RESEARCH COMMUNICATION

The ShBle resistance determinant from *Streptoalloteichus hindustanus* is expressed in *Haloferax volcanii* and confers resistance to bleomycin

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We have designed a gene cassette for expression of the bleomycin-resistance protein from *Streptoalloteichus hindustanus* (ShBle) in the extremely halophilic archaeon *Haloferax volcanii*, and shown that transformed haloarchaea are resistant to bleomycin. Recombinant ShBle was purified by a one-step affinity-chromatography procedure as a correctly folded, dimeric protein. ShBle thus

provides a useful haloarchaeal selectable marker and represents the first non-halophilic and soluble heterologous protein to be expressed in the Haloarchaea.

Key words: antibiotic resistance, Archaea, Haloarchaea, protein expression.

INTRODUCTION

Few antibiotic resistance determinants are available for *Haloferax volcanii* and other members of the extremely halophilic subgroup of the Archaea. This paucity is due to the intrinsic resistance of the Haloarchaea to the conventional antibacterials (such as ampicillin and tetracycline) that are commonly used in eubacterial genetics [1]. Two selectable markers currently in widespread use are novobiocin resistance, where mutations in the DNA gyrase gene (*gyrB*) confers resistance to novobiocin [2,3]; and mevinolin resistance, where an up-promoter mutation in the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase gene produces cells resistant to this drug [4]. A trimethoprim-resistance gene has also been developed and used [5,6] but, for unknown reasons, works only in *H. volcanii* (M. Mevarech, personal communication). The host ranges of the other two markers are also uncertain, as genetic work is usually restricted to only a few species (i.e. *Halobacterium salinarum*, *H. volcanii* and *H. hispanica*). In addition, all markers suffer from the potential disadvantage of recombination with the homologous chromosomal locus, as they are derived from chromosomal genes.

An antibiotic showing activity across a wide spectrum of organisms from bacteria through most eukaryotic species is bleomycin. The bleomycin (phleomycin) family of antibiotics are approximately 1400 Da molecular mass molecules which non-specifically cleave DNA strands by unknown mechanisms [7]. Several resistance determinants conferring bleomycin resistance have been isolated, the best characterized being ShBle from the soil streptomycete *Streptoalloteichus hindustanus*, which, in its active form, is a compact dimer of two 14 kDa subunits [8]. This homodimer binds bleomycin antibiotic within two clefts formed by the interaction of the two monomeric subunits in a 2:2 stoichiometric ratio [9]. ShBle is active when expressed in eubacterial, fungal, plant and mammalian species [9–12]. No archaeal homologue of this eubacterial gene is known. Thus we elected to express ShBle in haloarchaea and assess its ability to confer a bleomycin-resistant phenotype.

Expression of heterologous proteins in the haloarchaea is limited by a lack of tightly regulated expression-vector systems, and the problems associated with introduction of heterologous proteins into conditions of extremely high salt concentration (i.e. 2.5–6 M KCl; [13]). This latter factor has profound effects upon protein folding and stability, and only very few reports have shown that heterologous proteins can be expressed in a functional state in the Haloarchaea. A recent example was the expression of the seven transmembrane human *b₂*-adrenoceptor in *H. salinarum* and *H. volcanii*, where receptor activity was detected by binding of a specific antagonist [14], but others have found that over-expression can lead to extensive proteolysis within the cell [15]. Similarly, recombinant green fluorescent protein required fusion to bacteriorhodopsin before it could be functionally expressed in *H. salinarum* [16].

In the present work, we describe the design and construction of a gene cassette for expression of the ShBle resistance determinant in *H. volcanii*, and demonstrate that transformed cells are resistant to bleomycin. The ShBle protein could be isolated from haloarchaeal cells by a simple procedure, was of the predicted molecular mass and folded into the expected dimeric conformation.

EXPERIMENTAL

Reagents and molecular biology techniques

Vent DNA polymerase and all restriction enzymes were purchased from New England Biolabs, and used according to the manufacturer's instructions. T4 DNA ligase was from Gibco BRL (Melbourne, Australia). DNA fragment recovery and purification was by Bresaclean (Bresatec, Adelaide, SA, Australia). Bleomycin sulphate was purchased from ICN (Seven Hills, NSW, Australia). Standard molecular biological techniques were performed as described by Ausubel et al. [17].

Abbreviations used: ShBle, the bleomycin-resistance determinant from *Streptoalloteichus hindustanus*; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A.

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Construction of the haloarchaeal ShBle gene cassette

The *H. volcanii* ShBle gene cassette was produced by PCR from an ShBle DNA template previously isolated from *S. hindustanus* (JCM 3269); oligonucleotide primer design was based on the ShBle sequence reported in the GenBank database (accession number A31898). The 5' terminus was amplified and extended using the overlapping oligonucleotide primers #4562 (5'-TAC CAA GCT TTA GGG CCG GCG CGG GAA ACG TCG GAG CAT GGC CAA GTT GAC CAG TGC -3') and then #4878 (5'-ACC AAG CTT ATA ACT GGC CGC GGG AAA CGT CGG AGC ATG -3'), which incorporated the promoter sequence and a *Hind*III restriction site for cloning. The 3' terminus was amplified and extended using the reverse oligonucleotide primer #4563 (5'-GCT CTA GAA AAA AGT ACC TGC AGG GTA CCT CAT TTA TCA TCA TCA TCT TTA TAA TCT CCG TCC TGC TCC TCG GCC AC -3'), which included an *Xba*I restriction site. The isolated DNA fragment was cut with the restriction enzymes *Hind*III and *Xba*I, and ligated into the similarly cut *Escherichia coli*/*H. volcanii* shuttle vector pMLH3 [3]. Ligations were transformed into *E. coli* Tg-1, constructs checked by restriction analysis and DNA sequencing and plasmid DNA was passaged through *E. coli* JM110 [18] before transformation into *H. volcanii*.

H. volcanii transformation, growth and antibiotic sensitivity

H. volcanii DS70 was grown in 18% (w/v) salt water, modified growth medium as previously described [19]. The polyethylene glycol transformation method of Cline and Doolittle [20] was used to introduce plasmids into *H. volcanii* cells. For drug selection, novobiocin was added to solid or liquid media at 0.3 µg/ml and bleomycin was added at 4 µg/ml. Antibiotic minimum inhibitory concentration determinations were performed as described by Sahm and Washington [21].

Protein purification and Western blotting

For production of recombinant proteins, haloarchaeal cultures were grown in 1 litre of modified growth medium/novobiocin (0.3 µg/ml) at 37 °C with rotation at 200 rev./min for 72 h (late exponential phase). Cells were harvested by centrifugation at 12500 g for 15 min at 4 °C (Beckman JA-14), resuspended in 20 ml of 2 M NaCl/50 mM Tris/HCl (pH7.5) and sonicated for 3 × 15 s at setting 8 (Misonix XL). The soluble post-sonication (i.e. cellular) fraction was collected by centrifugation as above, diluted to 1 litre with distilled water (to reduce salt concentration and allow binding to the anti-FLAG resin, see below) and filtered through a 0.45 µm pore-size nitrocellulose membrane (Type HA; Millipore).

Recombinant proteins were purified by affinity chromatography using a Sepharose-linked M2 anti-FLAG antibody column (Kodak) [22] and elution with ImmunoPure™ gentle elution buffer (Pierce). Eluted proteins were dialysed against two changes of 2 litres of Tris-buffered saline containing 0.02% (w/v) sodium azide, concentrated by ultrafiltration over a 3000 kDa cutoff membrane (YM3, Diaflo; Millipore, North Ryde, NSW, Australia), and analysed by FPLC on a pre-calibrated Superose-12 HR10/30 column (Pharmacia Biotech).

Cell pellet and protein samples for Western-blot analysis were resuspended in SDS/PAGE sample buffer, subjected to SDS/PAGE (15% gels) and transferred by semi-dry transfer on to 0.2 µm nitrocellulose membranes (MSI, Westborough, MA, U.S.A.). Membranes were blocked in 1 × PBS containing 5% (w/v) Blotto before immunoblotting using the anti-FLAG M2

antibody (diluted 1:1000) and goat anti-mouse IgG (Fc)-horseradish peroxidase (Pierce), washed with PBS and then PBS containing 0.05% (v/v) Tween 20, and the proteins were revealed using the ECL® system (Amersham).

RESULTS

Design and construction of a *H. volcanii* gene cassette for expression of ShBle

H. volcanii cell growth was strongly inhibited by bleomycin concentrations above about 1 µg/ml (minimum inhibitory concentration of 1.25 µg/ml), making this antibiotic a suitable selective agent [7]. To determine whether the ShBle protein could be expressed in the Haloarchaea and thus render cells bleomycin resistant, a gene cassette containing the ShBle coding sequence was designed and constructed (Figure 1). Gene construction was by overlap PCR using specific oligonucleotide primers from a ShBle cDNA clone isolated from the genome of *S. hindustanus* (see the Experimental section). Haloarchaeal transcription initiation is dependent upon three upstream elements, the BRE (a two-adenine motif upstream of the TATA box), the TATA box (centred around -26/-27) and the initiator element, INR (for a review see [23]). The gene cassette was designed to incorporate these three upstream elements, and was based on the *H. volcanii* HMG-CoA reductase gene [4], except that a higher proportion of adenine and thymine residues were incorporated in order to increase transcription levels according to the promoter definition outlined by Soppe [23]. The initiator element (INR) was necessarily constrained by the incorporation of the ShBle-initiating methionine, but otherwise followed the HMG-CoA reductase gene sequence. The sequences of the TATA box and INR regions, as well as intervening sequences and the initial residues of the ShBle coding sequence, are shown in Figure 1(A).

The 3' end of the gene cassette incorporated the coding sequence for a single octapeptide FLAG tag (DYKDDDDK) prior to a TGA stop codon (Figure 1B). Incorporation of this sequence did not affect expression, folding or antibiotic binding of the ShBle protein when expressed in *E. coli* (S. D. Nuttall, unpublished work). A 22 nucleotide transcription termination

Figure 1 Gene cassette for expression of ShBle in *H. volcanii*

(A) Gene cassette 5' end. The TATA box and INR promoter elements are indicated, as are the *Hind*III restriction endonuclease site and the initiating ATG codon of the ShBle coding sequence. (B) Gene cassette 3' end, including the terminal residues of the ShBle coding sequence, glycine linker and the octapeptide FLAG tag. The transcription terminator is adapted from the *H. volcanii* DNA *gyrB* gene, and *Pst*I, *Kpn*I and *Xba*I restriction endonuclease sites are indicated.

Figure 2 Recombinant ShBle protein is expressed in *H. volcanii*

(A) Total cell fractions of *H. volcanii* cells, transformed with pMDS20 (negative control) or pHble1, were separated by SDS/PAGE through 15% (w/v) polyacrylamide Tris/glycine gels, transferred to nitrocellulose and immunodecorated using the anti-FLAG M2 antibody and a horseradish-peroxidase-conjugated secondary antibody. Approx. molecular masses (in kDa) are shown on the left. (B) Post-anti-FLAG M2 antibody column fraction analysed by SDS/PAGE through a 15% (w/v) polyacrylamide Tris/glycine gel and stained with Coomassie Brilliant Blue. (C) Post-anti-FLAG M2 antibody column fraction analysed by FPLC through a precalibrated Superose-12 gel-filtration column. ShBle was eluted at approx. 25 min, representing a molecular mass of approx. 30 kDa, which is consistent with that of the ShBle dimer. The A_{280} is given in arbitrary units.

motif was incorporated immediately following the translation stop codon, based upon the *H. volcanii* *gyrB* gene, but incorporating *Pst*I and *Kpn*I restriction endonuclease sites for future cloning purposes (Figure 1B). The gene cassette included terminal *Hind*III and *Xba*I restriction endonuclease sites, allowing cloning into the similarly cut *E. coli*/haloarchaea shuttle vector pMLH3 [3]. This cloning produced the plasmid pHble1, representing pMLH3 with the ShBle gene cassette replacing the mevinolin-resistance determinant.

ShBle confers resistance to bleomycin in transformed *H. volcanii* cells

Plasmid pHble1 was introduced into *H. volcanii* cells and novobiocin-resistant transformants were selected on solid media. Several colonies were then tested for their sensitivity to bleomycin by a standard minimum inhibitory concentration assay in liquid media. The minimum inhibitory concentration of *H. volcanii* DS70 (or the same strain carrying the novobiocin-resistance plasmid pMDS20) was 1.5–2 μ g/ml, whereas the pHble1 transformants showed minimum inhibitory concentrations of 10 μ g/ml bleomycin. Similar results were obtained on solid media and 4 μ g/ml was chosen as the most efficient concentration for selection. Plasmids of the expected size and restriction pattern could be isolated from these transformants when grown either under novobiocin (0.3 μ g/ml) or bleomycin (4 μ g/ml) selection.

Isolation and purification of ShBle protein from *H. volcanii* cells

The bleomycin resistance data presented above suggested that correctly folded and functional ShBle was being produced in haloarchaeal cells. To test this hypothesis, *H. volcanii* cell pellets were resuspended in SDS/PAGE buffer, electrophoresed through SDS/15% polyacrylamide gels, transferred to nitrocellulose membranes and subjected to Western-blot analysis with an anti-FLAG antibody. A single immunodecorated band corresponding to the ShBle-FLAG protein was observed at the expected molecular mass (approx. 14 kDa) in transformed cultures but not in cultures where vector (pMDS20) alone was present (Figure 2A). This band co-migrated with recombinant ShBle protein produced in an *E. coli* system (results not shown). There was no evidence of protein degradation occurring (Figure 2).

ShBle is a compact protein and, being of eubacterial origin, should be resistant to dilution of the haloarchaeal high-salt medium. Thus *H. volcanii* cellular fractions were purified, diluted with distilled water to reduce the salt concentration of the solution and applied to an M2 anti-FLAG antibody-Sepharose column. After washing, a single anti-FLAG antibody-reactive protein peak was eluted from the column, corresponding to ShBle (Figure 2B). FPLC analysis of this sample through a precalibrated gel-filtration column showed a single peak that was eluted at approx. 25 min, which corresponds to a protein of 28–30 kDa molecular mass (Figure 2C). This represents the ShBle dimer and again mimics the results obtained for the *E. coli*-produced protein. To establish conclusively that the isolated protein was ShBle, eight cycles of N-terminal amino acid sequencing were performed on material eluted from the FLAG column. The N-terminal sequence data showed that only one protein species was present (AKLTSVP...) and this sequence corresponded to the ShBle protein. However, the N-terminal initiating methionine residue had been removed, indicating that post-translational processing had occurred.

DISCUSSION

We have demonstrated that the eubacterial resistance protein, ShBle, can be expressed as a functional protein in the cytoplasm of the extremely halophilic archaeon *H. volcanii*, where the intracellular salt concentration (mainly KCl) is about 3 M. We believe that this is only the third non-halophilic protein to be expressed in the Haloarchaea and the first where the expressed protein is within the cytosolic fraction. The previously expressed human b_2 -adrenoceptor and bacteriorhodopsin-green fluorescent protein fusions both required the heterologous protein to be membrane associated [14,16]. The ability of ShBle to attain a functional conformation in *H. volcanii* may be due to its

ability to fold into a compact dimer in high salt solution. We predict that other less compact eubacterial or eukaryotic proteins may have greater difficulty in attaining and maintaining such a correctly folded conformation. The N-terminal sequencing data presented in the present study clearly indicates that processing of the protein N-terminus by removal of the N-terminal methionine residue occurs. This has also been observed for expression of the Haloarchaeal β -galactosidase gene *bgaH* (M. L. Dyall-Smith, unpublished work). Removal of the N-methionine residue does not affect the ability of ShBle to dimerize and suggests that one less residue in the ShBle N-terminal arm [24] is not detrimental to the interaction between ShBle monomers.

The ShBle gene cassette combined with bleomycin provides a fourth combination of selective agent/selectable marker for Haloarchaeal molecular genetics after the novobiocin-, mevinolin- and trimethoprim-resistance genes. This should be invaluable in cases where use of these latter resistance determinants is unsuitable. Moreover, because of its relatively high expression levels and selectable phenotype, the ShBle protein represents an ideal candidate for the study of the expression of heterologous proteins in the Haloarchaea.

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