Use of a halobacterial \textit{bgaH} reporter gene to analyse the regulation of gene expression in halophilic archaea

Dagmar Gregor and Felicitas Pfeifer

The \textit{bgaH} reading frame encoding a β-galactosidase of ‘\textit{Haloferax alicantei}’ was used as a reporter gene to investigate three different promoter regions derived from \textit{gvpA} genes of \textit{Haloferax mediterranei} (\textit{mc-gvpA}) and \textit{Halobacterium salinarum} (\textit{c-gvpA} and \textit{p-gvpA}) in \textit{Halofex volcanii} transfectants. The fusion of \textit{bgaH} at the start codon of each \textit{gvpA} reading frame (\textit{A1–bgaH} fusion genes) caused transcriptional problems in some cases. Transformants containing constructs with fusions further downstream in the \textit{gvpA} reading frame (\textit{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 \textit{gvpA} genes: the \textit{cA–bgaH} fusion gene was completely inactive, the \textit{mcA–bgaH} transformants showed low amounts of products, whereas the \textit{pA–bgaH} fusion gene was constitutively expressed in the respective transformants. The transcription of each \textit{A–bgaH} gene was activated by the homologous transcriptional activator protein GvpE. The \textit{cGvpE}, \textit{pGvpE} and \textit{mcGvpE} proteins were able to activate the promoter of \textit{pA–bgaH} and \textit{mcA–bgaH}, whereas the promoter of \textit{cA–bgaH} was only activated by \textit{cGvpE}. Among the three GvpE proteins tested, \textit{cGvpE} appeared to be the strongest transcriptional activator.

\textbf{Keywords:} \textit{Haloferax volcanii}, β-galactosidase reporter gene, gene regulation

\section*{INTRODUCTION}

Halophilic archaea are model organisms to study archaeal gene regulation \textit{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 \textit{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 \textit{et al}., 1993). Two additional factors, namely the TATA-box-binding protein TBP and TFB (the archaeal homologue of transcription factor TFII B) are sufficient for the initiation of basal transcription (Hausner \textit{et al}., 1996; Qureshi \textit{et al}., 1995; Thomm, 1996; Reeve \textit{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 \textit{in vitro} transcription studies using recombinant factors and RNA polymerase from the thermophilic archaean \textit{Sulfolobus} (Qureshi & Jackson, 1998; Bell \textit{et al}., 1998, 1999). So far, \textit{in vitro} transcription systems have been established for some methanogenic and thermophilic archaea, but not for halophilic archaea. The genome sequences of \textit{Methanococcus jannaschii} and \textit{Methanobacterium thermotrophicum} contain single genes for TBP and TFB proteins, whereas halophilic archaea harbour multiple gene copies for both TBP and TFB (Ng \textit{et al}., 1998; Baglia \textit{et al}., 2000; Thompson \textit{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 \textit{gvpDEFGHIJKLMNOP} that cluster in a genomic region called the vac region. Three different vac regions have been characterized, two of these are found in \textit{Halobacterium}

\begin{itemize}
  \item Abbreviations: \textit{gvp}, gas vesicle protein gene; Gvp, gas vesicle protein.
\end{itemize}
**METHODS**

**Growth conditions.** *Escherichia coli* strains DH5α, 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 µg 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 µl 100 µM MnCl₂. Transformants were selected on agar plates containing 0.2 µg novobiocin ml⁻¹ and/or 6 µg 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*-BanHI (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*-BanHI 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 XbaI–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-XbaI 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

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence*</th>
<th>Position†</th>
</tr>
</thead>
<tbody>
<tr>
<td>bggH-NcoI</td>
<td>CATTGTCATGGGAGTTGGCTCTG</td>
<td>2356–2380*</td>
</tr>
<tr>
<td>bggH-BamHI</td>
<td>GTAGACGGAGATCCGGTGTTATGAC</td>
<td>4571–4549*</td>
</tr>
<tr>
<td>cA-XbaI</td>
<td>CTTGTTCTGATAGTTGTAC</td>
<td>6087–6117†</td>
</tr>
<tr>
<td>cA-NcoI</td>
<td>CCGCAAGCCCATGGAGTCTGG</td>
<td>6237–6213†</td>
</tr>
<tr>
<td>pA-XbaI</td>
<td>CTTCCGATCTAGATTGCGAC</td>
<td>13–33†</td>
</tr>
<tr>
<td>pA-NcoI</td>
<td>CTTGCGCCATGGTGATCTGG</td>
<td>155–131†</td>
</tr>
<tr>
<td>mca-XbaI</td>
<td>CCAACTATCTAGATTTTGAC</td>
<td>415–131†</td>
</tr>
<tr>
<td>mca-NcoI</td>
<td>CCGCAGACCATGGAATCCTGG</td>
<td>4070–4092†</td>
</tr>
<tr>
<td>pE1</td>
<td>CGGAGATGTTGGATCATGTCGG</td>
<td>4093–4112†</td>
</tr>
<tr>
<td>pE2</td>
<td>GATCTTCCGTATCTGGAGTCTAGTATGGAAC</td>
<td>2309–2275†</td>
</tr>
<tr>
<td>mcE1</td>
<td>CGGAGATGTTGGATCATGTCGG</td>
<td>5936–5959†</td>
</tr>
<tr>
<td>mcE2</td>
<td>GCCGTAAGTATCAAGATTCG</td>
<td>6547–6525†</td>
</tr>
<tr>
<td>pAcA</td>
<td>CCGGAAAACGATGTGATTTGCCACAGTCGTTGCCG</td>
<td>6167–6172†</td>
</tr>
</tbody>
</table>

* Sequences underlined or in italic indicate unique sites.
† a, bggH sequence; b, c-vcg sequence; c, mc-vcg sequence; d, p-gveACNO sequence; e, p-gveD–M sequence.

fragments spanning the ATG start codon of gepA 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-gveA gene as template. The resulting megaprimer included 21 bp of the p-gveA promoter sequence fused to the TATA box of the c-A promoter fragment; this megaprimer was used together with primer cA-NcoI in the second PCR to amplify the entire pAcA promoter fragment.

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 bggH, whereas 600–700 bp Acc651/NcoI fragments containing the respective gveP reading frame were used to generate the gveP-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 bggH RNA probe was synthesized with the T3/T7 system using the 2.2 kb NcoI–BamHI bggH 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 (bggH gene), X94688 (c-vcg region of Hb. salinarum PHH4), X64701 (mc-vcg region of Hf. mediterranei), X64729 (p-gveACNO) and X55648 (p-gveD-FCN1). The latter two sequences derive from the p-vcg region on plasmid pH1 of Hb. salinarum PHH1 (formerly Hb. halobium NRC817).

RESULTS

Three different gveA 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 gveA mRNA leader regions are almost identical except for a 4 nt sequence at position 5–8 of the mRNA, and the
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. alienantei’ also contains a 34 nt leader region with no obvious Shine–Dalgarno motif (Holmes & Dyall-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 15-nt-larger pA, cA and mcA fragments (see Fig. 1b). The 2203 bp bgaH reading frame, amplified as a Ncol/BamHI 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 original bgaH gene (cE, Fig. 2). The transformants containing the original bgaH construct pMLH32 (Holmes & Dyall-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...
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**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 contain 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.

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 kbp 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...
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 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.

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 PgpE as the weakest and CgpE 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 CgpE 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 CgpE 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...
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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.

<table>
<thead>
<tr>
<th>Transformant</th>
<th>Specific β-galactosidase activity [mU (mg protein)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
</tr>
<tr>
<td>mcA–bgaH</td>
<td>6</td>
</tr>
<tr>
<td>pA–bgaH</td>
<td>13</td>
</tr>
<tr>
<td>cA–bgaH</td>
<td>ND</td>
</tr>
<tr>
<td>pAcA–bgaH</td>
<td>ND</td>
</tr>
</tbody>
</table>

![Fig. 5.](image)

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 → C) was introduced 5’ to the ATG start codon during the construction of the Ncol 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 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/cE 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-gvpDEFGHILJKLM 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–bgaH 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. Mitogenesis 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 fix genes 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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