Influence of salt on the transcription of the gas-vesicle genes of *Haloferax mediterranei* and identification of the endogenous transcriptional activator gene

Richard Röder and Felicitas Pfeifer

Author for correspondence: Felicitas Pfeifer. Tel: +49 6151 162957. Fax: +49 6151 162956. e-mail: Pfeifer@biol.bio.th-darmstadt.de

The transcription of the 14 gvp genes of the gas-vesicle-encoding mc-vac region was investigated, using RNA from 25% and 15% (w/v) salt cultures of the moderately halophilic archaeon *Haloferax mediterranei*. Transcription occurred only from two promoters, located in front of the mc-gvpA and mc-gvpD genes. In both cultures transcripts spanning the entire mc-gvpD€FGHIJKLM transcription unit were formed only during the exponential growth phase. Amounts of these transcripts were larger in the 25% salt culture, in which the 20 kb mc-gvpD mRNA and large amounts of 1.3 kb and 0.45 kb partial mc-gvpD transcripts were also synthesized during the stationary phase. The levels of the mc-gvpD transcripts and of the 324 nt mc-gvpA mRNA increased in parallel during the stationary phase of the 25% salt culture. Only under these conditions were mRNAs spanning the entire mc-gvpACNO transcription unit observed, and gas-vesicles were formed. Investigation of the influence of the mc-gvpDE genes on both mc-vac promoters in transformants revealed that by themselves they were nearly inactive. The addition of mc-gvpE, however, resulted in a high level of constitutively produced mc-gvpA and mc-gvpD mRNA, indicating a transcriptional activator function for the mc-gvpE product.

**Keywords:** *Haloferax mediterranei*, gas-vesicle genes, archaea, gene regulation, transcriptional activator

**INTRODUCTION**

The moderately halophilic archaeon *Haloferax mediterranei*, isolated from salterns near Alicante, Spain (Rodriguez-Valera et al., 1983), synthesizes gas-vesicles during the stationary phase when grown in media containing 17–30% (w/v) salt. Cells grown in 15% salt medium are gas-vesicle-free (Englert et al., 1990). Gas-vesicles are also formed by *Halobacterium salinarium* and various bacteria, for example cyanobacteria (for a review, see Walsby, 1994). Gases dissolved in the cytoplasm diffuse freely through the ribbed gas-vesicle wall, which consists of a single hydrophobic protein, named GvpA.

In the case of halophilic archaea, 14 genes are involved in gas-vesicle synthesis, which are clustered in a genomic region, such as the p-vac and the c-vac region of *Hb. salinarium* (located on a plasmid and on the chromosome, respectively) and the mc-vac region of *Hf. mediterranei* (Horne et al., 1991; Jones et al., 1991; Englert et al., 1992a). In these regions, the gvp genes are arranged as two units: mc-gvpACNO, and upstream and oppositely oriented, mc-gvpDEFGHIJKLM. The boundaries of the mc-vac region have been defined by transformation experiments using the Vac+ species *Haloferax volcanii* as recipient: the lack of mc-gvpO or mc-gvpM leads to Vac− transforms (Englert et al., 1992b). Similar results have also been obtained with the p-vac region of *Hb. salinarium* PHH1 (Offner et al., 1996). The involvement of only ten gvp genes in the homologous p-vac region of plasmid pNRC100 was reported by DasSarma et al. (1994); however, some of the results obtained by insertional mutations conflict with data obtained in our laboratory (see Offner et al., 1996). In the cyanobacterium *Calothrix* sp. two gvpA homologues (gvpA1 and gvpA2) and gvpC form one transcription unit (Damerval et al., 1987), whereas in *Anabaena flot-aguac*
multiple copies of the *gvpA* gene were detected, and four additional genes similar to the other halobacterial *gvp* genes *gvpN*, *gvpI*, *gvpK* and *gvpF/L* are located further downstream of this unit (see Walsby, 1994; Hayes & Powell, 1995). The exact number of genes involved in gas-vesicle formation of cyanobacteria has not yet, however, been determined.

In all cases, the *gvpA* and *gvpC* genes encode the only gas-vesicle structural proteins known to date: the major 8 kDa hydrophobic protein GvpA and the more hydrophilic 42 kDa GvpC, which appears to be located on the gas-vesicle surface. GvpC possibly stabilizes the overall structure, as shown for gas-vesicles of cyanobacteria (Hayes et al., 1992). Halobacterial GvpC proteins are detectable in gas-vesicle preparations, but a stabilizing function has not yet been determined (Halladay et al., 1993; Englert & Pfeifer, 1993). The functions of the other *gvp* genes and their products still need to be elucidated, but there is strong evidence that mc-gvpD is involved in the repression of gas-vesicle synthesis: *Hf. volcanii* transformants containing the mc-vec region with a deletion within mc-gvpD (ΔD) overproduce gas-vesicles, and the addition of the mc-gvpD gene on a second vector construct reduces their numbers back to wild-type levels (Englert et al., 1992b; Pfeifer et al., 1994). Additional support for such a function comes from the investigation of the p-vec region of *Hb. salinarum*: here, artificially large amounts of the 5'-terminal part of the p-gvpD mRNA result in the lack of the p-gvpF-M mRNA and a reduction in the number of gas-vesicles (Offner & Pfeifer, 1995).

Transcriptional analysis of the mc-vec region indicates the strong 324 nt mc-gvpA mRNA, which appears together with minor mRNAs spanning mc-gvpAC, mc-gvpACN and mc-gvpACNO in the early stationary phase, 40 h prior to the detection of gas-vesicles (Englert & Pfeifer, 1993). The mc-gvp-D-M genes appear to be organized in two separate transcription units: a 4.8 kb mRNA spanning mc-gvpF-M occurs predominantly during the exponential growth phase, whereas 1.9 and 1.1 kb mc-gvpD transcripts appear during the stationary phase (Englert et al., 1992a). Two divergent promoters are located in front of mc-gvpA and mc-gvpD; however, a third promoter within mc-gvpE, as found in the p-vec region of *Hb. salinarum*, could not be detected (Englert et al., 1992a; Englert, 1992; Offner & Pfeifer, 1995). Salt-dependent gas-vesicle formation in *Hf. mediterranei* has only been investigated in the case of the mc-gvpA mRNA: its level increases sevenfold in a culture grown in 25% salt medium (producing gas-vesicles), as compared to a 15% salt culture, in which cells are gas-vesicle-free (Englert et al., 1990).

In the present report, we determined all transcripts derived from the mc-vec region in *Hf. mediterranei*, using RNA from cells grown in both 25% and 15% salt media. In addition, the regulation of the two mc-vec promoters was analysed with respect to the influence of the mc-gvpDE genes. Halobacterial transformants carrying subfragments of the mc-vec region were then constructed, and transcripts investigated by Northern analyses.

**METHODS**

**Growth of *Hf. mediterranei* and *Hf. volcanii*.** *Hf. mediterranei* R4 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM 14117) and *Hf. volcanii* WFD11 was from W. F. Doolittle. *Hf. mediterranei* growth medium contained either 4 M NaCl (25% salt medium) or 2.3 M NaCl (15% salt medium), 60 mM MgSO₄, 20 mM KCl, 50 mM Tris/HCl pH 7.5 and 1.5% (w/v) peptone (Oxoid). *Hf. volcanii* growth medium contained either 3.4 M NaCl (25% salt medium) or 1.9 M NaCl (15% salt medium), 150 mM MgSO₄, 40 mM KCl, 50 mM Tris/HCl pH 7.5 and 1.5% peptone. Liquid cultures were grown at 37 °C and aerated on a gyratory shaker at 250 r.p.m. Colonies were cloned on growth media solidified with 1.8% (w/v) agar at 42 °C.

**Transformation of *Hf. volcanii* WFD11.** *Hf. volcanii* was transformed with DNA constructs containing the mc-vec region or part of it. All constructs contained the halobacterial plasmid pWL102 (Lam & Doolittle, 1989). The 10177 bp *PstI–BglI* (BP) construct (Englert et al., 1992b) carried the entire mc-vec region; the 9259 bp ΔD construct (Englert et al., 1992b) was a variant of the BP construct, carrying a 918 bp Xhol deletion within the mc-gvpD gene; the A construct contained the 623 bp *EcoRI–MluI* mc-gvp(C)A(D) fragment (containing the mc-gvpA gene and parts of mc-gvpC and mc-gvpD cloned into pWL102; the A+D construct contained the 2586 bp *EcoRI–DraI* mc-gvp(C)A(D) fragment cloned into pWL102; the A+D+E construct contained the 3310 bp *EcoRI–Clal* mc-gvp(C)ADE(F) fragment cloned into pWL102; the A+D+E construct was a variant of the A+D+Δ construct, carrying a 918 bp Xhol deletion within the mc-gvpD gene. Prior to transformation of *Hf. volcanii*, each construct was passaged through the *E. coli* IAM strain GM1674 (Palmer & Marinus, 1994) to avoid a halobacterial restriction barrier (Holmes et al., 1991). Transformation was done as described by Pfeifer & Ghahraman (1993) using 15% salt medium (in which transformants are most easily obtained), and transformants were selected on agar plates containing 6 µg mevinolin ml⁻¹. The presence of the desired construct in each transformant was verified by Southern analyses. Transformants were subsequently grown and analysed in 25% salt medium, in order to ensure the expression of the mc-vec region.

**Isolation of total RNA from cultures of *Hf. mediterranei* and *Hf. volcanii* and transcript analyses.** Total RNA was isolated from *Hf. mediterranei* grown in 25% or 15% salt medium by lysis in guanidine thiocyanate buffer and ultracentrifugation through a caesium chloride cushion, according to Ausubel et al. (1988). RNA from *Hf. volcanii* transformants was isolated using the RNaseasy kit from Qiagen, followed by DNase I digestion. Transformant RNA was isolated from 50 ml exponential-growth-phase cultures (OD₆0₀ 0.1–0.2), or from 5 ml stationary-phase cultures (OD₆0₀ > 2).

Northern analyses involved electrophoresis of RNA on denaturing, formaldehyde-containing 1.2% (w/v) agarose gels, followed by transfer to nylon membranes (Ausubel et al., 1988): 10 µg of each RNA sample was used in the case of *Hf. mediterranei*, but only 5 µg RNA from *Hf. volcanii* transformants because the pWL102 vector constructs are present in multiple copies in each cell. Strand-specific RNA probes were synthesized using the following fragments cloned in pBluescript as templates: the 849 bp *EagI–NruI* gvpN/O probe, the 367 bp *EcoRI–XmI* gvpA probe, the 304 bp *MluI–XhoI* S'gvpD probe, the 610 bp *SalI–SalI* probe from the middle of mc-gvpD (Englert et al., 1992a), the 580 bp *NraI–KpnI* gvpD/E probe, the 414 bp
Transcription of halobacterial gas-vesicle genes

Fig. 1. Growth of Hf. mediterranei in 25% (■) and 15% (○) salt cultures. Gas vesicles were only formed in the 25% salt culture and the arrow indicates their appearance in sample 4 at the beginning of the stationary phase. Samples were taken for the isolation of RNA at the time points indicated by numbers 1–5. Samples 1 (OD600 ~ 0.5), 2 (OD600 ~ 1.5) and 3 (OD600 ~ 2) were from the exponential growth phase, samples 4 (OD600 3–4) and 5 were from the stationary phase.

RESULTS

Growth of Hf. mediterranei in media containing 25% or 15% salt and isolation of total RNA

Cells of Hf. mediterranei grown in media containing 25% or 15% salt were examined for the formation of gas-vesicles by phase-contrast microscopy. Samples for the isolation of RNA were taken from both cultures throughout growth: sample 1 was from the mid-exponential growth phase, samples 2 and 3 were from the late exponential growth phase, and samples 4 and 5 were from the stationary phase (Fig. 1). In the 25% salt culture, light-refractile bodies (i.e. gas-vesicles) were first observed at the beginning of the stationary phase (see Fig. 1, sample 4) and up to three such inclusions were observed inside most cells 9 h later. No gas-vesicles were formed during growth in the 15% salt medium (Rodriguez-Valera et al., 1983; Englert et al., 1990; and this paper). At time point 5, the OD600 of the 25% salt culture decreased, and many cell fragments were observed. This was probably due to problems with the particular batch of the carbon source used for the medium. Therefore, samples 1–4 of both cultures were used for RNA extraction and for the investigation of mc-vac transcripts by Northern and S1 analyses.

Transcription of the mc-gvpACNO genes

From each sample of the 25% or 15% salt culture, 10 µg RNA was separated electrophoretically on denaturing agarose gels and hybridized to strand-specific RNA probes synthesized from either of the two DNA strands of the mc-gvpA gene (Fig. 2) or the mc-gvpNO genes. No antisense transcripts were observed with the respective probes (data not shown).

Transcripts formed in the 25% salt culture. Northern analysis with the gvpA probe detected a 0.32 kb transcript in all four samples taken throughout growth (see Fig. 2).
Fig. 2. Northern analyses of the transcription of the mc-vac region in Hf. mediterranei grown in 25% (b-d) or 15% (e-g) salt cultures. (a) The mc-vac region, with the mc-gvpACNO genes and the oppositely oriented mc-gvpDEFGHIJKLM genes, is shown at the top. The arrows represent the two mc-vac promoters. The probes used in the Northern analyses (gvpA, 5′gvpD and gvpL) are indicated as black bars below the respective genes. (b-g) Northern analyses: (b, e) gvpL probe, (c, f) 5′gvpD probe, (d, g) gvpA probe. Lanes 1–4 contain RNA samples (10 μg) taken at the time points 1–4, respectively, indicated in Fig. 1. The size of the hybridizing mRNAs is indicated in kb.

Comparison of these data revealed the following differences in the transcription of the mc-gvpACNO genes of Hf. mediterranei grown in media containing 25% or 15% salt: the 0.32 kb mc-gvpA mRNA increased markedly during the late exponential growth phase of the 25% salt culture (Fig. 2, samples 1 and 2), whereas a comparable increase was delayed in the 15% salt culture for 22 h, until the stationary phase (Fig. 2, samples 3 and 4). In the 25% salt culture only, mc-gvpA mRNA synthesis was accompanied by the formation of substantial amounts of the 1.8 kb, 2.4 kb and 3.0 kb mc-gvpACNO mRNAs during early stationary phase, and gas-vesicles were produced.

Transcription of the mc-gvpDEFGHIJKLM genes

For the Northern analyses of transcripts of mc-gvpDEFGHIJKLM, strand-specific RNA probes were derived from the 5′-terminal part of the mc-gvpD gene (see Fig. 2, 5′gvpD probe), from the middle of mc-gvpD, from the 3′ end of mc-gvpD and part of mc-gvpE, from mc-gvpF and from mc-gvpL (gvpL probe). No antisense RNAs were detected with any of these probes (data not shown). We present here only the analyses with the 5′gvpD probe and the gvpL probe, since they indicate all transcripts found in this region.

Transcripts formed in the 25% salt culture. The main signals observed with the 5′gvpD probe corresponded to transcripts of 2.0 kb, 1.3 kb and 0.45 kb (Fig. 2). They appeared only weakly in the RNA of sample 1, but hybridized strongly to RNA from samples collected during late exponential and stationary phase. The amount of the 2.0 kb mc-gvpD mRNA was lower at the beginning of the stationary phase (sample 4). The 1.3 kb and 0.45 kb RNAs, covering only parts of mc-gvpD, appeared in large amounts together with the mc-gvpA mRNA (see above).
Weak transcripts of 3.0 kb and up to 7 kb were observed mainly during the exponential growth phase (samples 1 and 2). The 3.0 kb transcript was long enough to span the mc-gvpDE genes, and the 7 kb mRNA spanned the entire mc-gvpDEFGHJKLM gene cluster. The gvpL probe detected only the 7 kb transcript during the exponential growth phase (Fig. 2). The detection of the 7 kb transcript with the probes from both ends of mc-gvpD-M showed that this region forms a single transcription unit, and no additional promotor is located in mc-gvpE as reported by Englert et al. (1992a). This was also indicated by the results obtained with three other probes from the mc-gvpDEF region (data not shown). The transcripts detected in Northern analyses with all the probes are summarized in Fig. 3.

**Transcripts formed in the 15% salt culture.** Similar Northern analyses using the 5'gvpD probe revealed small amounts of the 2.0 kb and 1.3 kb transcripts during the exponential growth phase; in the stationary phase the amount of the 2.0 kb transcript decreased (Fig. 2). The 0.45 kb RNA was detected only in very low amounts in the 25% salt culture, indicating multiple transcript 3' termini in a region between 210 nt and 481 nt downstream of the mc-gvpD start site (data not shown, Fig. 4) that could represent a transcriptional terminator or processing signal. The unprotected 5'-terminal part of the DNA probe was then degraded with S1 nuclease, and the length of the protected DNA strand determined on a sequencing gel. Multiple lengths of protected fragments were detected that occurred only in the S1 analysis with RNA from the 25% salt culture, indicating multiple transcript 3' termini in a region between 210 nt and 481 nt downstream of the mc-gvpD start site (data not shown, Fig. 4). The major stop sites occurred at positions +303, +364, +375 and +398 relative to the mc-gvpD start site, and at least 115 nt upstream of the palindromic sequence. For the determination of the 3' terminus, RNA from the late exponential growth phase of the 25% and 15% salt cultures was hybridized to an mc-gvpD fragment derived from the 5'-terminal region including the palindromic sequence. This fragment was radioactively labelled at the 5' end. The unprotected 5' terminal part of the DNA probe was then degraded with S1 nuclease, and the length of the protected DNA strand determined on a sequencing gel. Multiple lengths of protected fragments were detected that occurred only in the S1 analysis with RNA from the 25% salt culture, indicating multiple transcript 3' termini in a region between 210 nt and 481 nt downstream of the mc-gvpD start site (data not shown, Fig. 4). The major stop sites occurred at positions +303, +364, +375 and +398 relative to the mc-gvpD start site, and at least 115 nt upstream of the palindromic sequence. For the determination of the 3' terminus, RNA from the late exponential growth phase of the 25% and 15% salt cultures was hybridized to an mc-gvpD fragment derived from the 5'-terminal region including the palindromic sequence. This fragment was radioactively labelled at the 5' end. The unprotected 5' terminal part of the DNA probe was then degraded with S1 nuclease, and the length of the protected DNA strand determined on a sequencing gel. Multiple lengths of protected fragments were detected that occurred only in the S1 analysis with RNA from the 25% salt culture, indicating multiple transcript 3' termini in a region between 210 nt and 481 nt downstream of the mc-gvpD start site (data not shown, Fig. 4). The major stop sites occurred at positions +303, +364, +375 and +398 relative to the mc-gvpD start site, and at least 115 nt upstream of the palindromic sequence. The detection of more than one stop site was unexpected, but was verified by the use of a second DNA probe that revealed the same distribution of protected fragments.

**Transcription of halobacterial gas-vesicle genes**

![Schematic representation of the transcription of the mc-vac region in Hf. mediterranei](image)

**Fig. 3.** Schematic representation of the transcription of the mc-vac region in Hf. mediterranei. A genetic map of the mc-vac region is shown with both promotors represented as arrows. The bars depict the probes used for the investigation of mc-vac transcripts in Hf. mediterranei: gvpNO, gvpA, 5'gvpD, a probe from the middle of mc-gvpD also used in previous experiments (Englert et al., 1992a), gvpDE, gvpF and gvpL. Arrows below the map indicate the direction and relative strength of transcripts as observed in Northern analyses. Transcript lengths are given in kb. The transcripts are grouped according to their appearance during exponential and stationary phases of the culture.
**Fig. 4.** Palindromic region in the 5'-terminal part of mc-gvpD. (a) DNA sequence of this region (EMBL accession number X64701), numbered according to the distance from the transcriptional start site (+1) of mc-gvpD. The ATG start codon is indicated by bold letters. The MluI and Sau3AI restriction sites indicate the radioactively labelled 3' ends of the DNA fragments used for the determination of the 3' terminus of the 0.45 kb mc-gvpD RNA. Vertical arrows indicate the major stop sites observed (positions +303, +364, +375 and +398). Horizontal arrows indicate the location of palindromes (positions 4742-4900 of the mc-vac sequence, or +513 to +671 relative to the mc-gvpD transcriptional start site. (b) A possible secondary structure formed by this palindromic region, modified after a structure detected with the MFOLD program from the UWGCG package.

**Fig. 5.** Fragments of the mc-vac region used for the transformation of Hf. volcanii. The gvpA and the 5'gvpD probes used in Northern analyses are indicated by black bars below the respective mc-vac genes. Selected restriction sites defining the mc-vac subfragments are indicated. The mc-vac fragments used for transformation experiments are indicated by bars. The Xhol-deletion in the AD and A+AD+E construct (see text) is represented as a dotted line.
Transcription of halobacterial gas-vesicle genes

Fig. 6. Northern analyses of *Hf. volcanii* transformants. (a, b) Results obtained using the gvpA probe, (c, d) those obtained using the 5'gvpD probe (see Fig. 5 for location). RNA was from the *Hf. volcanii* transformants indicated at the top (see also Fig. 5). Five micrograms of RNA was applied from exponential (lanes e) and stationary (lanes s) phase cultures. The length of hybridizing transcripts is indicated in kbp.

Thus, the 0.45 kb RNA has no defined 3' terminus but rather represents a range of RNAs of different lengths.

Influence of mc-gvpDE on the activities of the mc-gvpA and mc-gvpD promoter

Transcript analysis revealed that the mc-vac region of *Hf. mediterranei* is transcribed from two divergent promoters, located in front of mc-gvpA and mc-gvpD. In order to investigate the endogenous regulation of the mc-vac region and to identify possible regulator genes, the activities of the two mc-vac promoters were assessed in *Hf. volcanii* transformants. The mc-vac subfragments used are shown in Fig. 5 and contained the promoter region plus different numbers of adjacent genes: the A construct contained the mc-gvpA gene and both promoters, the A+D construct contained mc-gvpA plus mc-gvpD, and the A+D+E construct contained mc-gvpA plus mc-gvpDE. The A+ΔD+E construct was a variant of the A+D+E construct that incurred a 918 bp deletion within mc-gvpD. This deletion causes strong gas-vesicle overproduction in transformants carrying the entire mc-vac region (ΔD transformant; Englert *et al.*, 1992b). As controls, *Hf. volcanii* transformants carrying the entire mc-vac region (BP construct; Englert *et al.*, 1992b) or the ΔD construct were investigated. Each of these fragments was ligated to the same vector plasmid. Transformants were grown in 25% salt medium, and total RNA was extracted from exponential and stationary phase cultures and analysed for mc-gvpA and mc-gvpD transcripts by Northern analyses, using the gvpA and the 5'gvpD probe (Fig. 6).

Northern analyses of the BP wild-type, and the gas-vesicle-overproducing ΔD transformant indicated that transcription from both promoters occurred mainly in the stationary phase in BP, but was constitutive in ΔD (Englert *et al.*, 1992b; and Fig. 6, BP and ΔD). Furthermore, the analysis of the ΔD transformant with the gvpA probe indicated a larger amount of mc-gvpACNO mRNAs than in the BP transformant. The ΔD transformant also contained larger amounts of the < 5.2 kb mc-gvpΔD-M mRNAs (Fig. 6, 5'gvpD probe). The ~2 kb transcript detected with the 5'gvpD probe corresponded to the mc-gvpΔDEF mRNA in the ΔD transformant. Thus, the mc-gvpEF genes were transcribed in ΔD throughout growth, and in larger amounts than in BP. Similar analyses were performed with the transformants carrying subfragments of the mc-vac region. The A and A+D transformants contained only traces of the 0.32 kb mc-gvpA mRNA in exponential and stationary phases (Fig. 6, gvpA probe). No mc-gvpD transcripts could be detected in the RNA of the A transformant, since these lacked the DNA sequences complementary to the 5'gvpD probe. However, low amounts of the 2.0 kb mc-gvpD mRNA and shorter transcripts were detected in the A+D transformant throughout growth (Fig. 6, 5'gvpD probe).
These results demonstrate that the mc-gvpA and the mc-gvpD promoters are only weakly active by themselves. Addition of mc-gvpE in the A+D+E transformant, however, led to the formation of large amounts of the 0.32 kb mc-gvpA mRNA throughout growth, as also seen in the A+ΔD+E transformant. In the case of the mc-gvpD promoter, high amounts of mc-gvpD transcripts were formed in the A+D+E and A+ΔD+E transformant as well. Both contained transcripts spanning mc-gvpDE in the exponential and stationary phases: as weak 2.9 kb mc-gvpDE mRNA in A+D+E, and as 2 kb mc-gvpΔDE mRNA in A+ΔD+E (also seen in ΔD, see above). These results demonstrate that mc-gvpE encodes the transcriptional activator of the mc-gvpA and probably also of the mc-gvpD promoter (thus activating its own transcription), and that an intact mc-gvpD gene has no influence on this mc-gvpE-mediated activation in the A+D+E transformant.

**DISCUSSION**

Gas-vesicle production in the moderately halophilic archaeon *Hf. mediterranei* depends on growth in media containing at least 17% salt and occurs in the stationary phase. This salt-dependent gas-vesicle formation has been shown to involve transcriptional regulation: the transcription of mc-gvpA, encoding the major gas-vesicle structural protein, correlates closely with the formation of gas-vesicles (Englert et al., 1990). In the present analysis, transcription of the mc-vac region was investigated during growth in 25% versus 15% salt medium. We did not differentiate between the effects of osmolarity and oxygen supply (salt decreases the solubility of O₂ in water), because the latter would have required the use of more sophisticated equipments than presently available.

The 14 mc-gvp genes are transcribed as two units (mc-gvpACNO and mc-gvpD-M) from two divergent promoters located in front of mc-gvpA and mc-gvpD (Englert et al., 1992a; and this report): in the present analysis the mc-gvpD-M genes were shown to represent a single transcription unit because long RNAs spanning this entire gene cluster were detected. No antisense RNAs could be detected in the mc-vac region. The two promoters of the mc-vac region were both active in cells grown in 25% or 15% salt media (although at different levels), despite the different Vac phenotypes observed in *Hf. mediterranei*. Major differences were that in the 25% salt culture (i) very large amounts of transcripts starting at the mc-gvpD promoter appeared in late exponential and stationary phases, comprising only mc-gvpD or parts of it, including a 0.45 kb transcript, and (ii) the earlier appearance of mc-gvpA mRNA led to the formation of mc-gvpACNO mRNAs, and finally to gas-vesicle synthesis. Thus, the mc-vac transcripts formed during gas-vesicle production included mc-gvpA and mc-gvpACNO mRNAs and (partial) mc-gvpD transcripts, which all appeared in high amounts during late exponential growth phase. The function of the latter transcripts during gas-vesicle synthesis is not clear. Interestingly, these transcripts stopped upstream of mc-gvpE, encoding the transcriptional activator gene of the mc-vac region.

Attempts to identify the 3′ end of the most abundant 0.45 kb RNA yielded no information about the mechanism of its generation, because different 3′ termini were detected. An extensive palindromic region was observed downstream of these multiple 3′ termini, but no relation with respect to transcriptional termination or processing could be defined.

The transcript pattern of the mc-vac region differs markedly from the transcription and expression of the plasmid-borne p-vac region of *Hb. salinarium* PH11, which is expressed from four promoters located in front of p-gvpA, p-gvpD, p-gvpF and p-gvpO. The p-vac region promotes gas-vesicle formation throughout the growth cycle: the p-gvpA mRNA occurs in all stages of growth together with smaller amounts of longer transcripts spanning p-gvpACNO (Horne et al., 1991; Englert et al., 1992a; Offner & Pfeifer, 1995). In addition, p-gvpO is also transcribed from its own promoter, underlining the importance of this gene for gas-vesicle synthesis (Offner et al., 1996). The p-gvpD-M cluster is transcribed as two units: the p-gvpDE mRNA occurs in the stationary phase, whereas the p-gvpFGHIJKLM mRNA appears only in the exponential growth phase, starting from an additional promoter located in p-gvpE (Offner & Pfeifer, 1995). It is interesting to note that the termination or processing observed behind and within mc-gvpD in the stationary phase in the mc-vac region results in the same exponential-growth-phase-dependent gvpF-M mRNA formation as in the p-vac region. In the constitutively expressed p-vac region, the gvpDE genes are not required for gas-vesicle synthesis (Offner & Pfeifer, 1995). The p-gvpA promoter is active by itself, thus the p-gvpE gene product might not be necessary. Therefore, an additional promoter located within p-gvpE could drive the expression of the p-gvpF-M gene cluster.

We also investigated the endogenous regulation of both mc-vac promoters in response to products of the adjacent mc-gvpDE genes. Earlier investigations indicated very low expression of the mc-gvpA gene when present by itself in *Hf. volcanii*, whereas the gene was expressed at a higher levels in transformants containing larger mc-vac sub-fragments (Englert et al., 1992b). In addition, these transformation experiments suggested a repressor function for the mc-gvpD gene and its product, since a mc-vac region containing an in-frame deletion within mc-gvpD (ΔD) resulted in a gas-vesicle overproducing transformant. This effect could be reduced to wild-type levels by the addition of mc-gvpD (Englert et al., 1992b; Pfeifer et al., 1994).

The constructs used in the present studies contained the mc-gvpA gene by itself, or mc-gvpA together with mc-gvpD, mc-gvpDE or mc-gvpΔDE. In the absence of mc-gvpE, both mc-vac promoters were nearly inactive, whereas the presence of the mc-gvpE gene (A+DE and A+ΔDE transformants) led to large amounts of mc-gvpA and mc-gvpD mRNAs. This indicates that mc-GvpE is a transcriptional activator for the mc-gvpA promoter, and probably also for the mc-gvpD promoter. A stability effect on the mc-gvpDE mRNA seems unlikely, since low
amounts of the mc-gvpD mRNA were already observed in the A + D transformant. In contrast to the growth-phase-dependent expression of mc-gvpA in transformants containing the entire mc-vac region (Englert et al., 1992b; and this report), in the A + D + E and the A + ΔD + E transformants the transcripts from both promoters were found during all stages of growth. This implies that there must be another mc-vac gene product inhibiting the GvpE-mediated activation of the mc-gvpA promoter during exponential growth. Such a function as previously assigned to the mc-gvpD gene product (see above); however, the results presented here demonstrate that mc-GvpD cannot mediate this effect by itself. Experiments to detect the additional factor inhibiting the activation of mc-gvpA in early growth stages are currently in progress; and the function of this protein possibly depends on the presence of GvpD. In this respect it is interesting that the GvpD protein contains a conserved motif (P-loop; Saraste et al., 1990) characteristic of the nucleotide-binding site for ATP/GTP-binding proteins.

ACKNOWLEDGEMENTS

Part of this work was done while the authors were guests in Wolfram Zillig's Abteilung at the Max-Planck-Institut für Biochemie in Martinsried. We thank him for hospitality and discussion in his group. Additional thanks for discussion are due to Kerstin Krüger, Sonja Offner and Andrea Mayr, and Peter Palm for help with the mp-old program. This work was financially supported by the Deutsche Forschungsgemeinschaft (PF 165/6-1).

REFERENCES


Received 18 October 1995; revised 29 January 1996; accepted 16 February 1996.