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ORIGINAL PAPER

Snaedis H. Bjornsdottir · Olafur H. Fridjonsson
Jakob K. Kristjansson · Gudmundur Eggertsson

Cloning and expression of heterologous genes in *Rhodothermus marinus*

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Abstract The construction of a shuttle cloning system for *Rhodothermus marinus* and *Escherichia coli* is described. It is based on the shuttle vector pRM3000, which contains a multiple cloning site as well as the shuttle marker *trpB* and TrpB$^-$ recipients of both species. The vector is stable and in 25 ± 2 and 91 ± 3 copies in *R. marinus* SB-1 and *E. coli* SDH-1, respectively. Three different *R. marinus* regulatory sequences of the *dnaJ, dnaK* and *recA* genes were integrated into pRM3000 to make the expression vectors pRM5100, pRM5200 and pRM5300, respectively. Genes encoding α- and β-galactosidase (*agaT* and *bgaT*) from *Thermus brockianus* were cloned in *R. marinus*. Expression of both recombinant genes in *R. marinus* was demonstrated. The *agaT* gene was used as a reporter to study transcription from the expression vectors. Induced expression by ten- and twentyfold was observed from the *dnaK* and *dnaJ* regulatory sequences, respectively, after a temperature shift from 63 to 77°C. This is the first report of cloning and expression of heterologous genes in *R. marinus* and the first study of promoter activity in the species.

Keywords *Rhodothermus marinus* · Shuttle vector · Expression vector · Reporter gene · Thermo-induced expression

Introduction

Rhodothermus marinus is a gram-negative, red-pigmented, aerobic rod which has been isolated from coastal geothermal regions (Alfredsson et al. 1988; Nunes et al. 1992; Petursdottir et al. 2000; Sako et al. 1996). It has attracted scientific interest because of its adaptation to habitats which are subject to severe changes in temperature, salinity and other environmental factors. Also, it is a potential source of thermostable enzymes of biotechnological interest. *R. marinus* secretes several extremely thermostable polysaccharide degrading enzymes which appear to be cell attached and exhibit optimal activity at about 85°C, exceeding the optimum for growth by 20°C (Bjornsdottir et al. 2006). Although many individual *R. marinus* genes and enzymes have been characterized, a great deal remains to be elucidated about the molecular biology of the species. Little information is currently available on gene expression in vivo and the *R. marinus* promoter structure has yet to be defined. If activities of interest, such as secretion and regulation of stress response are to be studied, methods for genetic manipulation are crucial.

Due to the lack of effective genetic methods for thermophiles, their genes are often expressed for analysis or increased production in a well known host such as *Escherichia coli* (Demirjian et al. 2001; Hough and Danson 1999). However, properties of gene products in their natural environment are not always reflected in an alternative host. Some products can only be assayed in the presence of appropriate components or as a part of a pathway in the native host. Moreover, genes from thermophiles are often poorly expressed in mesophiles. Different growth temperature or cytoplasmic conditions can cause improper folding of their products (Hidalgo et al. 2004) or more promiscuous activity and toxic effects (Adams et al. 1995). Difference in GC content and codon bias have also been shown to hamper expression in mesophiles (Ishida et al. 2002; Ishida and Oshima 1996; Te'o et al. 2000). Furthermore, inactive forms can be expected, particularly when complex proteins are produced in an alternative host. Therefore, the development of methods to clone and express genes in thermophiles is important when optimal production of their proteins is required.

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S. H. Bjornsdottir · G. Eggertsson (✉)
Institute of Biology, University of Iceland, Sturlugata 7,
IS-101 Reykjavik, Iceland
E-mail: gudmegg@hi.is
Tel.: +354-5254603
Fax: +354-5254069

O. H. Fridjonsson · J. K. Kristjansson · S. H. Bjornsdottir
Prokaria Ltd., Gylfaflot 5, IS-112 Reykjavik, Iceland

Genetic methods are usually restricted to closely related species. *R. marinus* belongs to the large and diverse bacterial phylum of *Bacteroidetes* (Garrity et al. 2004). Genetic tools for *E. coli*, such as vectors and selectable markers usually fail to function in *Bacteroidetes* (McBride and Kempf 1996; Salyers et al. 1999; Shoemaker et al. 1986). Techniques for genetic manipulation of *Bacteroides* species are well established and many of them are based on endogenous plasmids and transposons (Salyers et al. 1999). Vectors developed for *Bacteroides* have been successfully used in related bacteria of *Prevotella* (Shoemaker et al. 1991) and *Porphyromonas* (Maley et al. 1992). Other tools, such as a *Bacteroides* transposon and a selectable marker proved useful in the genera *Cytophaga, Flavobacterium, Flexibacter* and *Sporocytophaga* (McBride and Baker 1996). Cloning vectors for *Cytophaga* and *Flavobacterium* species were subsequently constructed from cryptic endogenous plasmids (Alvarez et al. 2004; McBride and Kempf 1996). *Rhodothermus* belongs to the *Crenotrichaceae* family of class *Sphingobacteria* along with four other genera (Garrity et al. 2004), which are all poorly known in terms of molecular biology. None of their members has been genetically manipulated, nor have species of *Thermonema*, the only known thermophilic relatives of *R. marinus* in the phylum *Bacteroidetes* (Garrity et al. 2004). If genetic tools are to be useful in *R. marinus*, they must function at its growth temperature of 55–77°C (Alfredsson et al. 1988). Consequently, methods developed for genetic manipulation of related genera are unlikely to function in *R. marinus*.

In a recent study we established a gene transfer system for *R. marinus*, which was based on selection for complementation of a stable tryptophan auxotrophy (Bjornsdottir et al. 2005). A hybrid plasmid, pRM100 composed of pUC18 and the cryptic *R. marinus* plasmid pRM21, was constructed. It contained the *R. marinus trpB* gene expressed from the promoter of the *R. marinus groESL* operon. The hybrid plasmid successfully transformed the recipient strain *R. marinus* SB-1 (*trpB*) to prototrophy at a high efficiency after electroporation and was used in optimization of conditions for transformation. The recipient, *R. marinus* SB-1, was a $TrpB^-$ nitrosoguanidine-derived mutant of a natural isolate which lacked the major *R. marinus* host-restriction system. The aim of this work was to develop convenient bifunctional shuttle vectors for transfer between *R. marinus* and *E. coli*, which allow the maintenance and expression of cloned genes in *R. marinus*. Also, to investigate the use of different genes as reporters of gene expression in *R. marinus*.

Materials and methods

Bacterial strains and plasmids

All genetic manipulation of *R. marinus* used the strain SB-1 (*trpB*) which was previously derived from *R. marinus* PRI 493 (Bjornsdottir et al. 2005). Both *R. marinus* PRI 493 and the type strain DSMZ 4252^T (Alfredsson et al. 1988) were used for isolation of genomic DNA. *E. coli* XL1-Blue (*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB* $lacI^qZ\Delta M15$ *Tn*10]) and DH5α ((Φ80*dlacZ*Δ*M15*) *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 deoR* λ^- Δ(*lacZYA-argF*)*U169*) were used for molecular cloning. The plasmid vectors pUC19 (Yanisch-Perron et al. 1985) and pET23b (Novagen, Madison, WI) were used as well as pCR®4-TOPO® combined with competent One Shot® TOP10 cells (Invitrogen, Carlsbad, CA). The hybrid plasmid pRM100 is described elsewhere (Bjornsdottir et al. 2005). Other strains and plasmids were constructed during this work.

Culture conditions and growth measurements

Liquid cultures of *R. marinus* were grown with shaking (100 rpm) in a waterbath (Innova 3100, New Brunswick Scientific, Edison, NJ) at 63–79°C. Medium 162 was used (Degryse et al. 1978) with 1% NaCl and 0.053% NH_4Cl. It also contained either 0.25% tryptone and 0.25% yeast extract (non-selective medium, R) or 0.2% soluble starch, 0.2% casaminoacids and vitamin mixture (Degryse et al. 1978) (selective medium, RSC). Both media were solidified with 2.5% agar and plates were incubated at 65°C. *E. coli* strains were grown at 37 or 42°C in LB medium or minimal medium A (Miller, 1972) with 0.2% glucose, 0.2% casaminoacids and vitamin B1 (25 μg/ml) (MMcas, selective medium). Tryptophan or indole (50 μg/ml), ampicillin (50–100 μg/ml) or chloramphenicol (6.25–25 μg/ml) were added to the media when needed. Cultures were diluted into preheated media at 1:100 when growth curves were monitored and at 1:10 when temperature shifts were performed.

Three different methods were used to monitor growth of liquid cultures. Increase in biomass was estimated by measuring the optical density at 600 nm (Novaspec II, Amersham Biosciences, Piscataway, NJ, USA) and the specific growth rate constant (μ) was calculated. Increase in colony forming units (CFU) was estimated by spreading appropriate dilutions on RSC plates. Increase in total numbers was monitored using microscopic enumeration. Samples were vortexed and fixed (1% glutaraldehyde) before counting in the Petroff Hausser Chamber (Hausser Scientific Partnership, Horsham, PA, USA) using 400× magnification (Nikon Eclipse 800).

Molecular cloning techniques

Conventional techniques were used for molecular cloning (Ausubel et al. 1989; Sambrook and Russell 2001). Genomic DNA was prepared using the MasterPure™ DNA Purification Kit (Epicentre, Madison, WI, USA). Restriction enzymes and DNA modification enzymes were purchased from New England Biolabs (Ipswich, MA, USA). Gel purifications were performed using GFX™ PCR DNA and Gel Band Purification Kit

(Amersham Biosciences) and plasmids were purified using QIAprep® Spin Miniprep Kit (Qiagen, Hilden, Germany). Screening for plasmid content was performed as described (Law and Crickmore 1997). Transformation was achieved using the Gene Pulser II system (Bio-Rad, Hercules, CA, USA). *E. coli* was treated according to the manufacturer's instructions and *R. marinus* using conditions described elsewhere (Bjornsdottir et al. 2005). Transformation efficiency was estimated as the number of transformants per microgram of DNA and transformation frequency as the proportion of viable cells that were transformed.

Oligonucleotides, PCR amplifications and sequence analysis

The oligonucleotides used in this work are listed in Table 1. PCR reactions used Dynazyme (Espoo, Finland) and Platinum® Pfx DNA Polymerase (Invitrogen) for direct amplification from colonies or genomic DNA, respectively. The nucleotide sequences of plasmids and PCR products were determined using an Applied Biosystems 3730 DNA analyzer and the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystesm, Foster City, CA, USA). PCR products were treated with ExoSap-It™ (Amersham Biosciences) prior to sequencing. DNA sequences were analyzed using Sequencher version 4.0.5 (Genes Codes Corporation, Ann Arbor, MI, USA) and Clone Manager version 7.0.3 (Scientific & Educational Software, Durham, NC, USA).

Table 1 Oligonucleotides used in this work

Oligonucleotide	Sequence
AgaTF	GGAATTCCATATGCGGGTAAAGGTGGG
AgaTR	GGAATTCCATATGTTAAAGAAGGG GGGCATAGTC
BgaTF	GGAATTCCATATGCTAGGTGTTTG CTACTAC
BgaTR	GGAATTCCATATGTTAACCCTCCTC CCAAACC
DnaJF	GGTACCGATCTCCGCGTCGATTATGC GCTG
DnaJR	CATATGTCCTTACCTCCGCTCGCAAC
DnaKF	GGTACCTAAGATACAAACCCGACGC GAAAA
DnaKR	CATATGCGTTCGCTGCTCTGG
McsA	GGTACCGATATCCATATGGCTAGCGA CGTCTCTAGA
McsB	TCTAGAGACGTCGCTAGCCATATGGAT ATCGGTACC
OriF	CACCTCTTCGGAAGGCCGCGTTGCTGG CGTTTTTCCAT
OriR	CACCTCTTCGTTCCAGACCCCGTAG AAAAGATCA
RecAF	GGTACCTACAGACCGGTGTCGCTCCG GTT
RecAR	CATATGTGTTGCGCTTGATCAGAT GGTTG
rmseq2	GACAGCAGCGGTAAAAGC
rmseq3	TCTCCGCGATACCATATTTGC
rmseq6	ATCGCTGGCTGGCCCGTGTC
rmseq7	CTTGCGATAACTCCCATTATCAC
rmseq9	CGGGCCATCCTCTACG
trpRF2	ATGTCGACCGCCGAGACC
trpRR2	TTACATATACCGTGCAATGG

McsA and mcsB were denatured at 96° for 1 min and allowed to anneal by slowly cooling to room temperature. They were subsequently used in blunt-end cloning for introduction of a multiple cloning site. Other oligonucleotides were used in PCR and sequencing reactions. Restriction sites used for cloning are underlined

Determination of plasmid copy number

Relative plasmid copy numbers were determined by the method described by Projan et al. (1983). Images of ethidium bromide-stained gels were obtained using Gel Doc 2000 and analyzed with the Quantity One 4.2 software (both from BioRad). Determinations were performed in triplicates and for at least three strains.

Isolation of TrpB$^-$ mutants

Escherichia coli DH5α was treated with ethyl methane sulphonate (EMS) and penicillin (both from Sigma-Aldrich, St. Louis, MO, USA) as previously described (Miller 1972). Cells were plated on LB medium after an enrichment in MMcas medium and incubation with penicillinase (MP Biomedicals, Irvine, CA, USA). Single colonies were replica plated on MMcas plates with tryptophan or indole added. Those that only grew in the presence of tryptophan were collected, grown overnight in LB medium and appropriate dilutions were spread on MMcas plates with and without tryptophan for analysis of reversion frequencies.

Enzyme assays

Colony screening for α- and β-galactosidase activity was performed by histochemical staining. Immobilization of single colonies on Hybond N+ nylon membrane discs (Amersham Biosciences) was used when screening for α-galactosidase. The discs were incubated at 80°C for 30 min on filter paper saturated with 0.1 M potassium phosphate buffer (pH 6.5) containing 6-bromo-2-napthyl-α-D-galactopyranoside (0.5 mg/ml) (MP Biochemicals). They were subsequently transferred to filter paper saturated in phosphate buffer containing Fast Blue RR (1.3 mg/ml) (Sigma-Aldrich) until purple colour developed. Screening for β-galactosidase was performed by pouring a soft agar overlay (R- or LB medium containing 0.7% agar) containing X-gal (40 μg/ml) (Sigma-Aldrich). Plates were incubated at 80°C until blue colour developed.

Cells from *R. marinus* cultures harvested for enzyme assays were washed and resuspended in 0.1 M potassium phosphate buffer (pH 6.5). Crude extracts were prepared by sonification and the cell debris was removed by centrifugation. Activities of α- and β-galactosidase were assayed in crude extracts and in culture supernatants,

which were concentrated using Amicon Ultra Centrifugal Filter Devices (30.000 MWCO) (Millipore, Bedford, MA, USA). All activity measurements of crude extracts were performed after incubation at 80°C for 10 min. α-Galactosidase activity was determined by measuring the hydrolysis rate of *para*-nitrophenyl-α-D-galactoside, (4 mg/ml), 13.3 mM in phosphate buffer as previously described (Ganter et al. 1988). One unit (U) of activity is defined as the amount of enzyme which liberates 1 μmol *p*-nitrophenol per minute under the given assay conditions. β-Galactosidase activity was determined by using *para*-nitrophenyl-β-D-galactoside, 13.3 mM, using the same conditions as assays for α-galactosidase. The protein concentration of samples was estimated by the dye-binding assay of Bradford (Bradford 1976) using bovine serum albumin as a standard and the commercial Bio-Rad reagent. Incubation of substrates with lysozyme treated cells (phosphate buffer pH 6.5 containing 1 mg/ml lysozyme, 25 mM EDTA and 0.1% Triton X-100) was used for direct assays of strains.

Detection of β-galactosidase activity on non-denaturing 10% PAGE was performed as described (Manchenko 1994) using X-gal and Nitro-blue-tetrazolium (Sigma-Aldrich). The proteins in the crude extracts were also analyzed by SDS-PAGE on 10% gels as described (Laemmli 1970) and stained with Coomassie blue.

RT-PCR and northern analysis

Total RNA was isolated from exponentially growing cells using the RiboPure™-Bacteria Kit (Ambion, Austin, TX, USA) and treated with deoxyribonuclease I (Fermentas, St. Leon-Rot, Germany) as recommended by the manufacturer. RT-PCR was performed using 250 ng RNA as a template with the One Step RT-PCR Kit (Qiagen) and the RiboLock™ Ribonuclease Inhibitor (Fermentas). The intensities of ethidium bromide-stained RT-PCR products on agarose gels were estimated using GelDoc 2000 and the Quantity One 4.2 software (BioRad). Northern analysis used in vitro labeling with digoxygenin-11-UTP and T7 RNA polymerase and chemiluminescence detection (DIG Northern Starter Kit, Roche Diagnostics, Penzberg, Germany). An RNA probe was synthesized using the system and the *agaT* gene cloned in pET23b. Total cellular RNA (1 μg) was separated on a 1% formaldehyde-agarose gel followed by transfer and crosslinking to Hybond N+ nylon membranes using UVC500 Ultraviolet Crossliner (both from Amersham Biosciences).

Results

Construction of the *R. marinus*–*E. coli* shuttle vector pRM3000

Unique restriction sites were added to the hybrid plasmid pRM100 (7.0 kb) previously reported (Bjornsdottir et al. 2005) and its molecular size was reduced in order to transform it into a convenient shuttle vector. First, a multiple cloning site (MCS) containing restriction sites for *Kpn*I, *Eco*RV, *Nde*I, *Nhe*I, *Aat*II and *Xba*I was integrated into pRM100. The complementary oligos, mcsA and mcsB (Table 1), were annealed and ligated blunt in 500× molar excess into an end-filled *Eco*NI site of pRM100. Plasmids were isolated from 28 ampicillin resistant colonies after transformation of *E. coli* XL-1 Blue with the ligation mixture. One plasmid, designated pRM200, was found by restriction digests to contain the MCS. The plasmid was subsequently resolved by *Hin*dIII to its three fragments, pUC18 (2.7 kb), pRM21 containing the MCS (3.0 kb), and the *trpB* marker fragment (1.4 kb). The two latter fragments were religated before transforming *R. marinus* SB-1. Plasmids, designated pRM2000, were isolated from resulting transformants and their linearization by *Kpn*I, *Eco*RV, *Nde*I, *Nhe*I, *Aat*II and *Xba*I was confirmed. Next, the *ori* region from bp 847–1486 of pUC18 was amplified with the primers oriF and oriR (Table 1) which added *Ear*I sites at each end. The product was ligated to the *Ear*I site of pRM2000 before transforming *E. coli* SDH-1 ($TrpB^-$, see the following section). The resulting Trp^+ transformants harboured the plasmid pRM3000. Orientation of the fragments in the plasmid was verified by sequencing (rmseq primers, Table 1) and is shown in the physical map in Fig. 1.

Recipient strains for pRM3000

The $TrpB^-$ *R. marinus* strain SB-1 was previously isolated (Bjornsdottir et al. 2005). Its *trpB* mutation was mapped by sequencing during this work. The primers trpRF2 and trpRR2 (Table 1) were designed after the GenBank sequence AY454303 and used in amplifying the gene from the genomic DNA of *R. marinus* SB-1 and its parental strain PRI 493. The products were cloned in pCR®4-TOPO® and sequenced with M13 universal primers. Sequence comparison revealed a nonsense mutation at bp 676 (CGA → TGA) which would cause a truncated product of 225 instead of 402 amino acids. *R. marinus* SB-1 was transformed with the shuttle vector pRM3000 and Trp^+ colonies appeared on selective (RSC) plates after 3–4 days. Transformation efficiency was estimated as $1.3 \pm 0.3 \times 10^7/\mu g$ of DNA and transformation frequency as 7 ± 3%. Approximately 27% of Trp^+ transformants were found to harbour pRM3000 by direct screening for plasmid content. However, the vector appeared to be stably maintained in those strains. Cultures from individual transformants were propagated for over 80 generations in liquid in the absence of selection (R-medium). Aliquots were regularly spread on selective and non-selective plates and no difference in numbers of CFU was observed. Furthermore, the vector was successfully prepared from 20 single Trp^+ colonies subjected to restriction analysis. No rearrangements were found. The copy number of pRM3000 in *R. marinus* SB-1 was determined as 25 ± 2

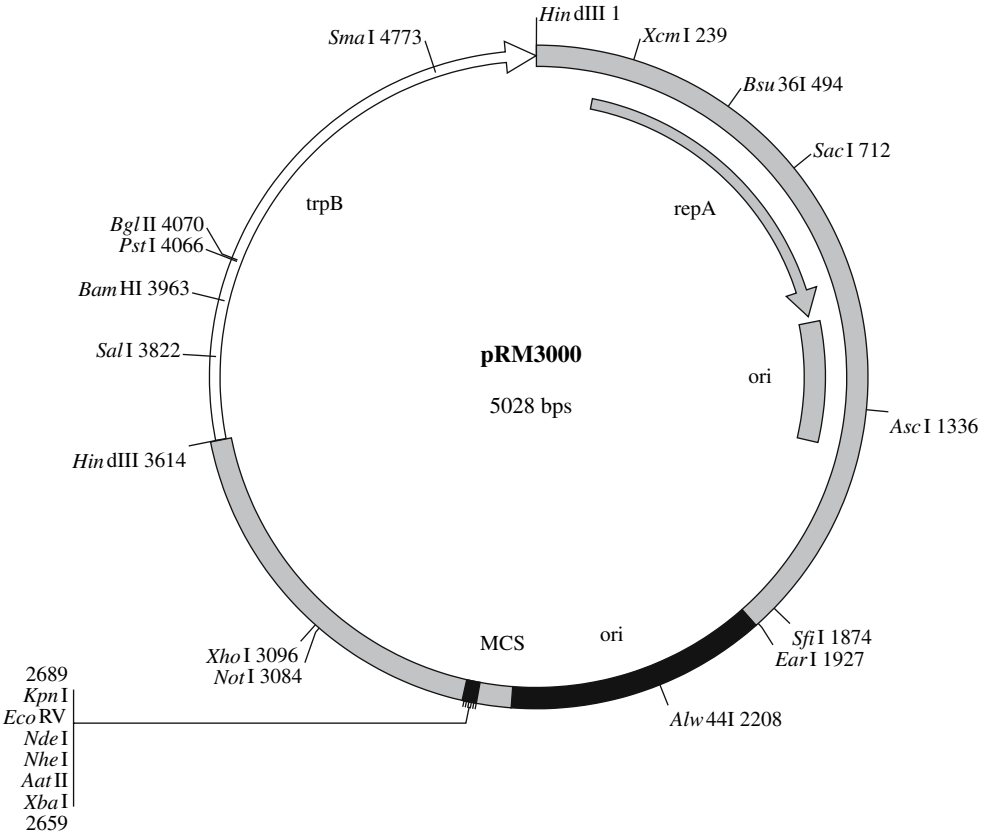

Fig. 1 A physical map of the shuttle vector pRM3000. The vector is based on the *R. marinus* plasmid pRM21. It is shown gray with its features, the *ori* region (1,100–1,440) and the *repA* gene (160–1,080). The *trpB* marker fragment (3,614–5,028) which is shown *white*, is derived from the hybrid plasmid pRM100. The *ori* for *E. coli* (1,935–2,573) is *black* and derived from pUC19. A custom sythesized multiple cloning site (*MCS*) (2,659–2,694) is also shown *black*. Some single restriction sites are shown as well as *Hin*dIII sites

per chromosome in late exponential cells grown in the selective medium (RSC) at 65°C.

Growth of *R. marinus* SB-1 in the selective medium supplemented with tryptophan was examined. First, its optimum and maximum growth temperatures were determined by monitoring the turbidity of liquid cultures grown at 63–79°C. Figure 2a shows estimates of growth rates at each temperature. Fastest growth, a doubling time of approximately 58 min ($\mu = 0.72 \pm 0.02\ h^{-1}$), was observed at 67°C and growth ceased at 79°C. The same result was obtained for *R. marinus* PRI 493. Next, a growth curve for *R. marinus* SB-1 at the optimum temperature was obtained (Fig. 2b). Parallel estimates of CFU numbers yielded highly irregular results. Therefore, cell number at each interval was estimated by direct cell counts (Fig. 2b). The maximum cell number determined was $2.5 \pm 0.3 \times 10^9$ cells/ml at OD_{600} of 2.2. Microscopic examination revealed heavy cell aggregation in freshly diluted cultures but the aggregation subsided as the cultures became denser. Therefore, estimation of cell numbers in samples taken at OD_{600} of less than 0.1 was impossible, except for the initial sample at T_0. A linear relationship between cell number and OD_{600} was obtained. Thus, cell number was found to be equivalent to $1.1 \times 10^9 \times (OD_{600}) + 3.0 \times 10^7$. This equation is valid for a culture grown in selective medium with an OD_{600} larger than 0.1.

The *E. coli* cloning strain DH5α was treated with EMS and penicillin in order to obtain a $TrpB^-$ mutant suitable as a recipient for pRM3000. The mutant strain SDH-1 with a low reversion frequency (9×10^{-9}) was chosen as a recipient. Sequence analysis and comparison of *trpB* genes from *E. coli* SDH-1 and DH5α revealed an ochre mutation at bp 934 (CAA → TAA) causing formation of a truncated protein of 311 instead of 347 amino acids. The *trpB* mutation of *E. coli* SDH-1 was complemented by pRM3000. It transfomed the strain at an efficiency of $2.4 \pm 0.7 \times 10^7$ per μg of DNA and at a frequency of $10 \pm 3\%$. Trp^+ colonies appeared on selective plates after 2 days. The vector was identified in every Trp^+ colony screened for plasmid content. The stability of pRM3000 was not examined under non-selective conditions. However, it has repeatedly been isolated from cells grown in non-selective (LB) medium. Neither loss of the vector nor rearrangements have been observed. Analysis found pRM3000 in 91 ± 3 copies per chromosome in *E. coli* SDH-1 grown at 37°C to late exponential phase. Growth of untransformed *E. coli* SDH-1 was examined in both LB and selective (MMcas) medium supplemented with tryptophan. Its doubling times were found to be approximately 21 min ($\mu = 1.95 \pm 0.02\ h^{-1}$) and 60 min ($\mu = 0.69 \pm 0.03\ h^{-1}$), respectively.

Construction of the shuttle expression vectors pRM5100, pRM5200 and pRM5300

Genomic sequences upstream of the *dnaJ, dnaK* and *recA* genes of *R. marinus* (GenBank no. AF145250; AF145251 and AF026690) were integrated into pRM3000 to allow

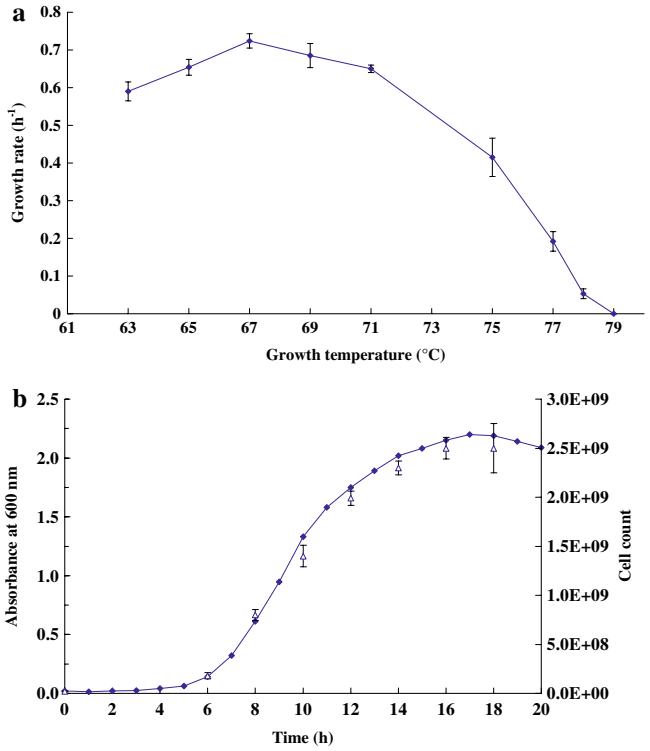

Fig. 2 Growth of *R. marinus* SB-1 in selective medium. **a** Effect of temperature on growth rate, μ, which was estimated from turbidimetry measurements at 600 nm. Values are averages of estimations from 2–6 cultures at each temperature. *Vertical bars* represent standard errors. **b** Growth at optimal temperature of 67°C. Turbidity and direct microscopic counts were used to monitor the increase in biomass (*closed circles*) and cell numbers (*open triangles*), respectively. Values for cell numbers are averages from eight determinations and *vertical bars* represent standard errors. Enumeration of cells in samples taken at 2 and 4 h was not possible due to cell aggregation

the construction of transcriptional fusions by cloning into the MCS. First, 200 bp upstream regions of the *dnaJ* and *dnaK* genes and the known 90 bp region upstream of the *recA* gene were amplified from the chromosome of *R. marinus* DMSZ 4252^T with primers dnaJF and dnaJR, dnaKF and dnaKR and recAF and recAR, respectively (Table 1). The primers added *Kpn*I sites to the 5′-ends and *Nde*I sites to the 3′-ends of each product which was subsequently isolated from pCR®4-TOPO® after cloning and sequencing and ligated to *Kpn*I/*Nde*I digested pRM3000. Trp$^+$ *E. coli* SDH-1 transformants were screened for the upstream regions by colony-PCR using primers rmseq6R and rmseq7F (Table 1). The positive transformants were grown in selective medium and vectors (5.2 kb) containing the *dnaJ* and *dnaK* upstream regions and a vector (5.1 kb) containing the *recA* upsteam region were isolated. They were designated pRM5100, pRM5200 and pRM5300, respectively. All of the vectors transformed *E. coli* SDH-1 and *R. marinus* SB-1 to efficiencies comparable to that of pRM3000 and were found in 83 ± 7 and 28 ± 3 copies in the species, respectively.

Cloning of heterologous reporter genes in *R. marinus*

Heterologous genes were introduced into the shuttle vectors to assess their use for gene cloning and expression in *R. marinus*. A gene from the *B. stearothermophilus* plasmid pNW33N (De Rossi et al. 1994) encoding a chloramphenicol acetyl transferase (*cat*) proved unsuitable as a reporter in *R. marinus* as its expression appeared to hinder growth. The *cat* gene was cloned in *R. marinus* SB-1 using the shuttle vector pRM3000 and the expected number of Trp$^+$ transformants appeared after 4 days. However, only very small Trp$^+$ transformants were observed after prolonged incubation when the introduced *cat* gene was carried by the expression vectors. Resistant colonies of *R. marinus* SB-1 grew after transfer to non-selective medium containing chloramphenicol and incubation for several days at 60–65°C. Their resistant phenotype could not be attributed to the presence of the *cat* gene as all attempts to amplify it or to isolate plasmids from the colonies were unsuccessful.

Two genes from *Thermus brockianus* encoding α- and β-galactosidase were examined as reporters of gene expression. Both genes, *agaT* of 1431 bp and *bgaT* of 1942 bp (GenBank No. AF135398), were amplified from the chromosome of *T. brockianus* using the primers agaTF and agaTR and bgaTF and bgaTR, respectively (Table 1). They were subsequently introduced into *Nde*I sites of all the shuttle vectors. The cloning was performed using *E. coli* SDH-1 as a recipient. Resulting Trp$^+$ transformants were screened for α- and β- galactosidase activity using histochemical staining during incubation at 80°C. Activity of α-galactosidase was detected in colonies harbouring *agaT* in transcriptional fusions with the *dnaJ* and *recA* upstream sequences while no activity was seen in colonies harbouring *dnaK* transcriptional fusions. Activity of β-galactosidase was detected in *E. coli* SDH-1 harbouring all the transcriptional fusions. Both types of staining showed the relative strength of expression from the *R. marinus* upstream sequences in *E. coli* SDH-1 as *dnaJ* (pRM5100) > *recA* (pRM5300) > *dnaK* (pRM5200). The staining detected no galactosidase activity in untransformed *E. coli* SDH-1 or in cells harbouring the recombinant genes in pRM3000 (no upstream region), which were identified using colony-PCR and sequencing.

Next, *R. marinus* SB-1 was transformed with shuttle vectors isolated from *E. coli* SDH-1. Trp$^+$ transformants were selected and screened for plasmid content. Liquid cultures of positive transformants grown in selective medium at 65°C were lysed and the extracts were directly assayed for galactosidase activity towards *p*-nitrophenol substrates at 80°C. Comparison of results from different transformants immediately demonstrated three facts about the expression of galactosidase in *R. marinus* SB-1. First, a different expression profile of the reporter genes was observed as compared to *E. coli* SDH-1. The relative expression from transcriptional fusions was found to be *dnaK* (pRM5200) > *dnaJ* (pRM5100) ≈ *recA* (pRM5300) for both recombinant

genes. Secondly, for each type of fusion, a greater activity of α- than β-galactosidase was detected. Finally, a background activity of both types was detected in untransformed *R. marinus* SB-1 and in transformants harbouring recombinant genes in pRM3000 (no upstream sequence). However, the background of β-galactosidase activity was around tenfold that of α-galactosidase. Figure 3 shows the activity of β-galactosidase in extracts of *R. marinus* SB-1 (lanes 1–3) as well as in extracts of strains containing recombinant *bgaT* in pRM5100 (lane 4), pRM5200 (lane 5) and pRM5300 (lane 6). Three active protein bands were detected in the recipient and an additional smaller band of different intensities was seen in the strains harbouring the three expression vectors. The above results demonstrated expression of both recombinant *T. brockianus* genes under the control of the *dnaJ, dnaK* and *recA* regulatory sequences in *R. marinus* SB-1. Next, expression of *agaT* from the three regulatory sequences was further investigated by measuring specific α-galactosidase activity in cell-free extracts.

Expression of *agaT* under heat shock

In order to examine whether the expression of *agaT* in *R. marinus* SB-1 was influenced by heat shock, cultures were subjected to temperature shifts. For comparison, expression of *agaT* in *E. coli* SDH-1 was examined. Cultures were shifted from 63 to 77°C and from 37 to 42°C, respectively, and samples were taken at regular intervals to measure α-galactosidase activity in cell-free extracts. As shown in Figure 4a, the specific activity for *R. marinus* SB-1 containing *dnaJ* and *dnaK* transcriptional fusions (expression vectors pRM5100 and pRM5200) was found to rise sharply during cultivation for 10 h after the temperature shift. For *E. coli* SDH-1, activity was barely detectable except for the strain containing *agaT* fused to the *dnaJ* regulatory sequence (pRM5100), which also reached a maximum after 10 h (Fig. 4b). Table 2 compares specific α-galactosidase activities before and after temperature shifts. Clear induction was seen in *R. marinus* SB-1 strains containing *agaT* fused to the *dnaJ* and *dnaK* regulatory sequences compared to no temperature shift (cultivation at 63°C). An approximately twentyfold and tenfold increase in activity was measured, respectively. Maximal α-galactosidase activity obtained was 2.38 ± 0.2 U/mg for SB-1/pRM5200/*agaT*, containing the *dnaK* regulatory sequence, after a shift from 63 to 77°C. This activity corresponds to a recombinant protein concentration of almost 1% of total soluble protein as V_{max} for AgaT for *p*NP-α-galactose was previously determined to be about 300 U/mg applying the same assay conditions (Fridjonsson et al. 1999). A faint band of approximately 55 kDa, corresponding to the size of AgaT was seen in SDS-PAGE analysis of crude extracts from the strain (data not shown). No activity was detected in culture supernatants. The enzyme assays showed no backgound activity of α-galactosidase for *E. coli* SDH-1 and very low activity for *R. marinus* SB-1. No background was detected after temperature shifts (Table 2). In *R. marinus* SB-1, the highest observed expression level of *agaT* was from the *dnaK* regulatory region, both before and after temperature shifts (Fig. 4a, Table 2). Conversely, the highest expression of *agaT* in *E. coli* SDH-1 was obtained from the *dnaJ* transcriptional fusion (Fig. 4b, Table 2).

To further demonstrate transcription of heterologous *agaT*, RT-PCR and Northern analysis were performed. Total RNA was harvested from *R. marinus* SB-1 after the temperature shift from 63 to 77°C. Both the gene specific primers (agaTF and agaTR, Table 1) and an RNA probe synthesized in vitro detected *agaT* transcripts in cellular RNA (Fig. 5a, b, respectively). Both analyses demonstrated the lack of *agaT* transcripts in untransformed *R. marinus* SB-1 and SB-1 containing *agaT* in pRM3000 (Fig. 5a lanes 2–3, Fig. 5b lanes 1–2). They also agreed with activity measurements on the relative strength of expression from the three *R. marinus* regulatory sequences after the temperature shift (compare Fig. 5a lanes 4–6, Fig. 5b lanes 3–5 to Fig. 4a). Densitometry measurements on the RT-PCR products allowed estimation of the relative amount of *agaT* transcripts from the *dnaJ, dnaK* and *recA* regulatory sequences as about 3:15:1 in *R. marinus* SB-1. The Northern analysis also detected *agaT* transcripts in *E. coli* SDH-1 (Fig. 5b). The copy numbers of expression vectors containing *agaT* were found to be 58 ± 3 and 14 ± 1 per chromosome in *E. coli* SDH-1 and *R. marinus* SB-1, respectively, and to remain stable after heat shock.

Discussion

The development and characterization of a cloning and expression system for *R. marinus* has been achieved. The system described is based on vectors replicating in recipient strains of both *R. marinus* and *E. coli* and contain a variety of cloning sites for introduction of DNA of

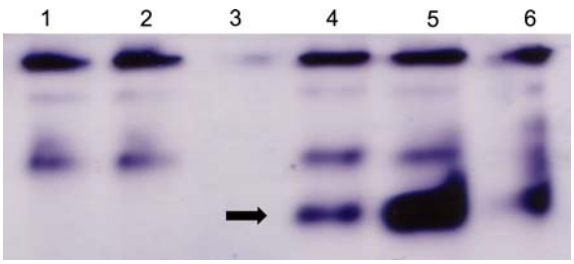

Fig. 3 Native-PAGE analysis (10%) of β-galactosidase activity in extracts of *R. marinus* SB-1. Activity was visualized using X-gal and NBT as described in Materials and methods. *Lane 1* SB-1; *lane 2* SB-1, cleared extract; *lane 3* SB-1, cellular fraction; *lane 4* SB-1/pRM5100/*bgaT*; *lane 5* SB-1/pRM5200/*bgaT*; *lane 6* SB-1/pRM5300/*bgaT*. The bands attributed to the recombinant β-galactosidase are indicated with an *arrow*

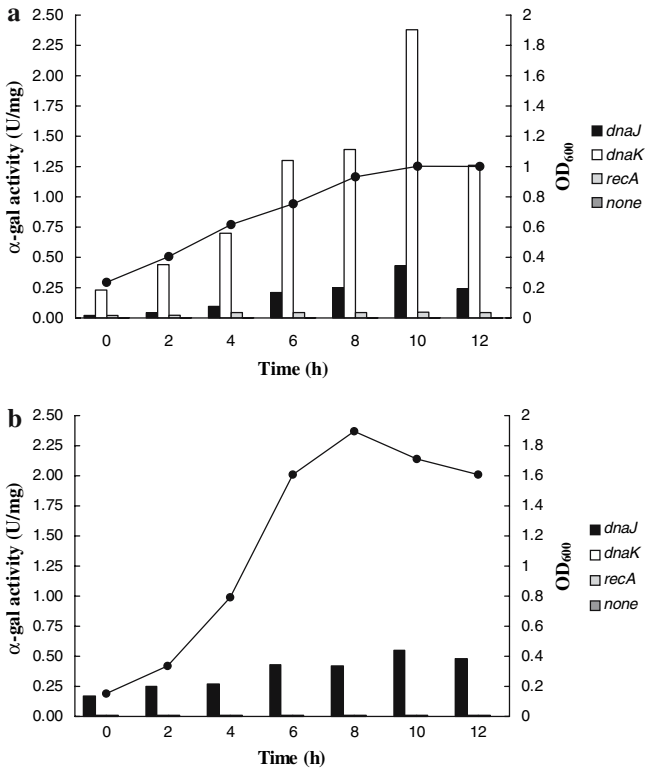

Fig. 4 Increase in α-galactosidase activity during incubation after temperature shifts. **a** Activity of *R. marinus* SB-1 after a temperature shift from 63 to 77°C. **b** Activity of *E. coli* SDH-1 after a temperature shift from 37 to 42°C. Precultures were diluted tenfold into selective media and samples were harvested every 2 h for determination of α-galactosidase activity as described in Materials and methods. The measured OD_{600} value of each sample is shown. *Black*, *white* and *gray bars* represent α-galactosidase activity of strains harbouring *agaT* expressed from pRM5100 (*dnaJ* promoter), pRM5200 (*dnaK* promoter) and pRM5300 (*recA* promoter). *Dark gray bars* represent activity of the recipients *R. marinus* SB-1 and *E. coli* SDH-1. Values are averages of three replicate samples from four experiments for a total of 12 data points. Standard error is less than 10%

interest. Conveniently, the system allows cloning to be performed in *E. coli* before intact sequenced recombinant plasmids are introduced into *R. marinus*. Furthermore, expression vectors were constructed in which a cloned gene can be placed under control of three different *R. marinus* regulatory sequences. Highest activity of the reporter AgaT produced in *R. marinus*, corresponding to approximately 1% of total cellular protein, was observed when the *dnaK* upstream region was used to regulate expression. For comparison, heterologous expression of the *agaT* from pBTac1 in *E. coli* JM109 yielded AgaT of approximately 1.5% of cellular protein (Fridjonsson et al. 1999).

The shuttle vectors appear to replicate stably in both *R. marinus* SB-1 and *E. coli* SDH-1. However, when *R. marinus* SB-1 was transformed with pRM3000 (5 kb), only about 27% of transformants were found to harbour the vector. The Trp^+ phenotype of the remaining colonies might be explained by homologous recombination and integration of the vector into the chromosome. Every Trp^+ transformant of the recombination deficient *E. coli* SDH-1 (*recA*) was found to contain the vector. When *R. marinus* SB-1 was transformed with expression vectors (6.7–7.1 kb) containing recombinant genes, about 75% of Trp^+ transformants were found to harbour them. Similar results were previously obtained with the hybrid plasmid pRM100 (7 kb) (Bjornsdottir et al. 2005). This fact indicated that plasmid size affected the prospect of cointegrate formation. Chromosomal integration of the shuttle vectors makes it difficult to assess plasmid stability based on the Trp^+ phenotype or the presence of vector-specific sequences. However, analysis of extrachromosomal DNA and highly consistent results from the expression of reporter genes carried by the vectors indicate that they are stably maintained in *R. marinus* SB-1.

At the onset of this work, sequences of around 30 *R. marinus* genes had been published (Bjornsdottir et al. 2006). We chose to integrate upstream sequences of the *dnaJ* and *dnaK* genes into pRM3000. Both genes are known to be subject to temperature regulation in other bacteria (Gross 1996; Osipiuk and Joachimiak 1997). For reference, the upstream sequence of the *recA* gene was also integrated. Minimum and maximum growth temperatures of 54°C and 77°C have been reported for *R. marinus* DSMZ 4252^T (Alfredsson et al. 1988). A maximum growth rate of 0.5 h^{-1} was observed at 65°C in R-medium. Another study reported minimum and maximum growth temperatures of 55 and 77–80°C, respectively, and an optimum at about 65–70°C with a maximum growth rate of over 0.6^{h-1} (Silva et al. 1999). In this work, growth of *R. marinus* SB-1 was examined in the selective starch-based (RSC) medium since knowledge of its growth characteristics was important for subsequent experiments. A maximum temperature of 78°C and a maximum growth rate of 0.72 h^{-1} at 67°C were determined. Aggregation in *R. marinus* cultures has previously been reported (Bjornsdottir et al 2005). Here, it was mainly seen in *R. marinus* cultures of low OD_{600} values. The aggregation might be due to formation of a capsule. A distinct slime capsule has been observed by ink staining of *R. marinus*, particularly of cells grown in starch (Alfredsson et al. 1988).

A convenient reporter is important for studies on genetic properties of *R. marinus*. Here, for the first time, heterologous genes, *agaT* and *bgaT*, were used to analyse expression in the species. An activity of both α-and β-galactosidase could be identified in colonies and assayed using *p*-nitrophenol derivatives as substrates. Activity of β-galactosidase has been detected in *R. marinus* DSMZ 4252^T (Gomes et al. 2000) while α-galactosidase activity was not detected without raffinose induction (Blucher et al. 2000). Here, a very low background of α-galactosidase was detected in *R. marinus* SB-1. Furthermore, AgaT was found to be a sensitive indicator of promoter activity. An additional feature of AgaT important to this work was its thermostability, i.e. its half-life of 17 h at 80°C (Fridjonsson et al. 1999). A gene encoding a less thermostable α-galactosidase from a thermophilic *Bacillus* strain (unpublished) was also

Table 2 α-Galactosidase activity of *R. marinus* SB-1 and *E. coli* SDH-1 after incubation for 10 h after temperature shifts

Strain	Promoter expressing recombinant *agaT*	α-Gal activity (U/mg)[a]				
		37°C[b]	42°C[b]	63°C[b]	75°C[b]	77°C[b]
E. coli SDH-1	none	0.00	0.00	ND	ND	ND
SDH-1/pRM5100/*agaT*	*dnaJ*	0.50	0.55	ND	ND	ND
SDH-1/pRM5200/*agaT*	*dnaK*	0.01	0.01	ND	ND	ND
SDH-1/pRM5300/*agaT*	*recA*	0.02	0.02	ND	ND	ND
R. marinus SB-1	none	ND	ND	0.01	0.00	0.00
SB-1/pRM3000/*agaT*	none	ND	ND	0.01	0.00	0.00
SB-1/pRM5100/*agaT*	*dnaJ*	ND	ND	0.02	0.25	0.43
SB-1/pRM5200/*agaT*	*dnaK*	ND	ND	0.23	1.59	2.38
SB-1/pRM5300/*agaT*	*recA*	ND	ND	0.02	0.05	0.04

ND not determined
[a]Activity in crude extracts was determined by measuring the rate of hydrolysis of *para*-nitrophenyl-α-D-galactoside at 80°C. Values are averages of three replicate samples from four experiments for a total of 12 data points. Standard error is less than 10%. One unit of activity is defined as the amount of enzyme that liberates 1 μmol of *p*-nitrophenol per min
[b]*E. coli* and *R. marinus* cultures were grown at 37 and 63°C, respectively, before dilution into fresh selective media and transfer to the indicated temperatures. Cells were harvested for enzyme assays after 10 h of incubation

cloned in the shuttle expression vectors. Activity of the recombinant enzyme was detected in *R. marinus* cells grown at 63°C but not in cells grown at 65°C. Another reason precluded the use of the *cat* gene as a reporter in *R. marinus*. Its expression appeared to exhibit toxic effects and to arrest growth of *R. marinus* as it could not be cloned using the expression vectors. Interestingly, no chloramphenicol resistant colonies were obtained despite numerous attempts to clone and express the *cat* gene in *Thermus thermophilus* (Park et al. 2004).

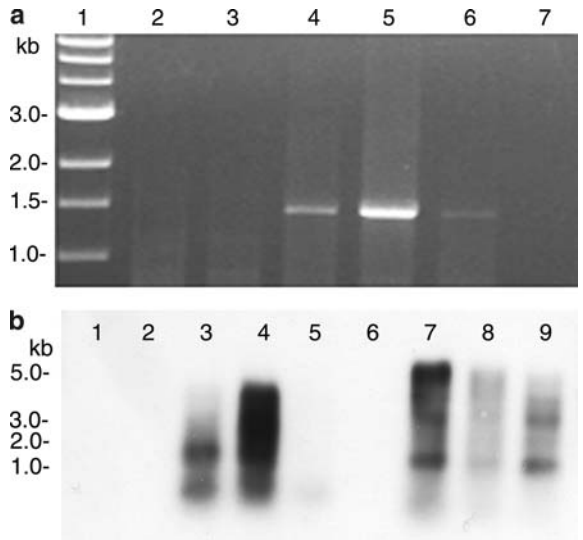

Fig. 5 Expression of recombinant *agaT* at the RNA level. Detection of *agaT* transcripts in RNA isolated after growth for 4 h after temperature shifts from 63 to 77°C and from 37 to 42°C for *R. marinus* SB-1 and *E. coli* SDH-1, respectively. **a** RT-PCR amplification from total cellular RNA (250 ng). *Lane 1* 1 kb size marker; *lane 2 R. marinus* SB-1 (untransformed); *lane 3* SB-1/pRM3000/*agaT*; *lane 4* SB-1/pRM5100/*agaT*; *lane 5* SB-1/pRM5200/*agaT*; *lane 6* SB-1/pRM5300/*agaT*; *lane 7* negative control. **b** Northern analysis of total cellular RNA (1 μg). *Lane 1* SB-1 (untransformed); *lane 2* SB-1/pRM3000/*agaT*; *lane 3* SB-1/pRM5100/*agaT*; *lane 4* SB-1/pRM5200/*agaT*; *lane 5* SB-1/pRM5300/*agaT*; *lane 6* SDH-1 (untransformed); *lane 7* SDH-1/pRM5100/*agaT*; *lane 8* SDH-1/pRM5200/*agaT*; *lane 9* SDH-1/pRM5300/*agaT*. Indication of molecular size is based on migration of an RNA ladder (New England Biolabs)

The heat shock response in *R. marinus* DSMZ 4252^T has been examined by two-dimensional gel electrophoresis (Thorolfsdottir 1999). However, results on induction of the *dnaJ* and *dnaK* genes were inconclusive. Another study found a 2.5 fold increase in the amount of mannosylglycerate synthase after a temperature shift from 65 to 75°C for 2 h (Borges et al. 2004). Results obtained in this study confirm that the *R. marinus dnaJ* and *dnaK* promoters regulate transcription through heat induction. Interestingly, different expression profiles for the promoters were seen in *R. marinus* and *E. coli* with the reporters which agreed on the same relative strength of the three *R. marinus* regulatory sequences. The strongest regulatory region for *R. marinus*, that of the *dnaK* gene, proved to be the least effective in *E. coli*. However, as long upstream sequences (90–200 bp) were used in the transcriptional fusions, different sequence elements could be acting as promoters in the different species. With a reporter system for *R. marinus*, experiments can now be performed to define the minimal promoter sequences of the *dnaJ* and *dnaK* genes and of other regulatory sequences. With better knowledge of promoter structure and function in *R. marinus* it is expected that the efficiency of expression of cloned genes can be considerably increased.

In conclusion, we have defined a cloning and expression system which can be used for expression of heterologous and homologous genes in the *R. marinus*. Furthermore, combined with the identified reporter genes, the system can be used for systematic studies of promoter activity in the species.

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