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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*

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ΔD transformants containing all 14 *gvp* genes of *Haloferax mediterranei* required for gas vesicle formation except for *gvpD* are gas vesicle overproducers (Vac^{++}), whereas ΔD/D transformants containing the *gvpD* reading frame under ferredoxin promoter control on a second construct in addition to ΔD did not form gas vesicles (Vac^{-}). The amino acid sequence of GvpD indicates three interesting regions (a putative nucleotide-binding site called the p-loop motif, and two basic regions); these were altered by mutation, and the resulting $GvpD_{mut}$ proteins tested in $\Delta D/D_{mut}$ transformants for their ability to repress gas vesicle formation. The exchange of amino acids at conserved positions in the p-loop motif resulted in Vac^{++} $\Delta D/D_{mut}$ transformants, indicating that these $GvpD_{mut}$ proteins were unable to repress gas vesicle formation. In contrast, a $GvpD_{mut}$ protein with an alteration of a non-conserved proline in the p-loop region (P41A) was still able to repress. The repressing function of the various GvpD proteins was also investigated at the promoter level of the *gvpA* gene. This promoter is only activated during the stationary phase, depending on the transcriptional activator protein GvpE. Whereas the Vac^{++} ΔD transformants contained very high amounts of *gvpA* mRNA predominantly in the stationary growth phase, the amount of this transcript was significantly reduced in the Vac^{-} transformants ΔD/D and $\Delta D/D_{P41A}$. In contrast, the Vac^{++} $\Delta D/D_{mut}$ transformants harbouring $GvpD_{mut}$ with mutations at conserved positions in the p-loop motif contained large amounts of *gvpA* mRNA already during exponential growth, suggesting that this motif is important for the GvpD repressor function during this growth phase. The GvpD mutants containing mutations in the two basic regions were mostly defective in the repressing function. The $GvpD_{mut}$ protein containing an exchange of the three arginine residues 494RRR496 to alanine residues was able to repress gas vesicle formation. No *gvpA* mRNA was detectable in this transformant, demonstrating that this GvpD protein was acting as a strong repressor. All these results imply that the GvpD protein is able to prevent the GvpE-mediated *gvpA* promoter activation, and that the p-loop motif as well as the two basic regions are important for this function.

Keywords: halophilic archaea, gas vesicles, repressor, gene regulation

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

The GenBank accession number for the mc-vac sequence is X64701.

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INTRODUCTION

The moderately halophilic archaeon *Haloferax mediterranei* produces gas vesicles during the stationary growth phase when grown in media containing 17–30% (w/v) salt, whereas cells grown in 15% salt media are gas vesicle free (Rodriguez-Valera *et al*., 1983; Englert *et al*., 1990). Gas vesicles are watertight, gas-permeable proteinaceous structures that allow organisms such as cyanobacteria or halophilic archaea to float in their watery environment (reviewed by Walsby, 1994). The ribbed envelope is formed by the 7–8 kDa protein GvpA; the GvpC protein is a minor constituent that strengthens the gas vesicle structure and plays a role in shape determination (Hayes *et al*., 1992; Englert & Pfeifer, 1993; Halladay *et al*., 1993; Kinsman *et al*., 1995; Offner *et al*., 1996).

Gas vesicle formation of *Hf. mediterranei* involves 14 *gvp* genes located in the so-called mc-vac region (mc = *mediterranei* chromosomal) that are arranged as two clusters, namely mc-*gvpACNO* and, upstream and oppositely oriented, mc-*gvpDEFGHIJKLM* (Englert *et al*., 1992a; see Fig. 1). The boundaries of the mc-vac region have been defined by transformation experiments using the Vac$^-$ species *Haloferax volcanii* as recipient (Englert *et al*., 1992b). Two similar vac regions are found in the extremely halophilic archaeon *Halobacterium salinarum* PHH1, which contains the so-called p-vac region on plasmid pHH1, and a second *gvp* gene cluster named c-vac in the chromosome (Englert *et al*., 1992a). A vac region almost identical to p-vac has been reported on plasmid pNRC100 of *Hb. salinarum* NRC-1 (Jones *et al*., 1991; DasSarma *et al*., 1994; Ng *et al*., 1998). The requirement of each *gvp* gene for gas vesicle formation has been investigated in more detail for both plasmid-encoded vac regions (DasSarma *et al*., 1994; Offner & Pfeifer, 1995; Offner *et al*., 1996, 2000). These studies indicate that *gvpD* and *gvpE* encode proteins presumably involved in the regulation of gas vesicle formation.

Two promoters located in front of mc-*gvpA* and mc-*gvpD* (the mcA and mcD promoter) drive the expression of the mc-vac region of *Hf. mediterranei*, leading to a complex pattern of transcription (Englert *et al*., 1992a; Röder & Pfeifer, 1996). Minor amounts of the 7 kb mc-*gvpDEFGHIJKLM* and the 3·0 kb mc-*gvpDEF* mRNAs occur together with 2·0 kb and 1·3 kb mc-*gvpD* transcripts during a short period in exponential growth, whereas the mc-*gvpACNO* cluster encoding the two gas vesicle structural proteins is not expressed at this time (see Fig. 1). During the stationary growth phase the 2·0 kb mc-*gvpD* mRNA and large amounts of the 1·3 kb and 0·45 kb transcripts occur. Also at this time, the mc-*gvpACNO* cluster is expressed, leading to large amounts of the 0·32 kb mc-*gvpA* mRNA and minor amounts of various mc-*gvpACNO* cotranscripts (Röder & Pfeifer, 1996). The strong activity of the mcA and mcD promoters during the stationary growth phase depends on the transcriptional activator GvpE encoded by the second gene in the mc-*gvpD–M* gene cluster. The GvpE

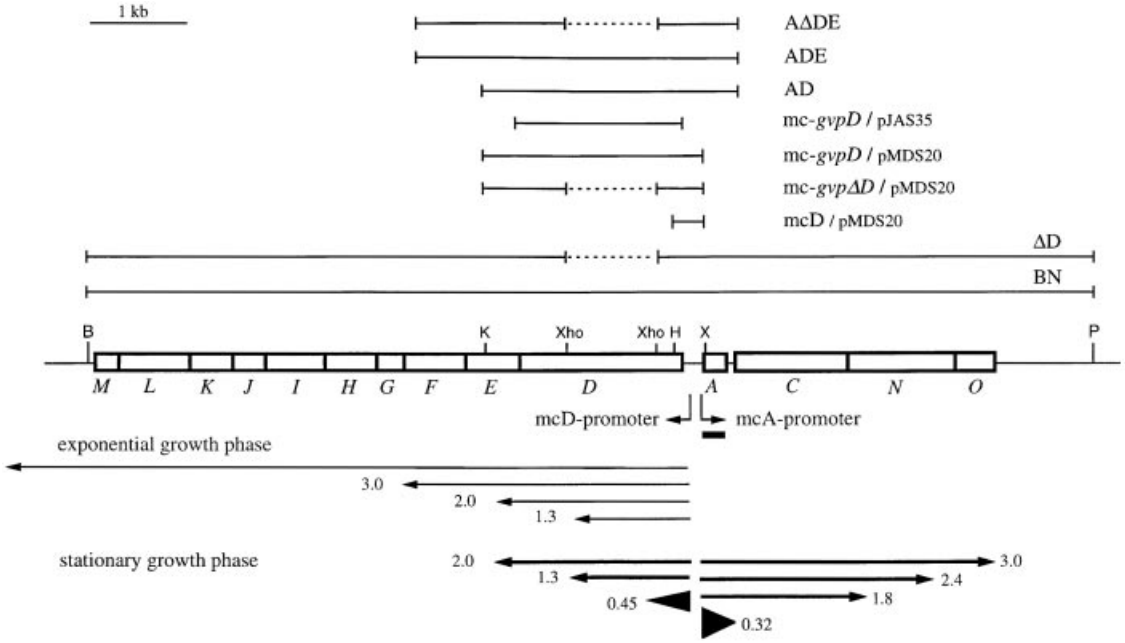

Fig. 1. Genetic map of the mc-vac region in *Hf. mediterranei* and schematic representation of the transcripts and constructs used for transformation. The mc-*gvp* genes are depicted as boxes labelled *A* and *C* through *O*. Vertical lines indicate relevant restriction sites used for cloning (B, *Bam*HI; H, *Hpa*I; K, *Kpn*I; P, *Pst*I; X, *Xcm*I; Xho, *Xho*I). The bar below the mc-*gvpA* gene represents the probe used for Northern analyses. Arrows below the map indicate the direction and relative strength of transcripts grouped according to their appearance during growth of the culture (Röder & Pfeifer, 1996). Lines above the map depict the various constructs used for transformation experiments.

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proteins encoded by all three archaeal vac regions resemble a basic leucine zipper protein (Krüger *et al.*, 1998), with structural similarities to eukaryotic gene regulators such as GCN4 in yeast (Ellenberger *et al.*, 1992). In the case of the mc-vac region, the mcA promoter by itself is almost inactive, and only active in a transformant containing mc-*gvpA* and mc-*gvpE* (Röder & Pfeifer, 1996).

Transcription in archaea depends on a single DNA-dependent RNA polymerase comprising 12 subunits that are homologous to the subunits of the eukaryotic RNA polymerase II. In addition, the archaeal promoter consists of a TATA box centred around position −28 upstream of the transcription start site, and the initiation of transcription requires the TATA box binding protein TBP and the transcription initiation factor TFB homologous to the eukaryotic transcription factor TFIIB (Hausner *et al.*, 1996; Qureshi *et al.*, 1995; Thomm, 1996). Multiple divergent genes encoding the transcription initiation factor TFB have been identified in *Hf. volcanii* (Thompson *et al.*, 1999). A second DNA element (TFB recognition element, BRE) located adjacent to the TATA box is also shared by archaea and eukaryotes (Lagrange *et al.*, 1998; Qureshi & Jackson, 1998). Despite the eukaryotic RNA polymerase and promoter structure, most archaeal gene regulator proteins are of the bacterial type. Examples are a repressor protein involved in the regulation of nitrogen fixation in *Methanococcus maripaludis* (Cohen-Kupiec *et al.*, 1997), and the regulator of arginine fermentation in *Hb. salinarum* (Ruepp & Soppa, 1996; Soppa *et al.*, 1998). The only example of a transcriptional activator similar to a eukaryotic-type regulator appears to be the basic leucine zipper protein GvpE (Krüger *et al.*, 1998).

In contrast to GvpE, the product of the mc-*gvpD* gene participates in the repression of gas vesicle formation: *Hf. volcanii* transformants containing an mc-vac region with a deletion in mc-*gvpD* (ΔD transformants) overproduce gas vesicles (Vac^{++} phenotype) in such a way that the discoid *Hf. volcanii* cells turn into spheres (Englert *et al.*, 1992b). The addition of the mc-*gvpD* gene on a second vector construct ($\Delta D/D_{native}$ transformant) reduces the amount of gas vesicles to the wild-type level, suggesting a repressor function of GvpD (Pfeifer *et al.*, 1994). Northern analyses demonstrate that ΔD transformants contain significantly higher amounts of all mc-vac transcripts (Röder & Pfeifer, 1996). Since large amounts of the 6 kb mc-*gvp*ΔD–M mRNA containing the mc-*gvpE* reading frame are present, the overproducer phenotype of the ΔD transformant could also be the result of an increased amount of GvpE.

The amino acid sequences of GvpD proteins of all three vac regions of halophilic archaea indicate interesting features: these proteins contain near the N-terminus a conserved p-loop motif ($_{36}$LYNGAPGTGKT$_{46}$) found in GTP/ATP-binding proteins such as adenylate kinase, RecA, Ras, G-proteins and elongation factors (Saraste *et al.*, 1990). In these proteins, ATP or GTP is bound in the p-loop sequence, and the γ-phosphoryl group is hydrolysed either by the nucleotide-binding protein itself or by an additional enzyme (Smith & Rayment, 1996; Skovgaard *et al.*, 1998). The conserved amino acid sequence of the classical mononucleotide-binding fold (kinase 1 motif) is GxxGxGKT/S (x = any amino acid). The lysine residue binds the negatively charged β- and γ-phosphoryl groups, whereas the serine (or threonine) residue is involved in the magnesium binding (Deyrup *et al.*, 1998). The mutagenesis of one conserved amino acid leads to a reduction in activity, or even to the loss of function (Konola *et al.*, 1994; Skovgaard *et al.*, 1998).

In this study, we investigated the cause of the gas vesicle overproducing phenotype of ΔD transformants in more detail. The putative p-loop motif in GvpD was mutagenized and the resulting mutants tested in $\Delta D/D_{mut}$ transformants to detect GvpD mutant proteins that are unable to reduce the amount of gas vesicles. Similar experiments were also employed to investigate the functional importance of two basic regions within GvpD. In addition, the various GvpD mutant proteins were tested for their ability to repress the formation of mc-*gvpA* mRNA.

METHODS

Growth of *Hf. volcanii*. *Hf. volcanii* WFD11, used for transformation experiments, was obtained from W. F. Doolittle (Halifax, Canada). The *Hf. volcanii* growth medium contained 3 M NaCl, 150 mM $MgSO_4$, 40 mM KCl, 10 nM $MnCl_2$, 25 mM Tris/HCl pH 7·2, 0·05 % (w/v) $CaCl_2$, 0·5 % (w/v) tryptone and 0·3 % (w/v) yeast extract. Liquid cultures were grown at 37 °C and 180 r.p.m., whereas colonies were grown on medium solidified with 1·8 % (w/v) agar at 42 °C.

Transformation of *Hf. volcanii* WFD11. The 9·259 bp ΔD construct contains the entire mc-vac region inserted in pWL102, but has incurred a 918 bp *Xho*I deletion within the mc-*gvpD* gene (Englert *et al.*, 1992b). The ADE and AΔDE constructs have been previously described (Röder & Pfeifer, 1996). *Hf. volcanii* ΔD transformants were transformed with different variants of mc-*gvpD* inserted in pMDS20 (Holmes *et al.*, 1991), or in the expression vector pJAS35 (Pfeifer *et al.*, 1994). The mc-*gvpD*/pMDS20 construct contained a 2002 bp *Xcm*I–*Kpn*I fragment (see Fig. 1), the mc-*gvp*ΔD construct contained the same fragment except for the 918 bp *Xho*I deletion, and the mcD construct carried a 320 bp *Xcm*I–*Hpa*I fragment containing the mcD promoter region plus 111 bp of the 5′ part of mc-*gvpD*. The mc-*gvpD*/pJAS35 construct harboured the mc-*gvpD* reading frame amplified as a 1651 bp PCR fragment using the primers CCAAAGTGCGTCATCCATGGCCC (positions 4296–4318; *Nco*I site spanning the ATG start codon underlined) and CCTCGATGAGCGGTACCATCTGTC (positions 5971–5948; *Kpn*I site introduced underlined), and inserted as an *Nco*I–*Kpn*I fragment in pJAS35. *Hf. volcanii* transformants containing the entire mc-vac region as a BN construct (Englert *et al.*, 1992a) were used as control. Prior to the transformation of *Hf. volcanii*, each construct was passaged through the *Escherichia coli* Dam^- strain GM1674 (Palmer & Marinus, 1994) to avoid a halobacterial restriction barrier (Holmes *et al.*, 1991). Transformation was done as previously described (Pfeifer & Ghahraman, 1993), and transformants were selected on agar plates containing 6 μg mevinolin ml^{-1} (for the selection of ΔD in pWL102) and 0·2 μg novobiocin ml^{-1} (for the selection of pMDS20 or pJAS35). The presence of the desired constructs in

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Table 1. Oligonucleotides used to amplify and mutagenize mc-*gvpD* sequences, and resulting mutations in GvpD

Name	Oligonucleotide*	Position†	Mutation in Gvpd
gvpD/Bam + Nco	GACCAAAGTGCGGGATCC*ATG*GCCCCACCAAACC	4294–4327	—
gvpD/*Hin*dIII	AGTGTCAGCTTTAAGCTTTTTCCGAAGTCC	6000–5970	—
MutP1	CCCGGCGCTGCGTTAACGAGCAGC	4436–4413	G39A
MutP2	CGTCTTTCCCGTTGCCGGCGCTCC	4449–4425	G42A
MutP3	GAACAGCGTCTTTGCCGTTCCCGGG	4455–4430	G44A
MutP4	CGTGAACAGCGTCTCTCCCGTTCCGG	4458–4432	K45E
MutP5	CTTTCCCGTTCCCGCCGCTCCGTTACG	4446–4419	P41A
MutP6	GAACAGCGTCTTNNCCGTNNCCGGCGCNNCGTTAACGAGCAG	4455–4414	EDD
AAAA	CTAGTCGGAGATACGCGGCCGTTGCTCCCGCTCGTCTTCTGC	4942–4900	AAAA
AEAE	CTAGTCGGAGATACTCGGCCGTTTCTCCCGCCTCGTCTTCTGC	4942–4900	AEAE
2-AAA	AATACGGACTCCGGCCAACGCCTCTAGTGCGAGATACCGGCGCGT	4965–4921	2-AAA; 2-AAAR
3-AAA	GGAACCGAACTCGCAGCGGCGGCGGACCGCG	5779–5809	3-AAA; 3-ADA
BamD	GTGCGGGATCCATGGCCCCACC	4301–4322	—
HinD	GAGCCGTTCAAGCTTTCACACCATCTCC	5938–5964	—

* Underlined nucleotides indicate mutations compared to the respective m-vac sequence. The start codon (ATG) of the *gvpD* reading frame is shown in italic.

† The GenBank accession number of the mc-vac sequence is X64701.

each transformant was controlled by Southern analyses using the internal 918 bp *Xho*I fragment derived from mc-*gvpD*, and vector-specific probes that were produced using either two 0·1 kb *Sac*I fragments derived from the *gyrB* gene in pJAS35, or an 1·8 kb *Eco*RV fragment derived from the HMG-CoA reductase gene in pWL102. Both probes were labelled by the random priming method using the DIG DNA Labeling Kit from Roche.

Site-directed mutagenesis of the mc-*gvpD* reading frame. The mutations in the mc-*gvpD* reading frame were achieved by two consecutive PCR reactions, where the product of the first PCR (the 'megaprimer' containing the desired mutation) was used in the second PCR together with a third primer. The sequences of the oligonucleotide primers used for the amplification are given in Table 1. The wild-type mc-*gvpD* reading frame was amplified using *Hf. mediterranei* DNA as template and the oligonucleotides gvpD-Bam + Nco and gvpD-*Hin*dIII as primers. The *Bam*HI site next to *Nco*I, and the *Hin*dIII site near the 3′ terminus, were used to insert the 1681 bp mc-*gvpD* fragment into the *E. coli* vector pBluescript.

For the construction of mutations in the p-loop motif the primer gvpD/Bam + Nco was used with one of the oligonucleotides MutP1 through MutP6 (see Table 1) and *Hf. mediterranei* DNA as template, resulting in the amplification of the respective 200 bp megaprimer. Each megaprimer was used in a second PCR together with the T7 primer and mc-*gvpD*-pBluescript SK+ as template for the amplification of the 1·7 kb mc-*gvpD* reading frame. For the mutagenesis of the basic region 1 (position 201–215 in the GvpD sequence), different megaprimers were synthesized using the primer gvpD/Bam + Nco together with either the AAAA or the AEAE primer (Table 1), resulting in the amplification of a 649 bp fragment. The oligonucleotide gvpD/Bam + Nco together with primer 2-AAA amplified a 672 bp fragment. The basic region 2 (positions 494–496 in GvpD) is located near the 3′-terminus of mc-*gvpD* and was mutagenized using the primer pair 3-AAA plus gvpD/*Hin*dIII, resulting in a 211 bp megaprimer. Each megaprimer was used together with the gvpD/*Hin*dIII primer (in the case of basic region 1), or the gvpD/Bam + Nco primer (in the case of basic region 2) for the second PCR that amplified the 1·7 kb mc-*gvpD* fragment containing the desired mutations.

The mutated mc-*gvpD* fragments were cloned as *Bam*HI–*Hin*dIII fragments into pBluescript and the mutations were confirmed by DNA sequence analyses. Unexpectedly, two additional mutations leading to D2-AAAR instead of D2-AAA, and D3-ADA instead of D3-AAA, were observed, most likely due to additional mutations in the oligonucleotides used for PCR. Each mutated mc-*gvpD* reading frame was inserted into the halobacterial expression vector pJAS35 using the *Nco*I/*Kpn*I sites, and used for the transformation of *Hf. volcanii*.

Isolation of RNA from *Hf. volcanii* and transcript analyses. RNA from *Hf. volcanii* transformants was isolated according to the single-step method of Chomczynski & Sacchi (1987). RNA from transformants in the exponential growth phase was isolated from cultures at OD_{600} 0·3–0·4 (OD_{600} measurements were made in a Beckman spectrophotometer), whereas RNA from stationary-phase cells was isolated from cultures at $OD_{600} \geqslant 2$. Northern analyses involved electrophoresis of 5 or 10 μg RNA on denaturing, formaldehyde-containing 1·2% (w/v) agarose gels, followed by transfer to nylon membranes (Ausubel *et al.*, 1988). Strand-specific RNA probes were synthesized using a 918 bp *Xho*I fragment derived from mc-*gvpD*, or a 367 bp *Xcm*I–*Eco*RI fragment from mc-*gvpA* cloned in pBluescript, as template for the T3/T7 polymerase system of Stratagene. The RNA was labelled using the DIG RNA Labeling Kit from Roche. Northern hybridization was generally carried out as described by Ausubel *et al.* (1988), but the hybridization solution contained 10% (w/v) dextran sulfate (Sigma), 1% (w/v) SDS, and 0·5% (w/v) skim milk powder.

Isolation of proteins, Western analysis and production of antisera. Total proteins from various *Hf. volcanii* transformants were isolated from exponential-phase (OD_{600} 0·3–0·4) and from stationary-phase ($OD_{600} > 2·0$) cultures.

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Samples (25 ml of exponential- and 3 ml of stationary-phase cultures) were centrifuged at 12000 ***g*** and resuspended in 400 µl TE buffer containing 0·5 µl DNase I (1 mg ml^{-1}).The suspension was dialysed against 10 mM Tris/HCl, pH 7·2, for 12 h at 4 °C, and centrifuged for 20 min at 12000 ***g*** for membrane removal. The protein content was determined by the Bradford method. Ten micrograms of protein from each sample was separated on 15 % Tricine SDS-polyacrylamide gels (Schägger & von Jagow, 1987). Proteins were transferred to nitrocellulose membranes (Biotrace NT 0·45 µm, Pall Gelman Sciences) for 1 h at 100 V and 4 °C using the Midget Multiblot System (Pharmacia) in a buffer containing 25 mM Tris/HCl, pH 8·3, and 192 mM glycine. The filters were incubated in blocking buffer (1 % BSA, 0·1 % Tween 20 in PBS) overnight at room temperature. The GvpD protein was detected by antiserum raised against His-tagged GvpD protein in a dilution of 1:1000 in blocking buffer for 1 h at room temperature. The filters were washed with blocking buffer and incubated with the second antibody. The ECL detection system (Amersham) was used to detect the hybridizing antibodies. The filters were exposed onto X-ray film for 10–15 min.

For formation of antibodies to the GvpD protein the *gvpD* reading frame was amplified by PCR using the two oligonucleotides BamD and HinD (Table 1) and inserted into the His-tag expression vector pQE8 (Qiagen). The His-tagged fusion protein was synthesized in *E. coli* and purified using metal chelate chromatography. Rabbits were injected with 100 µg purified protein contained in 1 ml solution (500 µl 100 mM Na_2HPO_4, pH 8·0, and 500 µl Freund's adjuvant), and boostered three times after 2 weeks each (performed by Eurogentec, Seraing).

Analysis of DNA sequence data. DNA sequence determination was done according to the Sanger method using the Sequi-Therm EXCEL II Long-Read DNA Sequencing Kit-LC protocol (Biozym). The fragments were separated using a LI-COR DNA sequencer at 1200 V, 35 mA and 50 °C for 7 h. The GenBank accession number of the mc-vac sequence is X64701.

RESULTS

The *gvpD* gene is involved in the repression of gas vesicle formation

Hf. volcanii ΔD transformants containing the mc-vac region with a 918 bp deletion in mc-*gvpD* overproduce gas vesicles (Englert *et al.*, 1992b), and this Vac^{++} phenotype can be reverted by the addition of mc-*gvpD* present on a second vector construct (Pfeifer *et al.*, 1994). To investigate the cause of the reduction of gas vesicle formation in $\Delta D/D_{native}$ transformants in more detail, different mc-*gvpD* variants were constructed (see Fig. 1). The mc-*gvpD*/pMDS20 construct contained the mc-*gvpD* gene including the endogenous promoter; this construct had been used for the initial ΔD/D transformant (Pfeifer *et al.*, 1994). mc-*gvp*ΔD/pMDS20 was the same construct, but had a 918 bp *Xho*I deletion in mc-*gvpD* (encoding non-functional GvpD), mc-*gvpD*/pJAS35 harboured the mc-*gvpD* reading frame expressed by the strong *fdx* promoter, and the mcD construct contained the mc-vac promoter region plus 111 bp derived from the 5′ part of mc-*gvpD* (Fig. 1). Transformants of *Hf. volcanii* were constructed containing ΔD and one of these various D constructs, and the Vac phenotype was inspected on agar plates. *Hf. volcanii* lacks gas vesicles and forms red translucent colonies, whereas those of ΔD transformants are turbid white (gas vesicle overproduction, Vac^{++}) (Englert *et al.*, 1992b; Fig. 2, top row). $\Delta D/D_{native}$ transformants form pink colonies due to a normal amount of gas vesicles (Vac^{+}), similar to mc-vac transformants (Fig. 2). The cells of the transformants can also be inspected by phase-contrast microscopy, where gas vesicles are seen as light-refractile particles inside the cells. ΔD cells appear as bright white spheres completely filled with

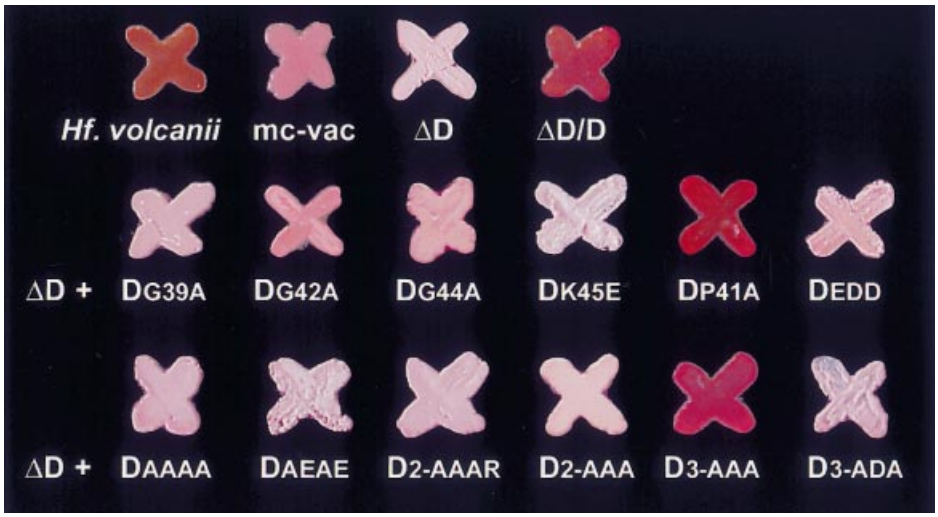

Fig. 2. Vac phenotypes of *Hf. volcanii* transformants on agar plates. The top row shows colonies of *Hf. volcanii* and control transformants. Gas vesicle overproducing (Vac^{++}) colonies (ΔD) are pink-white and opaque, Vac^{+} colonies (mc-vac) are pink and turbid, and gas vesicle negative (Vac^{-}) colonies (*Hf. volcanii* or ΔD/D) are red and translucent. The middle row shows colonies of transformants containing $\Delta D/D_{mut}$ constructs with mutations in the p-loop region, whereas the bottom row shows colonies harbouring $\Delta D/D_{mut}$ constructs with mutations in one of the two basic regions of GvpD.

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		mutations	designation	Vac phenotype of ΔD/D or ΔD/Dmut transformants
p-loop region:	36	LVNGAPGTGKT 46	D	-
		...A.....	DG39A	++
		.....A....	DG42A	++
		.....A..	DG44A	++
		.....E.	DK45E	++
		.....A.....	DP41A	-
		...E..D.D..	DEDD	++
basic region 1:	201	RGRTRRYLRLEKLRG 221	D	-
		A.A.AA.....	DAAAA	++
		A.E.AE.....	DAEAE	++
		.....A.A..A.	D2-AAA	++
		.....A.A..AR	D2-AAAR	++
basic region 2:	492	ELRRRADR 499	D	-
		..AAA...	D3-AAA	-
		..ADA...	D3-ADA	++

Fig. 3. Mutations in the p-loop and the two basic regions. The designations of the respective mutant constructs are given, and the Vac phenotype of the transformants is indicated.

light-refractile bodies, whereas *Hf. volcanii* cells are discoid (Pfeifer *et al*., 1994).

As expected, the control transformants ΔD plus the 'empty' vector pMDS20 (or pJAS35) were Vac^{++}, whereas the addition of mc-*gvpD*/pMDS20 (native GvpD) revealed Vac^{+} transformants (Pfeifer *et al*., 1994). The ΔD/mc-*gvp*ΔD (which encodes non-functional GvpD) and the ΔD/mcD (promoter region) transformants were both Vac^{++}, demonstrating that a defective GvpD, or additional copies of mc-vac promoter (which could compete for the GvpE activator protein), did not reduce the amount of gas vesicles in the ΔD transformants (Fig. 2, and data not shown). In contrast, the colonies of ΔD transformants containing mc-*gvpD*/pJAS35, where the *fdx* promoter promotes large amounts of mc-*gvpD* transcripts, were red, transparent and without gas vesicles (ΔD/D, Fig. 2, top row). These results demonstrated that the mc-*gvpD* reading frame, and thus the GvpD protein, was the reason for the reduced amount of gas vesicles.

The amino acid sequence of GvpD indicates three interesting regions that could be important for the repressor function: a putative p-loop motif located near the N-terminus (positions 36–46), and two basic regions (positions 201–222 and 494–499) that might function in DNA binding. These three regions were mutagenized and tested in $\Delta D/D_{mut}$ double transformants for their ability to reduce gas vesicle formation.

Importance of the p-loop motif for the GvpD repressor function

The putative p-loop motif in GvpD has the sequence $_{36}$LVNGAPGTGKT$_{46}$ (Fig. 3). The codons encoding the conserved amino acids (underlined) were altered by site-directed PCR mutagenesis, and inserted into the vector pJAS35 for expression under *fdx* promoter control. $\Delta D/D_{mut}$ double transformants were produced and tested for the ability to reduce gas vesicle overproduction. If the mutant GvpD proteins were unable to reduce gas vesicle formation in the ΔD transformant, the altered amino acids were important for the GvpD repressor function.

gvpD mutant genes were constructed encoding $GvpD_{mut}$ proteins with a G39A, G42A, G44A or K45E mutation (Fig. 3). In addition, a *gvpD* mutant was produced encoding GvpD that contained an alteration of the non-conserved proline P41 residue to an alanine. A sixth *gvpD* mutant was obtained using a degenerate primer for PCR mutagenesis: the $GvpD_{mut}$ encoded contained three alterations in the p-loop motif, namely G39E, G42D and G44D (D_{EDD}; Fig. 3). Each alteration in mc-*gvpD* was confirmed by DNA sequence analyses. The mutated reading frames were then transferred into the expression vector pJAS35 and used to transform ΔD transformants. As control, the ΔD transformant was complemented with the mc-*gvpD*/pJAS35 construct. Transformants appeared after 5–7 d on agar plates and were inspected for gas vesicle formation (Fig. 2). As expected, the ΔD/D transformants formed red and translucent colonies (Fig. 2, top row). The $\Delta D/D_{mut}$ transformants $\Delta D/D_{G39A}$, $\Delta D/D_{G42A}$, $\Delta D/D_{G44A}$, $\Delta D/D_{K45E}$ and $\Delta D/D_{EDD}$ (each mutation encodes GvpD protein altered in conserved amino acids of the p-loop motif) were Vac^{++}, implying that all these $GvpD_{mut}$ proteins were unable to repress gas vesicle overproduction (Fig. 2, middle row). Only the $\Delta D/D_{P41A}$ transformant was Vac^{-}, suggesting that the GvpD protein that had incurred an exchange of the non-conserved P41 to alanine was still functional (Fig. 2).

Northern analyses were performed to ensure that each mutated mc-*gvpD* gene was indeed transcribed. Total RNA was isolated during the exponential and stationary growth phases of the transformants and the 918 bp internal *Xho*I fragment derived from mc-*gvpD* was used as probe, since it is absent in the ΔD construct and hybridizes only with transcripts derived from the entire mc-*gvpD* reading frame (see Fig. 1). RNA of *Hf. volcanii* WFD11 (containing no mc-vac sequences) and of the ΔD transformant did not indicate any transcripts, whereas the mc transformant (containing the mc-vac region) indicated the expected mc-*gvpD* transcripts (Fig. 4a, top). The RNA of each double transformant contained mc-*gvpD* transcripts that were slightly smaller due to the lack of the 83 nucleotide mRNA leader that was cut off during the fusion of the reading frame to the *fdx* promoter in the vector pJAS35. The 0·3 kb mc-*gvpD* transcript occurred in stationary growth phase only (Fig. 4a), similar to the 0·45 kb transcript of the mc-vac region in *Hf. mediterranei* (Röder & Pfeifer, 1996). These Northern analyses demonstrated that each of the mc-*gvpD* reading frames in pJAS35 was transcribed.

The presence of the GvpD protein in these transformants was assessed by Western analysis using a GvpD-specific antiserum (Fig. 4b). The antiserum reacted with the His-tagged, 60 kDa GvpD protein isolated from *E. coli*, and also with the GvpD protein in lysates of the ΔD/D

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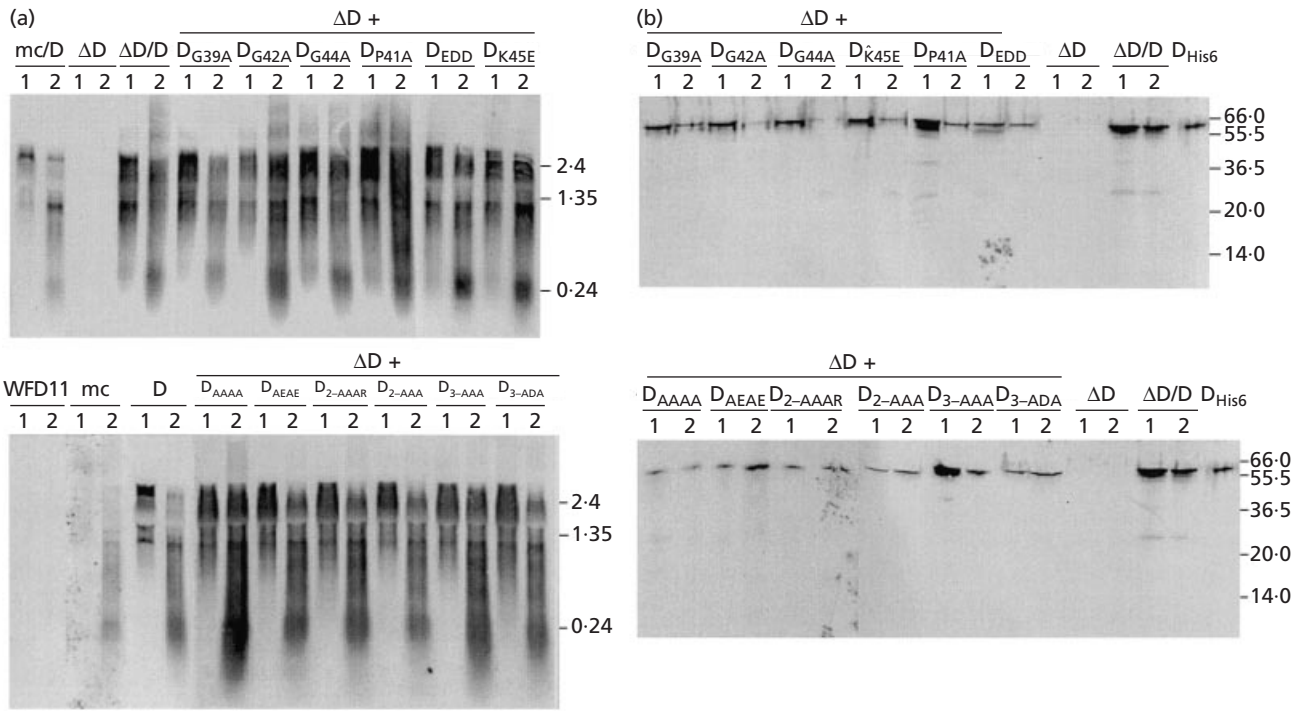

Fig. 4. (a) Northern analyses to assess the transcription of the mc-*gvpD* reading frame in pJAS35. Total RNA from *Hf. volcanii* WFD11 and from the various transformants isolated during the exponential (1) and stationary (2) growth phase was separated on 1·2 % agarose gels. The designation of the constructs contained in the various transformants is given on top. Northern analyses were done with 5 μg (upper blot) or 10 μg (lower blot) RNA per sample. A 918 bp *Xho*I fragment derived from the mc-*gvpD* gene was used as probe. The RNA marker sizes (in kb) are given on the right. (b) Western analyses to confirm the presence of the GvpD protein in the transformants. Proteins were isolated from the various transformants during the exponential (1) and stationary (2) growth phase. Ten microgram samples of total protein from cytoplasmic fractions was separated on 15 % Tricine SDS-polyacrylamide gels, blotted onto nylon membrane and hybridized with the GvpD-specific antiserum as described. The protein marker sizes (in kDa) are given on the right.

transformant. No reaction was observed with proteins derived from the ΔD transformant, indicating that the reaction of the antiserum was specific for GvpD (Fig. 4b). Each of the $\Delta D/D_{mut}$ transformants tested contained the 60 kDa $GvpD_{mut}$ protein, demonstrating that all mc-*gvpD* mRNAs were indeed translated and that the GvpD protein was present in the cells. Thus, each Vac phenotype observed with the transformants was due to the $GvpD_{mut}$ protein.

Importance of the two basic regions for the GvpD repressor function

Two conserved basic regions located in the GvpD sequence at position 201–215 (region 1) and position 494–499 (region 2) were also altered. Mutations in region 1 resulted in the mutant proteins D_{AAAA}, D_{AEAE} and $D_{2\text{-}AAA}$, and mutations in region 2 resulted in the $D_{3\text{-}AAA}$ protein (Fig. 3). Two additional alterations in mc-*gvpD* were obtained by chance and led to an alteration of the amino acid residues G215R ($D_{2\text{-}AAAR}$) and R495D ($D_{3\text{-}ADA}$) in GvpD (Fig. 3). Each of these mutated mc-*gvpD* fragments was inserted into pJAS35, and the resulting constructs were used to transform ΔD transformants. The phenotype of each $\Delta D/D_{mut}$ double transformant was again monitored by inspecting the colonies on agar plates (Fig. 2, bottom row). None of the GvpD mutants D_{AAAA}, D_{AEAE}, $D_{2\text{-}AAA}$, $D_{2\text{-}AAAR}$ and $D_{3\text{-}ADA}$ was able to reduce the Vac^{++} phenotype of $\Delta D/D_{mut}$ to Vac^{-}, indicating that these GvpD mutants were unable to repress gas vesicle formation. In contrast, the $D_{3\text{-}AAA}$ protein was active in repression, since the $\Delta D/D_{3\text{-}AAA}$ transformant did not contain gas vesicles (Fig. 2). The transcription of the mc-*gvpD* reading frame in pJAS35 was again analysed by Northern analysis to ensure the expression of each construct (Fig. 4a, bottom). In each case, mc-*gvpD* transcripts of 0·3, 1·2 and 3·0 kb were detected. Western analysis using the GvpD-specific antiserum indicated that GvpD (or $GvpD_{mut}$) proteins were present in each transformant (Fig. 4b, bottom).

Effect of $GvpD_{mut}$ on the mcA promoter activity

The effect of the *gvpD* mutations on the reduction of the gas vesicle formation could occur at the mcA promoter level. Northern analyses were carried out to investigate the amount of transcripts starting at the mcA promoter present in the ΔD construct during the growth of each

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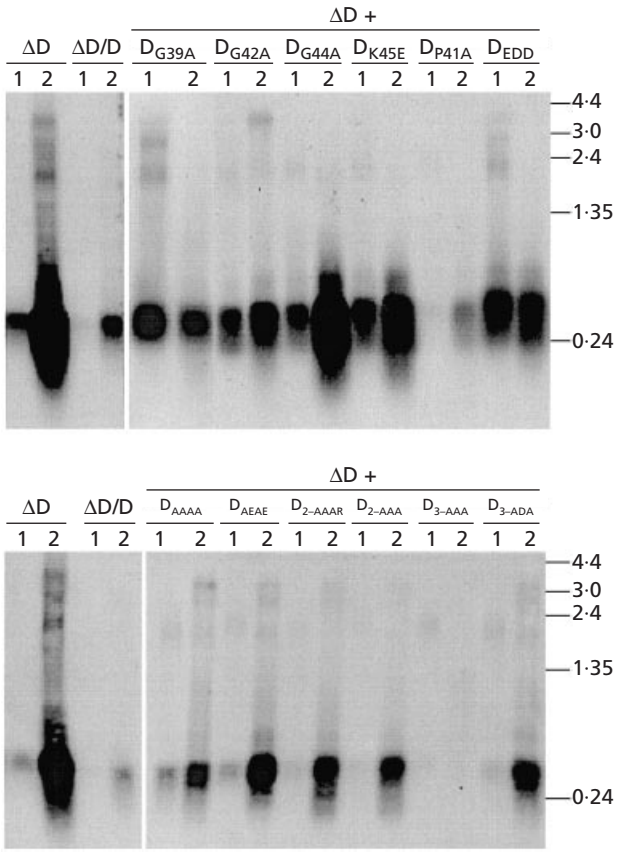

Fig. 5. Northern analyses to investigate mc-*gvpA* transcripts in ΔD, ΔD/D, and the various $\Delta D/D_{mut}$ transformants. Samples (5 µg) of RNA of the various transformants isolated during the exponential (1) and stationary (2) growth phase were separated on 1·2% agarose gels and hybridized with an mc-*gvpA*-specific probe. The RNA marker sizes (in kb) are given on the right. The exposure times of the upper and lower blots are slightly different, as visible for the control transformants ΔD and ΔD/D shown on the left.

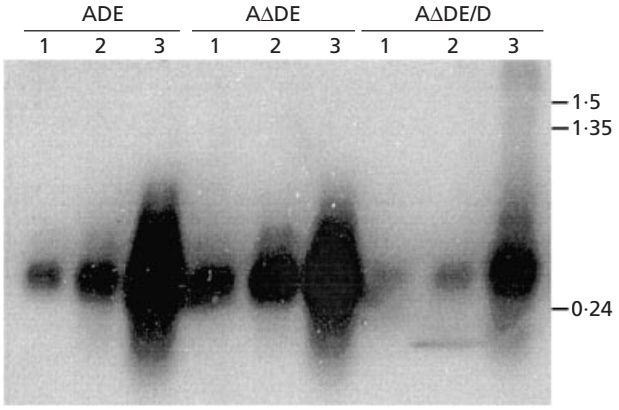

Fig. 6. Northern analyses to investigate mc-*gvpA* transcripts in ADE, AΔDE and AΔDE/D transformants. Samples (5 µg) of RNA of the various *Hf. volcanii* transformants isolated during (1) early exponential (OD_{600} 0·2–0·3), (2) mid-exponential (OD_{600} 0·45–0·6) and (3) stationary growth phase ($OD_{600} > 2$) was separated on a 1·2% agarose gel and hybridized with an mc-*gvpA*-specific probe. The RNA marker sizes (in kb) are given on the right.

transformant (Fig. 5). The ΔD transformant contains high amounts of the 0·32 kb mc-*gvpA* mRNA, especially during the stationary growth phase. In the ΔD/D transformant, the overall amount of mc-*gvpA* mRNA was significantly reduced, suggesting that GvpD is directly or indirectly involved in the regulation of the mcA promoter activity (Fig. 5, top and bottom). Inspection of the mc-*gvpA* transcripts in $\Delta D/D_{mut}$ transformants containing mutations in the p-loop motif of GvpD revealed high amounts of the mc-*gvpA* transcripts during all growth phases in each Vac^{++} transformant (Fig. 5, top). In contrast, the Vac^{-} transformant $\Delta D/D_{P41A}$ showed strongly reduced amounts of mc-*gvpA* mRNA, similar to ΔD/D.

The $\Delta D/D_{mut}$ transformants containing mutations in the two basic regions of GvpD revealed high amounts of mc-*gvpA* mRNA in the stationary growth phase only. In contrast to the ΔD/Dp-loop transformants, relatively low amounts of mc-*gvpA* mRNA were present during the exponential growth phase of these transformants (Fig. 5, bottom). The Vac^{-} transformant $\Delta D/D_{3\text{-}AAA}$, however, did not contain detectable amounts of mc-*gvpA* mRNA, indicating that the $GvpD_{3\text{-}AAA}$ protein conferred a complete repression of the mcA promoter.

Effect of GvpD mutations on the mcA promoter on the AΔDE construct

To determine whether the regulation of the mcA promoter involves other products of *gvp* genes besides GvpE and GvpD, we used a construct containing the mc-*gvpA* gene together with mc-*gvpDE* (ADE construct), and the AΔDE construct, where the mc-*gvpD* gene had incurred the same 918 bp internal deletion as found in ΔD (Röder & Pfeifer, 1996). ADE transformants contained high amounts of mc-*gvpA* mRNA, especially in the stationary growth phase, whereas slightly higher amounts of this transcript during exponential growth were observed in transformants containing the AΔDE construct (Fig. 6, and Röder & Pfeifer, 1996). The double transformant AΔDE/D containing the mc-*gvpD* reading frame under the control of the strong *fdx* promoter in addition to the AΔDE construct indicated a significantly reduced amount of mc-*gvpA* mRNA (Fig. 6). Thus, a low mcA promoter activity can be achieved by a high expression of the mc-*gvpD* gene.

DISCUSSION

The results presented in this report prove that the repression of gas vesicle formation in ΔD/D transformants was caused by the GvpD protein and not by the additional mc-vac promoter sequences that could compete for the GvpE activator. The strong *fdx* promoter in the expression vector pJAS35 presumably led to high amounts of GvpD that were sufficient to abolish gas vesicle formation, whereas the addition of the mc-

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gvpD gene under endogenous promoter control resulted in a gas vesicle level as observed in the wild-type (Pfeifer *et al*., 1994, and this report).

The p-loop motif and basic regions of GvpD are required for the repressing function

Various mutant GvpD proteins were designed to investigate the importance of these conserved regions with respect to the GvpD repressor function. Each mutation of a conserved residue (G39A, G42A, G44A or K45E) in the p-loop region resulted in an inactive GvpD protein when tested in $\Delta D/D_{mut}$ double transformants. In contrast, $GvpD_{mut}$ proteins with an alteration of the unconserved proline residue P41A within the p-loop region were still able to repress the gas vesicle formation in $\Delta D/D_{P41A}$ transformants. These results imply that ATP- or GTP-binding and hydrolysis might be important for the repressor function of GvpD, and experiments are under way to proof that GvpD can indeed bind mononucleotides.

Similar investigations of mutations in two basic regions in GvpD demonstrated that the exchange of arginine and lysine residues in basic region 1 completely abolished the repressing function: the GvpD mutants D_{AAAA}, D_{AEAE} and $D_{2\text{-}AAA}$ were unable to reduce the amount of gas vesicles in $\Delta D/D_{mut}$ transformants. This basic region 1 might be involved in DNA binding of GvpD; this needs to be investigated in more detail. In contrast, the alterations introduced in basic region 2 ($_{494}RRR_{496}$) revealed ambiguous results: an alteration of RRR to AAA revealed a $GvpD_{mut}$ protein that still functioned in repression. Since a positive charge in this region was obviously not required for the repressor function, this result suggested that region 2 is not involved in DNA binding. In contrast, changing the RRR to ADA residues and introduction of a negatively charged amino acid abolished the GvpD repressor function.

GvpD is involved in the reduction of the amount of mc-*gvpA* mRNA

The question whether GvpD is acting as repressor at the transcriptional level was further addressed by studying the effect of *gvpD* mutations on the activity of the mcA promoter. ΔD transformants contain high amounts of mc-*gvpA* mRNA, especially during stationary growth (Englert *et al*., 1992b; Röder & Pfeifer, 1996). Northern analyses indicated that the presence of the native *gvpD* gene in $\Delta D/D_{native}$ transformants resulted in a drastic reduction of the amount of mc-*gvpA* mRNA. The same reduction was seen with the Vac^- $\Delta D/D_{mut}$ transformants encoding the GvpD proteins D_{P41A} or $D_{3\text{-}AAA}$. The $\Delta D/D_{3\text{-}AAA}$ transformant even completely lacked mc-*gvpA* mRNA, suggesting that this mc-*gvpD* mutation gave rise to a 'super'-repressor. In contrast, the $D_{3\text{-}ADA}$ protein containing an aspartate in place of an alanine was inactive with respect to repression of the mcA promoter.

Different results were observed with the various $gvpD_{mut}$ genes that were unable to reduce the Vac^{++} phenotype in transformants. Despite the Vac^{++} phenotype, the mRNA patterns differed from that observed for the ΔD transformant. Transformants producing $GvpD_{mut}$ with alterations in the p-loop motif indicated significantly higher amounts of mc-*gvpA* mRNA during exponential growth, and the mcA promoter remained similarly active during stationary growth. The overproduction of gas vesicles in these transformants was possibly due to the early activation of the mcA promoter. Taking into account that the GvpD protein by itself cannot activate the mcA promoter (since the AD construct does not enable the cell to produce mc-*gvpA* mRNA; Röder & Pfeifer, 1996), this result must be due to the action of the GvpE activator, and this activation occurs earlier in these transformants compared to the mcA promoter activation in ΔD transformants. In contrast, alteration of a non-conserved amino acid in the p-loop region had no effect on the GvpD function. These results suggested that nucleotide binding (and hydrolysis?) was required to prevent the activation of the mcA promoter by GvpE during exponential growth. The Vac^{++} transformants containing mc-*gvpD* genes encoding proteins with mutations in the basic regions showed a slight reduction in the mc-*gvpA* amount compared to ΔD, but this amount of mRNA was still enough to reveal the overproduction of gas vesicles in these transformants.

The effect of mc-*gvpD* mutations on the mcA promoter was also studied in ADE, AΔDE and AΔDE/D transformants to determine whether additional *gvp* genes are involved. While the amount of mc-*gvpA* mRNA was high in AΔDE and only slightly lower in ADE transformants, the amount of this transcript was significantly reduced by the addition of the mc-*gvpD* gene under *fdx* promoter control in the AΔDE/D double transformant. This result is most likely due to an earlier and stronger synthesis of GvpD compared to the ADE transformants. These data imply that the repressing function of GvpD involved no additional Gvp proteins (besides possibly GvpE). It is not known, so far, whether GvpD acts directly or indirectly at the mcA promoter, since it is also possible that GvpD inactivates GvpE (depending on a functional p-loop) or acts at the mRNA level by reducing the amount of GvpE produced during exponential growth.

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