APPLIED AND ENVIRONMENTAL MICROBIOLOGY, May 1995, p. 1770–1775 Vol. 61, No. 5
0099-2240/95/$04.00+0

Insertional Mutations in the Hydrogenase *vhc* and *frc* Operons Encoding Selenium-Free Hydrogenases in *Methanococcus voltae*

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Received 4 October 1994/Accepted 10 February 1995

***Methanococcus voltae*, which contains four different gene groups that encode [NiFe]-hydrogenases, was transformed with integration vectors to achieve polar inactivation of two of the four hydrogenase operons that encode the selenium-free enzymes Vhc and Frc. Transformants which were selected by their acquired puromycin resistance showed site-specific insertions in either the *vhc* or *frc* operon by single crossover events. Southern hybridization revealed tandem integrations of whole vectors in the *vhc* operon, whereas only one vector copy was found in the *frc* operon. Northern (RNA) hybridizations showed a *pac* transcript of defined size, indicating strong termination in front of the hydrogenase genes downstream. In spite of the apparent abolition of expression of selenium-free hydrogenases through these polar insertions, they were not lethal to cells upon growth in selenium-deprived minimal medium, which we had previously shown to strongly induce transcription of the respective operons in *M. voltae*. Instead, like wild-type control cultures, transformants responded to selenium deprivation only with a reduction in growth rate. We conclude that loss of the potential to express a selenium-free hydrogenase can nevertheless be balanced by very small amounts of selenium hydrogenases under laboratory conditions in which the hydrogen supply is not likely to be a limiting growth factor.**

Hydrogenases are essential enzymes in the methanogenic archaeon *Methanococcus voltae*, which can gain its energy by the reduction of carbon dioxide with hydrogen. In our previous studies, biochemical and genetic approaches were taken in order to elucidate the reaction mechanism and synthesis regulation of hydrogenases in *M. voltae* (16, 22, 23). It was shown that *M. voltae* harbors four gene clusters that encode two [NiFe]-hydrogenases and a corresponding set of [NiFeSe]-hydrogenases (9). By investigating their transcription, we showed that the trace element selenium is involved in negative regulation of selenium-free [NiFe]-enzymes (1). Similar influences of selenium or metal availabilities on gene expression in other methanogens had previously been reported (10, 13).

The absence of any mutations within the set of negatively regulated hydrogenase operons in *M. voltae* indicated their essential role as backup systems, allowing cells to react to changing selenium availability in their environment, notably selenium deprivation. By insertion mutagenesis, we wanted to elucidate whether inactivation of the operons that encode selenium-free hydrogenases makes selenium deprivation a lethal condition.

So far, gene inactivation in *M. voltae* has been most easily achieved with integrating plasmids (7), since transposons are not available. These plasmids are based on *Escherichia coli* plasmid vectors which, in addition to their selection marker for this eubacterium, contain a puromycin resistance gene that is derived from *Streptomyces alboniger* and flanked by an *M. voltae* promoter and the transcription terminator of its *mcr* transcription unit (15). They integrate mostly site specifically (7) when a homologous chromosomal sequence is inserted next to the *pac* transcription unit just described. Chromosomal segments as short as 300 bp suffice for homologous integration of the plasmid into the chromosome (9a), while integration via the shorter terminator sequence has not been observed. If the chromosomal DNA fragment is an internal gene fragment that lacks both its 5′ and 3′ ends, integration leads to premature termination of transcription of this gene and genes which might be located downstream in a common transcription unit. We have used this insertion technique to interrupt operons that encode selenium-free hydrogenases.

Our present data indicate that the insertions obtained within encoding operons are not lethal under laboratory conditions, i.e., optimal hydrogen supply, even at very low selenium concentrations. This indicates that small amounts of selenium-containing hydrogenases allow sufficient compensation for the loss of selenium-free enzymes, which are normally induced under the culture conditions employed. In their natural habitat, these cells would thus be able to induce selenium-free enzymes as a backup system under conditions in which the residual selenium enzymes still guaranteed sufficient energy metabolism.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1.

Stock cultures and media. The complex medium used was similar to the complex medium described before (26), with 2 g of vitamin-free Casamino Acids. Vitamins were omitted. For stock cultures, transformation, and genomic DNA preparation, *M. voltae* cells were grown in 10 ml of complex medium in 100-ml serum bottles at 37°C with gentle shaking. Agar medium was complex medium supplemented with 1.3% (wt/vol) Bacto agar (Difco Laboratories, Augsburg, Germany). For transformation, agar was supplemented with 7.5 μg of puromycin dihydrochloride (Sigma, Deisenhofen, Germany) per ml. The anaerobic chamber used for plating and transformation procedures contained 78% N_2, 20% CO_2, and 2% H_2. For growth rate determinations, the previously described defined medium (1) was used with 10 μM sodium selenite when required. All media were reduced and dispensed in an anaerobic chamber that contained 95% N_2 and 5% H_2. Agar medium was prepared and poured as previously described (17).

Without added selenium, defined medium contained maximally 3 nM selenium, as determined by atomic absorption spectrometry. This value is the lower limit of detection in water and therefore an upper estimate of the true selenium content. In order to minimize the selenium concentration, all sulfates normally added were replaced by chlorides. In addition, chemically synthesized cysteine (purchased from Merck, Darmstadt, Germany) was used to eliminate random selenium contamination of natural cysteine obtained from yeast or keratin, which are frequent sources of commercial cysteine. Therefore, we believe that the actual selenium concentration in the depleted medium was less than 1 nm.

Cells were grown in 500-ml serum bottles with a maximal volume of 40 ml of

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
Strains		
E. coli Dh5α	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17* (r_K^- m_K^+) *supE44* k^- *thi-1 gyrA relA1*	Laboratories
M. voltae		
DSM 1537	Wild type	German Collection of Microorganisms
vhc3	*M. voltae* strain with an insertion of pvhc1.1 in the *vhc* hydrogenase operon	This study
frc3	*M. voltae* strain with an insertion of pfrc2.2 in the *frc* hydrogenase operon	This study
Plasmids		
pUC BM21	pUC19 derivative with extended multiple cloning site	Boehringer
Mip1	*M. voltae* integration vector	7
pfrc2.2	Integration vector that carries a 2.2-kb *Eco*RI fragment of the *frc* operon	This study
pvhc1.1	Integration vector that carries a 1.17-kb *Eco*RV-*Eco*RI fragment of the *vhc* operon	This study
pT7T3α19::*frc*	Plasmid with a 2.2-kb *Eco*RI insert of the *frc* operon used as a probe	S. Halboth
pT7T3α19::*vhc*	Plasmid with a 3.2-kb *Eco*RI insert that consists of part of the *frc* operon, the *frc-vhc* intergenic region, and nearly all of the *vhc* sequences	S. Halboth

defined medium at 2,000 hPa. Optical density at 578 nm was determined after reducing aliquots of cultures with sodium dithionite.

Protoplast formation and transformation. *M. voltae* was protoplasted and transformed as described previously (18), with the following modifications: 10 μg of pvhc1.1 or 50 μg of pfrc2.2 in 50 μl of Tris-EDTA buffer was added to 1×10^9 to 2×10^9 protoplasts in 2 ml of complex medium that contained 1% bovine serum albumin (BSA). Cultures were transferred to 100-ml serum bottles and incubated at 30°C. A control culture without plasmid DNA was treated similarly. After 21 h of gentle shaking at 30°C, cultures were spun down and resuspended in 1 ml of complex medium with BSA. Aliquots (100 and 800 μl) of the control were plated on selective agar medium in order to quantify spontaneously resistant cells. One hundred microliters of transformant cultures was diluted and spread on nonselective agar medium in order to determine plating efficiency. The same volumes of plasmid-containing cultures were spread on selective agar medium. After 3 days of incubation at 37°C, colonies on nonselective agar medium were counted for plating efficiency determination. Transformant colonies arose within 10 days of incubation.

Construction of vectors. The *pac* expression unit, which contained the puromycin transacetylase gene under the control of the promoter of the *M. voltae* methyl coenzyme M reductase transcription unit and was bordered by the corresponding terminator (11, 15), was derived from Mip1 (7). It was inserted as an *Eco*RV-*Kpn*I fragment into a *Hin*dII-*Kpn*I-digested *E. coli* vector, pUC BM21 (Boehringer, Mannheim, Germany). Then a 2.2-kb *Eco*RI fragment which contained parts of the *M. voltae frc* hydrogenase operon (Fig. 1) was inserted into the single *Eco*RI restriction site of pUC::*pac* to obtain the integration vector pfrc2.2. In all of the clones investigated, the *frc* fragment was inserted in the same direction as the resistance marker. For construction of the integration vector pvhc1.1, a 1.17-kb fragment that contained parts of the *vhc* hydrogenase operon (Fig. 1) was inserted into *Eco*RV-*Eco*RI-digested pUC BM21. Then a 1.7-kb *Eco*RI fragment that contained the *pac* expression unit from Mip1 was inserted into the single *Eco*RI restriction site of pUC::*vhc* to obtain pvhc1.1. Two clones of pvhc1.1 with opposite orientations of the *pac* unit were obtained. Plasmids were transformed into *E. coli* DH5α cells by standard methods (4, 19).

DNA and RNA preparations. Plasmid DNA was extracted from *E. coli* with the Qiagen plasmid kit (Diagen, Düsseldorf, Germany). *M. voltae* genomic DNA was prepared as previously described (1, 21). Total RNA was prepared by the single-step method (3).

Southern analysis. For filter hybridization (24), 1 to 3 μg of digested DNA was separated on 0.8% (wt/vol) agarose gels and transferred to Hybond-N membranes (Amersham, Braunschweig, Germany) by employing the manufacturer's protocol. All hybridizations were performed under stringent conditions with 50% formamide at 37°C. Filters were washed stringently with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) for 10 min at 37°C and subsequently at 60°C and with 0.1× SSC–0.1% SDS at 60°C.

Northern (RNA) analysis. Three to eight micrograms of total RNA was separated on denaturing 1.2% (wt/vol) agarose gels which contained 1.0% (vol/vol) formaldehyde (8). Then gels were rinsed in DEPC-treated water (3 min twice), and RNAs were transferred to nylon filters (Hybond-N) by capillary blotting with 10× SSC (19). RNAs were fixed on membranes by baking at 80°C for 30 min and subsequent UV cross-linking. Some of the filters were treated with methylene blue to stain *M. voltae* rRNA. Filters were hybridized under the stringent conditions described above at 42°C. Washing was done twice with 2× SSC–0.1% SDS (5 min each) at 42°C and twice with 0.2× SSC–0.1% SDS at 56°C for 15 min.

Radioactive labelling. Probes were labelled (5) with random hexanucleotide primers (pdN6; Boehringer). The *pac* gene (a 0.9-kb *Pst*I-*Nco*I fragment derived from Mip1), which has a high G+C content (12), was labelled with [α-^{32}P]dGTP (3,000 Ci/mmol; Hartmann Analytics, Braunschweig, Germany). Plasmids were labelled with [α-^{32}P]dATP (3,000 Ci/mmol; NEN DuPont, Dreieich, Germany). The following plasmids were used in hybridizations: pT7T3α19::*frc* (containing the 2.2-kb *Eco*RI fragment used for construction of pfrc2.2 [Fig. 1]), pT7T3α19::*vhc* (containing a 3.2-kb *Eco*RI fragment that consists of part of the *frc* operon, the *frc-vhc* intergenic region, and most of the *vhc* genomic DNA sequences), and pUC BM21.

Sequencing. Dideoxy sequencing (20) of constructs was done by using Sequenase version 2.0 and standard primers from U.S. Biochemicals, Bad Homburg, Germany.

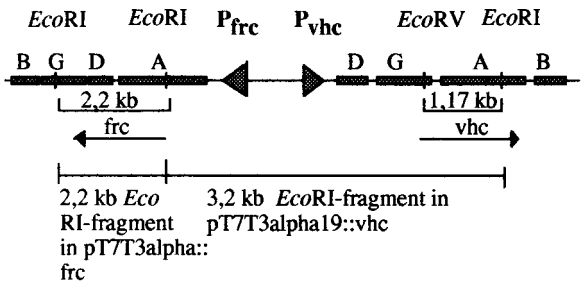

FIG. 1. Locations of vector-encoded *vhc* and *frc* inserts within their respective operons. The corresponding genes are identified as A, B, D, and G. P_{frc} and P_{vhc} are the putative promoters of these operons. Arrows indicate transcriptional directions. The 2.2-kb *frc* fragment and the 1.17-kb *vhc* fragment were used for the construction of pfrc2.2 and pvhc1.1, respectively. The locations of plasmid-encoded inserts used as probes in hybridization experiments are indicated.

RESULTS

A 10-μg mixture of both pvhc1.1 constructs was introduced into protoplasted *M. voltae* cells. The plating efficiency for this culture on nonselective agar medium was approximately 10%. Ten to twenty spontaneously resistant colonies per 100 μl of control culture occurred when plated on selective agar medium with 7.5 μg of puromycin per ml. Ten to one hundred colonies per 100 μl of transformant culture were obtained on selective agar medium. These colonies were bigger (in size) than those of spontaneously resistant mutants. For preparation of genomic DNA and restriction analysis, clones obtained after transformation by pvhc1.1 were grown in liquid complex medium. Genomic DNA from *M. voltae* wild-type cells was used as a negative control for Southern hybridization. Genomic DNAs were digested with suitable restriction enzymes, which are known to cut inside the integration vector and/or within adjacent genomic DNA sequences (see Fig. 2 and 3). Restriction analysis revealed that the integration vector had inte-

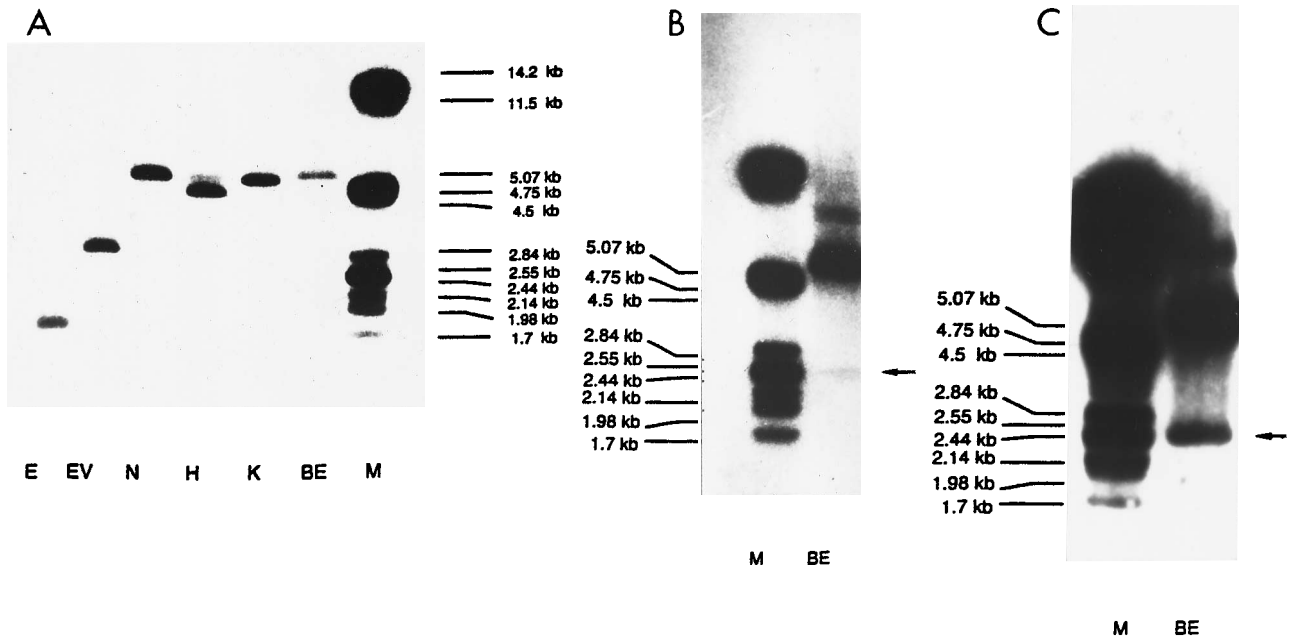

FIG. 2. Southern hybridization analysis of the vhc3 integration mutant. (A) Autoradiogram of Southern hybridization of vhc3 genomic DNA probed with the *pac* gene. Genomic DNA was extracted from culture after serial transfers in selenium-deficient medium. DNA was digested with *Eco*RI (E), *Eco*RV (EV), *Nde*I (N), *Hin*dIII (H), *Kpn*I (K), and *Bst*EII (BE). λ DNA digested with *Pst*I was used as a size marker (M). The corresponding sizes of restriction fragments are shown in Fig. 3. (B) Autoradiogram of panel A exposed for 60 h. Only the *Bst*EII digest of vhc3 DNA (BE) and the marker lane (M) are shown. After longer (15 times) exposure, the smaller *Bst*EII fragment (compare with Fig. 3), indicated by an arrow, was detectable on this blot. (C) Southern hybridization of vhc3 genomic DNA derived from the first cultures in complex medium. DNA was digested with *Bst*EII (BE) and hybridized with the *pac* probe. The smaller *Bst*EII fragment, as depicted in Fig. 3, is indicated by an arrow. λ DNA digested with *Pst*I was used as a molecular weight marker.

grated by site-specific recombination into the homologous hydrogenase DNA sequence. Except for one clone which carried the *pac* expression unit as a tandem repeat (data not shown), all clones had an identical restriction pattern. In Fig. 2A, Southern hybridization of one representative clone (vhc3) with the *pac* probe is shown. Neither *pac* nor plasmid probes hybridized with wild-type genomic DNA (not shown in Fig. 2A).

On the basis of the sizes of restriction fragments of vhc3 transformant genomic DNA, an integration pattern for the mutant (Fig. 3), showing integration of pvhc1.1 into homologous *vhc* DNA sequences and intrachromosomal amplification of the vector, was deduced. To investigate this amplification, genomic DNA was digested with *Bst*EII and probed with *pac*. *Bst*EII has a single recognition sequence within *pac* and the *vhcG* sequence. Two fragments of the predicted sizes occurred on this blot, but the smaller one was detectable only after longer film exposure (Fig. 2B). Amplification was again evident when DNA was cut with *Nde*I, which has a single site in pUC and the upstream intergenic region that is located between the promoters in front of the *vhc* and *frc* operons (Fig. 3). Finally, vhc3 DNA was digested with *Bam*HI-*Pst*I (not shown in Fig. 2A) because *Bam*HI cuts just in front of *pac* only, whereas there are two *Pst*I sites (one is 5′ of the *Bam*HI site and the other is within the *vhcA* genomic sequence). All of the expected fragments were obtained (Fig. 3). The smaller *Nde*I fragment (Fig. 3) was again visible only after prolonged exposure of the autoradiogram (data not shown). From the larger *Bst*EII and *Nde*I fragments, we concluded that pvhc1.1 had integrated into its target sequence at least twice. Site-specific integration was additionally proven by digestion with *Bst*EII and *Nde*I, which had no recognition sequences within the *vhc* region used for vector construction. The other restriction fragments which were probed with *pac* showed the arrangements of the various vector components, in particular, an inverted *pac* unit with respect to the putative promoter P_{vhc} (Fig. 3).

According to the pattern in Fig. 3, transcription of *vhcA* had to be impaired. The 5′ terminal copy of *vhcA* is incomplete because its 3′ end is missing in pvhc1.1. The copy of *vhcA* at the 3′ end is intact but separated from its promoter.

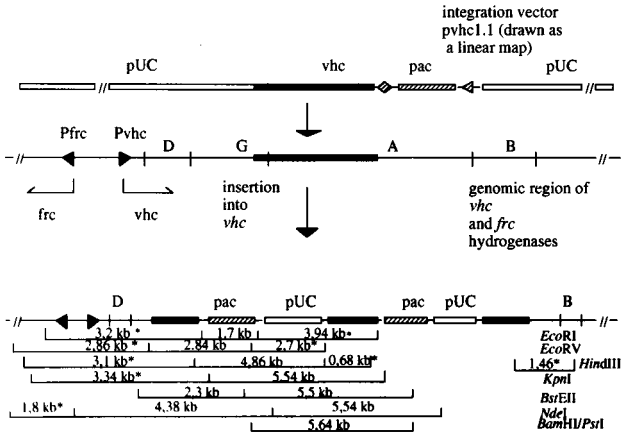

FIG. 3. Scheme of pvhc1.1 integration into the *vhc* operon. pUC sequences are drawn as open bars. Black bars represent homologous *vhc* regions. The *pac* expression unit is represented by hatched bars (for simplification, regulatory elements are not shown in the integration pattern). P_{frc} and P_{vhc} are the putative promoters which are located in the intergenic region between the *vhc* and *frc* operons. The D, G, A, and B genes of the *vhc* operon are drawn to scale. Restriction fragments (as derived from Fig. 2A) are shown below the scheme. Fragments that were obtained with the pT7T3α19::*vhc* probe are indicated by asterisks. Signals which were detectable with both *pac* and plasmid probes are not marked separately.

From the location of the *pac* probe, we predicted nearly equal signal strengths for the two *Bst*EII and two *Nde*I restriction fragments. This was indeed seen for Southern hybridizations performed with genomic DNAs at the beginning of serial transfers (data shown for *Bst*EII digest in Fig. 2C). The vhc3 genomic DNA in Fig. 2A was prepared from a culture that had been serially transferred 15 times in defined medium without selenium. The transferred inoculum was 5% each time. From restriction analyses of primary transformant cultures in complex medium, we conclude that the integration pattern was not altered. However, the relative intensity of the short 2.3-kb *Bst*EII fragment decreased. The same was true for the 4.38-kb *Nde*I fragment (data not shown). To investigate potential DNA rearrangements in vhc3 with respect to these faint signals, the Southern blot (corresponding to Fig. 2A) was additionally hybridized with pT7T3α19::*vhc* (data not shown). The corresponding locations and sizes of these restriction fragments are shown in Fig. 3 (indicated by asterisks). All of the expected restriction fragments in vhc3 genomic DNA were detected. A 3.34-kb *Kpn*I fragment (Fig. 3) proved that no noticeable deletions had taken place upstream of the insertion. This fragment could have been obtained only by the indicated arrangement because there was a single *Kpn*I recognition sequence downstream of the terminator contained in the expression unit. No other restriction fragments were obtained with *pac* or plasmid probes. Therefore, a second integration of pvhc1.1 in a different chromosomal location could be ruled out. PCR analysis, in which the 5′ region of the first *pac* copy, including the upstream promoter region, was investigated, confirmed this arrangement of vhc3 DNA (Fig. 3). From these findings, we conclude that the integration vector was strongly amplified during serial transfers of vhc3. As a consequence, larger *Bst*EII and *Nde*I fragments occurred much earlier during film exposure than did smaller fragments because of the increased copy number of the *pac* gene.

From pfrc2.2 transfection into *M. voltae*, 100 colonies per 900 μl of plated culture were obtained. When the same volume from a control culture was plated on selective agar medium, 21 spontanously resistant colonies grew up. After transformation, the plating efficiency on nonselective agar medium was approximately 14%. Five clones were chosen for Southern hybridization. All of them had site-specific insertions within the *frc* operon. Figure 4 shows the restriction analysis of genomic DNA from a representative clone (frc3). The radioactively labelled *pac* gene was used as a probe. As was the case with *vhc* mutants, genomic DNA from frc3 was prepared from a culture that had been transferred several times in selenium-deficient medium. From the data of respective Southern hybridizations, a scheme for pfrc2.2 insertion into the corresponding hydrogenase sequences was deduced (Fig. 5). In contrast to vhc3, no amplification took place. Only one *Bst*EII fragment was detectable (Fig. 5) because of a restriction site within the resistance gene and one site in the adjacent *vhc* operon (compare Fig. 3 and 5). Genomic hydrogenase sequences and the *pac* gene would be transcribed in the same direction, as shown by the arrangement of the other restriction fragments. The locations of vector elements within the *frc* locus were confirmed by a second hybridization with the plasmid, as described above (Fig. 5). The smaller *Nde*I fragment in Fig. 4 could not be assigned to known *Nde*I sites. Double digests, a *Bam*HI digest and hybridization with *pac*, revealed a new *Nde*I site upstream of the expression unit within the pUC sequence, which did not occur in all cells from frc3 cultures (the restriction fragments and *Nde*I site are shown in Fig. 5). Southern hybridizations of frc3 genomic DNA, prepared from the first cultures in complex medium, revealed that this mutation had occurred earlier under nonselective conditions for pUC BM21 maintenance. This neutral mutation was not present in all cells from frc3 cultures (compare, e.g., the largest *Nde*I fragment [Fig. 5]).

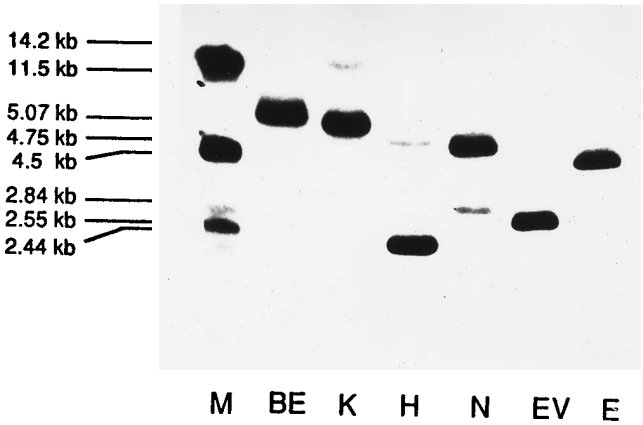

FIG. 4. Autoradiogram following Southern hybridization of frc3 genomic DNA with the *pac* probe. frc3 genomic DNA was digested with *Eco*RI (E), *Eco*RV (EV), *Nde*I (N), *Hin*dIII (H), *Kpn*I (K), and *Bst*EII (BE). *Pst*I-digested λ DNA was used as a molecular weight marker (M). DNA was hybridized and washed under the stringent conditions described in Materials and Methods. The resulting sizes and locations of frc3 restriction fragments are shown in Fig. 5.

From the integration pattern of pfrc2.2, it can be concluded that transcription of *frcG* and *frcB* should be impaired. The upstream copy of *frcG* is incomplete, whereas the downstream copy, which is restored by recombination, is separated from the *frc* promoter by termination downstream of the *pac* gene.

As previously described (1), transcription of the operons

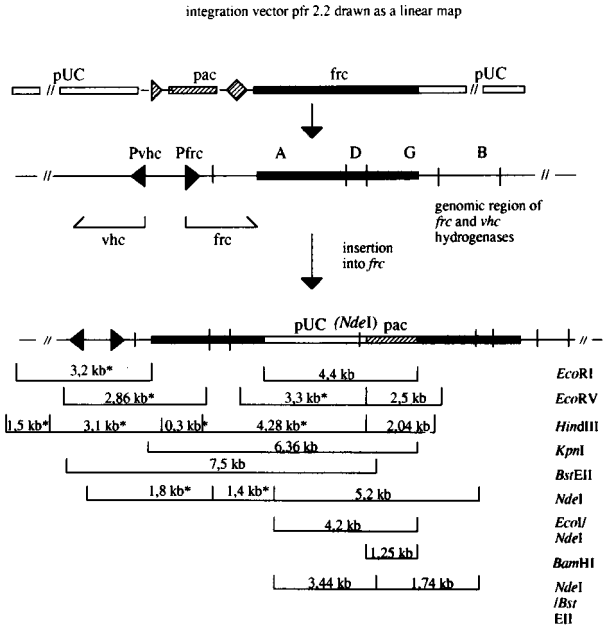

FIG. 5. Scheme of pfrc2.2 integration into the *frc* operon. The symbols used in Fig. 3 are used here for vector elements and the genomic site of vector insertion. Restriction fragments which were obtained from Southern analysis (Fig. 4) are shown below the scheme. Additional fragments which were detected with the pT7T3α19::*vhc* probe are indicated by asterisks. *Eco*RI-*Nde*I, *Bst*EII-*Nde*I, and *Bam*HI fragments which hybridized with the *pac* probe (data not shown in Fig. 4) are depicted below the scheme. Additional fragments which were detected because of the adventitious new *Nde*I site are not shown.

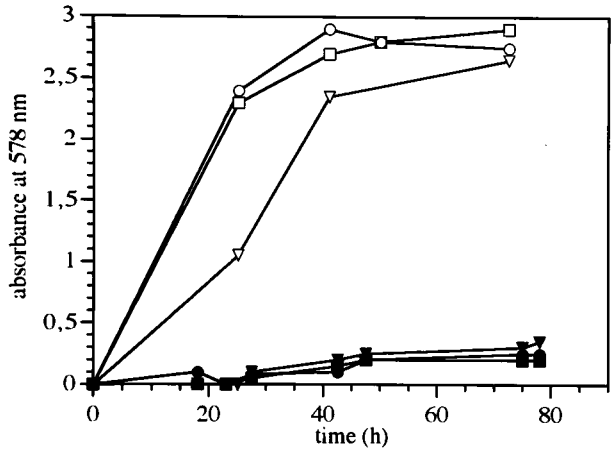

FIG. 6. Growth of wild-type and mutant *M. voltae* strains in defined medium with (open symbols) and without (filled symbols) added selenium. Cell density was measured as the A_{578}. The wild type, mutant vhc3, and mutant frc3 are symbolized by circles, squares, and triangles, respectively.

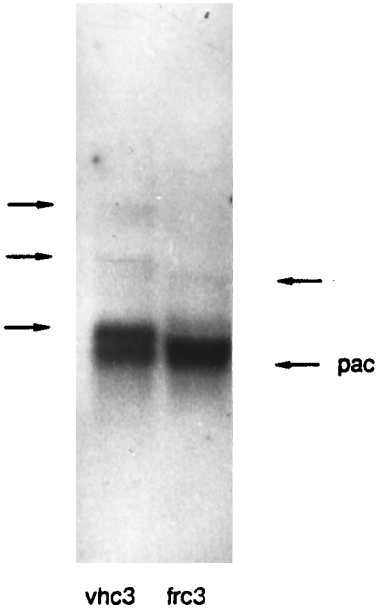

FIG. 7. Northern hybridization of total RNAs derived from vhc3 and frc3 mutants. In both RNAs, transcripts of equal size (identified as *pac*) were detected with the *pac* probe. Fragments which are larger than the *pac* transcript are indicated by arrows. Larger transcripts were not due to unspecific hybridization with rRNA, as was proven by staining the same filters with methylene blue. The putative locations of these transcripts are shown in Fig. 8.

investigated above was strongly induced when selenium was withdrawn. Therefore, we expected insertions in either *vhc* or *frc* to be lethal to cells under selenium depletion or at least to affect growth rates under these conditions. The growth rates of vhc3 and frc3 cultures, which were transferred several times in complex medium without selenium, were already reduced after the second transfer. Absorption values for subsequently transferred cultures (up to the 13th transfer) were similar to those shown in Fig. 6. Remarkably, a wild-type culture which had been transferred to the same medium showed reduced growth that was similar to that of mutants (Fig. 6). Upon readdition of selenium to cultures, their growth rates returned to their levels before selenium deprivation.

Two possibilities with respect to the nonlethality of insertions were considered. Either termination by the vector-encoded terminator was not as effective as predicted, and thus, the disrupted genes were translated from hybrid transcripts that started at a promoter in the vector sequence and functioned in *M. voltae*; or in spite of selenium deficiency, the trace element content was sufficient for the translation of selenocysteine-containing hydrogenases.

Transcription was investigated by Northern hybridization of total RNAs that had been extracted from vhc3 and frc3 cultures with the *pac* probe. A distinct signal was detected in both RNAs (Fig. 7); it was smaller than the 16S rRNA of *M. voltae*. It belonged to the 1.0-kb transcript of the *pac* gene which had been induced from the *mcr* promoter and terminated by the *mcr* terminator (expression unit derived from Mip1). Larger species (indicated by arrows in Fig. 7) of frc3 and vhc3 RNAs were also weakly probed with the *pac* sequence. They were identified as hybrid transcripts that started within the pUC sequences at random promoters, since they were also probed with pUC BM21 (data not shown). The strong signal obtained with vhc3 RNA, which is slightly larger than the *pac* transcript itself, most likely started directly downstream of the *mcr* promoter from pUC sequences that served as a fortuitous promoter in *M. voltae* (Fig. 8). With regard to this transcript, which is missing in frc3 RNA, it is worth mentioning that the pUC backbone in the vhc3 arrangement has an orientation that is opposite of that of the frc3 integration site (indicated by the pUC *Nde*I site in Fig. 8). The possible locations of larger transcripts are shown in Fig. 8. Total RNAs from vhc3 transformants were additionally hybridized with a plasmid that contained nearly all of the *vhc* sequences (Fig. 1). Both in RNA extracted from cells grown with selenium and in RNA extracted from cells deprived of selenium, no transcripts other than those probed by pUC were detected (data not shown). With total RNAs from frc3 transformants which had been hybridized with pUC and a plasmid-encoded 2.2-kb *frc* fragment (Fig. 1 and data not shown), an additional transcript was seen upon selenium deprivation of cultures. Figure 8 depicts all of the putative transcripts according to interrupted *vhc* and *frc* loci. The distinct signals obtained from all Northern hybridiza-

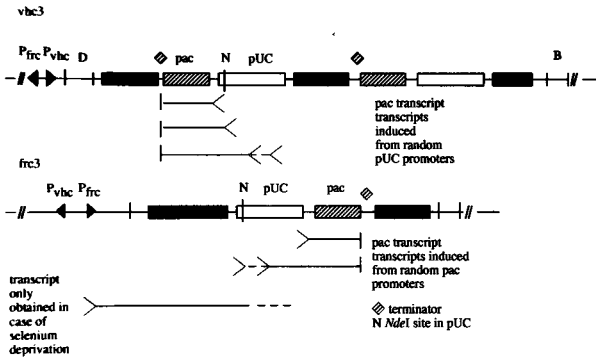

FIG. 8. Locations of transcripts within the interrupted *vhc* and *frc* operons of *M. voltae*. For vector elements and genomic sequences, the same symbols were used as in Fig. 3 and 5. Interrupted arrows indicate the putative locations of transcripts which were visualized with the *pac* probe and/or pUC BM21. The promoter of the *vhc* operon (P_{vhc}) and the promoter of the expression unit (indicated by the direction of the *pac* transcript) point toward each other. In frc3, the promoter of the *frc* operon (P_{frc}) is oriented in the same direction as the strong promoter in front of the *pac* gene. N, the position of the original pUC *Nde*I restriction site as a marker for the orientation of pUC within the indicated locus. The transcript which starts at P_{frc} was detected with a pUC probe or the pT7T3α19::*frc* probe (Fig. 1).

tions strongly indicated effective transcriptional termination by the *mcr* terminator, which is located downstream of the *pac* gene within the expression unit.

DISCUSSION

The coordinately regulated *vhc* and *frc* operons from *M. voltae* were disrupted by insertions of integration vectors. Up to now, homologous recombination between the genes of interest has been the only means to introduce *M. voltae* DNA or foreign DNA into cells (2, 7, 14, 18). We obtained insertions by recombination between homologous sequences which were parts of their respective operons (Fig. 1).

In view of the considerable lengths of these insertions, it was considered unlikely a priori that a readthrough transcript that led to the expression of genes downstream would be synthesized. In addition, in the case of the frc3 insertion, transcription would terminate at the *mcr* terminator that follows the *pac* selection cassette. Multiple integration in the vhc3 mutant strengthens the general argument presented above. In this case, transcription from the *mcr* promoter of the inserted plasmid forms antisense RNA, which certainly further reduces the chance of formation of a readthrough transcript.

We had previously found that selenium-free hydrogenases were induced upon selenium deprivation. Therefore, transformants that carried polar insertions in the operons that encoded these hydrogenases could have been expected to be unable to survive upon selenium withdrawal. However, although their growth was markedly reduced under this condition, their reaction did not differ from the behavior of wild-type cells (Fig. 6), and mutants also recovered after readdition of selenium.

The Northern blot shown in Fig. 7 gave no indication of readthrough transcripts in these operons, a possibility which cannot, however, be completely ruled out. We consider this an unlikely explanation for the survival of these cells. Instead, we assume that the residual selenium content of the medium, which might even be the result of constant leakage of the trace element from the glassware used (6), has allowed low-level constitutive production of selenium-containing hydrogenases that is sufficient to sustain the slow growth of cells in the absence of selenium-free enzymes under laboratory conditions. Future measurements of specific hydrogenase activities in extracts of selenium-limited wild-type cells and insertion mutants of the described types may indicate whether the total hydrogenase activities in these two cases differ.

Under laboratory conditions, the hydrogen supply probably does not limit the growth of *M. voltae*, in contrast to the situation in its natural habitat (25). It is therefore entirely possible that under natural conditions, which cannot be mimicked in pure cultures, the loss of genetic information for selenium-free hydrogenases is indeed conditional lethal.

The ability of cells to induce selenium-free enzymes at selenium levels that are sufficient for low-level production of selenium-containing enzymes would also allow efficient transition from growth with selenium to growth without this trace element in the natural habitat upon a change in the selenium supply. This is important for cell survival since the concentration of soluble selenium fluctuates and can drop below concentrations of 1 nM, especially in oxygen-free soils and sediments (6).

ACKNOWLEDGMENTS

We thank G. B. Patel for communicating the transformation procedure to us prior to its publication.

This work was supported by the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

REFERENCES

1. **Berghöfer, Y., K. Agha-Amiri, and A. Klein.** 1994. Selenium is involved in the negative regulation of the expression of selenium-free [NiFe] hydrogenases in *Methanococcus voltae*. Mol. Gen. Genet. **242:**369–373.
2. **Bertani, G., and L. Baresi.** 1987. Genetic transformation in the methanogen *Methanococcus voltae* PS. J. Bacteriol. **169:**2730–2738.
3. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
4. **Cohen, S. N., A. C. Y. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA **69:**2110–2114.
5. **Feinberg, A. P., and B. Vogelstein.** 1983. A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **132:**6–13.
6. **Fishbein, L.** 1991. Metals and metalloids, and their ions and compounds, p. 1153–1190. *In* E. Merian (ed.), Metals and their compounds in the environment. VCH Verlagsgesellschaft mbH, Weinheim, Germany.
7. **Gernhardt, P., O. Possot, M. Foglino, L. Sibold, and A. Klein.** 1990. Construction of an integration vector for use in the archaebacterium *Methanococcus voltae* and expression of a eubacterial resistance gene. Mol. Gen. Genet. **221:**273–279.
8. **Gilman, M.** 1987. Preparation and analysis of RNA, p. 4.0.1–4.1.6. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology, vol. 1. John Wiley and Sons, Inc., New York.
9. **Halboth, S., and A. Klein.** 1992. *Methanococcus voltae* harbours four gene clusters potentially encoding two [NiFe] and two [NiFeSe] hydrogenases, each of the cofactor F_{420}-reducing or F_{420}-non-reducing types. Mol. Gen. Genet. **233:**217–224.

9a. **Jarrell, K. F., and A. Klein.** Unpublished data.

10. **Jones, J. B., and T. Stadtman.** 1981. Selenium-dependent and selenium-independent formate dehydrogenase of *Methanococcus vannielii*: separation of the two forms and characterization of the selenium-independent form. J. Biol. Chem. **256:**656–663.
11. **Klein, A., R. Allmansberger, M. Bokranz, S. Knaub, B. Müller, and E. Muth.** 1988. Comparative analysis of genes encoding methyl coenzyme M reductase in methanogenic bacteria. Mol. Gen. Genet. **213:**409–420.
12. **Lacalle, R. A., D. Pulido, J. Vara, M. Zalacain, and A. Jiménez.** 1989. Molecular analysis of the pac gene encoding a puromycin N-acetyl transferase from *Streptomyces alboniger*. Gene **79:**375–380.
13. **May, H. D., P. S. Patel, and J. G. Ferry.** 1988. Effect of molybdenum and tungsten on synthesis and composition of formate dehydrogenase in *Methanobacterium formicicum*. J. Bacteriol. **170:**3384–3389.
14. **Micheletti, P. A., K. A. Sment, and J. Konisky.** 1991. Isolation of a coenzyme M-auxotrophic mutant and transformation by electroporation in *Methanococcus voltae*. J. Bacteriol. **173:**3414–3418.
15. **Müller, B., R. Allmansberger, and A. Klein.** 1985. Termination of a transcription unit comprising highly expressed genes in the archaebacterium *Methanococcus voltae*. Nucleic Acids Res. **13:**6439–6445.
16. **Muth, E., E. Mörschel, and A. Klein.** 1987. Purification and characterization of an 8-hydroxy-5-deazaflavin-reducing hydrogenase from the archaebacterium *Methanococcus voltae*. Eur. J. Biochem. **169:**571–577.
17. **Patel, G. B., C. G. Choquet, J. H. E. Nash, and G. D. Sprott.** 1993. Formation and regeneration of *Methanococcus voltae* protoplasts. Appl. Environ. Microbiol. **59:**27–33.
18. **Patel, G. B., J. H. E. Nash, B. J. Agnew, and G. D. Sprott.** 1994. Natural and electroporation-mediated transformation of *Methanococcus voltae* protoplasts. Appl. Environ. Microbiol. **60:**903–907.
19. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
20. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
21. **Sitzmann, J., and A. Klein.** 1991. Physical and genetic map of the *Methanococcus voltae* chromosome. Mol. Microbiol. **5:**505–513.
22. **Sorgenfrei, O., A. Klein, and S. P. J. Albracht.** 1993. Influence of illumination on the electronic interaction between ^{77}Se and nickel in active F_{420}-non-reducing hydrogenase from *Methanococcus voltae*. FEBS Lett. **332:**291–297.
23. **Sorgenfrei, O., D. Linder, M. Karas, and A. Klein.** 1993. A novel very small subunit of a selenium containing [NiFe] hydrogenase of *Methanococcus voltae* is posttranslationally processed by cleavage at a defined position. Eur. J. Biochem. **213:**1355–1358.
24. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:**503–517.
25. **Thauer, R. K.** 1990. Energy metabolism of methanogenic bacteria. Biochim. Biophys. Acta **1018:**256–259.
26. **Whitman, W. B., E. Ankwanda, and R. S. Wolfe.** 1982. Nutrition and carbon metabolism of *Methanococcus voltae*. J. Bacteriol. **149:**852–863.