regardless of whether the plasmid DNA is methylated at 5′-GATC-3′ sites by *E. coli* Dam methylase (Fig. 4F). Therefore, Δ*mrr* strains remove the need to passage plasmid DNA through an *E. coli dam* mutant before transformation (21). Not only does this save time, it also reduces the risk of incurring mutations during growth in *E. coli dam* mutants, which are deficient in DNA mismatch repair (27). Furthermore, *H. volcanii* Δ*mrr* strains may be transformed directly with a DNA ligation, which permits the cloning of protein overexpression constructs that are toxic to *E. coli*.

We urge caution regarding the use of Δ*mrr* strains in routine genetic experiments, since they have not been phenotyped extensively and deletion of the *mrr* gene might have pleiotropic effects. For example, *Salmonella enterica* serovar Typhimurium mutants with a constitutively active *mrr* gene display increased basal SOS induction (1). Some restriction modification systems act as selfish mobile genetic elements that resist their loss from the host, leading to cell death (25). However, *mrr* is unlikely to have been acquired by lateral gene transfer, since the synonymous codon usage of this gene is identical to the genome average (data not shown). We consider it more probable that Mrr acts in cellular defense against foreign DNA. Furthermore, we have demonstrated that Δ*mrr* strains are fit for purpose when used in protein overexpression (Fig. 4G), thereby bringing closer the goal of an archaeal model organism that is suitable for both biochemistry and genetics.

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S.L., K.W., M.M., and T.A. wrote the paper; M.M. and T.A. designed the experiments; S.B., K.W., and T.A. performed the microbiological and biochemical experiments; S.L. carried out the mass spectrometry; and S.L. and T.A. analyzed the data.

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