Jessica Blanton is a biology major at Amherst College. During the summer of her senior year, she conducted research at Lawrence Berkeley National Laboratory as a participant in the DOE Science Undergraduate Laboratory Internship program. For her internship she worked on a transformation system for the archae Sulfolobus solfataricus. This research was presented as a poster at the 2005 AAAS Meeting in Washington, DC. The following fall she stayed on at LBNL working on in vitro expression of human DNA repair proteins. She is now conducting her undergraduate thesis in the field of plant molecular phylogenetics, and will graduate in the winter of 2005. After graduating she plans to spend a year as a Research Assistant before pursuing a doctorate in the biological sciences.

Steve Yannone is currently a staff scientist at Lawrence Berkeley National Laboratory in the department of Molecular Biology. He completed his undergraduate studies in biochemistry at the University of California at Riverside, his Ph.D. in biochemistry at UC Irvine, and his postdoctoral training at Los Alamos National Laboratory. Steve began his research career with an undergraduate research scholarship to study insect kairomones in the department of entomology at UC Riverside. His graduate work was focused on cellular stress responses and redox status in the nitrogen fixing bacteria Azotobacter vinelandii. His graduate studies lead him into the field of DNA repair and he now leads an active research program at LBNL studying the biochemistry of mammalian DNA repair enzymes.

Jill Fuss is a postdoctoral fellow at Lawrence Berkeley National Laboratory in the laboratories of Priscilla K. Cooper and John A. Tainer. She received her Ph.D. in Molecular and Cell Biology from the University of California, Berkeley in 2001. Jill first became interested in basic science research as an undergraduate during a summer internship at the National Institutes of Health in Bethesda, MD. As a graduate student, Jill worked mainly in the field of DNA replication using biochemistry and cell biology to characterize a mammalian DNA polymerase. She is currently studying the structural biology of DNA repair. Jill is an NIH Ruth L. Kirschstein National Research Service Award Fellow and is a recipient of the 2002 Department of Energy Office of Science Outstanding Mentor Award. When not in the lab, Jill enjoys driving her diesel Volkswagen that runs on vegetable oil.

MODELING DNA REPAIR: APPROACHING *IN VIVO* TECHNIQUES IN THE HYPERTHERMOPHILE *SULFOLOBUS SOLFATARICUS*

Jessica Blanton, Jill Fuss, Steven M. Yannone,
John A. Tainer, Priscilla K. Cooper

ABSTRACT

Archaea are found in some of the most extreme environments on earth and represent a third domain of life distinct from Eukarya and Eubacteria. The hyperthermophilic archaeon *Sulfolobus solfataricus*, isolated from acidic hot springs (80°C, pH 3) in Yellowstone National Park, has emerged as a potential model system for studying human DNA repair processes. Archaea are more closely related to Eukarya than to Eubacteria, suggesting that archaeal DNA repair machinery may model the complex human system much more closely than that of other prokaryotes. DNA repair requires coordinated protein-protein interactions that are frequently transient. Protein complexes that are transient at extreme temperatures where archaea thrive may be more stable at room temperature, allowing for the characterization of otherwise short-lived complexes. However, characterization of these systems in archaea has been limited by the absence of a stable *in vivo* transformation and expression system. The work presented here is a pilot study in gene cloning and recombinant protein expression in *S. solfataricus*. Three genes associated with DNA repair were selected for expression: MRE11, PCNA1, and a putative CSB homologue. Though preparation of these recombinant genes followed standard methods, preparation of a suitable vector proved more challenging. The shuttle vector pSSV64, derived from the SSV1 virus and the *E. coli* vector pBSSK+, was most successfully isolated from the DH5α *E. coli* strain. Currently, alternative vectors are being designed for more efficient genetic manipulations in *S. solfataricus.*

INTRODUCTION

If left unrepaired, damage to an organism's DNA from both exogenous sources, such as UV radiation, and endogenous sources, such as the reactive oxygen species produced from metabolism, can cause permanent genetic changes. Mutations and chromosomal changes are often detrimental, leading to defects in function. It is because of these defects that understanding DNA maintenance and repair mechanisms is important for medical research, notably in studying cancer and degenerative diseases.

One means of investigating repair mechanisms is through comparative organismal studies. This is especially applicable for

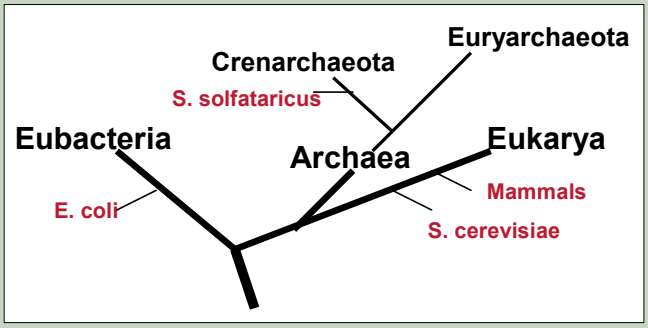

Figure 1. Phylogenetic relationship between the three domains of life. Archaea and Eukarya diverge after the split from Eubacteria.

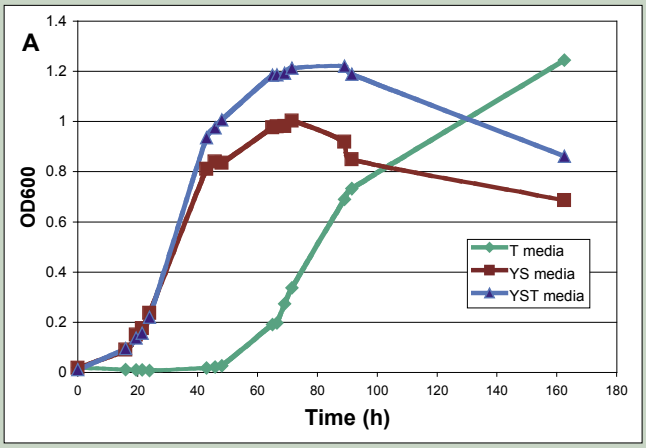

Figure 2. (A) *S. solfataricus* growth during 170h. (B) Semi-logarithmic plot of *S. solfataricus* growth during 170 h at 80°C.

DNA repair research, since genome maintenance is of such high importance that these mechanisms are found highly conserved or analogous across all life. These studies can also provide clues as to the evolutionary path of mammalian DNA repair and damage response. One such model organism under study is *Sulfolobus solfataricus*, a species representing the third domain of life—the archaea.

Archaea are prokaryotes, but the divergence between Archaea and Eukarya lies after the split from Eubacteria (Figure 1), therefore archaea are more closely related to eukaryoes than bacteria [1]. Many of these microbes are extremophiles, living in some of the harshest conditions on earth, up to temperatures of 113°C and to pHs below 1.0 [2]. Though they maintain internal pHs much closer to 7.0, the internal temperature matches that of the environment [3]. In this extreme heat, archaea perform cellular functions, including the difficult task of keeping their genome intact at temperatures shown ordinarily to drastically increase the error rate during DNA replication [4,5].

Archaeal repair mechanisms are of high interest to the scientific community for a number of reasons. Archaeal DNA transcription shares characteristics with that of both Eubacteria and Eukarya [1]. This implies archaea may be an intermediate system that could illuminate the evolution of eukaryotic DNA repair [6,7,8]. Also, some archaea are hyperthermophiles and employ arsenals of proteins that function at high temperatures. These proteins should be very stable at room temperature, relative to those of mesothermophiles, and working with such proteins may allow researchers to more easily trap their dynamic complexes [6].

S. solfataricus is a hyperthermophilic archaeon found in Yellowstone hot springs that has optimum growing conditions of 80°C, pH 3 [2]. This organism is a good candidate for a model archaeal system because it is practical for laboratory use relative to other extremophiles (growth temperature below boiling and pressure at 1 atmosphere) and the annotated genome has been published [9,10]. Previous studies have characterized *S. solfataricus* DNA repair pathway genes by expressing and extracting the proteins from *E. coli* hosts. This is in part because of the high degree of knowledge, experience, and molecular biological tools developed for the *E. coli* system, but more pointedly because of the absence of a stable archaeal transformation system.

In order to clone genes into archaea, viral vectors are currently being developed that are infective, expressible, and stable. SSV1 is one such archaeal virus that has been highly characterized, from which the vector pSSV64, inducible with either UV radiation or mitomycin C, has been produced [11, 12, 13].

The research presented here represents a series of pilot studies of *in vivo* expression of supposed *S. solfataricus* DNA repair genes, with the intent to extract *S.so* protein complexes. Three genes associated with DNA repair were selected for study. The first gene, an MRE11 homologue, is involved with DNA double-strand break repair. The protein structure has been solved from the archaeon *Pyrococcus furiosus* [14]. PCNA-1 is a well-characterized "DNA

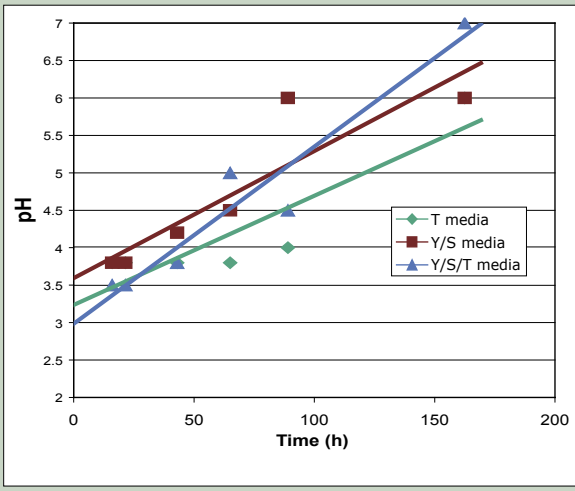

Figure 3. Acidity of *S. solfataricus* cultures over 170 h at 80°C. Lines represent a linear regression of the data.

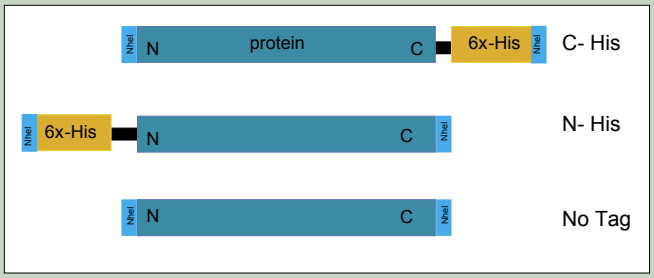

Figure 4. Orientation of the histidine tag (6xhis) for each insert. NheI restriction endonuclease sites will be used to clone the genes into the pSSV64 shuttle vector.

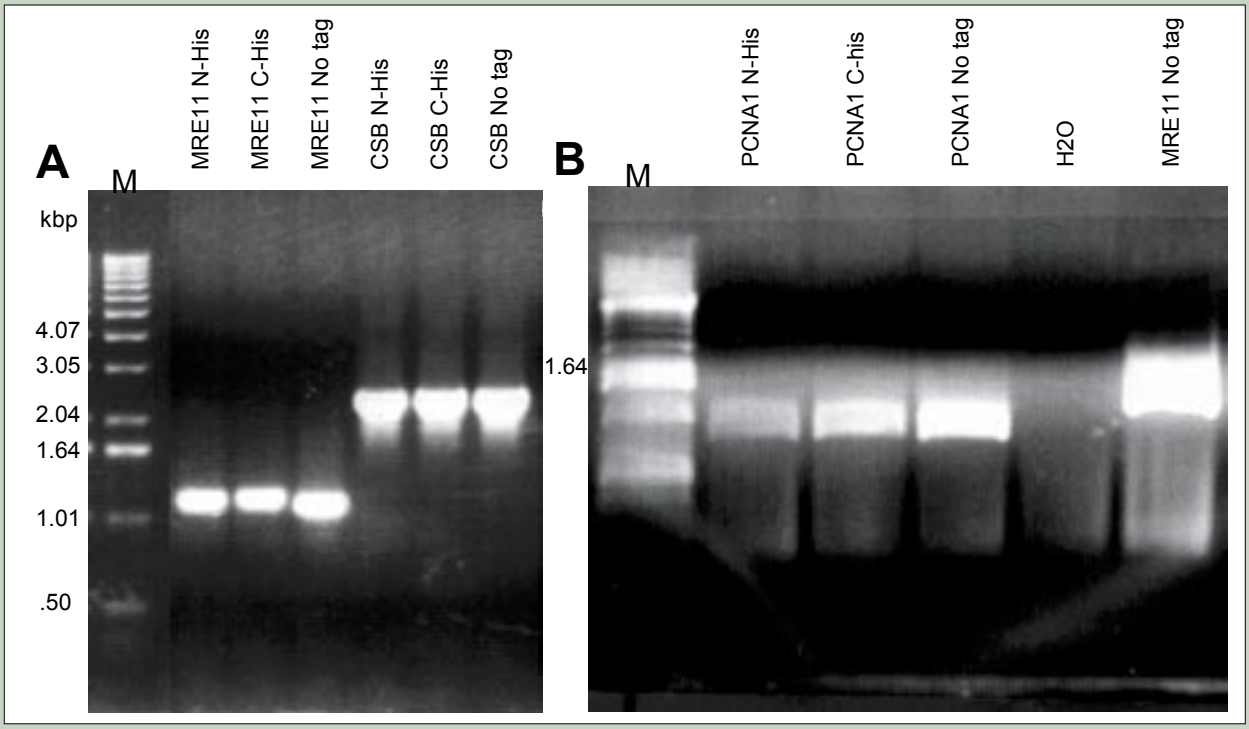

Figure 5. (A) PCR of MRE11 and CSB constructs performed at 65°C. (B) PCR of PCNA-1 construct performed at 60°C. Controls using either no primers or Mre11 primers were performed as negative and positive controls, respectively.

clamp" from *S. solfataricus,* of which the homologues increase the processivity of DNA polymerase in other organisms and has been well characterized from expression in *E. coli* but not yet expressed in *S. solfataricus* [15]. Lastly, a SWI/SNF ATPase (putatively a CSB-like protein referred to here simply as CSB) was chosen. It is a critical protein for transcription-coupled repair in mammals, and of which little is known in archaea [16]. These studies will contribute to developing *S. solfataricus* as a model organism, opening the door for the study of many thermostable proteins. Because of the phylogenetic relationship of archaea to eukaryotes, characterization of these proteins will in turn aid in understanding the dynamic protein complexes of our own repair systems.

MATERIALS AND METHODS

Materials

Archaeal strain *S. solfataricus* P2 and pSSV64 viral vector were provided by Ken Stedman. Five bacterial *E. coli* cell lines were used: TOP10F', GeneHOG, Stbl3, DH5α (all four Invitrogen) and SURE 2 (Stratagene). A shuttle vector pCR2.1-TOPO (Invitrogen) was used for *E. coli* transformation of PCR products. One restriction enzyme, Nhe1 (NEB), was used.

S. solfataricus cell growth

S. solfataricus P2 cells were grown aerobically in 50 ml liquid cultures at 80°C. Media was prepared in accordance with Zillig *et. al* [17]. Three different media were used containing either 0.2% tryptone, 0.1% yeast extract and 0.2% sucrose (Y/S/T media); or 0.1% yeast extract and 0.2% sucrose (Y/S/T media); or only 0.2% tryptone (T media) as a carbon source. All media contained 0.3% ammonium sulfate, 0.07% glycine, 0.05% potassium hydrogen phosphate, 0.01% potassium chloride, 0.01% magnesium chloride, and 0.005% calcium nitrate. To a final volume of 1L, the following volumes of 1% solutions were added: 244µl sodium borate, 90 µl manganese chloride, 11 µl zinc sulfate, 2.5 µl cupric sulfate, 1.5 µl sodium molybdate, 1.5 µl vanadyl sulfate, 0.5 µl cobalt chloride, and 0.5 µl nickel sulfate. The carbon source was added, the media was adjusted to pH 3.2 with a 1:1 dilution of sulfuric acid in water, autoclaved, and stored at 4°C after opening. All solutions were prepared as percent weight volume (w/v) except where indicated.

Cell growth and culture pH was monitored by Optical Density measurements at 600nm (OD_{600}) and indicator paper respectively.

S. solfataricus total DNA isolation

Total DNA was prepared by phenol extraction as according to Stedman *et al.*, 1999 [12], using 50 ml of *S. solfataricus* growing at log phase (OD_{600}=0.323). Purity and concentration were determined by $OD_{260/280}$ measurements.

Construction of MRE11, CSB and PCNA1 inserts

A six-histidine tag and NheI restriction sites were introduced to MRE11, CSB and PCNA1 genes during Polymerase Chain Reaction (PCR) amplification with Taq polymerase, using *S. solfataricus* genomic DNA as template. The primers were checked against hairpins and dimers, and for ideal annealing temperature with

DNASTAR. All PCR products were confirmed on a 1% agarose gel with 0.5µg/ml ethidium bromide.

PCR products were adenylated for TOPO TA cloning by adding 1µl of 100mM dATP, 0.5µl Taq polymerase to each reaction in a total volume of 50µl (Invitrogen). Each insert was cloned into the pCR2.1-TOPO vector and transformed into One Shot TOP10F' *E. coli* cells (Invitrogen).

Transformed cells were plated on LB plates selecting for ampicillin resistance and grown overnight. Overnight liquid cultures were inoculated from single colonies and the plasmid DNA was isolated as described using Wizard *Plus* SV Minipreps DNA Purification System as per the manufacturer's instructions (Promega). All constructs were verified by restriction digest with Nhe1 and by sequencing.

Inserts were cut from the plasmid by NheI digestion, and purified by gel extraction (GeneClean - Bio 101).

Preparation of pSSV64

The pSSV64 vector was transformed into the four *E. coli* strains, which were plated on LB + 100µg/ml ampicillin and grown overnight. Liquid cultures of LB + 50µg/ml ampicillin were inoculated with single colonies and grown overnight at 25°C for 24 hours. Isolation of pSSV64 was performed by an alkaline lysis extraction. Pellets from overnight cultures were lysed with 300µl of a 1% SDS, 200mM sodium hydroxide solution and neutralized with 300µl of a 3M potassium acetate (pH 5.5) solution. Cell debris and genomic DNA was pelleted out by a 15 min centrifugation at 14000 x g. The aqueous phase (850 µl) was centrifuged again and a final volume of 800µl supernatant containing the pSSV64 vector was extracted. The pSSV64 vector was precipitated with 800µl isopropanol and centrifuged 30 min at 14000 x g. After removing the supernatant the pellet was washed with 500µl 70% (v/v) ethanol and centrifuged 5 min at 14000 x g. This ethanol wash and centrifugation was repeated three times to extract salts. The pellet was dried overnight and dissolved in 40µl H_20. The vector was verified on a 0.5% agarose gel with 0.5µg/ml ethidium bromide.

RESULTS

Culture growth

S. solfataricus grew faster in Y/S and Y/S/T media (peak OD_{600} at 80 h) than the T media (peak OD_{600} at final timepoint at 170 h) (Figure 2A). Though the peak density of the Y/S media (OD_{600} = 1.0) did not reach that of the Y/S/T media (OD_{600} = 1.2), the T media surpassed both with a final OD_{600} of 1.3. The doubling times calculated from the growth curve (Figure2B) were 6.7 h for the Y/S/T media, 5.8 h for the Y/S media, and 6.0 h for the T media. No significant difference in generation times between media or correlation with carbon abundance was noted**.** Over time, all three cultures rose in pH. The Y/S/T media showed the most drastic increase while the T was the slowest to rise (Figure 3).

Insert construction

Design of the MRE11, CSB and PCNA1 primers involved the insertion of a six-histidine tag. To ensure accessibility of the histidine tag on the protein, three constructs were made of each protein, changing the orientation of the tag (Figure 4). The PCR reaction was run with an annealing temperature of 65°C. The MRE11 and CSB reactions produced product (figure 5A), but the PCNA1 reactions were successful only when the annealing temperature was lowered to 60°C (figure 5B). Sequences are shown in Table 1.

For more efficient cloning into the viral vector, the PCR products were adenylated and cloned into the pCR2.1-TOPO vector, amplified and then liberated by Nhe1 digestion. These digests

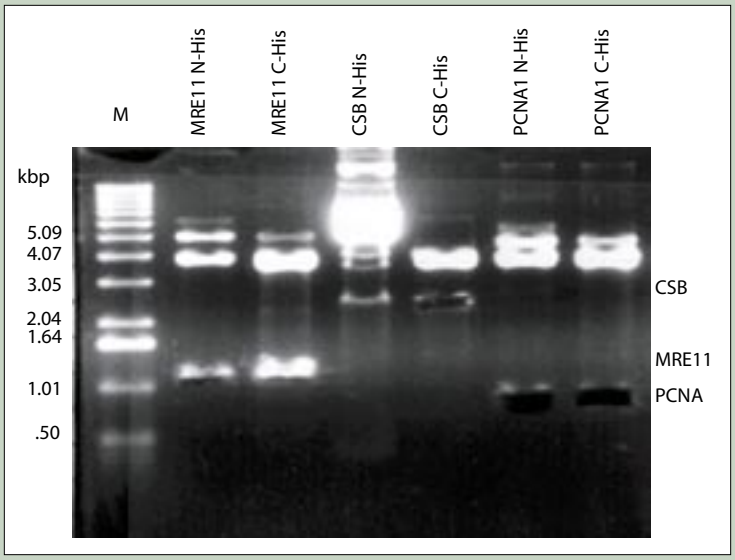

Figure 6. Gel separation of NheI digestion. Inserts were removed from gel for gel purification. Fragments were resolved on a 1.0 % agarose gel with 0.5µg/ml EtBr.

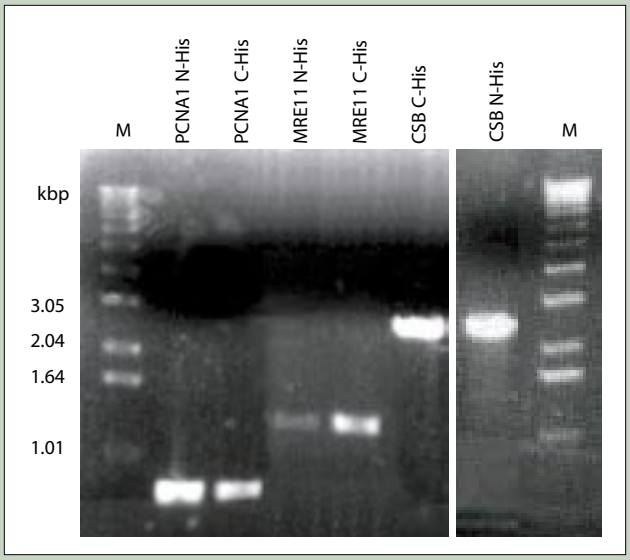

Figure 7. GeneClean gel purification confirmation of inserts. Purified fragments were resolved on a 1.0 % agarose gel with 0.5µg/ml EtBr.

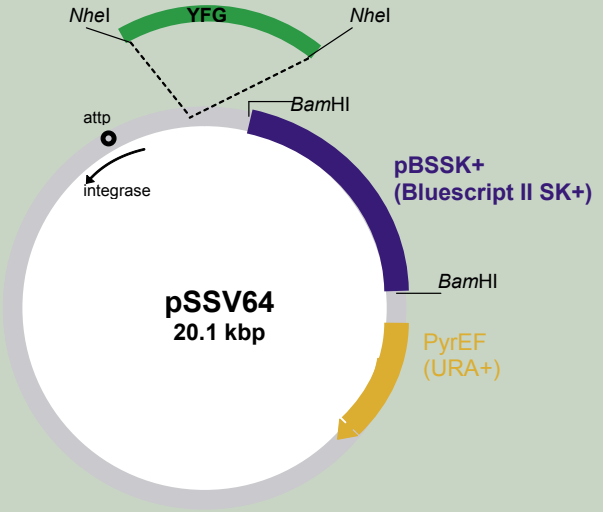

Figure 8. pSSV64 vector. Bluescript plasmid was inserted for bacterial replication, ampicillin resistance, blue/white screening. URA+ complementation from the pyrEF gene [13].

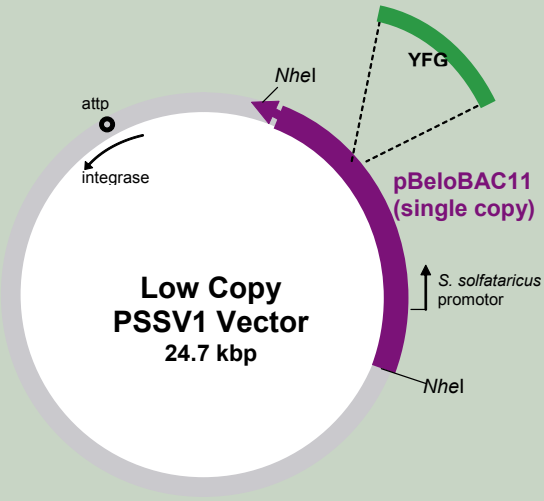

Figure 10. Proposed single copy viral shuttle vector designed with pBeloBAC11 bacterial plasmid and SSV1 virus for future insert expression of your favorite gene (YFG).

were run out on a 1.0% agarose gel (figure 6) to isolate the inserts. These bands were cut from the gel and purified (figure 7).

pSSV64 isolation

The pSSV64 vector (Figure 8) is a large construct comprised mainly of the archaeal virus, SSV1. The plasmid pBSSK+ was inserted for replication in bacteria, ampicillin resistance and blue/white selection [13]. The *pyr*EF gene provides complementation for *S. solfataricus* URA- mutants.

Because of the large size of this vector, pSSV64 has a much higher recombination rate than the smaller, commonly used bacterial plasmids. To ensure isolation of full-length vector four strains of *E. coli* GeneHOG, Stbl3, SURE 2 and DH5α were selected for transformation and extrachromosomal DNA isolated from these *E. coli* strains was run against a Hind λ DNA digest marker. Only the DH5α showed significantly more product in the 20.1 kbp band than

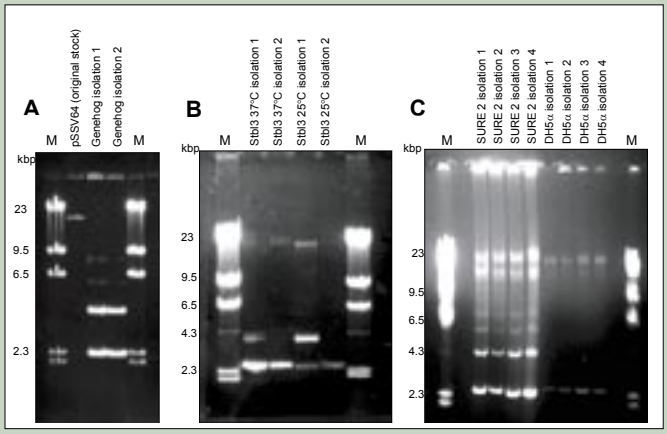

Figure 9. Isolation of pSSV64 from E. coli. (A) GeneHOG grown at 37°C, (B) Stbl3 grown at 37°C and 25°C, (C) SURE 2 and DHFα grown at 25°C. Run against a HIND λ DNA marker on a 0.5% agarose gel with 0.5μg/ml EtBr.

in any smaller band. The GeneHOG cells produced no noticeable amount of the full 20.1 kbp vector; instead, four smaller bands were seen (Figure 9A). The other three cell lines produced similar small bands to the GeneHOG but also produced the full-length pSSV64 (Figures 9B,C).

DISCUSSION AND CONCLUSIONS

Growing S. solfataricus

Overall it was found that the richer media (Y/S and Y/S/T) provided faster culture growth, though the T media may potentially reach the highest densities. Growth in the Y/S and Y/S/T media began to fall off immediately after peaking, which may be due to the rise in pH over time, making the environment too neutral for *S. solfataricus*. This rise may be caused by the accumulation of waste products and from cell lysis, which would release internal cell contents (maintained at a neutral pH in living cells) into the media. Continually buffering growth media with 10 mM citrate could counter this effect.

Insert preparation follows standard methodologies

In preparing the selected gene for ligation into the shuttle vector, standard genomic template isolation, PCR, cloning, and purification methods were performed successfully. This indicates that preparation of *S. solfataricus* genes requires no more development than is normally used for bacterial gene preparation.

Vector isolation is ill suited to E. coli hosts

Four strains of E. coli were transformed with the pSSV64 vector, and extrachromosomal DNA isolated from these cultures were too small to be the full viral vector. This indicates a high recombination

Gene	Direction	Tag	Sequence (5' to 3')
Mre11	For.	N-term 6x-His	GAGCTAGCATGCATCATCATCATCATCATGTACAAATTTTACATATTTCTGATACTC
	Rev.	-	GAGCTAGCTTATAAGTTAACACCTGTTAACTTTTTTAATATC
	For.	-	GAGCTAGCATGGTACAAATTTTACATATTTCTGATACTC
	Rev.	C-term 6x-His	GAGCTAGCTTAATGATGATGATGATGATGTAAGTTAACACCTGTTAACTTTTTTAATATC
CSB	For.	N-term 6x-His	GAGCTAGCATGCATCATCATCATCATCATTGGATAGTCCATGGCGTATGGGAC
	Rev.	-	GAGCTAGCTTATATGATATCGAATAAGATGACTGCATGAG
	For.	-	GAGCTAGCATGTGGATAGTCCATGGCGTATGGGAC
	Rev.	C-term 6x-His	GAGCTAGCTTAATGATGATGATGATGATGTATGATATCGAATAAGATGACTGCATGAG
PCNA-1	For.	N-term 6x-His	GAGCTAGCATGCATCATCATCATCATCATATATATCTTAAATCTTTTGAAAGG
	Rev.	-	GAGCTAGCTTAAACTTTTGGAGCTAATAAATAAG
	For.	-	GAGCTAGCATGATATATCTTAAATCTTTTGAAAGG

Table 1. Primer oligonucleotide sequences.

rate by the bacteria, producing smaller sized plasmids. Of these four strains, the DH5α strain proved most successful in producing substantial amounts of full-length viral DNA (20kbp) versus any smaller recombinant isolates. This difficulty in isolation, even when using strains constructed specifically for use with large or unstable DNA (Stbl3 and SURE 2) was probably due to the extremely large size of the pSSV64 vector (4 times the size of average bacterial vectors) and also to the high copy nature of the pBSSK+ bacterial plasmid insert. Though DH5α is a standard laboratory strain, it does carry the *recA1* mutation, reducing homologous recombination ability and presumably aiding the retention of the full size of the pSSV64 vector. However, the other three strains as well carry *rec* mutations, leaving their high recombination rate unexplained.

Further Directions

It will be possible to continue with protein expression using the vector isolated from the DH5α strain by ligating the genes of interest directly into an NheI digested vector sample. The 2kbp contaminant fragment seen in the isolation will likely not interfere with protein expression because it will be insufficient to support viral growth. The resulting construct will be electroporated into *S. solfataricus* DS522 cells, complementing the URA- defect of the strain for transformant screening. This is a self-spreading vector that should propagate through a culture entirely, not causing cell death but retarding growth. Replication and expression of this vector will be induced with UV radiation and protein purification will be attempted by standard purification methods.

A second method of obtaining sufficient quantities of pSSV64 will be to infect a *S. solfataricus* population and isolate the vector directly from the archaea. Alternatively, a second vector has been designed to alleviate some of the complications of pSSV64 (Figure 10). This vector would use the single copy plasmid pBeloBAC11, instead of the high copy pBSSK+, requiring less energy from the bacterium to maintain it. This vector will contain a *S. solfataricus* promoter for gene expression and lack the *pyr*EF nutritional marker.

If these three proteins can be successfully expressed in *S. solfataricus*, it will open the door for further protein expression and characterization of not only the DNA repair pathways, but other processes as well.

ACKNOWLEGEMENTS

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