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Overexpression and purification of halophilic proteins in *Haloferax volcanii*

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Halophilic enzymes function optimally at high salt concentrations and are active at low water availability. Such conditions are encountered at elevated concentrations of solutes such as salts and sugars, and at high concentrations of organic solvents. However, expression in heterologous hosts such as *Escherichia coli* can cause problems, since halophilic proteins typically misfold and aggregate in conditions of low ionic strength. We have harnessed the sophisticated genetic tools available for the haloarchaeon *Haloferax volcanii*, to develop a system for the overexpression and purification of halophilic proteins under native conditions.

Key words: protein overexpression, His-tag, archaea, *Haloferax volcanii*, halophile

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Halophilic archaea offer an unparalleled resource of enzymes that are tolerant of high concentrations of salt and organic solvents.[1-3] Halophilic microorganisms found in hypersaline lakes such as the Dead Sea are able to grow in the presence of molar concentrations of salt.[4] There are two biological strategies to cope with a high salt environment: halophilic bacteria maintain an osmotic balance of their cytoplasm with the medium by accumulating organic solutes, whereas halophilic archaea (haloarchaea) accumulate high cytoplasmic concentrations of salt (typically potassium). The latter strategy mandates that intracellular enzymes are able to function in high salt concentrations; this is achieved by a reduction in overall hydrophobicity and an increase in acidic residues on the protein surface.[5-7] The high number of negative charges coordinates a network of hydrated cations, thereby maintaining the protein in solution via a salt-enriched solvation shell.

Since halophilic enzymes are adapted to function at high salt concentrations, they are active at low water availability. Such conditions are encountered at elevated concentrations of solutes such as salts and sugars, and at high concentrations of organic solvents. It is well known that halophilic enzymes have significantly greater activity and stability in organic solvents than equivalent enzymes from non-halophilic organisms.[1] For example, catalase from *Halobacterium cutirubrum* exhibits up to threefold stimulation of activity by 0.5–1.5 M monovalent salts and by 2–4 M organic solvents, such as ethylene glycol, glycerol and dimethylsulfoxide.[8]

Halophilic proteins usually feature an excess of acidic amino acids, the negative surface charge is critical to solubility in high salt.[6,7] This can pose problems for expression in heterologous hosts such as *Escherichia coli*, since halophilic proteins typically misfold and aggregate in conditions of low ionic strength. Purification of halophilic enzymes from *E. coli* has often relied on the recovery of insoluble protein from inclusion bodies, followed by denaturation and refolding in hypersaline solutions.[9] To circumvent this problem, we have developed a system for the expression of halophilic proteins in the haloarchaeon *Haloferax volcanii*.[10]

Hfx. volcanii is the organism of choice for haloarchaeal genetics.[11,12] It has simple growth requirements (aerobic conditions, optimum temperature 45°C) and is easy to culture in the laboratory.[12] As a halophile, it is extremely resilient to contamination—few organisms can survive in 2.5 M NaCl. This is a significant advantage for industry, since it circumvents the need for

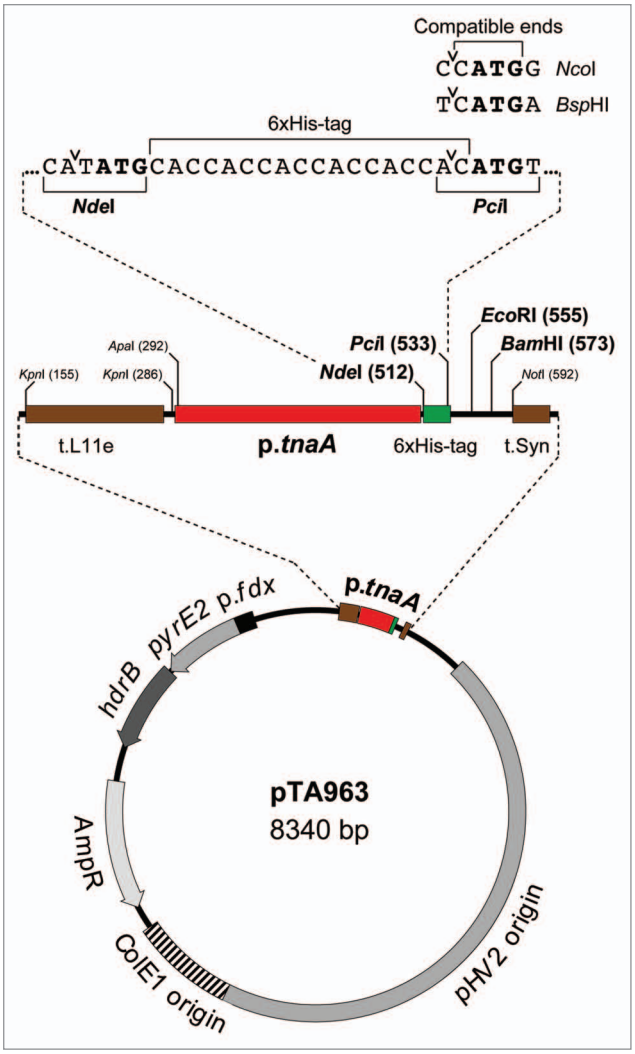

Figure 1. Conditional overexpression vector pTA963. pTA963 utilizes the tryptophan-inducible promoter p.*tnaA*. For N-terminally 6xHis tagged proteins, the 5' end of the gene is ligated with the *Pci*I site located downstream of a $(CAC)_6$ tract. *Pci*I-compatible ends are generated by *Nco*I and *Bsp*HI, and are used where the second codon starts with G and A, respectively. For expression of native proteins, the 5' end of the gene is ligated with the *Nde*I site downstream of the promoter.[10]

sterile growth conditions. Another feature of haloarchaea that make them attractive to industry is the ease of lysis upon addition of water, reducing the cost of protein purification.[12] Amongst halophiles, *Hfx. volcanii* can grow across a wide range of salinities, from 12–24% salt (w/v), approximately 1.8–3.5 M NaCl. It is therefore ideally placed for investigating halophilic proteins from other organisms, which might grow at different salt concentrations.

Hfx. volcanii can grow in defined media, which has enabled the development of a wide range of selectable markers.[11] The genetic toolbox for *Hfx. volcanii* also includes gene knockout methods, plasmids with high and low copy-number replication origins, and the tryptophan-inducible *tnaA* promoter.[13-16] We have made significant improvements to transformation protocols, by generating strains deficient in the Mrr restriction endonuclease.[10] Mrr cleaves foreign DNA that is methylated at 5'-GATC and reduces the efficiency of transformation by 50-fold. The Δ*mrr* strains allow direct and efficient transformation of *Hfx. volcanii* (>10^7 transformants/μg DNA) without the need to passage DNA through an *E. coli dam* mutant, they are ideal for generating expression libraries.

The overexpression system we have developed consists of a highly-engineered host strain H1209 (Δ*pyrE*2 Δ*hdr*B Δ*mrr Nph-pit*A), and a corresponding plasmid pTA963 for inducible overexpression of His-tagged proteins.[10] pTA963 utilizes the p.*tnaA* tryptophanase promoter of *Hfx. volcanii*, genes under control of the p.*tnaA* promoter show rapid and strong induction of expression upon addition of ≥1 mM tryptophan.[16] For expression of proteins with an N-terminal hexahistidine (6xHis) tag, a $(CAC)_6$ tract was incorporated in pTA963 (**Fig. 1**), and a polylinker ensures that cloning is facile. To insulate the gene from read-through transcription, the expression cassette was flanked by two transcriptional terminators (t.L11e and t.Syn). The vectors are based on pTA230 and use the pHV2 replication origin that maintains the plasmid at a copy number of ~6 per genome equivalent.[13] Selection is based on the *pyrE2* and *hdrB* markers, which allow growth of the host strain H1209 on media lacking uracil and thymidine, respectively; the latter marker allows the plasmid to be maintained in rich media (Hv-YPC), thus maximizing protein overexpression.[13,14]

In *Hfx. volcanii* H1209 the *pitA* gene is replaced with its orthologue from *Natronomonas pharaonis*.[10] PitA is a fusion of chlorite dismutase-like and monooxygenase-like domains, it is unique to haloarchaea and in almost all species features a histidine-rich linker.[17] Owing to this histidine-rich region, PitA is the major contaminant of His-tagged recombinant proteins purified from *H. volcanii* by immobilized metal affinity chromatography. We were unable to delete *pitA* gene by standard techniques, indicating that it is essential.[13,14] Instead, we replaced *pitA* with its orthologue from the haloalkaliphile *N. pharaonis*, which does not feature a high number of histidines in the central linker region. The *H. volcanii* strain H1209 with *Nph-pitA* gene replacement shows no contamination by PitA of His-tagged recombinant proteins, thus improving protein yield and purity.

We have used this overexpression system to purify several halophilic proteins from *Hfx. volcanii*, including the DNA repair proteins RadA and RadB.[10] The protocol is simple and robust, and compatible with the molar concentrations of salt required for the stability of halophilic proteins.

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