Genetic Identification of Three ABC Transporters as Essential Elements for Nitrate Respiration in *Haloferax volcanii*

Christian Wanner and Jörg Soppa

Biozentrum Niederursel, Institut für Mikrobiologie, J. W. Goethe-Universität D-60439 Frankfurt, Germany

Manuscript received March 22, 1999
Accepted for publication May 13, 1999

ABSTRACT

More than 40 nitrate respiration-deficient mutants of *Haloferax volcanii* belonging to three different phenotypic classes were isolated. All 15 mutants of the null phenotype were complemented with a genomic library of the wild type. Wild-type copies of mutated genes were recovered from complemented mutants using two different approaches. The DNA sequences of 13 isolated fragments were determined. Five fragments were found to overlap; therefore nine different genomic regions containing genes essential for nitrate respiration could be identified. Three genomic regions containing genes coding for subunits of ABC transporters were further characterized. In two cases, genes coding for an ATP-binding subunit and a permease subunit were clustered and overlapped by four nucleotides. The third gene for a permease subunit had no additional ABC transporter gene in proximity. One ABC transporter was found to be glucose specific. The mutant reveals that the ABC transporter solely mediates anaerobic glucose transport. Based on sequence similarity, the second ABC transporter is proposed to be molybdate specific, explaining its essential role in nitrate respiration. The third ABC transporter is proposed to be anion specific. Genome sequencing has shown that ABC transporters are widespread in Archaea. Nevertheless, this study represents only the second example of a functional characterization.

ARCHAEA form a third monophyletic domain of living organisms apart from Bacteria and Eukarya (Woese *et al.* 1990). Many archaeal components involved in replication, transcription, and translation are more closely related to eukaryal than to bacterial proteins. During recent years considerable progress has been made toward understanding the archaeal basal transcription apparatus (for a review see Soppa 1999). In short, Archaea share with Eukarya the TATA box and a TFB recognition element as major promoter elements, the basal transcription factors TATA box-binding protein and transcription factor B, which bind to these DNA elements, and a multicomponent RNA polymerase.

In contrast to the increasing knowledge about basal transcription, only a little information about regulation of transcription is available. As many components of the archaeal central metabolism are more closely related to bacterial than to eukaryal enzymes, it seems interesting to study how "Bacteria-like" genes are regulated in organisms with a "Eukarya-like" transcription apparatus. A variety of model systems that will allow study of transcriptional regulation in Archaea emerge (*e.g.*, Ruepp and Soppa 1996; Yang *et al.* 1996; Cheung *et al.* 1997; Cohenkupiec *et al.* 1997; Kuo *et al.* 1997; Sorgenfrei *et al.* 1997; Krüger *et al.* 1998; Soppa *et al.* 1998). Several distinct regulatory mechanisms appear to be involved. To broaden the view, we aim at establishing the nitrate respiration regulon of *Haloferax volcanii* as an additional example. Nitrate respiration has been extensively studied with Bacteria, and a vast body of knowledge has been accumulated concerning both the enzymes involved and the regulation of transcription of the corresponding genes (for reviews see Stewart 1988, 1993; Zumft 1997; Lin and Stewart 1998). Therefore, results for archaeal nitrate respiration can readily be compared with those for bacterial nitrate respiration. However, to date, little information about archaeal nitrate respiration is available. It was reported that several haloarchaeal species are able to grow anaerobically with nitrate (Oren and Trüper 1990), and nitrate reductases or their soluble domains of three species have been identified (Hochstein and Lang 1991; Alvarezossorio *et al.* 1992; Bickelsandkötter and Ufer 1995). However, in none of these cases have the genes coding for these enzymes been reported. To characterize nitrate respiration of *H. volcanii*, the very general approach of complementation of nitrate respiration-deficient mutants with a genomic library was chosen. Until now this approach has been possible only for a limited number of archaeal species as it requires a high transformation frequency. The advantage of this approach is that it allows access to all components involved, highly regulated structural genes as well as poorly expressed constitutive genes for regulatory proteins. The isolation of nitrate respiration-deficient mutants has been described previously (Soppa 1998). Here, we report on the complementation of mu-

Corresponding author: Jörg Soppa, J. W. Goethe-Universität, Biozentrum Niederursel, Institut für Mikrobiologie, Marie-Curie-Str. 9, D-60439 Frankfurt, Germany. E-mail: soppa@em.uni-frankfurt.de

tants, isolation of wild-type copies of essential genes, and the characterization of three genomic regions coding for ABC transporters involved in nitrate respiration.

ABC transporters couple the hydrolysis of ATP to solute transport across biological membranes (for reviews see Boos and Lucht 1996; Schneider and Hunke 1998). ABC transporters form a widespread protein family present in Archaea, Bacteria, and Eukarya. The ABC transporter genes from seven completely sequenced genomes have been collected and cataloged into 25 ortholog groups (Tomii and Kanehisa 1998). In contrast to the vast knowledge of primary sequences, only a minor fraction of ABC transporters have been functionally characterized, and the annotated "substrate specificities" of many ABC transporters rest solely on primary sequence similarities. Before this study, the trehalose/maltose transporter of *Thermococcus litoralis* was the only archaeal ABC transporter with an experimentally proven substrate specificity (Horlacher *et al.* 1998). In addition, the multidrug-efflux system of *H. volcanii* can be assumed to be an ABC transporter (Komatsubara *et al.* 1996), but the gene sequence is not known.

ABC transporters are typically composed of two membrane subunits and two ATP-binding subunits, both of which can be either homodimeric or heterodimeric. In addition, the genes for two or more subunits can be fused, and the functions are then fulfilled by protein domains of fusion proteins instead of different polypeptides.

The ATP-binding subunit is typically characterized by several well-conserved sequence motifs: a Walker site A (GXXGXGKS/T; X represents any amino acid), a helical domain, a linker peptide (LSGGQQ/R/KQR), a Walker site B (hhhhD; h represents a hydrophobic amino acid), and a switch region with a conserved histidine. Membrane subunits typically contain five to six membrane-spanning helices and a so-called EAA-loop ~100 amino acids from the C terminus (EAAXXLGX$_{11}$LP). Many bacterial ABC transporters contain, in addition, periplasmic solute-binding proteins, which carry the substrate to the transport complex. Binding proteins from gram-negative bacteria are soluble in the periplasm, whereas binding proteins from gram-positive bacteria are bound to the cytoplasmic membrane by a lipid anchor or by electrostatic forces.

MATERIALS AND METHODS

Organisms: *H. volcanii* WR340 was obtained from Moshe Mevarech (Tel Aviv University, Tel Aviv, Israel). *Escherichia coli* XL-1 Blue MRF′ and *E. coli* SCS110 were obtained from Stratagene (Heidelberg, Germany).

Media and growth conditions: *H. volcanii* WR340 was grown at 42° either in complex medium as described by Cline *et al.* (1989) or in synthetic medium as described by Mevarech and Werczberger (1985), additionally supplemented with 0.2 g/liter histidine. For anaerobic growth, sodium nitrate was added at a final concentration of 50 mm, and either glucose or pyruvate were added at a final concentration of 25 mm. Solid media contained 1.2% agar and the plates were sealed in a plastic bag to minimize evaporation. For selective growth, the media contained 0.1 mg/liter novobiocin. *E. coli* XL-1 Blue MRF′ and SCS110 were grown in SOB media (Hanahan 1985). For selective growth, the medium was supplemented with 100 mg/liter ampicillin. Solid media contained 1.2% agar.

Molecular biological methods: General molecular biological methods were performed according to Sambrook *et al.* (1989) or according to the manufacturer's instructions. Restriction enzymes and other DNA-modifying enzymes were purchased from Boehringer (Mannheim, Germany) and MBI Fermentas (St. Leon-Rot, Germany). Oligonucleotides were purchased from ARK Scientific (Darmstadt, Germany). *E. coli* was transformed by electroporation. Plasmids were isolated using JET-star ion exchange columns from GENOMED (Bad Oeynhausen, Germany). DNA sequences were determined using the BigDye Terminator RR Mix and the ABI Prism sequencer 377 (PE Applied Biosystems, Weiterstadt, Germany).

Construction of a genomic library and complementation of nitrate respiration-deficient mutants: Genomic DNA from wild-type *H. volcanii* was isolated as described previously (Rosenshine *et al.* 1987). The kinetics of cleavage with the restriction endonuclease *Hpa*II was determined analytically and conditions that resulted in a high fraction of partially cleaved DNA fragments 3–5 kbp in length were derived. After preparative cleavage, the fragment mixture was separated electrophoretically and fragments of the desired size range were isolated with the QIAEX II gene extraction kit (QIAGEN, Hilden, Germany). They were ligated into the suicide vector pBN1 (Nowosad 1991) cleaved with *Cla*I, which yields *Hpa*II-compatible ends. pBN1 contains a novobiocin-resistance gene (Holmes and Dyall-Smith 1991), which allows the selection of transgenic *H. volcanii* after transformation.

H. volcanii has a restriction system that recognizes GATC-methylated DNA, and consequently the transformation frequency using unmethylated DNA is 1000-fold higher than that using dam-methylated DNA (Holmes *et al.* 1991). Therefore, the genomic library was passed through the *dam*-negative *E. coli* strain SCS110 before transformation of *H. volcanii* mutants. Transformation of *H. volcanii* was performed essentially as described (Cline *et al.* 1989). After transformation of a nitrate respiration-deficient mutant, an aliquot was used to determine the number of independent novobiocin-resistant clones by plating serial dilutions on selective plates. The remaining mixture of transformed cells was grown aerobically in liquid media containing novobiocin (0.1 μg/ml) to select for cells that had stably integrated a plasmid library entry into the chromosome. Subsequently, an aliquot was used to inoculate a culture placed under anaerobic conditions to select for complemented mutants with restored capability to grow via nitrate respiration. Growth was recorded colorimetrically with a Klett photometer (Klett Manufacturing Co., New York).

Reisolation of wild-type copies after complementation: Two strategies were used to reisolate wild-type copies of the genes defective in the nitrate respiration-deficient mutants (see results). In both cases, genomic DNA of the complemented mutants was isolated and cleaved with restriction endonucleases at a DNA concentration of 50 ng/μl. After endonuclease inactivation, the DNA fragments were religated at a DNA concentration of 5 ng/μl, resulting in intramolecular circularization. The fragment mixture was used to transform *E. coli*. Only religated fragments containing the vector pBN1 and haloarchaeal genomic DNA flanking the integration site were able to yield stable ampicillin-resistant transformants. Plasmids were isolated from several independent clones and characterized by mapping with restriction endonucleases. Complemen-

tation of the respective nitrate respiration-deficient *H. volcanii* mutants allowed us to identify plasmids with haloarchaeal genomic fragments including wild-type copies of the mutated genes. After this functional test, the inserts of selected plasmids were sequenced on both strands by primer walking.

Northern blot analysis: For Northern blot analysis, total RNA was isolated from wild-type cells according to Chomczynski and Sacchi (1987). The RNA was separated using formaldehyde-containing gels (Sambrook *et al.* 1989) along with unlabeled PCR fragments corresponding to the respective probes (see below), which were used as standards to determine detection limit. RNA and standards were transferred to uncharged nylon membranes (QIAGEN) by downward capillary blotting (Chomczynski 1992). Labeled probes were generated using a PCR approach including digoxigenin-11-dUTP purchased from Boehringer. After hybridization, digoxigenin-containing bands were visualized using an alkaline phosphatase-coupled anti-digoxigenin antibody (Boehringer) and the chemiluminescent substrate CPD-star (Tropix/PE Applied Biosystems) according to the manufacturer's instructions.

Databases, computer programs, and internet sites: The EMBL nucleotide sequence database (Stoesser *et al.* 1999), the SWISSPROT and TREMBL protein sequence databases (Bairoch and Apweiler 1999), and the PROSITE database (Hofmann *et al.* 1999) were used for sequence comparison and sequence motif detection. The programs FASTA (Pearson 1990), SRS (Etzold *et al.* 1996), ClustalW (Higgins *et al.* 1994) and MacVector (Eastman Kodak Chemical Company, New Haven, CT) were applied for sequence comparison, sequence retrieval, multiple sequence alignments, and DNA and protein sequence analysis. The following internet sites were frequently used:

http://srs.ebi.ac.uk:5000/—for sequence retrieval
http://www2.ebi.ac.uk/services.html—for similarity searches, pattern searches, and multiple sequence alignments
http://www.expasy.ch/—for protein sequence analysis
http://www.genome.ad.jp/kegg/kegg2.html—for ortholog group identification.

Nucleotide sequence accession numbers: The nucleotide sequences have been deposited in the EMBL database under the accession nos. AJ238879 (glucose-specific ABC transporter), AJ238878 (presumed molybdate transporter), and AJ238877 (presumed anion transporter).

RESULTS AND DISCUSSION

Characterization of nitrate respiration-deficient mutants: Nitrate respiration-deficient mutants were isolated using an optimized 5-bromo-2′-deoxyuridine selection procedure as previously described (Soppa 1998). More than 40 mutants of three different phenotypic classes were obtained. The first mutant class is composed of mutants of the null phenotype, which are totally unable to grow with nitrate as the terminal electron acceptor. Mutants of the second class are able to grow with nitrate respiration, albeit with a reduced growth rate compared to the wild type. Mutants of the third class are characterized by a mutant-specific lag phase of 1–3 days after inoculation before they start nitrate respirative growth with the same growth rate as the wild type. All mutants grow aerobically at rates indistinguishable from the wild type. Figure 1 shows anaerobic growth curves of selected mutants in comparison to the wild type.

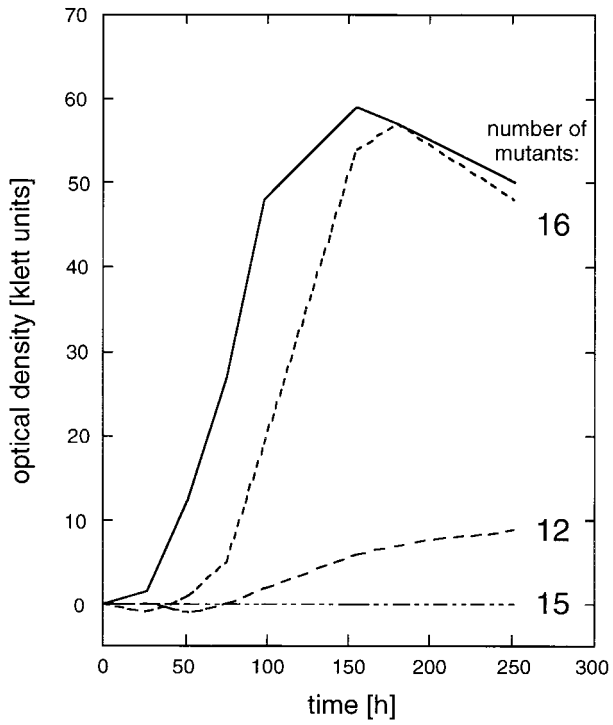


Figure 1.—Phenotypes of nitrate respiration-deficient mutants. Cultures were inoculated with aerobically grown cells and incubated anaerobically with nitrate as the terminal electron acceptor. Growth curves of the wild type (solid line) and selected mutants (broken lines) are shown. Mutants unable to grow with nitrate respiration (null phenotype), with a reduced growth rate, and with a late onset of growth are indicated by different broken line types. The total numbers of mutants obtained for each phenotypic class are included.

Construction of a genomic library of *H. volcanii*: The restriction endonuclease *Hpa*II was chosen for library construction. It has the recognition sequence G′CGC and upon total digestion cleaves the GC-rich genome of *H. volcanii* into fragments of an average size below 0.5 kbp. For a statistical representation of the haloarchaeal genome, the conditions for partial cleavage were optimized and a fragment mixture with a size distribution of 3–5 kbp was isolated. It was cloned into the suicide vector pBN1 (Nowosad 1991). Transformation of *E. coli* yielded 2.6×10^6 independent transformants. Analysis of randomly chosen clones revealed that 90% of the library entries contained a genomic insert of the expected size. Thus, the library represents the genome of *H. volcanii* with a probability of >99.8%.

Complementation of mutants: The genomic library was passed through the *E. coli* strain SCS110 to circumvent the restriction system of *H. volcanii* (see materials and methods). Subsequently it was used for transformation of *H. volcanii* mutants. For stable transformation, plasmids have to integrate into the genome via homolo-

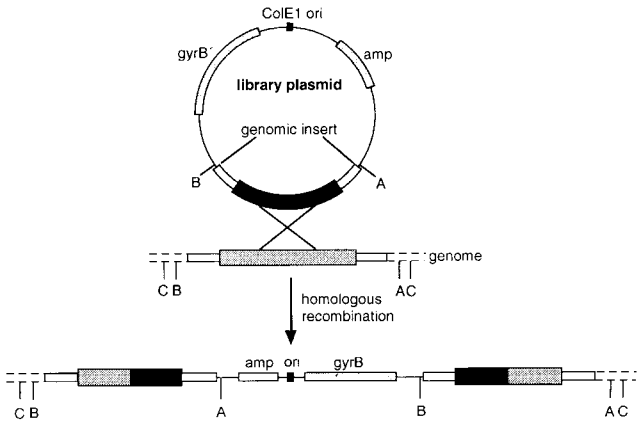


Figure 2.—Complementation of mutants with a genomic library in a suicide vector. As indicated, the library plasmid containing the wild-type copy (in black) of a mutated gene (in gray) integrates in the chromosome via homologous recombination. After integration, the vector is bordered by two fusion genes, one of which carries the site of mutation. The restriction endonucleases used for reisolation of wild-type copies are indicated (A to C, see text). Relevant parts of the suicide vector are shown, *i.e.*, the replication origin and the resistance genes used for selection in *E. coli* (amp, ampicillin resistance) and *H. volcanii* (gyrB′, novobiocin resistance).

gous recombination at the site of the cloned genomic fragment (see Figure 2). Two consecutive aerobic cultures of the cell mixtures in novobiocin-containing medium were used to select for stable transformants. By plating of aliquots the average combined frequency of transformation and recombination was determined to be 2×10^4 per microgram of DNA. Anaerobic cultures in nitrate-containing liquid media were used to select for mutants complemented with wild-type copies of the inactivated genes. The resulting mixtures of complemented cells were plated on solid media, and the nitrate respiration-positive phenotype of randomly chosen clones was checked. In this study, 15 mutants of the null phenotype were chosen for complementation, and the isolation of complemented clones was successful in all cases.

Isolation of wild-type copies of mutated genes: In the genome of complemented mutants the integrated vector pBN1 is bordered by a wild-type copy and a mutated copy of a gene essential for nitrate respiration (see Figure 2). Two strategies were used to reisolate wild-type copies. The first approach made use of restriction enzymes, which cut at a single site at the border of the integrated pBN1 and at an unknown site within the genome (sites A or B in Figure 2). Genomic DNA of complemented mutants was cleaved with these enzymes in separate reactions, religated in dilute solutions, and the resulting fragment mixture was used to transform *E. coli*. This approach yielded pairs of plasmids for each mutant, one containing genomic DNA left of the integration site, the other containing genomic DNA right of the integration site. The plasmids were used to retransform the respective *H. volcanii* mutants. Complementation to the nitrate respiration-positive phenotype allowed the identification of plasmids with wild-type copies of essential genes defective in the mutants. This approach was successful in 6 out of the 15 cases. The approach will fail if the restriction sites A or B are very close to the integration site, allowing the cloning of only very small genomic fragments without enough wild-type information, or if the restriction sites are very far from the integration site, inhibiting cloning in *E. coli*.

In the remaining nine cases an alternative strategy was chosen. Genomic DNA of complemented mutants was cleaved with enzymes that do not cut within pBN1 (enzymes C in Figure 2). Emphasis was put on enzymes with AT-rich recognition sequences with a low average cutting frequency in the GC-rich genome. After religation in dilute solution, the fragment mixtures were used to transform *E. coli*. This approach leads to plasmids that contain genomic DNA flanking the integrated vector on both sides. After isolation from *E. coli*, plasmid sizes were checked, and plasmids of 8–10 kbp were chosen for retransformation of the respective *H. volcanii* mutants. Again, complementation of mutants to a nitrate respiration-positive phenotype revealed cloning of wild-type copies of the mutated genes. This approach was successful in the remaining nine cases.

Characterization of isolated genomic regions: For each mutant 1 plasmid was chosen for sequence determination of the cloned genomic fragment. Before sequencing it was confirmed that the plasmid can complement the respective mutant. Until now the genomic fragments from 13 different plasmids were sequenced. Comparison of the sequences showed that 5 plasmids carried overlapping fragments from the same genomic region, whereas the remaining plasmids were from different parts of the genome. Therefore, in total, fragments from nine genomic regions carrying essential genes for nitrate respiration have been cloned. Here we present the data on three genomic regions that include genes for *A*TP *b*inding *c*assette transporters (ABC transporters). The characterization of the six additional genomic regions is presented elsewhere. Mutants, plasmids, and relevant genes discussed below are summarized in Table 1.

A glucose-specific ABC transporter: Plasmid pCW292 was isolated from the complemented mutant 170/5 using the second approach. It contains an insert of ~3.8 kbp. Two partial open reading frames (ORFs) with haloarchaeal codon usage were detected (Figure 3A).

Partial ORF 1 is similar to ATP-binding subunits of ABC transporters. A total of 313 C-terminal amino acids can be deduced from the partial gene sequence, which is larger than the 250 amino acids of a monomeric ATP-binding protein. The most similar protein is an ATP-binding subunit of an ABC transporter of *Archaeoglobus fulgidus* with 45% of identical amino acids (AF0887), which is annotated to be ribose specific based on its

TABLE 1

Plasmids, mutants, and relevant genes

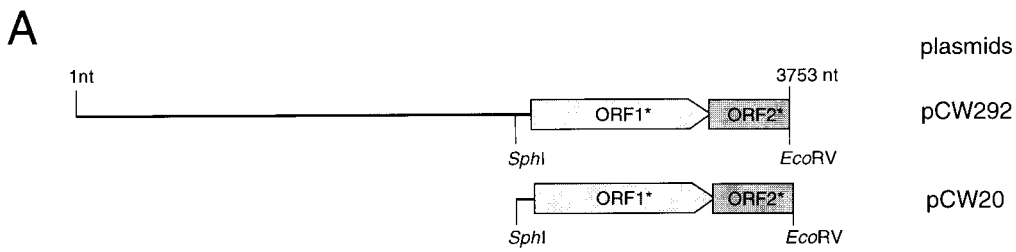
Plasmid	Parent	Complemented mutant	Insert size (bp)	Relevant proteins encoded within the insert
pCW292		170/5	3753	ATP-binding and permease subunit of a glucose-specific ABC transporter (partial sequences)
pCW20	pCW292	170/5	1646	ATP-binding and permease subunit of a glucose-specific ABC transporter (partial sequences)
pCW8		168/6	3526	Permease subunit of an ABC transporter with presumed molybdate specificity Benzaldehyde dehydrogenase
pCW10	pCW8	None	1736	Benzaldehyde dehydrogenase
pCW15	pCW8	168/6	1789	Permease subunit of an ABC transporter with presumed molybdate specificity
pCW55		167/30	2882	ATP-binding and permease subunit of an ABC transporter Deduced protein with similarity to a conserved hypothetical archaeal protein (partial)
pCW30	pCW55	167/30	2074	ATP-binding and permease subunit (partial) of an ABC transporter

similarity with the *E. coli* ribose transporter RsbA. Most proteins in the protein sequence databases with similarity to the haloarchaeal protein are annotated as sugar-specific ABC transporters and they belong to ortholog group 2 (Tomii and Kanehisa 1998). Figure 3B shows an alignment of the deduced haloarchaeal protein with the most similar database matches. The majority of ortholog group 2 ABC transporters contain an ATP-binding protein of ~500 amino acids, twice the size of a monomeric subunit. Therefore, the haloarchaeal protein shares a typical feature of this group. The two domains of these natural dimers are functionally not equivalent. The sequence motifs are much more conserved in the N-terminal domains than in the C-terminal domains (Figure 3B). In the C-terminal domains the lysine of Walker box A is not at the consensus position (GKT/S), the conserved aspartate is missing in Walker box B, and helical domain and switch region are not recognizable. This pattern is conserved in the C-terminal domain of the haloarchaeal sugar transporter (Figure 3B).

Directly downstream of ORF1 a second partial ORF was found. Both ORFs overlap by four nucleotides, indicating that they are cotranscribed and that the gene products are involved in the same process. ORF2 codes for a polypeptide of 140 amino acids. The most similar protein is a permease subunit of an ABC transporter of *A. fulgidus* (52% identity), which is annotated to be ribose specific (AF0888). The ATP-binding subunit (see above) and the permease subunit of the *A. fulgidus* ribose ABC transporter are encoded by adjacent genes. The majority of database entries with similarity to the haloarchaeal protein deduced from ORF2 are permease subunits of sugar-specific ABC transporters and belong to ortholog group two. A multiple sequence alignment with the most similar database entries is shown in Figure 3C. The amino acid composition of the deduced protein shows the high abundance of alanine, leucine, and valine residues that is typical for haloarchaeal membrane proteins (Soppa *et al.* 1993). Together these residues comprise 50.7% of the deduced permease subunit, a much higher value than the average value of 26.0% for soluble haloarchaeal proteins. A hydropathic profile allows the prediction of three membrane-spanning helices within the first 140 amino acids, which is typical for permease subunits of ABC transporters (data not shown).

The two ORFs cover 1.5 kbp out of the 3.8 kbp of cloned genomic DNA. No further ORF with haloarchaeal codon usage could be detected within the remaining 2.3 kbp. To ascertain that mutant 170/5 has a defect within ORF1 or ORF2, the rest of the cloned genomic region was deleted from plasmid pCW292, yielding plasmid pCW20 (Figure 3A). It could still complement mutant 170/5, proving that the cloned ABC transporter is essential for nitrate respiration.

To elucidate the substrate specificity of this ABC transporter, *H. volcanii* wild type was grown anaerobically in synthetic medium with nitrate and different sugars as electron donors. It could not grow with ribose and most other sugars tested, but it could grow on glucose/nitrate and pyruvate/nitrate, respectively. Anaerobic growth of mutant 170/5 with pyruvate/nitrate was indistinguishable from the wild type, but it could not use glucose for nitrate respirative growth. However, the mutant can grow aerobically in synthetic medium using glucose as the sole energy source. These data show that (1) *H. volcanii* can couple glucose oxidation and nitrate reduction for anaerobic growth, (2) glucose is transported into the cell using an ABC transporter composed of at least ORF1 and ORF2, (3) during anaerobic growth no alternative glucose transport system is present, and (4) a different glucose transporter is present under aerobic



B

```
                box A                  linker peptide                box B
A.fu. .....29-LLGENGAGKTTLM-41.....134-NLSVGEEQRVEILKALFRDVKLLILDEPTAVLT-166.....
B.su. .....35-LLGENGAGKSTLM-47.....140-DISVGMQQRAEILKTLYRGADILIFDEPTAVLT-172.....
P.ho. .....35-LLGENGAGKSTLM-47.....193-DLPIGIQQRVEIIKALMANAKILILDEPTSVLT-171.....
T.pa. .....35-LLGENGAGKSTLM-47.....141-DITVGMQQRVEILKMLYRDARVLIFDEPTAVLA-173.....
E.co. .....35-LCGENGSGKSTLM-47.....142-DLGLGQQQLVEIAKALNKQVRLLILDEPTASLT-174.....
           * ****:**:***            :: :* :* .** * *   . :**:****: *:

ORF1* ----DRVTVLRDGENVGTVSTSDVSRADLAEMMVGREVLFTVDREKVAPGEPVLRARNVSATDDRG----------
A.fu. 201-DRITVLRRGRKVGTVITSETNERELTRMMVGRDIVLDITKPPSTPGREVLRVEDLWVRDERG----------
B.su. 237-DRVTVIRKGKGIKTLDVRDTNQDELASLMVGREVSFKTEKRAAQPGAEVLAIDGITVKDTRG----------
P.ho. 206-DRVTVLRRGEVVGVAKTKEVSERDLAKMMVQREIVMEIKKSPASPGNTVLRVEDLWVKDDRG----------
T.pa. 208-DRCTVLRRGACIGTVSVAEVGEERLVEMMVGHAVDYALPRASRKDGACVLEVRSLSVGARRVTSGQMWADASPSAA
E.co. 209-DTICVIRDGQHIGTRDAAGMSEDDIITMMVGRELTALYPNEPHTTGDEILRIEHLTAWHPVN----------
          *   *:* *  : .  .   .. :  :** : :     .     *  :*    : .

                box A
ORF1* --IEALSNVDLTVRRGEVVGIAGVSGNGQKELAEVIAGLRT-VSAGELTVDGRDLTNASPRTFIDNGVSFVPEDRNRYGS
A.fu. --NYAVKGVSFSIREGEIFGIAGVSGNGQKELEEAIAGLRK-VERGRIILDGKDITAKGVAERED--IAYIPEDRIGVGL
B.su. --IETVRDLSLSVKAGEIVGIAGVDGNGQSELIEAVTGLRK-TDSGTITLNGKQIQNLTPRKITESGIGHIPQDRHKHGL
P.ho. --LDAVKGVSFELKEGEILGIAGVQGNGQKELAEALAGIRR-IEKGRIELLGRDVTELSADKRYKAGLAYVPDSR-KVGL
T.pa. PRAYGVRAVSFQVRCGEILCITGVDGNGQSQLLEAIAGLVP-VSEGQILLDGCEIQRTSVRERVLRGVSYIPEDRRKHGL
E.co. RHIKRVNDVSFSLKRGEILGIAGLVGAGRTETIQCLFGVWPGQWEGKIYIDGKQVDIRNCQQAIAQGIAMVPEDRKRDGI
          :  :.: :: **:. *:*: * *:.:  : : *:      * : : * ::           :. :*:.*   *

                                                          linker peptide
ORF1* AGDLSVMHNAAMKDFREIRFGSGVTLDYGELRAHAEALVEAFDVRGVHDVTDATAGDLSGGNLQKLILAREISRDPDLLV
A.fu. APSLSVLENLMLKDFKK--YKKRFSLNFEEMEKRAMSLVEEFSVKT--PSLHSPAGTLSGGNIQRLILARELSRNPRLII
B.su. VLDFPIGENILLQSYYKKPYSALGVLHKGEMYKKARSLITEYDVRT--PDEYTHARALSGGNQQKAIIGREIDRNPDLLI
P.ho. VLDMSVVENVILTNLPS--VMRGRRISWEKANKIGKEIVERFDVLVS--SLSTPIKHLSGGNQQKVMVGREIIREPKVVI
T.pa. VLDFSVEENMVLRSYFRAPFARRGILDRRVIAQHAHALAKKFEVQSGALGCAVRARTLSGGNQQKVIIARELHRAPRLLI
E.co. VPVMAVGKNITLAALNK-FTGGISQLDDAAEQKCILESIQQLKVKTS--SPDLAIGRLSGGNQQKAILARCLLLNPRILI
      .  :.: .*  :              :                .*             ***** *: ::.* :   * :::

      box B
ORF1* ANQPTRGVDVGAIESIREAILDQRTEGTGVVLLSEDLDEIIDLSDRILVVYEGEVVYETTPEDADERIGLEMTG
A.fu. ASQPTRGLDVAGIDYVRRRLIEAAMRGSAVLLISEDLDEIFQLSDRIGVMYEGEIRGIVDRKNANYEKIGYLMAG
B.su. AAQPTRGLDVGAIEFVHKKLIEQRDAGKAVLLLSFELEEIMNLSDRIAVIFEGRIIASVNPQETTEQELGLLMAG
P.ho. VSEPTQGLDIGATEFIRKTLVSLRDQGKGILLISTDLDEILQLSDRIAVMYEGKIMAIGKVEEFTLEKLGLLMGG
T.pa. AAQPTRGLDLGAVQYVHRAIVAERNRGGAVLLFSLDMDEVLALADSIAVMYEGEIVGTVHACDATEQELGRLMSG
E.co. LDEPTRGIDIGAKYEIYKLINQLVQQGIAVIVISSELPEVLGLSDRVLVMHEGKLKANLINHNLTQEQVMEAALR
        :**:*:*:..   : . :      * .::::* :: *:: *:* : *:.**.:       :   :.:


ORF1* GGDATATAGAQVRGLGGSS 313
A.fu. GLEVAA------------ 495
B.su. STQKEAGKANG-------- 510
P.ho. VNA--------------- 498
T.pa. MRKKETAGKKTGVQG---- 533
E.co. SEHHVEKQSV--------- 513
```

Figure 3.—A glucose-specific ABC transporter. (A) Schematic overview of the genomic region isolated from complemented mutant 170/5 and a deletion variant. ORFs with halobacterial codon usage, relevant restriction sites, and plasmid names are shown. (B) Multiple sequence alignment of partial ORF1 with the five most similar database entries, all of which are ATP-binding subunits of ABC transporters. In the upper part the alignment of two regions with signature sequences from the N-terminal part of the five proteins is included where no sequence information for ORF1 is yet available. Typical sequence motifs are shown above the sequences. Conserved positions are indicated below the sequences by an asterisk (identical amino acids), a colon (conserved substitutions), or a dot (semiconserved substitutions). All arginines in ORF1 are underlined. Arginines in positions at which none of the other sequences has a positively charged amino acid (R or K) are boxed. In all figure legends the following abbreviations denote the host organism, annotated function, and accession number (in parentheses) of the proteins shown. A. fu., *A. fulgidus*, ribose-specific ABC transporter (O29375); B. su., *Bacillus subtilis*, hypothetical protein (O05253); P. ho., *Pyrococcus horikoshii*, sugar-specific ABC transporter (O59404); T. pa., *Treponema pallidum*, ribose-specific ABC transporter (O83341); E. co., *E. coli*, xylose-specific ABC transporter (P37388). (C) Multiple sequence alignment of partial ORF2 with the N-terminal regions of the five most similar database entries, all of which are membrane subunits of ABC transporters. Putative membrane-spanning helices are indicated above the sequences, and the conserved amino acids are indicated below the sequences. The lengths of the aligned parts and the total length of the five proteins are shown. A. fu., *A. fulgidus*, ribose-specific ABC transporter (O29374); B. su., *B. subtilis*, hypothetical protein (O05254); P. ho., *P. horikoshii*, hypothetical protein (O59405); B. bu., *Borrelia burgdorferi*, ribose-specific ABC transporter (O51621); T. pa., *T. pallidum*, ribose-specific ABC transporter (O83342).

conditions. This is in accordance with an earlier report that had shown that during aerobic growth glucose import by *H. volcanii* is catalyzed by a sodium/glucose-symporter (Tawara and Kamo 1991). Thus, two different principles are used under aerobic and anaerobic conditions, and differential production of the transport-

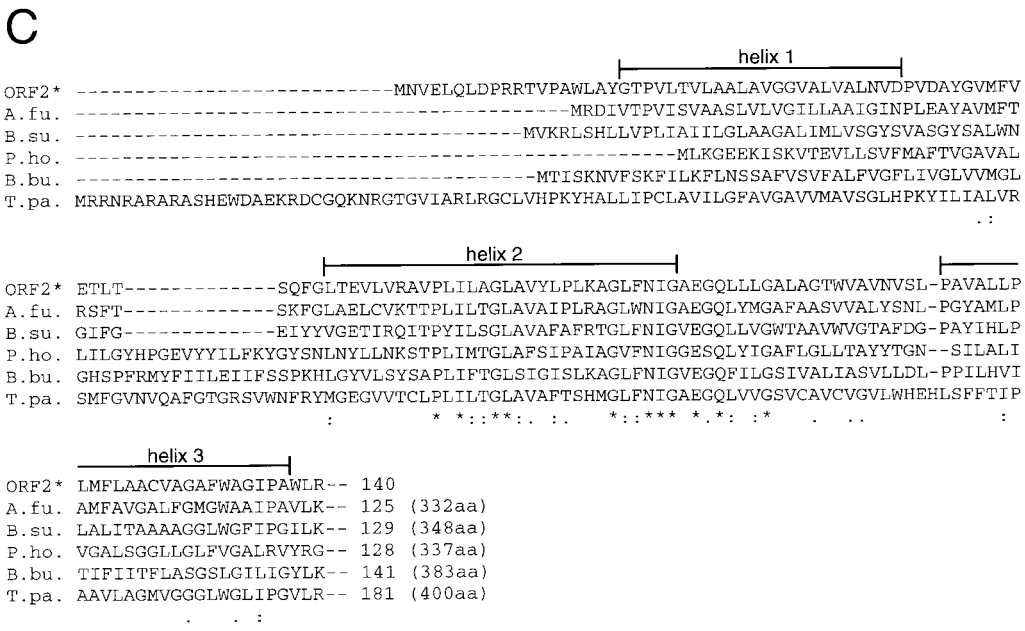


Figure 3.—*Continued.*

ers can be expected. ABC transporters can accumulate their substrates against much higher concentration gradients compared to solute symporters, which could explain the preference for an ABC transporter under anaerobic conditions.

An ABC transporter with presumed molybdate specificity: Genomic DNA (3.5 kbp) was recovered from the complemented mutant 168/6. Two genes with haloarchaeal codon usage were detected, coding for the permease subunit of an ABC transporter (ORF1) and a benzaldehyde dehydrogenase (ORF2; Figure 4A). To clarify which of the genes is essential for nitrate respiration, two smaller plasmids containing exclusively the gene for the permease subunit (pCW15) or for the dehydrogenase (pCW10) were constructed. Upon transformation of the mutant, only pCW15 could restore the wild-type phenotype, proving that the ABC transporter is indispensable for nitrate respiration. A 340-bp overlapping part of this gene was sequenced previously by Charlebois and colleagues (R. Farahani, J.-C. Imbeault, A. St. Jean, C. C.-Y. Chan, G. Allard and R. L. Charlebois, unpublished data; accession no. U95374) while studying the instability of the megaplasmid pHV3, showing that this essential gene is not encoded on the chromosome.

The amino acid composition of the permease subunit is typical for haloarchaeal membrane proteins with a high content of alanine, valine, and leucine residues (44.9%). A hydropathic profile allows the prediction of five membrane-spanning helices, which is typical for ABC transporters with an import function (data not shown). The deduced protein contains an EAA-loop that matches the consensus sequence well (Figure 4B).

The most similar database entry is the permease subunit of an ABC transporter from *A. fulgidus* (41% identity; AF0093), which is annotated to be sulfate specific due to its similarity to other ABC transporters. The highest similarity to a database entry with an experimentally proven function is to ModB of *E. coli* (Maupin-Furlow *et al.* 1995), the permease subunit of a molybdate-specific ABC transporter (36% identity). The 40 most similar database entries are annotated as molybdate-specific permease subunits, sulfate-specific permease subunits, or hypothetical proteins, respectively. Most of them belong to ortholog group 1-1. A possible role for a sulfate transporter in nitrate respiration is not obvious, whereas molybdate transport is essential, because nitrate reductases are molybdenum enzymes. Therefore, we propose that the permease subunit encoded on pCW15 is part of a molybdate-specific ABC transporter. It is unusual that a permease gene is not linked to a gene coding for an ATP-binding subunit. However, there is no open reading frame upstream of the permease gene, and downstream is the gene for the benzaldehyde dehydrogenase. Therefore, the permease subunit has to act in concert with an ATP-binding subunit that is encoded at a different location on the plasmid or on the chromosome.

An ABC transporter with presumed anion specificity: About 2.9 kbp of genomic DNA was recovered from the complemented mutant 167/30 using the first approach (Figure 5A). Three ORFs with haloarchaeal codon usage could be detected: the C-terminal part of an ORF coding for a conserved hypothetical archaeal protein of unknown function (ORF1) and two ORFs with coding capacity for the permease (ORF2) and the ATP-binding subunit (ORF3) of an ABC transporter. To elucidate whether the ABC transporter is essential for nitrate respiration, plasmid pCW30 was constructed. It contains only the ABC transporter genes but lacks the first open

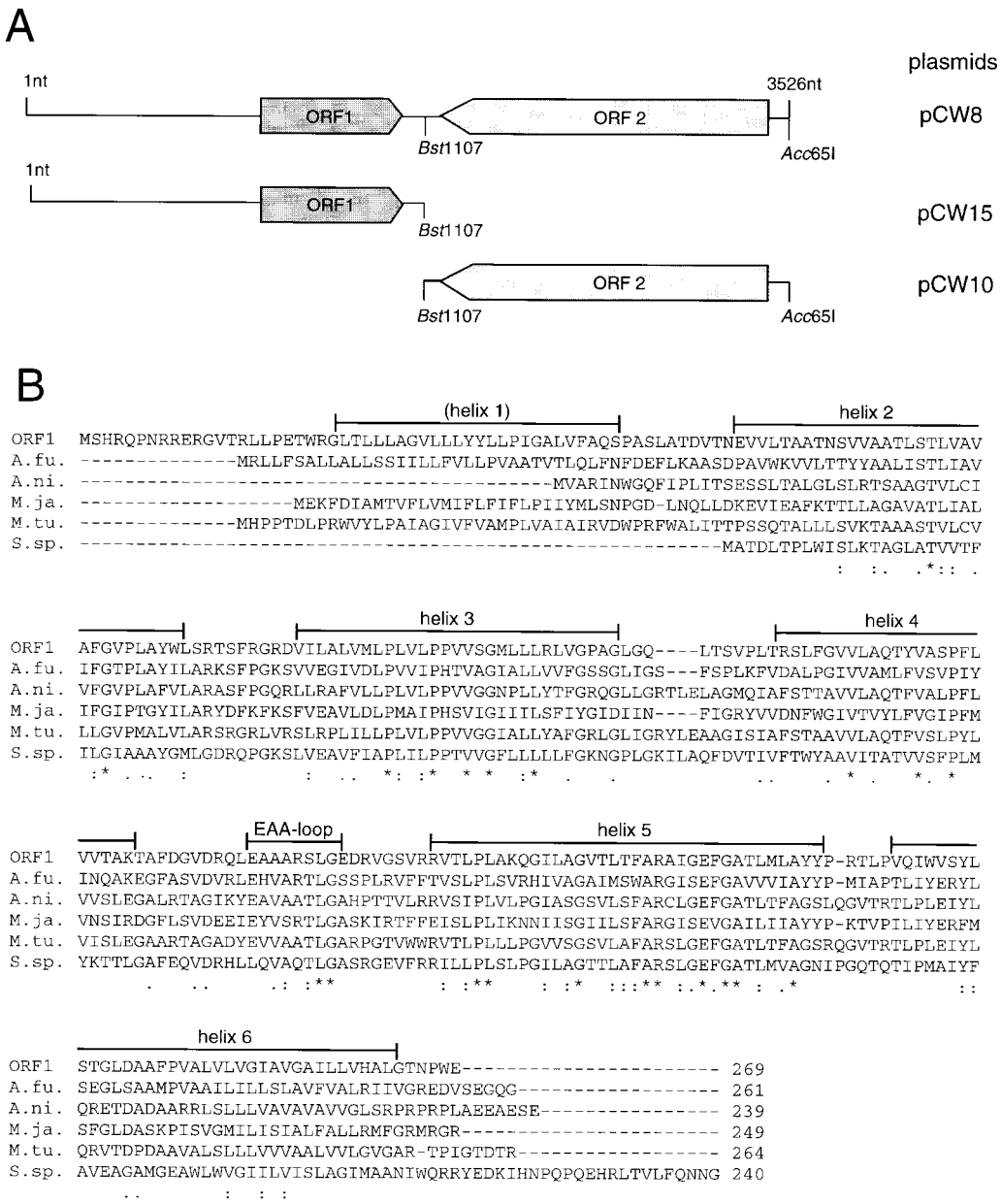


Figure 4.—An ABC transporter with presumed molybdate specificity. (A) Schematic overview of the genomic region isolated from complemented mutant 168/6 and two deletion variants. ORFs with halobacterial codon usage, relevant restriction sites, and plasmid names are shown. (B) Multiple sequence alignment of ORF1 with the five most similar database entries, all of which are membrane subunits of ABC transporters. Putative membrane-spanning helices and the EAA-loop are indicated above the sequences, and the conserved amino acids are indicated below the sequences (for definition of symbols see Figure 3B). A. fu., *A. fulgidus*, sulfate-specific ABC transporter (O30143); A. ni., *A. nicotinovorans*, molybdate-specific ABC transporter (O31230); M. ja., *Methanococcus jannaschii*, hypothetical protein (Q58763); M. tu., *Mycobacterium tuberculosis*, molybdate-specific ABC transporter (P95156); S. sp., *Synechocystis* sp., ABC transporter (Q55993).

reading frame (Figure 5A). Transformation of mutant 167/30 with pCW30 restored the wild-type phenotype, proving that nitrate respiration requires the ABC transporter activity.

The permease subunit is 232 amino acids long (Figure 5B). The amino acid composition is typical for haloarchaeal membrane proteins; the content of alanine, leucine, and valine residues is 39.2%. A hydropathic profile allows the prediction of five to six membrane-spanning helices (data not shown). The protein sequence contains an EAA-loop that matches the consensus sequence well. The database entry with the highest similarity is the permease subunit of an ABC transporter from *Methanobacterium thermoautotrophicum* (44% identity; MTH1731), which is proposed to be sulfate specific. Half of the 50 most similar database entries are annotated to be either sulfate or phosphate specific and belong to ortholog group 1-1 or 3-7, respectively.

A 289-amino-acid-long ORF with coding capacity for an ATP-binding subunit was found downstream of the permease gene. The two genes have an overlap of four nucleotides, indicating that they are cotranscribed. Database searches revealed that a high fraction of similar proteins are annotated to be phosphate specific and belong to ortholog group 3-7. A multiple sequence alignment of the ATP-binding subunit with the most similar database entries is shown in Figure 5B. As can be seen, the sequence motifs indicative for ABC transporters are well conserved in the haloarchaeal protein.

The substrate specificity of this ABC transporter is not clear. Primary sequences of both subunits indicate that it is anion specific. It seems possible that phosphate

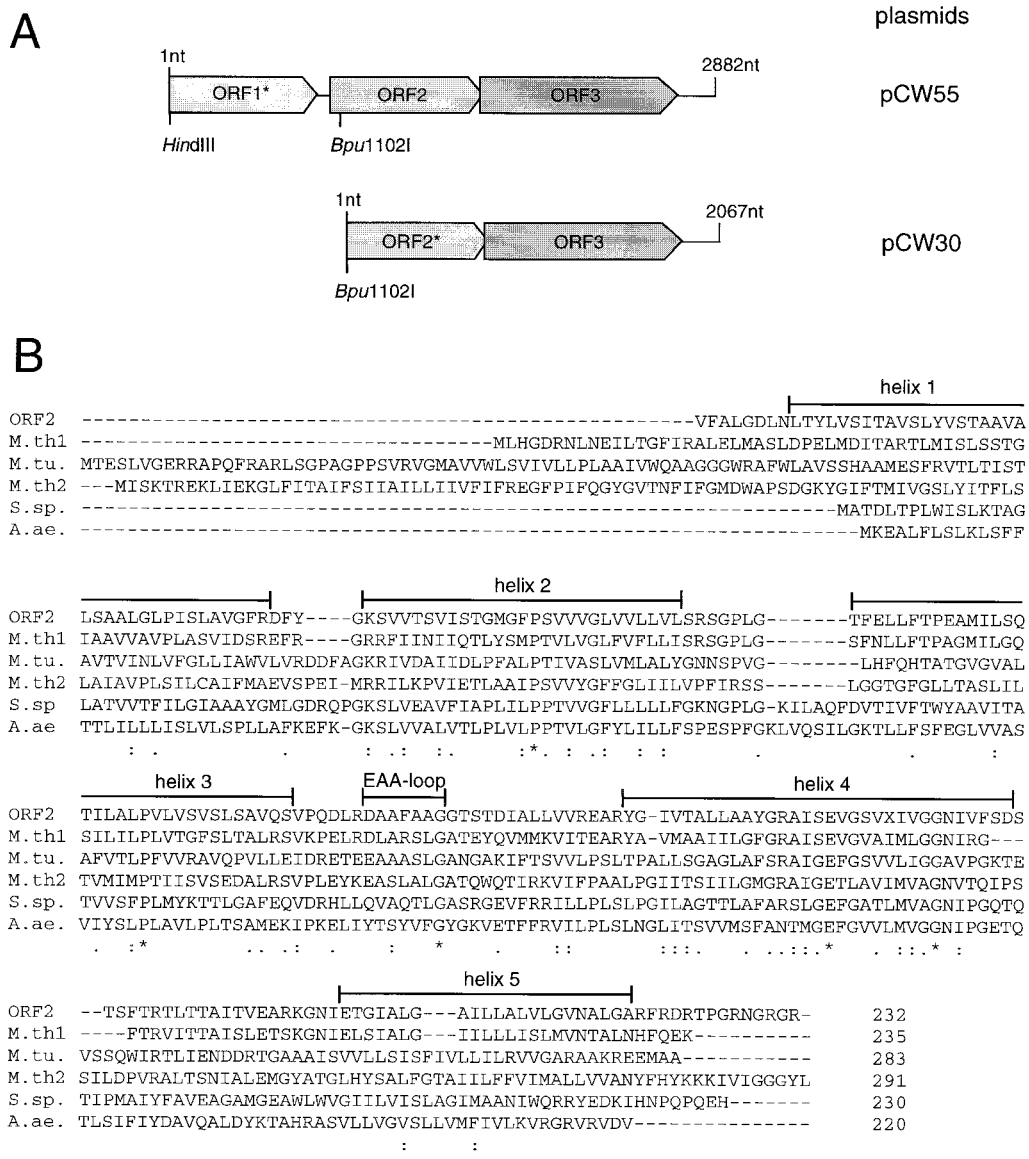


Figure 5.—An ABC transporter with presumed anion specificity. (A) Schematic overview of the genomic region isolated from complemented mutant 167/30 and a deletion variant. ORFs with halobacterial codon usage, relevant restriction sites, and plasmid names are shown. The deduced protein sequences of ORF2 and ORF3 are compared to homologous proteins in Figure 5, B and C. (B) Multiple sequence alignment of ORF2 with the five most similar database entries, all of which are membrane subunits of ABC transporters. Putative membrane-spanning helices and the EAA-loop are indicated above the sequences, and the conserved amino acids are indicated below the sequences (for definition of symbols see Figure 3B). M. th.1, *M. thermoautotrophicum*, sulfate-specific ABC transporter (O26578); M. tu., *M. tuberculosis*, hypothetical protein (P71745); M. th.2, *M. thermoautotrophicum*, phosphate-specific ABC transporter (O27762); S. sp., *Synechocystis* sp., ABC transporter (Q55993); A. ae., *Aquifex aeolicus*, molybdate-specific ABC transporter. (C) Multiple sequence alignment of ORF3 with the five most similar database entries, all of which are ATP-binding subunits of ABC transporters. Typical sequence motifs are shown above the sequences, and the conserved amino acids are indicated below the sequences (for definition of symbols see Figure 3B). All arginines in ORF3 are underlined. Arginines in positions at which none of other sequences has a positively charged amino acid (R or K) are boxed. A. ae., *A. aeolicus*, phosphate-specific ABC transporter (O67154); M. th., *M. thermoautotrophicum*, phosphate-specific ABC transporter (O27764); M. ja., *M. jannaschii*, phosphate-specific ABC transporter (Q58418); B. su., *B. subtilis*, hypothetical protein (P46342); E. co., *E. coli*, hypothetical ABC transporter (P37774).

transport is mediated by different transporters aerobically and anaerobically, comparable to glucose transport. Alternatively, nitrate transport would be another essential process for nitrate respiration.

An unusual arginine content of haloarchaeal ATP-binding subunits: Haloarchaeal soluble proteins have a very specific amino acid distribution as determined by the amino acid analysis of bulk haloarchaeal proteins (Reistad 1970) and the statistical analysis of primary sequences (Soppa *et al.* 1993). They are characterized by a high content of acidic amino acids (18.8%) and a reduced content of lysines (2.7%). This results in a high preference for acidic amino acids (difference of acidic minus basic residues) of 10.8% in contrast to proteins from nonhalophilic organisms, *e.g.*, enteric bacteria (1.9%). The high excess of acidic amino acids on the surface of soluble haloarchaeal proteins is essential for solubility at the high cytoplasmic salt concentrations of up to 5 m. Properties of haloarchaeal proteins have been reviewed by, *e.g.*, Lanyi (1974) and Eisenberg *et al.* (1992). Structure determinations of haloarchaeal proteins have revealed that the acidic residues build a network of tightly bound cation and water molecules at the surface (Dym *et al.* 1995; Frolow *et al.* 1996; Pieper *et al.* 1998). Both ATP-binding subunits described above have amino acid compositions that are essentially typical

C

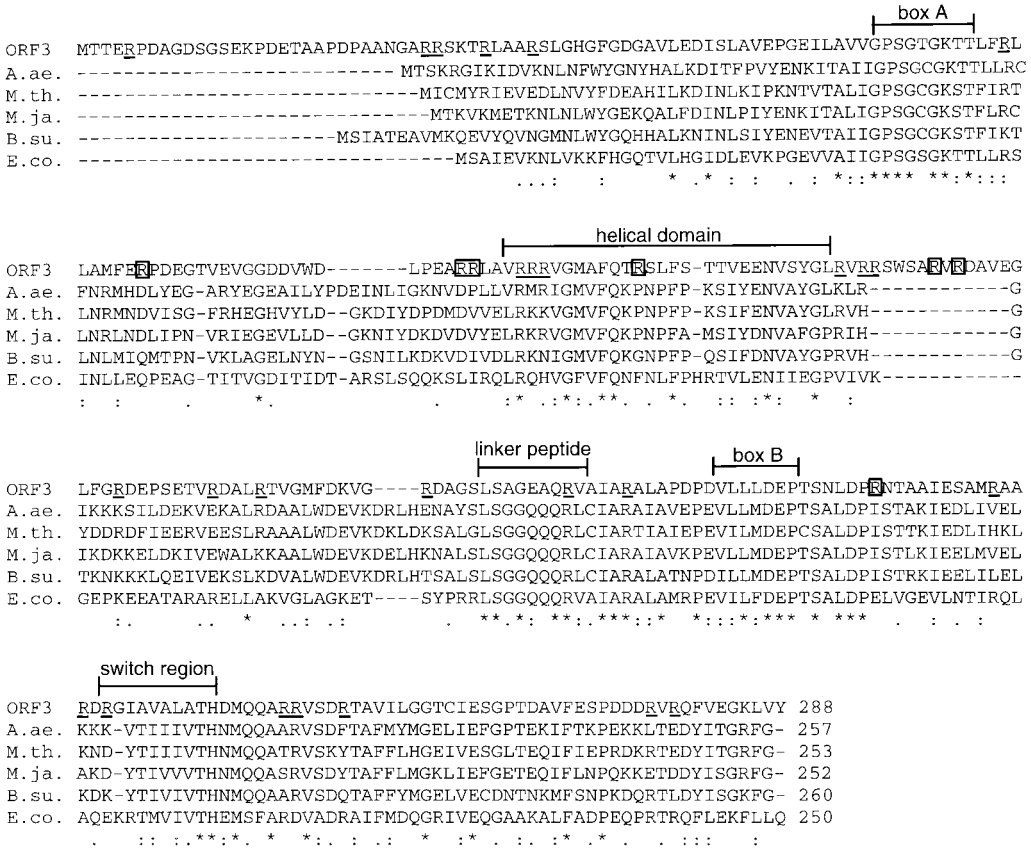


Figure 5.—*Continued.*

for soluble haloarchaeal proteins. However, their arginine contents of 9.3% (glucose transporter) and 11.5% (anion transporter) are much higher than the average arginine content of 5.3%. This results in a much lower preference for acidic amino acids (7.8 and 3.1%) than in average soluble proteins (10.8%).

The high arginine content of ~10% seems to be confined to haloarchaeal ATP-binding subunits. It is not found in the homologous ATP-binding proteins from Bacteria and other Archaea; *e.g.*, the average arginine content of the nonhaloarchaeal sequences shown in Figures 3C and 5C is 6%. Nor is it found in other soluble haloarchaeal proteins interacting with integral membrane proteins, *i.e.*, the chemotaxis signal transducers CheA and CheB (Rudolph and Oesterhelt 1995; Rudolph *et al.* 1995), which have an average content of 5.2%.

It is tempting to speculate that the high arginine content facilitates interactions with the negatively charged phospholipids and/or with the membrane subunit. In this respect it is noteworthy that in the anion transporter subunit the "haloarchaeal-specific arginines," which do not have counterparts in homologous proteins, are clustered around the helical domain (boxed in Figure 5C). The positive charge density of this region is extremely high: 11 out of 42 residues are arginines. On the basis of the analysis of suppressor mutations it was proposed earlier that this helical domain could be involved in the interaction of the ATP-binding subunits with the membrane subunits (Schneider and Hunke 1998). It will be interesting to see whether the high positive charge density holds true for the helical domain in the N-terminal part of the glucose transporter subunit, which is not yet available.

Transcription of ABC transporter genes: To investigate transcription of the ABC transporter genes, Northern blot analyses were performed using RNA isolated from aerobically and anaerobically grown cultures. No transcripts could be detected. Therefore, unlabeled PCR fragments generated from the respective genes were used as internal standards to determine the sensitivity of detection, and transcription from the gene for the cell surface glycoprotein (Sumper *et al.* 1990) was determined as a control. It was confirmed that transcription of all three ABC transporter genes is very low and prevents transcript detection with Northern blots (data not shown). It could be concluded that transcript levels of the five ABC transporter genes are at least 20-fold lower than the transcript level of the cell surface glycoprotein gene.

ABC transporters in Archaea: The first archaeal ABC transporters were discovered in 1996 (Jovell *et al.* 1996;

Xavier *et al.* 1996). The recent publication of four archaeal genome sequences has revealed that ABC transporters are widespread in Archaea. Until now, however, only one archaeal ABC transporter had been functionally characterized (Xavier *et al.* 1996; Horlacher *et al.* 1998). This seems worth mentioning as >400 bacterial and archaeal ABC transporter sequences are known and the annotated "function" of the vast majority rests solely on primary sequence similarities. Here we present experimental evidence that in *H. volcanii* at least three ABC transporters are involved in nitrate respiration and that one of them is glucose specific. To our knowledge this is the first glucose-specific ABC transporter described.

Conclusions: Complementation of *H. volcanii* mutants with a genomic library of the wild type turned out to be a very effective method for identification of essential genes for nitrate respiration. In total, nine genomic regions have been detected until now, at least one of which resides on a megaplasmid. As will be presented elsewhere, highly expressed genes that are either constitutively transcribed or upregulated during nitrate respiration, as well as genes encoding regulatory proteins, were found. Here we show that also poorly expressed genes could be detected, coding for three different ABC transporters involved in substrate and cofactor import. The glucose-specific ABC transporter is essential solely under anaerobic conditions. This is in accordance with an earlier study, which showed that under aerobic conditions glucose is imported via a sodium:glucose-symporter.

This work was supported by the Deutsche Forschungsgemeinschaft (DFG) through grant So264/3. J.S. is supported by the DFG through a "Heisenbergstipendium" (grant So264/4).

LITERATURE CITED

Alvarezossorio, M. C., F. J. G. Muriana, F. F. Delarosa and A. M. Relimpio, 1992 Purification and characterization of nitrate reductase from the halophile archaebacterium *Haloferax mediterranei*. Z. Naturforsch. Sect. C Biosci. **47:** 670–676.

Bairoch, A., and R. Apweiler, 1999 The SWISS-PROT protein sequence data bank and its supplement TrEMBL in 1999. Nucleic Acids Res. **27:** 49–54.

Bickelsandkötter, S., and M. Ufer, 1995 Properties of a dissimilatory nitrate reductase from the halophilic archaeon *Haloferax volcanii*. Z. Naturforsch. Sect. C Biosci. **50:** 365–372.

Boos, W., and J. M. Lucht, 1996 Periplasmic binding protein-dependent ABC transporters, pp. 1175–1209 in *Escherichia coli and Salmonella*, edited by F. C. Neidhardt. ASM Press, Washington, DC.

Cheung, J., K. J. Danna, E. M. O'Connor, L. B. Price and R. F. Shand, 1997 Isolation, sequence, and expression of the gene encoding halocin H4, a bacteriocin from the halophilic archaeon *Haloferax mediterranei* R4. J. Bacteriol. **179:** 548–551.

Chomczynski, P., 1992 One-hour downward alkaline capillary transfer for blotting of DNA and RNA. Anal. Biochem. **201:** 134–139.

Chomczynski, P., and N. Sacchi, 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:** 156–159.

Cline, S. W., L. C. Schalkwyk and W. F. Doolittle, 1989 Transformation of the archaebacterium *Halobacterium volcanii* with genomic DNA. J. Bacteriol. **171:** 4987–4991.

Cohenkupiec, R., C. Blank and J. A. Leigh, 1997 Transcriptional regulation in Archaea: *in vivo* demonstration of a repressor binding site in a methanogen. Proc. Natl. Acad. Sci. USA **94:** 1316–1320.

Dym, O., M. Mevarech and J. L. Sussman, 1995 Structural features that stabilize halophilic malate dehydrogenase from an archaebacterium. Science **267:** 1344–1346.

Eisenberg, H., M. Mevarech and G. Zaccai, 1992 Biochemical, structural, and molecular genetic aspects of halophilism. Adv. Prot. Chem. **43:** 1–62.

Etzold, T., A. Ulyanov and P. Argos, 1996 SRS: information retrieval system for molecular biology. Methods Enzymol. **266:** 114.

Frolow, F., M. Harel, M. Mevarech, J. L. Sussman and M. Shoham, 1996 Insight into protein adaptation to a saturated salt environment from the crystal structure of a halophilic 2Fe-2S ferredoxin. Nat. Struct. Biol. **3:** 452–458.

Hanahan, D., 1985 Techniques for transformation of *E. coli*, pp. 109–135 in *DNA Cloning—A Practical Approach.* edited by D. Glover. IRL Press, Oxford/Washington, DC.

Higgins, D., J. Thompson and T. Gibson, 1994 ClustalW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and matrix choice. Nucleic Acids Res. **22:** 4673–4680.

Hochstein, L. I., and F. Lang, 1991 Purification and properties of a dissimilatory nitrate reductase from *Haloferax denitrificans*. Arch. Biochem. Biophys. **288:** 380–385.

Hofmann, K., P. Bucher, L. Falquet and A. Bairoch, 1999 The prosite database: its status in 1999. Nucleic Acids Res. **27:** 215–219.

Holmes, M. L., and M. L. Dyall-Smith, 1991 Mutations in DNA gyrase result in novobiocin resistance in halophilic archaebacteria. J. Bacteriol. **173:** 642–648.

Holmes, M., S. D. Nuttal and M. L. Dyall-Smith, 1991 Construction and use of halobacterial shuttle vectors and further studies on Haloferax DNA gyrase. J. Bacteriol. **173:** 3807–3813.

Horlacher, R., K. B. Xavier, H. Santos, J. DiRuggiero and W. Boos, 1998 Archaeal binding protein-dependent ABC-transporter: molecular and biochemical analysis of the trehalose/maltose transport system of the hyperthermophilic archaeon *Thermococcus litoralis*. J. Bacteriol. **180:** 680–689.

Jovell, R. J., A. J. Macario and E. C. de Macario, 1996 ABC transporters in Archaea: two genes encoding homologs of the nucleotide-binding components in the methanogen *Methanosarcina mazei* S-6. Gene **174:** 1–4.

Komatsubara, M., A. Abe, R. Okumura, S. Miyauchi and N. Kamo, 1996 Doxoribicin-efflux pump in *Haloferax volcanii* is energized by ATP. FEMS Microbiol. Lett. **139:** 83–88.

Krüger, K., T. Hermann, V. Armbruster and F. Pfeifer, 1998 The transcriptional activator GvpE for the halobacterial gas vesicle genes resembles a basic region leucine-zipper regulatory protein. J. Mol. Biol. **279:** 761–771.

Kuo, Y. P., D. K. Thompson, A. St. Jean, R. L. Charlebois and C. J. Daniels, 1997 Characterization of two heat shock genes from *Haloferax volcanii*: a model system for transcription regulation in the Archaea. J. Bacteriol. **179:** 6318–6324.

Lanyi, J., 1974 Salt-dependent properties of proteins from extremely halophilic bacteria. Bacteriol. Rev. **38:** 272–290.

Lin, J. T., and V. Stewart, 1998 Nitrate assimilation by bacteria. Adv. Microb. Physiol. **39:** 1–30.

Maupin-Furlow, J. A., J. K. Rosentel, J. H. Lee, U. Deppenmeier, R. P. Gunsalus *et al.*, 1995 Genetic analysis of the *modABCD* (molybdate transport) operon of *Escherichia coli*. J. Bacteriol. **177:** 4851–4856.

Mevarech, M., and R. Werczberger, 1985 Genetic transfer in *Halobacterium volcanii*. J. Bacteriol. **162:** 461–462.

Nowosad, B., 1991 Etablierung und Optimierung von Transformationssystemen bei verschiedenen halobakteriellen Arten. Diploma Thesis, University of Regensburg, Germany.

Oren, A., and H. G. Trüper, 1990 Anaerobic growth of halophilic archaebacteria by reduction of dimethylsulfoxide and trimethylamine N-oxide. FEMS Microbiol. Lett. **70:** 33–36.

Pearson, W. R., 1990 Rapid and sensitive sequence comparison with FASTP and FASTA. Methods Enzymol. **183:** 63–98.

Pieper, U., G. Kapadia, M. Mevarech and O. Herzberg, 1998 Structural features of halophilicity derived from the crystal structure of dihydrofolate reductase from the dead sea halophilic archaeon, *Haloferax volcanii*. Structure **6:** 75–88.

Reistad, R., 1970 On the composition and nature of the bulk pro-

tein of the extremely halophilic bacteria. Arch. Microbiol. **208:** 353–360.

Rosenshine, I., T. Zusman, R. Werczberger and M. Mevarech, 1987 Amplification of specific DNA sequences correlates with resistance of the archaebacterium *Halobacterium volcanii* to the dihydrofolate reductase inhibitors trimethoprim and methotrexate. Mol. Gen. Genet. **208:** 518–522.

Rudolph, J., and D. Oesterhelt, 1995 Chemotaxis and phototaxis require a CheA histidine kinase in the archaeon *Halobacterium salinarium*. EMBO J. **14:** 667–673.

Rudolph, J., N. Tolliday, C. Schmitt, S. C. Schuster and D. Oesterhelt, 1995 Phosphorylation in halobacterial signal transduction. EMBO J. **14:** 4249–4257.

Ruepp, A., and J. Soppa, 1996 Fermentative arginine degradation in *Halobacterium salinarium* (formerly *Halobacterium halobium*): genes, gene products, and transcripts of the *arcRACB* gene cluster. J. Bacteriol. **178:** 4942–4947.

Sambrook, J., E. Fritsch and T. Maniatis, 1989 *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schneider, E., and S. Hunke, 1998 ATP-binding-cassette (ABC) transport systems: functional and structural aspects of the ATP-hydrolyzing subunits/domains. FEMS Microbiol. Rev. **22:** 1–20.

Soppa, J., 1998 Optimization of the 5-bromo-2′-deoxyuridine selection and its application for the isolation of nitrate respiration-deficient mutants of *Haloferax volcanii*. J. Microbiol. Methods **34:** 41–48.

Soppa, J., 1999 Transcription initiation in Archaea: facts, factors, and future aspects. Mol. Microbiol. **31:** 1295–1306.

Soppa, J., J. Duschl and D. Oesterhelt, 1993 Bacterioopsin, haloopsin, and sensory opsin I of the halobacterial isolate *Halobacterium* sp. strain SG1: three new members of a growing family. J. Bacteriol. **175:** 2720–2726.

Soppa, J., T. A. Link, A. Z. Mühlen, A. Ruepp and P. Vatter, 1998 Regulation of gene expression in *Halobacterium salinarum*: the arcRACB gene cluster and the TATA box-binding protein, pp. 249–263 in *Microbiology and Biogeochemistry of Hypersaline Environments*, edited by A. Ohren. CRC Press, Boca Raton, FL.

Sorgenfrei, O., S. Müller, M. Pfeiffer, I. Sniezko and A. Klein, 1997 The [NiFe] hydrogenase of *Methanococcus voltae*: genes, enzymes and regulation. Arch. Microbiol. **167:** 189–195.

Stewart, V., 1988 Nitrate respiration in relation to facultative metabolism in enterobacteria. Microbiol. Rev. **52:** 190–232.

Stewart, V., 1993 Nitrate regulation of anaerobic respiratory gene expression in *Escherichia coli*. Mol. Microbiol. **9:** 425–434.

Stoesser, G., M. A. Tuli, R. Lopez and P. Sterk, 1999 The EMBL nucleotide sequence database. Nucleic Acids Res. **27:** 18–24.

Sumper, M., E. S. Berg, R. Mengele and I. Strobel, 1990 Primary structure and glycosylation of the S-layer protein of *Haloferax volcanii*. J. Bacteriol. **172:** 7111–7118.

Tawara, E., and N. Kamo, 1991 Glucose transport of *Haloferax volcanii* requires the Na^+-electrochemical potential gradient and inhibitors for the mammalian glucose transporter inhibit the transport. Biochim. Biophys. Acta **1070:** 293–299.

Tomii, K., and M. Kanehisa, 1998 A comparative analysis of ABC transporters in complete microbial genomes. Genome Res. **8:** 1048–1059.

Woese, C. R., O. Kandler and M. L. Wheelis, 1990 Towards a natural system of organisms: proposal for the domains Archaea, Bacteria, and Eucarya. Proc. Natl. Acad. Sci. USA **87:** 4576–4579.

Xavier, K. B., L. O. Martins, R. Peist, M. Kossmann, W. Boos *et al.*, 1996 High-affinity maltose/trehalose transport system in the hyperthermophilic archaeon *Thermococcus litoralis*. J. Bacteriol. **178:** 4773–4777.

Yang, C. F., J. M. Kim, E. Molinari and S. DasSarma, 1996 Genetic and topological analyses of the bop promoter of *Halobacterium halobium*: stimulation by DNA supercoiling and non-B-DNA structure. J. Bacteriol. **178:** 840–845.

Zumft, W. G., 1997 Cell biology and molecular basis of denitrification. Microbiol. Mol. Biol. Rev. **61:** 533–616.

Communicating editor: F. Pfeifer