Extremophiles (2001) 5:153–159
Digital Object Identifier (DOI) 10.1007/s007920100189

ORIGINAL PAPER

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Thermoadaptation of a mesophilic hygromycin B phosphotransferase by directed evolution in hyperthermophilic Archaea: selection of a stable genetic marker for DNA transfer into *Sulfolobus solfataricus*

Received: December 8, 2000 / Accepted: February 12, 2001 / Published online: May 17, 2001

Abstract A mutated version of the hygromycin B phosphotransferase (hph_{mut}) gene from *Escherichia coli*, isolated by directed evolution at 75°C in transformants of a thermophilic strain of *Sulfolobus solfataricus*, was characterized with respect to its genetic stability in both the original mesophilic and the new thermophilic hosts. This gene was demonstrated to be able to express the hygromycin B resistance phenotype and to be steadily maintained and propagated also in other, more thermophilic strains of *S. solfataricus*, i.e., up to 82°C. Furthermore, it may be transferred to *S. solfataricus* cells by cotransformation with pKMSD48, another extrachromosomal element derived from the virus SSV1 of *Sulfolobus shibatae*, without any loss of stability and without affecting the replication and infectivity of this viral DNA. The hph_{mut} and the wild-type gene products were expressed at higher levels in *E. coli* and purified by specific affinity chromatography on immobilized hygromycin B. Comparative characterization revealed that the mutant enzyme had acquired significant thermoresistance and displayed higher thermal activity with augmented catalytic efficiency.

Key words Hygromycin B phosphotransferase · Genetic marker · Genetic stability · Thermostability · Thermophilicity · Shuttle vector · Gene expression

Communicated by G. Antranikian

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Presented at the Third International Congress on Extremophiles, Hamburg, 2000

Introduction

Over the past 20 years, a great deal of interest has been focused on Archaea because of their ability to flourish in the most extreme environments and hence provide a new generation of novel bioprocesses and microbial products of interest to industry (Aguilar 1996).

Most of the attention given to these applicative aspects has prevailed on the elucidation of the basic molecular cell biology of Archaea and has led to a delay in the realization of essential gene transfer strategies, appropriate vector/host transformation systems, mutant production, and screening methods (Ciaramella et al. 1995; Sowers and Schreier 1999). In contrast, because of the greater similarity to mesophilic relatives thermophilic Bacteria can be genetically manipulated as easily as the most extensively studied model system, *Escherichia coli*. In fact, some genetic tools for both aerobic genera (*Thermus* and *Bacillus*) (Koyama et al. 1990; Tamakoshi et al. 1997, 1999; De Rossi et al. 1994) and anaerobic genera (*Thermoanaerobacter* and *Thermoanaerobacterium*) (Mai and Wiegel 2000; Mai et al. 1997) have proven successful for the expression of foreign genes or the overexpression of endogenous genes. Nevertheless, the optimal growth temperature of thermophilic bacteria generally has an upper limit around 70°C; thus, a shift toward the more thermophilic archaeal expression systems (growth up to 113°C) (Blochl et al. 1997) would offer the greatest advantage for expanding the temperature limit for an efficient production of thermoproteins when their expression in mesophilic cells is seriously hampered. Even more interestingly, a more efficacious stabilization of mesophilic or moderately thermophilic proteins or enzymes can likely be achieved by directed evolution in this class of hosts.

Among the hyperthermophilic Archaea, the genus *Sulfolobus* (Brock et al. 1972) has been especially studied with regard to its physiological requirements (Grogan 1989) and the presence of several promising extrachromosomal genetic elements (Zillig et al. 1994, 1996, 1998), and the sequence of its genome has been completed (the entire

genomic sequence is available on the Internet at the MAGPIE site: http://niji.imb.nrc.ca/sulfolobus/).

More interestingly, some significant advances in the development of gene transfer systems have been made, and an encouraging first generation of shuttle vectors is currently available for *Sulfolobus* representatives. A hybrid plasmid, pAG21, derived from the *E. coli* plasmid pBR322 and containing both the *Sulfolobus solfataricus adh* gene as a genetic marker and rep (replication)-like sequences from the plasmid pGT5 of *Pyrococcus abyssi* (Erauso et al. 1996) as the replication origin, has been described as an effective shuttle vector between *E. coli* and both *Pyrococcus furiosus* and *Sulfolobus acidocaldarius* (Aravalli and Garret 1997). A viral shuttle vector, pKMSD48, containing the *E. coli* pBluescript plasmid inserted into an intact chromosome of the virus SSV1 was shown to be stable at a high copy number in both *E. coli* and *S. solfataricus* without affecting the viral viability and infective potency (Stedman et al. 1999).

In a previous report, we described an *E. coli*/*S. solfataricus* shuttle vector, the pEXSs, constructed by inserting the genomic replication sequence of *S. shibatae* (DSM 5389) virus particle SSV1 (Palm et al. 1991; Schleper et al. 1992) into the *E. coli* plasmid pGEM5Zf (+) and using a suitable mutant of the hygromycin B phosphotransferase gene from *E. coli* (Gritz and Davies 1983) as the selective transformation marker. In fact, to obtain a functional enzyme in vivo at otherwise prohibitively high growth temperatures of the *S. solfataricus* Gθ strain (optimum temperature, 75°C), we constructed a library of random mutant versions of the *hph* gene, and the sequence that was able to confer resistance was selected by transfer into *S. solfataricus* and recovery from the resistant transformant (hygB^r 2A). The hybrid *S. solfataricus/E. coli* DNA was transferred from *E. coli* to *S. solfataricus* and vice versa without undergoing any rearrangement or integration into the chromosomes of either host (Cannio et al. 1998).

In this article, we describe the efficacy of this *hph* mutant variant as a genetic marker for a broad host range, namely, its ability to confer resistance to different and more thermophilic strains of *S. solfataricus* in which it can be transferred and propagated in a similar stable fashion. Moreover, the hygB^r 2A transformant could be superinfected by the virus hybrid pKMSD48 without any loss of resistance phenotype and virus viability. Finally, the cloning and the expression in *E. coli* of both wild-type and mutant *hph* genes allowed comparative characterization of their catalytic efficiency, thermostability, and thermophilicity and purification of the enzymes by affinity chromatography on hygromycin B-coupled sepharose (Zalacain et al. 1987).

Material and methods

Strains, plasmids, enzymes, and chemicals

Escherichia coli TOP10F' (Hanahan 1983) and *E. coli Rb791* (Brent and Ptashne 1981) strains were used as host cells for plasmid propagation and for the expression of *hph* genes, respectively, and routinely cultured in Luria-Bertani (LB) medium with ampicillin (100 μg/ml).

Sulfolobus solfataricus Gθ selected by Cannio et al. (1998), P2 (DSM 1617), and MT4 (DSM 5833) strains supplied by Deutsche Sammlung von Mikroorganismen (DSM) (Braunschweig, Germany) were grown aerobically in Brock's basal salt medium containing 0.1% (w/v) yeast extract, 0.1% (w/v) casamino acids, and 0.1% (w/v) glucose and buffered at pH 4.0 at 75°, 80°, and 82°C, respectively. *S. solfataricus* recombinant clones were selected on the same medium containing hygromycin B 300 μg/ml (Calbiochem, San Diego, CA, USA) in Gelrite plates (Phytagel; Sigma, Milan, Italy), and propagated under selective growth conditions according to Cannio et al. (1998).

Plasmid vector pTRC99A was obtained from Pharmacia Biotech (Uppsala, Sweden). Plasmid vectors PHL1, containing the complete *E. coli hygromycin B phosphotransferase* coding sequence and pKMSD48 (Stedman et al. 1999), were kindly provided by Dr. L. Marzullo and Dr. K.M. Stedman, respectively. Isopropyl thiogalactoside (IPTG) and hygromycin B were supplied by Calbiochem. Restriction and modification enzymes were purchased from Roche (Basel, Switzerland), the Sequenase version 2.0 dideoxy sequencing kit from United States Biochemicals (Amersham, Buckinghamshire, England), and *Pfu* DNA polymerase from Stratagene (La Jolla, CA, USA). *Taq* DNA polymerase and DNA molecular size markers were obtained from Promega Biotec (Madison, WI, USA). Synthetic oligonucleotides were supplied by Primm (Milan, Italy), and radioactive materials were obtained from Amersham International (Milan, Italy).

Transformation of *Sulfolobus solfataricus* and selection and genetic characterization of recombinants

Sulfolobus solfataricus cells were made competent for electroporation and transformed with the vectors pEXSs (Cannio et al. 1998) or pKMSD48 following the procedure described by Schleper et al. (1992). All hyg B^R-resistant clones were checked at the DNA level. Total DNA was isolated according to Guagliardi et al. (1995). Extrachromosomal DNA was prepared from 100-ml aliquots of the cultures with the alkaline lysis method and the NucleoSpin Plus Plasmid Miniprep kit (Clontech, Palo Alto, CA, USA), and used to transform *E. coli* TOP10 F' cells to verify the presence and copy number of the plasmids (Cannio et al. 1998). Polymerase chain reaction (PCR) analysis was performed on total and extrachromosomal DNA using the specific *hph* primers:

hph-N-ter: 5′-GAGTCATGAAAAAGCCTGAACTCAC-3′

hph-C-ter: 5′-CCGCATGCTATTCCTTTGCCCTCGG-3′

containing the *Bsp*HI and *Sph*I restriction sites, which are underlined in the sequences. The reactions were carried out using 5 U Taq DNA polymerase (Promega), 1 M each primer, 1 μM dinucleoside triphosphate (dNTP), and appropriate buffer. The amplification products were analyzed by agarose gel electrophoresis. The Gθ strain was also

cotransformed with the viral vector pKMSD48 and the pEXSs vector in equimolecular ratio and plated onto solid medium containing hygromycin B, 300 μg/ml; ten single clones were grown in selective liquid medium and analyzed by plaque test according to Stedman et al. (1999), with Gθ as the indicator strain for infection and 75°C as the incubation temperature.

Gene expression and activity of the wild-type and mutant hygromycin B phosphotransferases

The coding sequences of the mutant and wild-type *hph* (hph_{mut} and hph_{wt}) were PCR amplified from the pEX2A (Cannio et al. 1998) and pHL1 plasmids, respectively, using Pfu DNA polymerase under standard conditions (25 cycles at 50°C annealing temperature), and the oligonucleotides mentioned earlier. The amplified DNAs were cloned into the pTrc99A plasmid, and *E. coli* transformants were selected on plates containing both ampicillin (100 μg/ml) and hygromycin B (50 μg/ml). Growth, IPTG induction, and lysis for protein extract preparations were performed as already described for the *S. solfataricus adh* genes by Cannio et al. (1996), using 90 mM Tris HCl, pH 7.5 as the lysis buffer. Protein extracts from *S. solfataricus* clone hyg B^r 2A (Cannio et al. 1998) were prepared by grinding the cells harvested from an early stationary phase (about 0.8 OD_{600}) with sand in Eppendorf tubes using a suitable conical pestle and suspending the proteins in the same *E. coli* lysis buffer.

Purification by affinity chromatography on Sepharose-6B-Hygromycin B of the HPH enzymes from their crude extracts followed the procedure described by Zalacain et al. (1987) for the protein isolated from *Streptomyces hygroscopicus*. The purification was qualitatively judged by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970), and proteins were detected by silver staining. The hygromycin B phosphotransferase assay was performed using antibiotic radiolabeling and the dot blot procedure described by Mohr (1989). The reaction mixture (180 μl), containing 90 mM Tris-HCl, pH 7.5, 177 mM ammonium sulfate, 17.7 mM magnesium sulfate, 0.275 mM ATP, 0.02 mM hygromycin B, and 0.10 μCi γ-[^{32}P]ATP and the protein samples, was incubated for 150 min at 37°C. The protein amounts assayed were 3–10 μg for the *E. coli* crude extracts, 30–50 μg of the hyg B^r 2A *S. solfataricus* protein extracts, and 3–5 ng of the proteins purified. Different aliquots of the reaction mixture were blotted using a BioRad Dot Blot apparatus (BioRad, Hercules, CA, USA), and the membranes NC and Whatman P-81, according to Mohr (1989), were rinsed in hot water (70°C), dried, and exposed to autoradiography for 3–16 h.

To determine the relative thermal activity of the two enzymes, the reaction mixtures containing the *E. coli* extracts were incubated at 50° or 60°C. The thermal resistance of the enzymes was investigated by heating *E. coli* cell extracts (1.5 mg/ml in reaction buffer) for 30 min at 50° and 60°C, and the residual activity was detected at 37°C as described. For quantitative assays, after transfer, the Whatman P-81 was dried and the spot areas were cut, placed in scintillation vials, and Cherenkov counted. Activity was expressed as the counts per minute of the labeled hygromycin B on filters produced by 1 μg of the enzyme preparations.

Results

In vivo stability of the *hph* gene as a genetic marker under conditions of increasing temperatures and viral infection

The *E. coli/S. solfataricus* shuttle vector pEXSs (Cannio et al. 1998) was used to transform different *S. solfataricus* strains, namely, MT4 (T_{opt}, 87°C) and P2 (T_{opt}, 82°C), which are more thermophilic than the Gθ strain used for the vector construction and the selection of the thermoadapted *hph* gene (hph_{mut}) at 75°C.

The pEXSs vector was found to transform all hosts with efficiency levels comparable to the Gθ strain (10^2–10^4 colonies per μg of DNA), thus demonstrating a broader transformation potency and the ability of hph_{mut} gene to confer the hyg B^r phenotype also at higher growth temperatures. In fact, the selection on hygromycin B proved a good test for the functionality of the HPH mutant (HPH_{mut}) enzyme up to 82°C, i.e., at the maximum temperature imposed by the growth on Gelrite plates of the most thermophilic strain tested. Furthermore, the presence of the pEXSs vector in hyg B^R-transformed clones and the genetic stability of the hph_{mut} gene were ascertained by performing PCR analysis (amplified sequence of 1,028 bp) on both total and extrachromosomal DNA using the specific primers hph-N-ter and hph-C-ter (Fig. 1).

The pEXSs vector and the antibiotic selection were not affected by and did not interfere with the replication and infectivity of an SSV1-derived virus pKMSD48 described by Stedman et al. (1999). After cotransformation of the Gθ cells with both the plasmid and the viral vectors, several hyg B^R clones were selected on hygromycin B plates, picked, and grown for several generations in liquid culture under constant antibiotic selection. The analysis performed on certain clones (Fig. 2 shows the results obtained for three of these clones) using a lawn of the Gθ cells as the indicator strain for viral infection, revealed turbid halos around one of three spotted cultures, thus demonstrating virus production by pEXSs transformants.

Characterization of mutant and wild-type hygromycin B phosphotransferase enzymes

The hph_{mut} gene was characterized by analyzing the gene product in comparison to the wild-type counterpart expressed in an *E. coli* strain. We assayed the hygromycin B phosphotransferase activity using a dot blot assay of the protein extracts incubated at 37°C with the antibiotic substrate and the cosubstrate γ-[^{32}P]ATP. Reaction mixtures were transferred onto phosphocellulose filters, and the

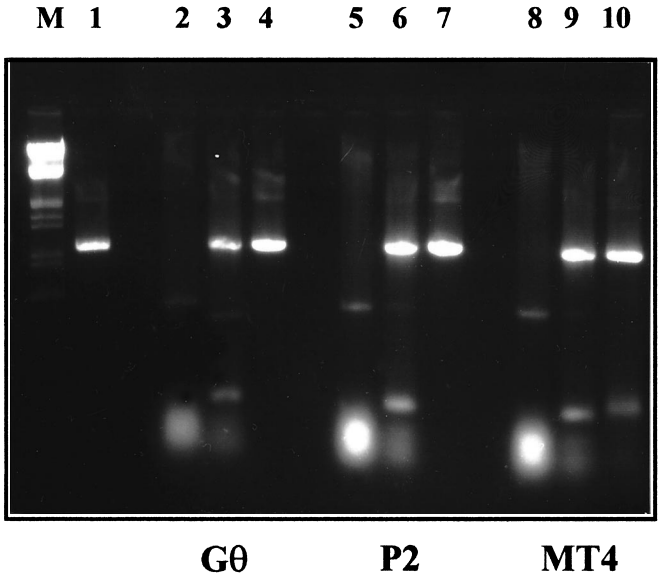

Fig. 1. Detection of the hygromycin B phosphotrasferase (*hph*) gene after transfer into increasingly thermophilic *Sulfolobus solfataricus* strains. The presence of the hph_{mut} in isolated pEXSs transformants of *S. solfataricus* Gθ, P2, and MT4 was ascertained by polymerase chain reaction (PCR) amplification with specific primers of the coding sequence in both total (*lanes 3, 6, 9*) and extrachromosomal (*lanes 4, 7, 10*) DNA and agarose gel analysis. *Eco*RI/*Hin*dIII-digested λDNA (*lane M*) and the amplification product of the hph_{mut} gene from the isolated pEXSs plasmid (*lane 1*) were used as the molecular weight marker and positive control for PCR, respectively; the total DNA of the untransformed strains was the negative control for the PCR experiment specificity (*lanes 2, 5, 8*)

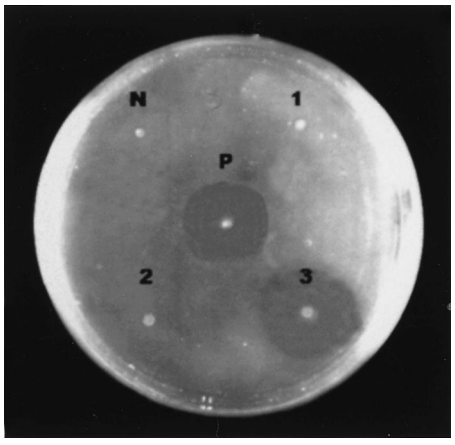

Fig. 2. Plaque test of *Sulfolobus solfataricus* Gθ cotransformed with the pKMSD48 virus and the pEXSs plasmid. Cultures of some clones isolated on hygromycin B selective medium after transfer of the two extrachromosomal DNAs were spotted onto a lawn of untransformed cells on plates. After growth, the halo formation around the colonized areas was visible for one of three clones tested (clone 3) and in the clone P transfected only with pKMSD48, indicating viral viability and hence successful transfection. No plaque was detectable around the untransformed clone N, used as a negative control

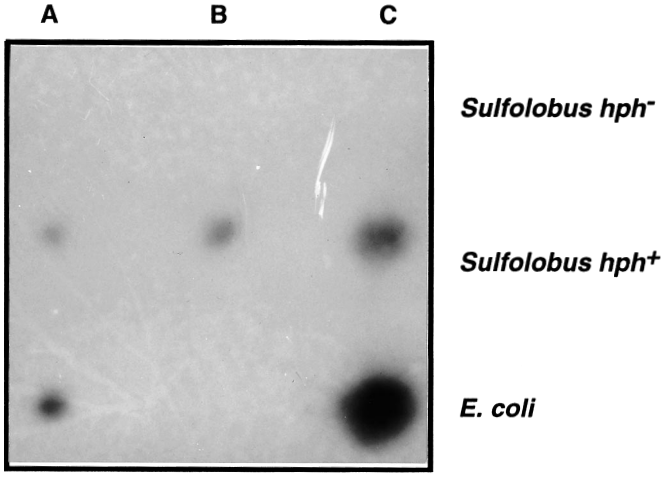

Fig. 3. Detection of hygromycin B phosphotransferase activity in pEX2A *Sulfolobus solfataricus* transformant. Hygromycin B was incubated in the presence of γ-[^{32}P]ATP with crude extracts from either *Sulfolobus* Gθ 2A transformant or a nontransformed strain. The strain Rb791 of *E. coli* transformed with and expressing the *hph* gene at high level was used as a positive control for the assay. The phosphorylated proteins were preimmobilized by filtration on nitrocellulose membrane. The specific labeled product was fixed on a P81 phosphocellulose filter and revealed by autoradiography. The amounts of proteins were 0.1 and 1 μg for the *Escherichia coli* extract (*A, C*) and 2, 5, and 20 μg for both hph^+ and hph^- *Sulfolobus* extracts (*A, B, C*)

phosphate-labeled product was seen as spots of different intensities on autoradiographic films (Mohr 1989).

The result of a standard assay is shown in Fig. 3. The different amounts of protein extracts from both *S. solfataricus* and *E. coli* hph^+ are indicated. The whole protein extract from an hph^- *S. solfataricus* strain was used as a negative control for the qualitative detection, which was found to be zero, of the background signal intensity. This experiment demonstrated the presence of a functional enzyme in *S. solfataricus*, although it is evident that, on average, a 20-fold excess of the *Sulfolobus* protein extract was necessary to detect signals comparable to those obtained with the *E. coli* hph^+ extracts. The difference in the amount of the two enzymes from the different sources was too limited for a comparative study but could be bypassed by studying the two gene products in a homogeneous overexpression system. Therefore, the mutant and wild-type versions of the *hph* gene were cloned into an *E. coli* expression vector under the control of a *lac*-derived promoter to obtain larger and more comparable amounts of both products. Clones transformed with both *hph* genes were able to grow onto a hygromycin B-selective medium, showing that the resistance determinant for *E. coli* was conserved by the mutant gene, i.e., the mutant gene was still able to confer resistance to the mesophilic host.

The protein extracts from two different clones were then assayed for HPH activity. Figure 4 shows the results of the assays performed at three different incubation temperatures. Higher thermal activity was observed for HPH_{mut} with an optimum that widened between 37°C (100%) and 50°C (97%) and showed significant retention at 60°C (82%), whereas HPH_{wt} activity showed a distinct gradual drop (100% at 37°C, 82% at 50°C, and 30% at 60°C). The indication of increased thermal resistance in the mutant enzyme was accurately investigated by performing the assay at 37°C after a 30-min incubation of the protein extracts at 55° and

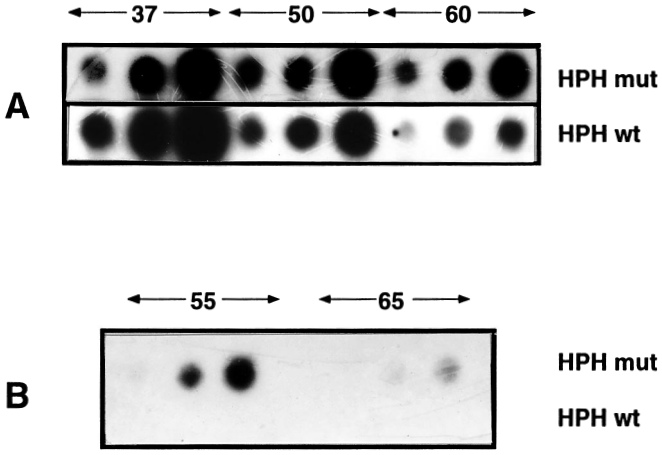

Fig. 4A,B. Thermoactivity and thermostability of the thermostabilized and wild-type *hph* gene products. Both hph_{wt} and hph_{mut} genes were overexpressed in *E. coli*, and 0.1, 0.5, and 1 μg HPH_{mut} protein extracts and fourfold correspondingly higher amounts of HPH_{wt} were assayed. Thermophilicity was determined at three different temperatures (37°, 50°, and 60°C) (**A**), and thermoresistance was evaluated as residual activity at 37°C after a 30-min heating at 55°C and 65°C (**B**)

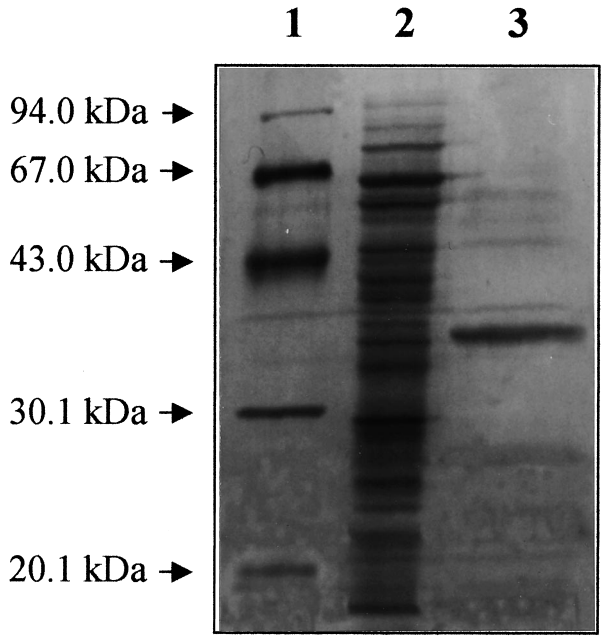

Fig. 5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the ligand affinity-purified recombinant HPH_{mut} enzyme: 10 μg of the total protein extracts from recombinant *E. coli* (*lane 2*) and 1 μg of the combined active fractions from the Sepharose-6B-hygromycin-B column (*lane 3*) were subjected to electrophoresis with molecular mass standards (*lane 1*), and proteins were detected by silver staining of the gel. Molecular weights are indicated by the respective *arrows*. Identical results were obtained for the recombinant wild-type enzyme

65°C. The mutant enzyme activity was still detectable after the 30-min heat treatment at 55° and 65°C, with a residual activity of 35% and 15%, respectively, whereas the wild-type counterpart was already completely inactivated at the lower temperature.

Both proteins were purified by specific hygromycin B affinity chromatography to a high degree of homogeneity (purification factor about 700 fold with a yield of 80%), as judged by the increase of the specific activity (280×10^6 versus 400×10^3 cpm μg^{-1}) and by SDS-PAGE. The results for the HPH_{mut} protein are shown in Fig. 5 (the identical data obtained for HPH_{wt} are not shown). The assays performed on the partially purified proteins confirmed the results obtained on crude extracts. Interestingly, this result highlighted the fact that the catalytic efficiency of the mutant enzyme was about fourfold higher than that of the wild-type counterpart.

Discussion

In the framework of a project aimed at the expression in hyperthermophilic Archea of homologous and heterologous genes encoding correctly folded and functional proteins of biotechnological interest, we described, as a preliminary study, the construction of a *S. solfataricus/E. coli* shuttle vector that allowed the selection of a mutant version (hph_{mut}) of the *hph* gene able to confer drug resistance to the thermophilic host. The alignment of hph_{mut} and wild-type *hph* coding sequences have revealed two effective point mutations (G155 → C and C156 → G producing Ser → Thr; G714 → T resulting in Trp → Cys) (Cannio et al. 1998). The relationship between these amino acid replacements and the biological activity at higher temperature acquired by the mutated enzyme was not clear even at a level of computational prediction; at present, three-dimensional (3-D) models of related enzymes suitable for a specific molecular modeling study are not available in the structure data banks.

Both the hph_{mut} sequence and the entire shuttle vector were demonstrated to be highly stable and maintained, not only in the original *S. solfataricus* used for the selection, but also in more thermophilic strains where they can still enable growth on the selection agent hygromycin B. The transformation of the different hosts tested by the hph_{mut} gene clearly indicates that its product remains functional at increasing temperatures from 75°C up to 82°C, and hence is expected to be a good candidate as a genetic marker at even higher temperatures upon further thermoadaptation. Therefore, the sequence responsible for autonomous replication of the virus SSV1 of *S. shibatae* contained in the pEXSs vector can also be recognized by some phylogenetically close *Sulfolobus* strains, and not only by the natural host and *S. solfataricus* P2, as previously reported. In addition, genetic stability of the pEXSs vector persists even when there is another hybrid *E. coli/S. solfataricus* shuttle vector, the viral SSV1 derivative pKMSD48 (Stedman et al. 1999), in the same cell, and it still confers drug resistance to the cotransformed host, which produces virus particles without any apparent decrease in the growth inhibitory effect of the indicator cells. As successfully developed for other organisms (Criswell and Bradshaw 1998), an efficient cotransformation vector system could prove useful for several studies and applications, such as (i) in vivo gene transfer and replacement; (ii) transcription

regulation to evaluate the transactivation of specific gene targets by putative transcription factors; and (iii) gene expression, when nonarchaeal sequences are to be translated and the coexpression of rare tRNA genes is necessary to bypass the marked difference in the codon usage (Wakagi et al. 1998).

The new phenotype hyg B^r acquired by *S. solfataricus* not only was a stable marker for the easy selection of transformants, but it also allowed the recovery of a functional mutant of a mesophilic protein by directed evolution, which was useful as a model for thermoadaptation in hyperthermophilic Archaea. The overexpression in *E. coli* of both wild-type (hph_{wt}) and mutated *hph* genes provided enough material for analysis and exceeded the limit of comparing enzymes produced by heterogeneous sources. The hph_{mut} gene retained the ability to confer resistance to hygromycin B to *E. coli* at the same drug concentration routinely used for the selection of hph_{wt} transformants, and thus the functionality of the encoded enzyme did not seem to be altered at 37°C. In fact, the thermal activity analysis confirmed that HPH_{mut} still had optimum activity at 37°C, but somehow the activity was extended over a wider range, up to 50°C. The prolonged incubation time required for the enzyme assay did not make it possible to exclude the presumed contribution of a higher thermal stability to this increase of thermophilicity.

HPH_{mut} showed a higher, although fairly moderate, thermoresistance, with significant residual activity detectable after 30-min incubation at 65°C; this result cannot in itself explain why this enzyme was still active at 82°C, the highest temperature used for in vivo tests in the MT4 *S. solfataricus* strain. Similarly, the data collected on the affinity-purified enzymes, which confirmed the results obtained on crude preparations, provided evidence of an improved catalytic efficiency of HPH_{mut}, but these were still not sufficient to rationalize the endurance of its activity in vivo in the thermophilic host. Extrinsic factors other than the two amino acid replacements found in the primary structure, such as posttranslational modifications, compatible solutes, molecular chaperones, and other heat shock factors present in the *S. solfataricus* cytosol (Trent et al. 1994), may be involved in preserving this enzyme from thermal denaturation and guaranteeing its performance in vivo.

To the best of our knowledge, this is the first description of a thermophilic archaeal expression system for the artificial evolution of a foreign gene from a mesophilic bacterium. It represents significant progress in the challenge of forcing proteins and enzymes to work in expanded limits of biocatalysis. The pEXSs shuttle vector, as well as the use of the two-vector transformation system described herein, shows the general feasibility of genetic manipulation of this important group of microorganisms, which should facilitate further genetic research and biotechnological exploitation of the *Sulfolobus* genus and related Crenarchaea.

Acknowledgments We acknowledge Dr. Anna D'Angelo for contribution to the work of protein purification. We also thank Dr. Kenneth M. Stedman for providing us the shuttle vector pKMSD48 and for helpful scientific discussion. This work was supported in part by grants from the EC projects, contracts BiO4-CT96-0488 and QLK3-CT-2000-00649, and by C.N.R. Target Project on Biotechnology.

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