Use of a halobacterial *bgaH* reporter gene to analyse the regulation of gene expression in halophilic archaea

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The *bgaH* reading frame encoding a β -galactosidase of '*Haloferax alicantei*' was used as a reporter gene to investigate three different promoter regions derived from *gvpA* genes of *Haloferax mediterranei* (mc-*gvpA*) and *Halobacterium salinarum* (c-*gvpA* and p-*gvpA*) in *Haloferax volcanii* transformants. The fusion of *bgaH* at the start codon of each *gvpA* reading frame (A1-*bgaH* fusion genes) caused translational problems in some cases. Transformants containing constructs with fusions further downstream in the *gvpA* reading frame (A-*bgaH*) produced β -galactosidase, and colonies on agar plates turned blue when sprayed with X-Gal. The β -galactosidase activities quantified by standard ONPG assays correlated well with the mRNA data determined with transformants containing the respective *gvpA* genes: the cA-*bgaH* fusion gene was completely inactive, the mcA-*bgaH* transformants showed low amounts of products, whereas the pA-*bgaH* fusion gene was constitutively expressed in the respective transformants. The transcription of each A-*bgaH* gene was activated by the homologous transcriptional activator protein GvpE. The cGvpE, pGvpE and mcGvpE proteins were able to activate the promoter of pA-*bgaH* and mcA-*bgaH*, whereas the promoter of cA-*bgaH* was only activated by cGvpE. Among the three GvpE proteins tested, cGvpE appeared to be the strongest transcriptional activator.

Keywords: *Haloferax volcanii*, β -galactosidase reporter gene, gene regulation

INTRODUCTION

Halophilic archaea are model organisms to study archaeal gene regulation *in vivo*. They are easy to grow, and a transformation system including vector plasmids conferring resistance to mevinolin or novobiocin is available (Lam & Doolittle, 1989; Holmes *et al.*, 1991). Transcription in archaea possesses fundamental similarities to eukaryal transcription: archaeal promoters contain a TATA box element centred 25–28 nucleotides upstream of the transcription start site, and gene expression involves a single DNA-dependent RNA polymerase comprising 12 subunits that are homologous to the core components of the eukaryal RNA polymerase II (Zillig *et al.*, 1993). Two additional factors, namely the TATA-box-binding protein TBP and TFB (the archaeal homologue of transcription factor TFIIB) are sufficient for the initiation of basal transcription (Hausner *et al.*, 1996; Qureshi *et al.*, 1995; Thomm,

1996; Reeve *et al.*, 1997). TFB recognizes a DNA sequence immediately upstream of the TATA box (the TFB recognition element BRE found in many archaeal genes) as demonstrated by *in vitro* transcription studies using recombinant factors and RNA polymerase from the thermophilic archaeon *Sulfolobus* (Qureshi & Jackson, 1998; Bell *et al.*, 1998, 1999). So far, *in vitro* transcription systems have been established for some methanogenic and thermophilic archaea, but not for halophilic archaea. The genome sequences of *Methanococcus jannaschii* and *Methanobacterium thermoautotrophicum* contain single genes for TBP and TFB proteins, whereas halophilic archaea harbour multiple gene copies for both TBP and TFB (Ng *et al.*, 1998; Baglia *et al.*, 2000; Thompson *et al.*, 1999).

We are using the genes encoding gas vesicles to study gene regulation in halophilic archaea. Gas vesicle formation involves the 14 genes *gvpDEFGHIJKLM* and *gvpACNO* that cluster in a genomic region called the vac region. Three different vac regions have been characterized, two of these are found in *Halobacterium*

Abbreviations: *gvp*, gas vesicle protein gene; Gvp, gas vesicle protein.

salinarum PHH1, namely the chromosomal c-vac region and the plasmid-borne p-vac region of the 150 kb plasmid pHH1 (Englert *et al.*, 1992a). In the case of the related strain *Hb. salinarum* NRC-1, two identical *gvp1* gene clusters corresponding to the p-vac region are present on the two large plasmids pNRC100 and pNRC200, and an additional *gvp2* gene cluster almost identical to c-vac is found on pNRC200 (DasSarma *et al.*, 1994; Ng *et al.*, 1998, 2000). The mc-vac region (*mediterranei* chromosomal) is found in the chromosome of *Haloferax mediterranei* (Englert *et al.*, 1992a). We use the designations c-*gvp*, p-*gvp* and mc-*gvp* to distinguish the various *gvp* genes according to the origin of the vac region. The c-vac region is expressed only in p-vac deletion strains such as *Hb. salinarum* PHH4 harbouring the 35 kb plasmid pHH4 (Pfeifer & Blaseio, 1989).

The *gvpACNO* gene cluster encodes the major structural protein, GvpA, of the gas vesicles, and GvpC, a protein involved in stabilization of the structure and in shape formation (Offner *et al.*, 1996). The products of the *gvpD* and *gvpE* genes are involved in regulation: GvpE is a transcriptional activator (Krüger & Pfeifer, 1996; Krüger *et al.*, 1998), whereas GvpD takes part in the repression of gas vesicle formation (Englert *et al.*, 1992b; Pfeifer *et al.*, 1994, 2001). These two Gvp proteins predominantly influence the promoter of the *gvpACNO* unit and thus determine whether and when gas vesicles are formed. The promoter of the p-*gvpA* gene of *Hb. salinarum* PHH1 has a high activity throughout growth, and transcription results in large amounts of p-*gvpA* mRNA plus minor amounts of p-*gvpACNO* cotranscripts (Horne *et al.*, 1991; Englert *et al.*, 1992a). In contrast, the promoter of the c-*gvpA* gene is active only during the stationary phase in *Hb. salinarum* PHH4 (Horne & Pfeifer, 1989), similar to the promoter of the mc-*gvpA* gene of *Hf. mediterranei* (Englert *et al.*, 1992a).

The activities of the c-*gvpA* and mc-*gvpA* promoters depend on the presence of the GvpE activator protein, as demonstrated by transformation experiments using *Haloferax volcanii* as recipient. This strain does not contain the *gvp* genes and offers a clean genetic background. Transformants harbouring the p-*gvpA* gene produce p-*gvpA* mRNA throughout growth, whereas the respective c-*gvpA* and mc-*gvpA* transformants contain no or minor amounts of *gvpA* mRNA. However, transformants harbouring c-*gvpA* plus c-*gvpE* (expressed under *fdx* promoter control) contain large amounts of c-*gvpA* mRNA and also GvpA protein (Krüger *et al.*, 1998). Similarly, mc-*gvpADE* transformants produce large amounts of mc-*gvpA* transcripts and GvpA protein (Röder & Pfeifer, 1996). These results demonstrate that GvpE is a transcriptional activator protein. Homology modelling of the C-terminal part of GvpE indicates an amphiphilic α helix that exhibits a similar structure as the one found in the basic leucine zipper (bZIP) activator protein GCN4 of yeast (Ellenberger *et al.*, 1992; Krüger *et al.*, 1998). A region adjacent to the N terminus consists of basic amino acids

and constitutes a putative DNA-binding site. Such bZIP proteins are transcriptional activators predominantly found in eukarya.

To investigate the basal and induced activities of the three different *gvpA* promoters in *Hf. volcanii* transformants in more detail, we tested a halobacterial β -galactosidase as reporter. The *bgaH* gene encoding this enzyme has been isolated from a 'superblue' mutant of '*Haloferax alicantei*' (Holmes *et al.*, 1997; Holmes & Dyall-Smith, 2000). *Hf. volcanii* lacks detectable β -galactosidase activity and is thus suitable for these studies. Transformants expressing the enzyme turn blue when sprayed with X-Gal on agar plates, and the β -galactosidase activity can be quantified by a standard assay hydrolysing ONPG. An application of this system for the investigation of various promoters in *Hb. salinarum* has recently been reported by Patenge *et al.* (2000).

In this report, *Hf. volcanii* transformants were produced containing fusions between the various *gvpA* promoter regions and the *bgaH* reading frame. Different fusion constructs were tested at the level of mRNA formation and at the level of β -galactosidase activity to gain insights into the amount of both products. β -Galactosidase activity did not always reflect the mRNA data, indicating that the fusion site is rather crucial for the translation of the *bgaH* reading frame. However, the A-*bgaH* fusion genes were sufficient to investigate *gvpA* regulation.

METHODS

Growth conditions. *Escherichia coli* strains DH5 α , XL-1 Blue (Stratagene) and GM1674, a *dam*-negative strain (Palmer & Marinus, 1994) were grown aerobically at 37 °C in LB broth (Sambrook *et al.*, 1989). For selection of transformants, ampicillin was added at a concentration of 100 μ g ml⁻¹. *Hf. volcanii* WFD11, lacking the endogenous plasmid pHV2 (Cline *et al.*, 1989), was grown in rich medium containing (l⁻¹): 175 g NaCl, 37 g MgSO₄·7 H₂O, 3·7 g KCl, 5 g Bacto tryptone, 3 g Bacto yeast extract, 25 ml 1 M Tris/HCl pH 7·2, 5 ml 10% CaCl₂·H₂O and 100 μ l 100 μ M MnCl₂. Transformants were selected on agar plates containing 0·2 μ g novobiocin ml⁻¹ and/or 6 μ g mevinolin ml⁻¹. Lovastatin (a derivative of mevinolin) was a gift from MSD Sharpe & Dohme.

Constructs used for transformation. The 2203 bp *bgaH* reading frame was amplified by PCR using the oligonucleotides *bgaH*-NcoI and *bgaH*-BamHI (Table 1), together with pMLH32 as template (Holmes & Dyall-Smith, 2000). The NcoI site of primer *bgaH*-NcoI included the ATG start codon of the *bgaH* reading frame, whereas the *bgaH*-BamHI primer was complementary to a sequence located 200 bp downstream of the *bgaH* stop codon. Each *gvpA* promoter region was amplified by PCR as a *Xba*I-NcoI fragment using synthetic oligonucleotides (Table 1) and subfragments of the three different vac regions inserted in *E. coli* plasmids as templates. For the fusion at the ATG start codon of *gvpA*, the respective A-*Xba*I primer was used, together with pA1-NcoI (amplification of the 109 bp pA1 promoter fragment), cA1-NcoI (109 bp cA1 promoter fragment), and mcA1-NcoI (119 bp mcA1 promoter fragment) (see Fig. 1). Slightly larger

Table 1. Synthetic oligonucleotides used for the amplification by PCR

Primer	Sequence*	Position†
<i>bgaH</i> - <i>NcoI</i>	CATTGTCCATGGCAGTTGGTGTCTG	2356–2380 ^a
<i>bgaH</i> - <i>BamHI</i>	GTGACGCGGATCCGCGTGTGTAC	4571–4549 ^a
<i>cA</i> - <i>XbaI</i>	CTTGTTCTAGATTTTGTAC	6087–6117 ^b
<i>cA1</i> - <i>NcoI</i>	CCGCCAAGCCCATGGAGTCTGG	6237–6213 ^b
<i>cA</i> - <i>NcoI</i>	GTTGCGCCATGGTGTATCTGG	6216–6197 ^b
<i>pA</i> - <i>XbaI</i>	CTCCGTATCTAGAAGTACGAC	13–33 ^d
<i>pA1</i> - <i>NcoI</i>	CTGCCAAGCCCATGGAATCTGGTTG	155–131 ^d
<i>pA</i> - <i>NcoI</i>	GGTTGCGCCATGGGTGATCTG	135–115 ^d
<i>mcA</i> - <i>XbaI</i>	CCAAACTATCTAGATGTTTGAC	4213–4192 ^c
<i>mcA1</i> - <i>NcoI</i>	CCGCCAGACCCATGGAATCGGGC	4070–4092 ^c
<i>mcA</i> - <i>NcoI</i>	TGAACCATGGTTGATCTGGG	4093–4112 ^c
<i>pE1</i>	CGGAGATGGTGGATCCATGGACGACTTGC	1690–1708 ^e
<i>pE2</i>	GATCTTCTGTCTGCAGGTACCGTATGTGTATAGG	2309–2275 ^e
<i>mcE1</i>	CGGAGATGGTGTGACCCATGGAAC	5936–5959 ^e
<i>mcE2</i>	GCCGTAAGTGTACAGATTATCGC	6547–6525 ^e
<i>pAcA</i>	CCGGAACGATGTGTATGGTTTCACCAGTCGTTTCGGC	6167–6127 ^b

* Sequences underlined or in italic indicate restriction sites.

† *a*, *bgaH* sequence; *b*, *c*-vac sequence; *c*, *mc*-vac sequence; *d*, *p-gvpACNO* sequence; *e*, *p-gvpD*-*M* sequence.

fragments spanning the ATG start codon of *gvpA* were amplified using the respective *A-XbaI* primer together with *pA-NcoI* (resulting in the 127 bp *pA* promoter fragment), *cA-NcoI* (137 bp *cA* promoter fragment) and *mcA-NcoI* (126 bp *mcA* promoter fragment). For the chimeric *pAcA* promoter fragment, a 60 nt megaprimer was amplified using the primers *cA-XbaI* and *pAcA* and the *c-gvpA* gene as template. The resulting megaprimer included 21 bp of the *p-gvpA* promoter sequence fused to the TATA box of the *c-gvpA* gene; this megaprimer was used together with primer *cA-NcoI* in the second PCR to amplify the entire *pAcA* promoter fragment.

Each promoter fragment was purified by agarose gel electrophoresis, cleaved with *XbaI*/*NcoI*, and ligated with the *bgaH* reading frame (cleaved with *NcoI* and *BamHI*) and the *XbaI*/*BamHI*-hydrolysed *E. coli* vector pBluescript II SK(+) (Stratagene). The exact fusion in each *A1-bgaH* and *A-bgaH* gene was confirmed by DNA sequence analysis. The *A1-bgaH* and *A-bgaH* genes were transferred as *XbaI*/*BamHI* fragments into the halobacterial vector plasmid pWL102 (Lam & Doolittle, 1989). For the expression of the *p-gvpE* and *mc-gvpE* reading frames, both were amplified by PCR using the primer pairs *pE1*/*pE2*, or *mcE1*/*mcE2* (Table 1). The PCR products were inserted into the halobacterial expression vector pJAS35 for expression under *fdx* promoter control (Pfeifer *et al.*, 1994). The *c-gvpE*-*pJAS* construct has already been described (Krüger *et al.*, 1998).

Transformation of *Hf. volcanii* WFD11. Prior to the transformation of *Hf. volcanii*, each construct was passaged through *E. coli* GM1674 (*dam*-negative) to avoid a halobacterial restriction barrier (Holmes *et al.*, 1991). Transformation was carried out as described previously (Pfeifer & Ghahraman, 1993). The presence and the amount of the desired plasmid(s) in each transformant was determined by Southern analyses using specific DNA probes. A 3.5 kb *HindIII*/*BamHI* fragment derived from pMLH32 was used for *bgaH*, whereas 600–700 bp *Acc65I*/*NcoI* fragments containing the respective *gvpE* reading frame were used to generate

the *gvpE*-specific probes. Each probe was labelled with digoxigenin using the DIG-Labeling Kit from Roche.

RNA isolation and transcript analysis. Total RNA was isolated by the method of Chomczynski & Sacchi (1987), or by using the RNeasy Kit from Qiagen, followed by DNase I digestion. For transcript analysis, 5 or 10 µg RNA was separated on denaturing, formaldehyde-containing 1.2% (w/v) agarose gels, followed by transfer to nylon membranes (Ausubel *et al.*, 1988). A strand-specific *bgaH* RNA probe was synthesized with the T3/T7 system using the 2.2 kb *NcoI*-*BamHI* *bgaH* fragment inserted in pBluescript as template. The probe was labelled with digoxigenin using the DIG RNA Labelling Kit obtained from Roche.

β-Galactosidase assay. β-Galactosidase activity in colonies was visualized by spraying the transformant colonies grown on agar plates with X-Gal (10 mg ml⁻¹). β-Galactosidase activity in cell lysates was measured using the ONPG assay as described by Holmes *et al.* (1997). The protein concentration was determined by the Bradford assay (Ausubel *et al.*, 1988) using BSA as standard.

Accession numbers of DNA sequences. These are as follows: U70664 (*bgaH* gene), X94688 (*c*-vac region of *Hb. salinarum* PHH4), X64701 (*mc*-vac region of *Hf. mediterranei*), X64729 (*p-gvpACNO*) and X55648 (*p-gvpDEFGHIJKLM*). The latter two sequences derive from the *p*-vac region on plasmid pHH1 of *Hb. salinarum* PHH1 (formerly *Hb. halobium* NRC817).

RESULTS

Three different *gvpA* genes encoding the major gas vesicle structural protein of *Hb. salinarum* and *Hf. mediterranei* were used for a comparative investigation of gene expression. These genes are transcribed as 340 nt mRNAs containing a 19–20 nt leader region (Horne & Pfeifer, 1989; Englert *et al.*, 1990). The *gvpA* mRNA leader regions are almost identical except for a 4 nt sequence at position 5–8 of the mRNA, and the

Fig. 1. (a) Promoter region of the three *gvpA* genes and of the pAcA hybrid, and (b) extent of the A1- and A-promoter fragments. (a) The start site of transcription (G) is labelled +1, and the ATG start codon is shown in bold. The TATA box centred around -28 is shown in bold. The consensus sequences of the BRE element and of the TATA box are given above (R = A or G, N = any base, W = A or T, Y = C or T). Sequences derived from the p-*gvpA* promoter in the pAcA-*bgaH* promoter are underlined. The nucleotide sequences around the ATG start codon used to introduce the *NcoI* site (CCATGG) during the construction of the A1-*bgaH* gene fusions are shown in italic. (b) Schematic representation of the *gvpA* promoter region, including the TATA box and the putative BRE element as well as the start sites of transcription (+1) and of translation (ATG). The bars below the map indicate the various oligonucleotides used for the PCR amplifications. The lines above the map represent the promoter fragments pA1, cA1 and mcA1, or the slightly larger fragments pA, cA and mcA.

nucleotides adjacent to the ATG start codon (Fig. 1a). No sequences reminiscent of a Shine–Dalgarno motif are present. The mRNA of the *bgaH* gene of '*Hf. alicantei*' also contains a 34 nt leader region with no obvious Shine–Dalgarno motif (Holmes & Dyll-Smith, 2000).

To investigate the regulatory elements that contribute to the expression of the *gvpA* genes in *Hf. volcanii* transformants in more detail, we amplified fragments containing the respective *gvpA* promoter regions, including the region transcribed into the mRNA leader (Fig. 1b). The first round of amplifications resulted in the fragments pA1, cA1 and mcA1 (109–119 bp) containing the ATG start codon of the respective *gvpA* gene within the *NcoI* cloning site. The second series of amplifications resulted in the 15-nt-larger pA, cA and mcA fragments (see Fig. 1b). The 2203 bp *bgaH* reading frame, amplified as a *NcoI*/*Bam*HI fragment, was fused to the respective A1- and A-promoter fragments at the ATG start codon of *bgaH*. The A1-*bgaH* fusions encoded the native β -galactosidase, whereas the A-*bgaH* fusions encoded a β -galactosidase protein with five additional amino acids derived from GvpA at the N terminus (MAQPD or MVQPD, see below). Constructs containing the A1-*bgaH* or A-*bgaH* fusion genes were used to transform *Hf. volcanii*. Transformants containing only the A-*bgaH* (or A1-*bgaH*) construct were used to monitor the basal amount of mRNA and β -

galactosidase activity, whereas transformants harbouring in addition one of the three different *gvpE*-pJAS constructs (i.e. A-*bgaH*/E or A1-*bgaH*/E transformants) were used to determine the GvpE-dependent activity of each *gvpA* regulatory region.

Reporter gene fusion at the ATG start codon of the *gvpA* reading frame

The various A1-*bgaH* and A1-*bgaH*/E transformants were analysed by Southern blotting for the presence of the desired construct(s). The desired transformants were streaked as cross-shaped colonies on agar plates and sprayed with X-Gal solution after growth to demonstrate β -galactosidase activity (Fig. 2). Colonies of *Hf. volcanii* are usually orange-red and retain this colour after X-Gal treatment, similar to *Hf. volcanii* containing the *c-gvpE* gene (cE, Fig. 2). The transformants containing the original *bgaH* construct pMLH32 (Holmes & Dyll-Smith, 2000) turned dark blue (*bgaH*, Fig. 2). Transformants containing the mcA1-*bgaH* construct (mcA1) formed brownish red colonies, consistent with the very low activity of the mc-*gvpA* promoter in transformants harbouring the mc-*gvpA* gene (Röder & Pfeifer, 1996). The mcA1-*bgaH*/cE transformants (mcA1-*bgaH* plus *c-gvpE*-pJAS) turned dark blue, demonstrating the induction of mcA1-*bgaH* expression by the cGvpE protein (mcA1/cE, Fig. 2). The cA1-*bgaH* transformants (cA1) remained orange-red, which is

Fig. 2. Colonies of *Hf. volcanii* transformants sprayed with X-Gal. The cells were grown as cross-shaped colonies on agar medium and sprayed with 10 mg X-Gal ml⁻¹. The constructs present in each transformant are indicated above the colonies. The designation 'bgaH' indicates the presence of plasmid pMLH32, and 'cE' indicates construct *c-gvpE*-pJAS. The respective A1-*bgaH* and A-*bgaH* fusion genes are inserted in pWL102. The origin of the promoter in the A1- and A-*bgaH* fusion genes is indicated by 'mc' (*mediterranei* chromosomal), 'c' (c-vac) and 'p' (p-vac).

indicative of the inactive *c-gvpA* promoter, similar to the complete lack of detectable amounts of *c-gvpA* mRNA in transformants containing the *c-gvpA* gene (Krüger & Pfeifer, 1996). However, colonies of the cA1-*bgaH*/cE transformants (cA1/cE) also remained orange-red, demonstrating that these cells did not contain detectable amounts of β -galactosidase activity (Fig. 2). This result was in contrast to the data obtained with *c-gvpA* + *c-gvpE*-pJAS transformants which produce large amounts of *c-gvpA* mRNA and GvpA protein (Krüger *et al.*, 1998). Also, transformants containing the pA1-*bgaH* construct (pA1) remained red although the *p-gvpA* gene is transcribed in *p-gvpA* transformants. Since the cA1-*bgaH* and pA1-*bgaH* genes contained the entire promoter and regulatory region, including the transcriptional start site of the respective *gvpA* gene, these results raised the question of whether the lack of β -galactosidase activity was caused by transcriptional or translational problems.

Fig. 3. Northern analysis to determine the presence of *bgaH* transcripts. RNA samples derived from exponential growth of the pA1-, mcA1- and cA1-*bgaH*, and mcA1-*bgaH*/cE and cA1-*bgaH*/cE transformants were separated on 1.2% agarose gels and hybridized with the 2.2 kb *NcoI*-*Bam*HI *bgaH* fragment as strand-specific probe. The RNA marker sizes (in kb) are given on the left. β -Galactosidase activity was also determined as indicated below the autoradiogram: -, (+) and + indicate the intensity of the blue colour (see Fig. 2).

Northern analyses were performed to monitor the A1-*bgaH* transcriptional levels. Total RNA was isolated from the transformants, electrophoretically separated on an agarose gel and hybridized with a *bgaH*-specific probe (Fig. 3). The 2.8 kb *bgaH* mRNA (and degradation products) was detected in pA1-*bgaH* and mcA1-*bgaH* transformants, whereas cA1-*bgaH* transformants did not contain detectable amounts of *bgaH* transcript. These results correlated with the earlier mRNA studies on the respective *gvpA* transformants. The mcA1-*bgaH*/cE and cA1-*bgaH*/cE transformants contained large amounts of *bgaH* mRNA, demonstrating that the transcription of both A1-*bgaH* genes was activated by cGvpE. Since the transcript formation in all of these transformants was as expected, the lack of β -galactosidase activity in case of the pA1 and cA1/cE transformants was likely due to a problem that occurred at the level of translation.

Reporter gene fusions within the *gvpA* reading frame

The second series of fusion genes (A-*bgaH*) contained the *bgaH* reading frame fused to the larger pA, cA or mcA promoter fragments. The fusion was generated in the *gvpA* reading frame, resulting in β -galactosidase proteins containing five amino acids of GvpA (MAQPD

Fig. 4. Growth curves and β -galactosidase activities determined for *mcA-bgaH* and various *mcA-bgaH/E* transformants. Each plot shows the growth curve (●) and the specific β -galactosidase activity (△) of transformants containing the *mcA-bgaH* gene (*mcA*), or *mcA* plus *mc-gvpE* (*mcE*), *c-gvpE* (*cE*) or *p-gvpE* (*pE*) in *pJAS35*. β -Galactosidase activities were determined by the ONPG assay.

in the case of the *p-gvpA* and *c-gvpA*, and MVQPD in the case of the *mc-gvpA* fusion). Transformants contained either the *A-bgaH* construct by itself (to determine the basal product levels), or together with one of the three *gvpE*-*pJAS* constructs. All transformants were analysed by Southern blotting for the presence of the desired plasmids. Colonies of each of these transformants were sprayed with X-Gal solution for the detection of β -galactosidase activity (Fig. 2, bottom). The *mcA-bgaH* transformants (*mcA*) were light blue, presumably due to low β -galactosidase production, the *cA-bgaH* transformants (*cA*) remained orange-red (no β -galactosidase production), and the *pA-bgaH* transformants (*pA*) were blue due to a higher amount of β -galactosidase activity. Each transformant containing one of the *A-bgaH* genes plus *c-gvpE*-*pJAS* showed a significant increase in blue colour: the activation was strongest with the *mcA-bgaH/cE* transformant (*mcA/cE*), but also the *cA-bgaH/cE* (*cA/cE*) and the *pA-bgaH/cE* transformants (*pA/cE*) turned dark blue (Fig. 2, bottom). Thus, the *A-bgaH* fusion genes revealed β -galactosidase activities according to the *gvpA* mRNA levels in the respective *gvpA* + *c-gvpE* (or + *mc-gvpE*) transformants (Röder & Pfeifer, 1996; Krüger *et al.*, 1998).

The expression of the different *A-bgaH* genes in transformants was investigated with each of the three GvpE proteins, and the specific β -galactosidase activities were quantified in samples taken throughout the growth period using the ONPG assay. The analyses of the transformants harbouring the *mcA-bgaH* construct by itself or including one of the three *gvpE* genes (*mcA*, *mcA/pE*, *mcA/cE*, and *mcA/mcE* transformants) is presented in Fig. 4; similar experiments were done with

all other transformants (data not shown). Since the β -galactosidase activities increased during exponential and early stationary phases, a mean activity was calculated from the activities determined in samples taken between 100 and 150 h of growth (Table 2). The highest specific β -galactosidase activity determined among the non-induced *A-bgaH* genes was found with *pA-bgaH* transformants; *mcA-bgaH* transformants showed a low activity, whereas *cA-bgaH* transformants did not contain detectable β -galactosidase activity (Table 2). The activation of each *A-bgaH* gene by GvpE was tested in the three different *A-bgaH/E* transformants. The various *mcA-bgaH/E* transformants demonstrated high β -galactosidase activities (ranging from 120 to 900 mU mg⁻¹), with *pGvpE* as the weakest and *cGvpE* as the strongest activator protein (Table 2). The *pA-bgaH* gene was also activated by all three GvpE proteins, but the expression of *cA-bgaH* was only achieved with the homologous *cGvpE* protein (*cA-bgaH/cE* transformants, Table 2). The specific β -galactosidase activity determined in the latter transformant was slightly higher than the basal β -galactosidase activity in the *pA-bgaH* transformants. None of the heterologous GvpE activator proteins was able to activate the *cA-bgaH* gene (Table 2). In each case, the *cGvpE* protein appeared to be the strongest transcriptional activator protein among the three GvpE proteins (Table 2).

Amount of *bgaH* mRNA and β -galactosidase activity throughout growth

The different *A-bgaH* and respective *A-bgaH/cE* transformants were investigated at the mRNA level for the expression of the *A-bgaH* gene. The cells were grown in

Table 2. Specific β -galactosidase activities of the various transformants

Values are means of samples taken between 100 and 150 h of growth. ND, Not detectable.

Transformant	Specific β -galactosidase activity [mU (mg protein) ⁻¹]			
	Basal	+ p- <i>gvpE</i>	+ mc- <i>gvpE</i>	+ c- <i>gvpE</i>
mcA- <i>bgaH</i>	6	120	480	900
pA- <i>bgaH</i>	13	200	170	240
cA- <i>bgaH</i>	ND	ND	ND	20
pAcA- <i>bgaH</i>	ND	4	15	65

Fig. 5. (a) Growth curve and β -galactosidase activities, and (b) Northern analysis to detect *bgaH* mRNA in the mcA-*bgaH*/cE transformant. (a) Samples were taken during growth (●), and the specific β -galactosidase activities (△) determined by the ONPG assay. The numbers above the growth curve indicate samples used for Northern analysis. (b) RNA isolated from samples 1–11 was used for Northern analysis and hybridized with a digoxigenin-labelled, *bgaH*-specific probe. RNA marker sizes (in kb) are given on the right.

25 % salt medium, and samples were taken during the exponential and stationary phases for RNA isolation and Northern analysis. The 2.8 kb *bgaH* mRNA (and degradation products) was detected in all transformants (data not shown). The mcA-*bgaH*/cE transformant was used to compare the amount of *bgaH* mRNA produced during growth with the β -galactosidase activities measured in the respective cell lysates. Eleven samples were taken throughout growth in 25 % salt medium, and RNA was isolated and hybridized with the *bgaH*-specific probe (Fig. 5). The *bgaH* mRNA was mainly detected in samples derived from exponential and early stationary phases (Fig. 5b, lanes 1–7). Only minimal amounts of *bgaH* mRNA were detectable after 185 h (Fig. 5b, lanes 8–11). The β -galactosidase activity was also determined for each sample and indicated an increase during the

exponential and the early stationary phase (up to 150 h), which paralleled the increase in the *bgaH* mRNA (Fig. 5a). However, despite the minor amounts of *bgaH* mRNA observed during the stationary phase, the β -galactosidase activities remained constantly high, indicating that the β -galactosidase protein was rather stable and remained active.

pAcA promoter to investigate the region required for GvpE activation

A chimeric *gvpA* promoter was constructed by substituting a region of 21 nt 5' to the TATA box of the cA promoter with the respective 21 nt derived from the pA promoter (see Fig. 1a) and the resulting pAcA-*bgaH* gene was tested for β -galactosidase activities and poss-

ible activation by the various GvpE proteins in transformants. Transformants containing the pAcA-*bgaH* construct did not produce detectable amounts of β -galactosidase activity, similar to transformants containing the cA-*bgaH* construct (Table 2). However, the transformants harbouring the pAcA-*bgaH* construct plus one of the three *gvpE*-pJAS constructs produced β -galactosidase, with pAcA-*bgaH*/pE exhibiting the lowest and pAcA-*bgaH*/cE the highest β -galactosidase activity (Table 2). Compared to the transformants containing the original pA- and cA-*bgaH* sequences, a threefold higher β -galactosidase activity was found compared to cA-*bgaH*/cE, but the pAcA-*bgaH*/cE transformant attained only 36% of the β -galactosidase activity of the pA-*bgaH*/cE transformant (Table 2). Thus, the pAcA promoter acquired the ability for activation by the heterologous GvpE activator proteins.

DISCUSSION

The promoter of the plasmid-encoded p-*gvpA* gene of *Hb. salinarum* PHH1 is highly active, whereas the respective c-*gvpA* and mc-*gvpA* promoters depend on the activation mediated by the GvpE activator proteins (Röder & Pfeifer, 1996; Krüger *et al.*, 1998). The *bgaH* reading frame encoding the β -galactosidase of '*Hf. alicantei*' (Holmes & Dyall-Smith, 2000) was used to investigate the basal and induced activities of the various *gvpA* promoter regions in *Hf. volcanii* transformants. Different fusion sites between the promoter fragments and *bgaH* were tested. One series of constructs contained the *bgaH* reading frame fused directly at the ATG start codon of the respective *gvpA* gene (pA1-, cA1- and mcA1-*bgaH*), whereas the second series (pA-, cA- and mcA-*bgaH*) contained the fusion site further downstream, resulting in β -galactosidase proteins containing five additional amino acids of GvpA at the N terminus.

Reporter gene fusions at the ATG start codon of the *gvpA* gene may lead to problems in translation

All A1-*bgaH* and A1-*bgaH*/cE transformants contained the expected amount of *bgaH* mRNA. The mcA1-*bgaH*, cA1-*bgaH* and mcA1-*bgaH*/cE transformants also showed β -galactosidase activity, indicating that the mRNA consisting of the *gvpA* mRNA leader and the *bgaH* reading frame was indeed translated. However, the pA1-*bgaH* and cA1-*bgaH*/cE transformants did not show detectable amounts of β -galactosidase activity despite the presence of the mRNA, suggesting that a problem occurred at the translational level. A single point mutation (T or A $\rightarrow$ C) was introduced 5' to the ATG start codon during the construction of the *NcoI* site for the fusion (Fig. 1a). Presumably, this alteration must be the reason for the lack of translation of these pA1- and cA1-*bgaH* mRNAs. However, a similar alteration was introduced in the mcA1-*bgaH* mRNA without preventing β -galactosidase formation. The leader sequences of the *gvpA* mRNAs are similar but not identical, and it is still unclear which regions are important for the initiation of translation. It is possible that the mutations destroyed a

signal important for translation initiation, or forced an mRNA secondary structure that masked the AUG start codon. Various stem-loop secondary structures are possible in the leader region that are currently under investigation. Regardless, these results imply that the fusion of the *bgaH* reporter gene should not be made at the start codon of the gene under investigation.

The β -galactosidase activity reflects the activity of the *gvpA* promoters

The larger promoter fragments used for the pA-, cA- and mcA-*bgaH* constructs revealed the expected mRNAs that, when present, were translated into β -galactosidase. The analysis of the product formation in each A-*bgaH* transformant indicated no mRNA and β -galactosidase in the cA-*bgaH* transformant, a low mRNA amount and β -galactosidase activity in the mcA-*bgaH*, and a high mRNA amount and β -galactosidase activity in the pA-*bgaH* transformant. These results reflected the activities of the various *gvpA* promoters in *gvpA* transformants (Röder & Pfeifer, 1996; Krüger *et al.*, 1998). Each promoter of the A-*bgaH* genes was stimulated at least by the homologous GvpE protein, indicating that the binding site of GvpE is contained within the promoter fragments used for the construction. The promoter of the mc-*gvpA* gene was the strongest activated promoter tested. Although the basal β -galactosidase activity was low in mcA-*bgaH* transformants, the activity was stimulated 80-fold by the homologous mcGvpE protein and 150-fold by the cGvpE protein. Among the various GvpE proteins tested with the mc-*gvpA* promoter, pGvpE appeared to be the weakest and cGvpE the strongest activator. The pA-*bgaH* construct was also stimulated by all three GvpE proteins in respective pA-*bgaH*/E transformants, but the overall induction was lower, except for the homologous pGvpE protein. Also, in this case, cGvpE appeared to be the strongest activator. It is possible that the single amino acid difference in the GvpA sequence near the N terminus results in a difference in the specific activity of the two β -galactosidase proteins (MVQPD in the case of mcA-*bgaH* and MAQPD in the case of cA- and pA-*bgaH*). However, the products of the pA-*bgaH* and the cA-*bgaH* genes are identical, and at least these specific β -galactosidase activities can be compared with each other.

Similar to the c-*gvpA* gene, the cA-*bgaH* fusion gene was completely inactive in transformants. Only the homologous cGvpE activator was able to induce mRNA and β -galactosidase formation, but the activity remained relatively low. The pGvpE and mcGvpE proteins were unable to induce the c-*gvpA* promoter, which could be due to a weaker activity, but the DNA sequence responsible for GvpE binding could also contribute. The inability of pGvpE to activate the c-*gvpA* promoter has already been recognized during heterologous complementation studies using the p-*gvpDEFGHIJKLM* unit of the p-vac region to complement the c-*gvpACNO* genes in *Hf. volcanii* transformants: no GvpA formation could be observed, suggesting that pGvpE cannot

activate the *c-gvpA* promoter (Offner *et al.*, 1998). This could also be the reason that gas vesicles of *Hb. salinarum* PHH1 are only formed by pGvpA: the pGvpE activator protein produced from the p-vac region is unable to stimulate the expression of the *c-gvpACNO* gene cluster present in the same cell. The latter *gvp* gene cluster is only activated by the homologous cGvpE activator protein.

Archaeal promoters consist of a TATA box centred around position -28 relative to the transcriptional start site, and many promoters contain the TFB recognition element BRE (consensus: RNWAAW, R = A or G, N = any base, W = A or T) (Bell *et al.*, 1999). The p-*gvpA* promoter contains a TATA box almost identical to the archaeal consensus, and also a highly conserved putative BRE element (Fig. 1a). These features could be the reason for the relatively high basal activity of the p-*gvpA* promoter, which does not depend on an activator protein for expression. In contrast, the inactive *c-gvpA* promoter shows less conservation in the TATA box, and no sequences reminiscent of a BRE element (Fig. 1a). Activation of this promoter required the cGvpE activator protein; neither pGvpE nor mcGvpE were sufficient for activation.

For a preliminary investigation of the DNA sequences required for GvpE stimulation, a chimeric pAcA promoter was tested for *bgaH* expression in transformants. This promoter fragment consisted mainly of *c-gvpA* sequences, but 21 nt 5' to the TATA box were substituted by the respective sequences derived from the p-*gvpA* promoter, including the almost perfect BRE element (Fig. 1a). The pAcA-*bgaH* transformants indicated no basal β -galactosidase activity, suggesting that the putative BRE element next to the TATA box was not sufficient for transcription initiation at this promoter. However, the pAcA-*bgaH* construct was induced by all three GvpE proteins in the respective transformants, and cGvpE was the strongest activator. It is still not known where the GvpE activator protein(s) bind in the promoter region for activation, but the fact that all GvpE proteins were able to activate the pAcA-*bgaH* gene suggested that the sequences upstream of the TATA box were important in this process. Mutagenesis of the entire promoter region is in progress to determine overall promoter strength and the nucleotides important for the activation by GvpE.

The β -galactosidase activity is more stable than the *bgaH* mRNA

The investigation of the amount of *bgaH* mRNA in the mcA-*bgaH*/cE transformant indicated a high amount of mRNA during the exponential and early stationary phases, and no *bgaH* mRNA in the late stationary phase. The increase in the β -galactosidase activity was in accordance with the increase in the mRNA during the exponential phase, but stayed at a high level during the stationary phase, implying that the β -galactosidase protein is rather stable. Thus, the *bgaH* reporter system is useful to determine the initial promoter activation.

However, since the *bgaH* mRNA and β -galactosidase stabilities deviate from each other, promoter deactivation studies are not possible. This observation contrasts with results presented by Patenge *et al.* (2000), where the promoter of the bacterio-opsin (*bop*) gene and various *fdx* promoter mutants were analysed with the *bgaH* reporter system in the related species *Hb. salinarum*. In the case of the *bop* promoter-*bgaH* fusion, the β -galactosidase activity remained at a high level for 96 h, but was reduced in the single sample taken after 120 h. However, other transformants contained stable amounts of β -galactosidase activity (Patenge *et al.*, 2000).

Overall, the *bgaH* reporter gene has turned out to be a useful tool for the investigation of promoter and regulatory activities at a more quantitative level. Although *Hf. volcanii* is only distantly related, halobacterial promoters derived from *Hf. mediterranei* and *Hb. salinarum* can be studied and compared to each other in this system. In further experiments, we hope to gain more insights into the activation of the various *gvpA* promoters by GvpE, and study the action of other regulatory proteins encoded by the vac region.

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REFERENCES

- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1988). *Current Protocols in Molecular Biology*, vol. 1. New York: Greene Publishing Associates and Wiley-Interscience.
- Baglia, N., Goo, Y. A., Ng, W. V., Hood, L., Daniels, C. & DaSarma, S. (2000). Is gene expression in *Halobacterium* NRC-1 regulated by multiple TBP and TFB transcription factors? *Mol Microbiol* **36**, 1184–1185.
- Bell, S., Jaxel, C., Nadal, M., Kosa, P. & Jackson, S. (1998). Temperature, template topology, and factor requirements of archaeal transcription. *Proc Natl Acad Sci USA* **95**, 15218–15222.
- Bell, S., Kosa, P., Sigler, P. & Jackson, S. (1999). Orientation of the transcription preinitiation complex in Archaea. *Proc Natl Acad Sci USA* **96**, 13662–13667.
- Chomczynski, P. & Sacchi, N. (1987). Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* **162**, 156–159.
- Cline, S. W., Schalkwyk, L. C. & Doolittle, W. F. (1989). Transformation of the archaeobacterium *Halobacterium volcanii* with genomic DNA. *J Bacteriol* **171**, 4987–4991.
- DasSarma, S., Arora, P., Lin, F., Molinari, E. & Yin, L. (1994). Wild-type gas vesicle formation requires at least ten genes in the *gvp* gene cluster of *Halobacterium halobium* plasmid pNRC100. *J Bacteriol* **176**, 7646–7652.
- Ellenberger, T. E., Brandl, C. J., Struhl, K. & Harrison, S. C. (1992). The GCN4 basic region leucine zipper binds DNA as a dimer of

- uninterrupted α helices: crystal structure of the protein–DNA complex. *Cell* **71**, 1223–1237.
- Englert, C., Horne, M. & Pfeifer, F. (1990). Expression of the major gas vesicle protein in the halophilic archaeobacterium *Haloferax mediterranei* is modulated by salt. *Mol Gen Genet* **222**, 225–232.
- Englert, C., Krüger, K., Offner, S. & Pfeifer, F. (1992a). Three different but related gene clusters encoding gas vesicles in halophilic archaea. *J Mol Biol* **227**, 586–592.
- Englert, C., Wanner, G. & Pfeifer, F. (1992b). Functional analysis of the gas-vesicle gene cluster of the halophilic archaeon *Haloferax mediterranei* defines the vac-region boundary and suggests a regulatory role for the *gvpD* gene or its product. *Mol Microbiol* **6**, 3543–3550.
- Hausner, W., Wettach, J., Hethke, C. & Thomm, M. (1996). Two transcription factors related with the eucaryal transcription factors TATA-binding protein and transcription factor IIB direct promoter recognition by an archaeal RNA polymerase. *J Biol Chem* **271**, 30144–30148.
- Holmes, M. L. & Dyall-Smith, M. (2000). Sequence and expression of a halobacterial β -galactosidase gene. *Mol Microbiol* **36**, 114–122.
- Holmes, M. L., Nuttall, S. D. & Dyall-Smith, M. (1991). Construction and use of halobacterial shuttle vectors and further studies on *Haloferax* DNA gyrase. *J Bacteriol* **12**, 3807–3813.
- Holmes, M. L., Scopes, R., Moritz, R., Simpson, R., Englert, C., Pfeifer, F. & Dyall-Smith, M. (1997). Purification and analysis of an extremely halophilic β -galactosidase from *Haloferax alicantei*. *Biochim Biophys Acta* **1337**, 276–286.
- Horne, M. & Pfeifer, F. (1989). Expression of two gas vacuole protein genes in *Halobacterium halobium* and other related species. *Mol Gen Genet* **218**, 437–444.
- Horne, M., Englert, C., Wimmer, C. & Pfeifer, F. (1991). A DNA region of 9 kb contains all genes necessary for gas vesicle synthesis in halophilic archaeobacteria. *Mol Microbiol* **5**, 1159–1174.
- Krüger, K. & Pfeifer, F. (1996). Transcript analysis of the c-vac region, and differential synthesis of the two regulatory gas-vesicle proteins GvpD and GvpE in *Halobacterium salinarum* PHH4. *J Bacteriol* **178**, 4012–4019.
- Krüger, K., Hermann, T., Armbruster, V. & Pfeifer, F. (1998). The transcriptional activator GvpE for the halobacterial gas vesicle genes resembles a basic region leucine-zipper regulatory protein. *J Mol Biol* **279**, 761–771.
- Lam, W. L. & Doolittle, W. F. (1989). Shuttle vectors for the archaeobacterium *Halobacterium volcanii*. *Proc Natl Acad Sci USA* **86**, 5478–5482.
- Ng, W. L., Ciufo, S., Smith, T. & 9 other authors (1998). Snapshot of a large dynamic replicon in a halophilic archaeon: megaplasmid or minichromosome? *Genome Res* **8**, 1131–1141.
- Ng, W. L., Kenney, S., Mahairas, G. & 14 other authors (2000). Genome sequence of *Halobacterium* species NRC-1. *Proc Natl Acad Sci USA* **97**, 12176–12181.
- Offner, S., Wanner, G. & Pfeifer, F. (1996). Functional studies of the *gvpACNO* operon of *Halobacterium salinarum* reveal that the GvpC protein shapes gas vesicles. *J Bacteriol* **178**, 2071–2078.
- Offner, S., Ziese, U., Wanner, G., Typke, D. & Pfeifer, F. (1998). Structural characteristics of halobacterial gas vesicles. *Microbiology* **144**, 1331–1342.
- Palmer, B. & Marinus, M. (1994). The *dam* and *dcm* strains of *Escherichia coli* – a review. *Gene* **143**, 1–12.
- Patenge, N., Haase, A., Bolhuis, H. & Oesterhelt, D. (2000). The gene for a halophilic β -galactosidase (*bgaH*) of *Haloferax alicantei* as a reporter gene for promoter analyses in *Halobacterium salinarum*. *Mol Microbiol* **36**, 102–113.
- Pfeifer, F. & Blaseio, U. (1989). Insertion elements and deletion formation in a halophilic archaeobacterium. *J Bacteriol* **171**, 5135–5140.
- Pfeifer, F. & Ghahraman, P. (1993). Plasmid pHH1 of *Halobacterium salinarum*: characterization of the replicon region, the gas-vesicle gene cluster and insertion elements. *Mol Gen Genet* **238**, 193–200.
- Pfeifer, F., Offner, S., Krüger, K., Ghahraman, P. & Englert, C. (1994). Transformation of halophilic archaea and investigation of gas-vesicle synthesis. *Syst Appl Microbiol* **16**, 569–577.
- Pfeifer, F., Zotzel, J., Kurenbach, B., Röder, R. & Zimmermann, P. (2001). A p-loop motif and two basic regions in the regulatory protein GvpD are important for the repression of gas vesicle formation in the archaeon *Haloferax mediterranei*. *Microbiology* **147**, 63–73.
- Qureshi, S. & Jackson, S. (1998). Sequence-specific DNA binding by the *S. shibatae* TFIIB homolog, TFB, and its effect on promoter strength. *Mol Cell* **1**, 389–400.
- Qureshi, S., Baumann, P., Rowlands, T., Khoo, B. & Jackson, S. (1995). Cloning and functional analysis of the TATA binding protein from *Sulfolobus shibatae*. *Nucleic Acids Res* **23**, 1775–1781.
- Reeve, J. N., Sandman, K. & Daniels, C. (1997). Archaeal histones, nucleosomes, and transcription initiation. *Cell* **89**, 999–1002.
- Röder, R. & Pfeifer, F. (1996). Influence of salt on the transcription of the gas-vesicle genes of *Haloferax mediterranei* and identification of the endogenous transcriptional activator gene. *Microbiology* **142**, 1715–1723.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Thomm, M. (1996). Archaeal transcription factors and their role in transcription initiation. *FEMS Microbiol Rev* **18**, 159–171.
- Thompson, D. K., Palmer, J. R. & Daniels, C. J. (1999). Expression and heat-responsive regulation of a TFIIB homologue from the archaeon *Haloferax volcanii*. *Mol Microbiol* **33**, 1081–1092.
- Zillig, W., Palm, P., Klenk, H.-P., Langer, D., Hüdepohl, U., Hain, J., Lanzendörfer, M. & Holz, I. (1993). Transcription in archaea. In *The Biochemistry of Archaea*, pp. 367–391. Edited by M. Kates. Amsterdam: Elsevier Science.

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