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Inactivation of the *selB* Gene in *Methanococcus maripaludis:* Effect on Synthesis of Selenoproteins and Their Sulfur-Containing Homologs

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The genome of *Methanococcus maripaludis* harbors genes for at least six selenocysteine-containing proteins and also for homologs that contain a cysteine codon in the position of the UGA selenocysteine codon. To investigate the synthesis and function of both the Se and the S forms, a mutant with an inactivated *selB* gene was constructed and analyzed. The mutant was unable to synthesize any of the selenoproteins, thus proving that the gene product is the archaeal translation factor (aSelB) specialized for selenocysteine insertion. The wild-type form of *M. maripaludis* repressed the synthesis of the S forms of selenoproteins, i.e., the selenium-independent alternative system, in selenium-enriched medium, but the mutant did not. We concluded that free selenium is not involved in regulation but rather a successional compound such as selenocysteyl-tRNA or some selenoprotein. Apart from the S forms, several enzymes from the general methanogenic route were affected by selenium supplementation of the wild type or by the *selB* mutation. Although the growth of *M. maripaludis* on H_2/CO_2 is only marginally affected by the *selB* lesion, the gene is indispensable for growth on formate because *M. maripaludis* possesses only a selenocysteine-containing formate dehydrogenase.

Within the genomic sequence of *Methanococcus jannaschii*, seven putative genes coding for selenocysteine-containing proteins were recognized (6, 37). Six of these genes could be correlated with ^{75}Se-labeled gene products, namely, formate dehydrogenase (FDH), heterodisulfide reductase, formylmethanofuran dehydrogenase, two subunits of the F_{420}-nonreducing hydrogenase, and one subunit of the F_{420}-reducing hydrogenase (37). Intriguingly, all of these putative selenoproteins are directly or indirectly involved in the methanogenic pathway (20, 30). Since *M. jannaschii* possesses only single genes for most of these functions on the chromosome, its growth is absolutely dependent on the presence of the trace element selenium in the medium (20).

In other species of *Methanococcus* this strict requirement is replaced by a facultative dependence; of these, *Methanococcus voltae* is the best characterized (27). This archaeon synthesizes a set of selenoproteins upon growth in the presence of selenium, but it also possesses the genetic capacity to express genes for the same functions but with a cysteine residue in the position of the selenocysteine (12). In the presence of selenium the genes for this backup system are repressed via an as-yet-unidentified mechanism (18).

A new addition to this list of such facultatively selenium-requiring organisms is *Methanococcus maripaludis*. As judged by the electrophoretic behavior of its proteins labeled with [^{75}Se], this organism contains the same set of selenoproteins as *M. jannaschii*; the identity of one of them, VhuD, was recently assigned (19). Alternative genes are present on the chromosome whose derived amino acid sequences are highly similar to the selenocysteine-containing ones but with a cysteine in the sequence position of selenocysteine (J. A. Leigh, unpublished data). The development of a powerful genetic system for *M. maripaludis* (33) has facilitated the elucidation of crucial mechanistic details for selenoprotein formation in *Archaea*, which bears striking similarities to the process in *Eukarya* (20). With the aid of this system the function of an RNA element in the 3′-nontranslated region of the mRNA as a SECIS element could be proven (19).

In the present communication we extend these studies and provide in vivo proof for the key role of a previously in vitro-characterized protein as a translation factor (aSelB) in selenoprotein synthesis (21). Moreover, the physiological role of selenoproteins in the metabolism of *M. maripaludis* and the regulatory pattern of the selenocysteine- and the cysteine-containing homologs are characterized.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. maripaludis* JJ (DSM 2067) was cultivated at 37°C in defined McSe medium. For the preparation of McSe medium, all metal sulfates of McN (36) were replaced by the respective chlorides without changing the concentration of the corresponding cations. For growth on formate, the medium contained 2% sodium formate; to keep the pH constant during growth, 80 mM morpholinepropanesulfonic acid (pH 6.8) was also added. Na_2SeO_3 was supplemented from a separate stock solution to a final concentration of 10 μM. Cysteine-HCl (15 mM) served as the sole reductant. The concentration of residual selenium in McSe medium was determined by atomic absorption spectroscopy after generation of Se hydrides to 0.1 μM (Flipo, Turciansce Teplice, Slovakia). Solid medium contained 1% (wt/vol) Bacto agar (Difco). Puromycin (2.5 μg/ml) was added for the growth of transformants carrying the *pac* resistance cassette (9). Cultures were pressurised every 12 h with 2.02×10^5 Pa of H_2/CO_2 (80:20), or 1.01×10^5 Pa of N_2/CO_2 (80:20) for growth on formate. Growth was monitored by following the optical density at 580 nm (OD_{580}) of the cultures and averaging the values of at least three parallel cultures.

Metabolic labeling of *M. maripaludis* with ^{75}Se-labeled selenite (specific activity, 0.19 Ci/mmol) and detection of labeled macromolecules were conducted as described previously (19).

Escherichia coli XL10 Gold-Kan (Stratagene) was grown either in double-

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concentrated liquid Luria-Bertani (LB) medium or on regular LB plates (22), both containing 50 μg of kanamycin/ml. For cultivation of transformants carrying derivatives of pIJA03, 100 μg of ampicillin/ml was added to the medium.

Disruption of the *selB* gene in *M. maripaludis* JJ. Standard molecular techniques were applied as described previously (2, 22). The primers oselB-1 (5′-CCTTTATATCTAAGTGCATATATAATCTAG-3′) and oselB-2 (5′-CTACCGCATGTTCTCCAAATAGTAT-3′) were deduced from preliminary genomic DNA sequences of *M. maripaludis* LL (Leigh, unpublished) to amplify the *selB* gene of *M. maripaludis* JJ by PCR. Both strands of the products from three independent PCRs were sequenced with a Dye Terminator cycle sequencer (Perkin-Elmer, Zaventem, Belgium). The GenBank accession number of the nucleotide sequence of the *selB* gene of *M. maripaludis* JJ is Y150167). From this sequence, the primers oJB1 (5′-GGAATTCGGTTTTTCAGCATTTAAAC-3′) and oJB2 (5′-GAAGTTAAAATACATCCTCG-3′) were deduced to obtain a 662-bp fragment of the *selB* gene by PCR that contained an *Eco*RI restriction site introduced on its 5′ end (the 3′ region of the PCR fragment contained an indigenous *Bgl*II restriction site). The *Eco*RI-*Bgl*II-digested fragment was ligated into pIJA03 (29) restricted with the same enzymes and transformed into XL10 Gold-Kan. The resulting plasmid was named pJKoB1. With the aid of the primers oJB3 (5′-GGGGTACCGAGGATGTATTTTAACTTC-3′) and oJB4 (5′-GACTAGCTAGCCCATCTTCTAAGCCTG-3′), a second, 618-nucleotide fragment of the *selB* gene was generated by PCR containing *Kpn*I and *Nhe*I restriction sites at the 5′ and 3′ end, respectively. After restriction with *Kpn*I and *Nhe*I, it was ligated with the identically treated pJKoB1 vector and propagated in XL10 Gold-Kan. The resulting plasmid was designated pJKoB2.

The pJKoB2 plasmid was linearized by digestion with *Eco*RI and *Nhe*I and the 2.6-kb DNA fragment enclosing the *pac* resistance cassette flanked by *selB* DNA sequences from *M. maripaludis* JJ was purified by agarose gel electrophoresis. *M. maripaludis* JJ was transformed with this linear DNA fragment as described previously (32) and plated on solid medium in serum bottles (31). Individual colonies were inoculated into McSe liquid medium and at an OD_{580} of 0.6 the cultures were diluted 10^6-fold and again plated to obtain a pure clone. Total DNA was isolated from clone JB14 by the CTAB (cetyltrimethylammonium bromide) method (2). *Xho*I-restricted chromosomal DNA was probed by Southern hybridization (22) with a *pac*-specific *Xba*I/*Nhe*I restriction fragment of pIJA03. To verify the disruption of the *selB* gene by the *pac* cassette, the primers oforw1 (5′-GAATCAGGGATCATATCTATCACCAAAAAATCAGTTC-3′), oforw2 (5′-CTGAAATTGCATCAACTTCGGCACACGATAAACTG-3′), and orev (5′-CCATCTTCTAAGCCTGTTTAATATTACCTTGTCACG-3′) were employed together with the Advantage GC2 PCR kit (Clontech, Heidelberg, Germany).

Immunoblotting analysis. Purified aSelB of *M. jannaschii* (the MJ0495 protein [21]) was used for immunization of a rabbit to produce aSelB-specific antibodies (Seqlab, Göttingen, Germany). Immunoglobulins devoid of unspecific cross-reactivity were prepared by affinity chromatography of crude α-aSelB serum. For this purpose, aSelB of *M. jannaschii* was immobilized on CNBr-activated Sepharose 4B (Amersham, Freiburg, Germany) according to the manufacturer's instructions and used as an affinity matrix. Antibody purification was carried out according to the manufacturer's instructions. Crude extracts of *M. maripaludis* were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE [16]), and the separated macromolecules were blotted on a nitrocellulose membrane. The blot was treated with the purified aSelB-specific antibodies as described previously (25). Peroxidase-conjugated protein A from *Staphylococcus aureus* (Bio-Rad, Munich, Germany) was used for detection with the ECL system (Roche, Penzberg, Germany).

For immunological detection of FDH subunits from *M. maripaludis*, antiserum directed against the FDH of *Methanobacterium formicicum* (13, 26) was employed at a 1:250 dilution. The serum was kindly donated by J. G. Ferry.

FDH activity staining of polyacrylamide gels. FDH activity in polyacrylamide gels was visualized employing a modification of a described method (24). All steps were carried out in an anaerobic glove box. Cell pellets (0.5 OD units) were lysed in sample buffer (0.01 U of DNase I/μl in separation buffer). After centrifugation, supernatants were separated anaerobically by electrophoresis in a 10% nondenaturing polyacrylamide gel (16) containing 0.1% SDS. The separation buffer consisted of 25 mM Tris (pH 8.8), 192 mM glycine, 10 mM sodium azide, 2 mM dithiothreitol, 0.1% SDS, and 5% glycerol. After electrophoresis, the gel was transferred to an anaerobic box that was sealed and purged with N_2 to remove residual hydrogen. The staining solution (60 mM potassium phosphate, pH 7.5; 1.5 mM methyl viologen; 20 mM sodium formate; 1 mM dithiothreitol) was added anaerobically and the developing blue stain was fixed by addition of 0.5 mM 2,3,5-triphenyltetrazolium chloride.

2D-PAGE. Separation of *M. maripaludis* proteins by two-dimensional PAGE (2D-PAGE) was carried out as described previously (10), with the following modifications: Immobiline dry strips (Amersham) with a length of 180 mm and a nonlinear pH gradient from 3 to 10 were used. Cells of *M. maripaludis* (corresponding to up to 3 mg of total protein) were lysed in 45 μl of water containing 1 U of DNase I. After centrifugation at 14,000 × *g*, 105 μl of sample solution (Immobiline dry strip kit; Amersham) was added to the supernatant to give a final sample volume of 150 μl. The sample was applied at the anode end of the gel, and electrophoresis was performed at 3,500 V for 20 h. After subsequent separation in an SDS–10% polyacrylamide gel (23), the proteins were stained with Coomassie blue.

Identification of proteins from 2D polyacrylamide gels. Proteins separated by 2D-PAGE were directly electroblotted onto an Immobilon-polyvinylidene difluoride membrane (Millipore). The membrane was stained with Coomassie blue and destained for 10 min with 10% (vol/vol) acetic acid before spots were excised for N-terminal amino acid sequence analysis. Sequencing was performed in a protein sequencer 492A (Applied Biosystems) according to the instructions of the manufacturer. The sequences obtained were used as a probe to search the databases for fitting proteins via the BLAST algorithm (1).

RESULTS

M. maripaludis JJ synthesizes at least eight selenium-containing macromolecules, two of which probably represent tRNAs and six of which represent selenocysteine-containing proteins (19). It has been speculated that most of the selenoproteins of this organism may be involved in methanogenesis, the path of energy conservation in methanogenic *Archaea*.

Disruption of the *selB* gene. To investigate the function of the selenoproteins, an *M. maripaludis* strain was constructed that was unable to synthesize selenoproteins. For this purpose, the selenocysteine-specific translation factor aSelB was chosen as the target because, analogous to the bacterial system, this protein is thought to be the central component in selenoprotein biosynthesis. The coding gene was identified within the genomic DNA sequences of *M. maripaludis* LL (Leigh, unpublished) on the basis of its high similarity to the *selB* gene from *M. jannaschii* (6, 21). With the sequence from strain LL as a lead, the homologous open reading frame from *M. maripaludis* JJ could be cloned and sequenced (see Materials and Methods). The deduced amino acid sequence of the gene product is 62% identical to that of the corresponding *M. jannaschii* protein and contains all of the primary structural features that qualify it as the archaeal selenocysteine-specific elongation factor (20).

For inactivation of SelB in *M. maripaludis* JJ, plasmid pJKoB2 was constructed, in which the *pac* cassette is flanked by 630- and 618-nucleotide portions of the coding region of *selB* (Fig. 1). The plasmid was linearized by restriction, and the linear DNA fragment containing the *selB* knockout construct was transferred into *M. maripaludis* JJ. To aquire puromycin resistance, two homologous recombination events have to take place, replacing the wild-type *selB* gene by the knockout construct (Fig. 1). One puromycin-resistant clone obtained was designated JB14 and examined further.

Characterization of JB14. By Southern hybridization it was confirmed that the *pac* cassette had inserted in single copy into the chromosome (data not shown). To subsequently verify its insertion into the *selB* gene, analytical PCR was performed with chromosomal DNA as a template and oligonucleotides oforw1, oforw2, and orev, respectively, priming within and upstream of the gene (Fig. 1). The size of the PCR products derived from JB14 corresponded to the expected size of the *selB* gene disrupted by the *pac* cassette (Fig. 2A), thus confirming its correct insertion into the chromosome.

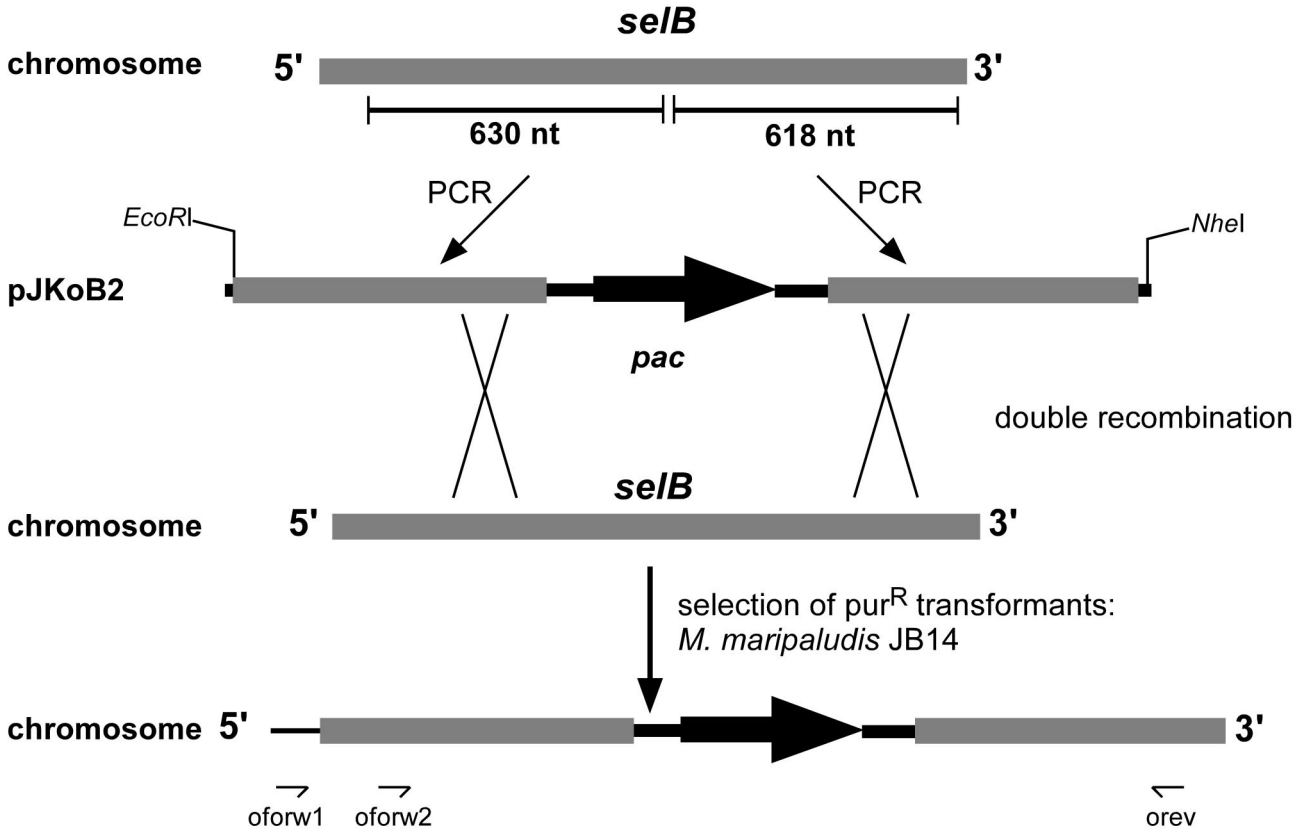

FIG. 1. Strategy for the disruption of the *selB* gene of *M. maripaludis* JJ. pJKoB2 was constructed by flanking the *pac* resistance cassette with sequences of *selB* generated by PCR. *M. maripaludis* JJ was transformed with linear DNA (*pac*-containing *Eco*RI-*Nhe*I fragment of pJKoB2) to obtain puromycin-resistant transformants through double recombination events.

To demonstrate the absence of the *selB* gene product, cell lysates of *M. maripaludis* JB14 and JJ were subjected to immunoblotting analysis (Fig. 2B). Affinity-purified antibodies directed against aSelB from *M. jannaschii* specifically reacted with a protein in an extract from *M. maripaludis* JJ that corresponds in size to that of the deduced *selB* gene product of this organism (Fig. 2B, first and second lanes). The amount of reacting protein did not vary with the selenium status of the

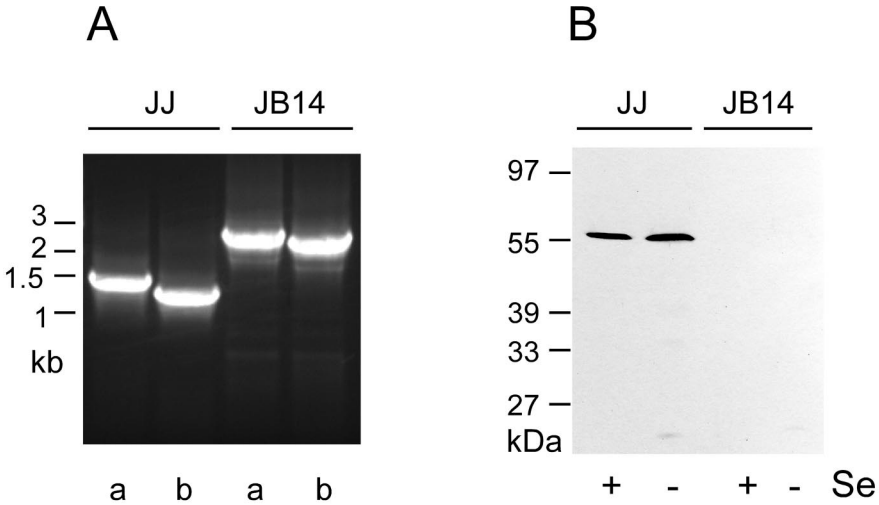

FIG. 2. Analysis of strain JB14. (A) PCR products generated with the primer pairs oforw1-orev (lanes a) and oforw2-orev (lanes b) by using genomic DNA of *M. maripaludis* JJ and JB14, respectively, as templates; numbers give the size of the DNA standards in kilobase pairs. (B) Immunoblotting analysis of *M. maripaludis* JJ and JB14. Cells were grown in media supplemented with 10 μM selenite (+Se) or without selenium supplementation (−Se), respectively. Extracts were separated by SDS-PAGE, blotted onto a nitrocellulose membrane, and probed with antibodies directed against aSelB from *M. jannaschii*. Molecular mass standards are indicated on the left.

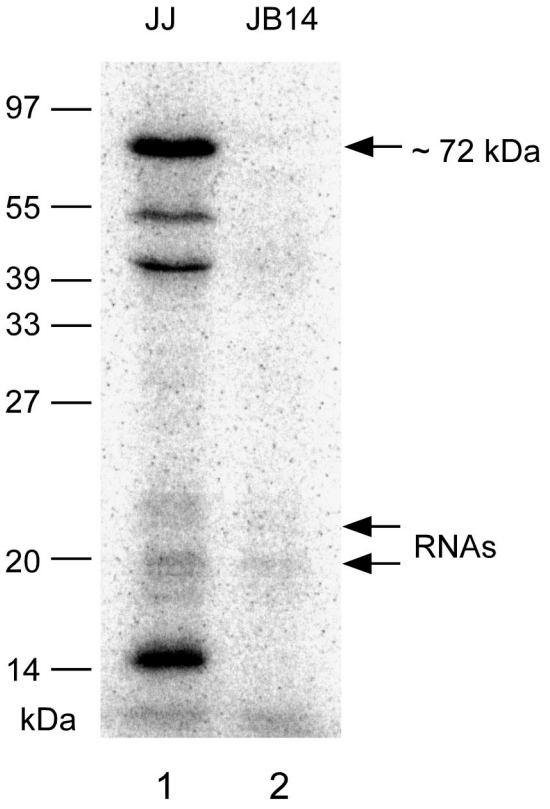

FIG. 3. Selenoprotein synthesis by *M. maripaludis* JB14. Autoradiograph of a SDS–12% PAGE gel after electrophoresis of cell lysates from ^{75}Se-labeled *M. maripaludis* JJ (lane 1) and JB14 (lane 2), respectively. The migration positions of the standard proteins are indicated on the left; the positions of the putative FdhA selenoprotein and of ^{75}Se-labeled RNAs are also indicated (see the text for details).

culture. Its synthesis, therefore, appears to be independent of the presence of selenium. However, regulation of *selB* expression by selenium concentrations of <0.1 μM cannot be ruled out because further reduction of the selenium concentration in the medium could not be achieved. Extracts from JB14 did not react with the antibodies under the same conditions, a finding which confirms the absence of aSelB in this strain (Fig. 2B, third and fourth lanes).

Next, the role of aSelB in selenoprotein synthesis in vivo was tested. *M. maripaludis* JJ and JB14 were metabolically labeled with [^{75}Se]-selenite. Crude extracts were separated by SDS-PAGE, and the selenium-containing macromolecules were visualized by phosphoimaging. As shown in Fig. 3, *M. maripaludis* JB14 is unable to synthesize the selenoproteins (lane 2) that are present in the wild type (lane 1). This finding demonstrates the crucial role of aSelB in archaeal selenoprotein biosynthesis. Intriguingly, however, disruption of *selB* was not lethal, a finding which excludes essential functions for any of the selenoproteins of *M. maripaludis* upon growth with hydrogen as the electron donor. Furthermore, the ^{75}Se-labeled RNA species (Fig. 3, arrows), albeit reduced in amount, were not absent in JB14; this finding suggests an aSelB-independent biosynthetic route for the seleno-modified RNAs, as had been shown for *E. coli* (28).

Analysis of the protein pattern of *M. maripaludis* JJ and JB14. It was plausible to assume that the selenoproteins of *M. maripaludis*, although dispensable for growth on H_2/CO_2, serve distinct metabolic functions. To investigate the effect of selenium on its hydrogenotrophic metabolism, the protein pattern was analyzed. *M. maripaludis* JJ and JB14 were grown on H_2/CO_2 with or without selenium supplementation; extracts were prepared and analyzed by 2D-PAGE. After the gels were stained with Coomassie blue, several proteins were identified that were synthesized at a significantly increased level in the absence of supplemented selenium or that disappeared when the medium was fortified with adequate amounts of selenium or vice versa; Fig. 4A and B display sections of the stained gels. When extracts from *M. maripaludis* JB14 were analyzed (Fig. 4C), it was found that the protein pattern of cells grown under selenium-limited and selenium-adequate conditions were identical. Interestingly, these patterns were exactly the same as those of wild-type cells cultivated under selenium-limited conditions.

The spots showing the most significant differences under selenium-adequate and selenium-reduced conditions were excised from the gels and subjected to N-terminal amino acid sequence analysis, and the proteins were identified by comparison with proteins in the databases by using the BLAST tool (Table 1). One protein that was preferentially synthesized in selenium-enriched medium was characterized as the homolog of the large subunit of the selenocysteine-containing Vhu hydrogenase, namely, VhuA. A protein preferentially formed in the medium without selenium supplementation is a homolog of the large subunit of the selenium-free hydrogenase Vhc, namely, VhcA. Intriguingly, selenium starvation also led to a significant increase in the level of two enzymes directly involved in the methanogenic pathway: N^5,N^{10}-methylenetetrahydromethanopterin dehydrogenase (Hmd) and formylmethanofuran:tetrahydromethanopterin *N*-formyltransferase (Ftr).

Physiology of JB14. To assess the consequences of the loss of selenoproteins in *M. maripaludis*, attempts were made to specify a phenotype for JB14 that differentiates it from the wild type. For this purpose, growth experiments were performed with the wild-type *M. maripaludis* and the *selB* mutant. When *M. maripaludis* JJ was cultivated with H_2/CO_2, no differences in growth could be observed when selenium was omitted from the medium or added to a concentration of 10 μM selenite (Fig. 5A, solid symbols). This finding is in contrast to reports on a stimulatory effect of selenium on the growth rate (15). The discrepancy might be due to the contaminating selenium in the medium (0.1 μM; see Materials and Methods). Strikingly, however, JB14 also showed growth behavior very similar to that of the wild type when cultivated on H_2/CO_2 (Fig. 5A, open symbols). The only difference was, apart from a somewhat prolonged lag phase, that the growth rate was decreased from 0.35 to 0.30 h^{-1}. The absence of selenoproteins therefore has only a marginal effect on the growth of this organism on H_2/CO_2.

When cultivated on formate, however, the growth of wild-type *M. maripaludis* was clearly stimulated by the addition of selenium to the medium (Fig. 5B). Strain JB14 did not grow on this substrate at all (Fig. 5B), thus demonstrating a crucial role of at least one selenoprotein in the utilization of this substrate. Identical results were obtained when the concentrations of tungstate and molybdate were varied (data not shown); these

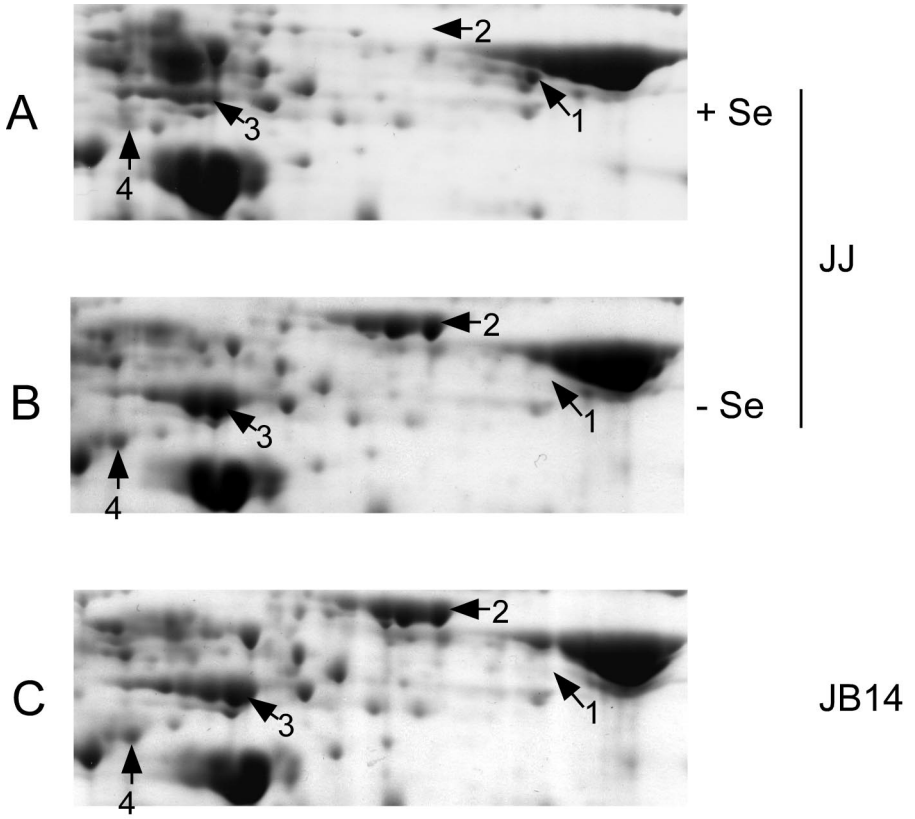

FIG. 4. Selenium-dependent protein synthesis by *M. maripaludis*. Cells of *M. maripaludis* were grown on H_2/CO_2 with (+Se) or without (−Se) selenite (10 μM) supplementation. Extracts were separated by 2D-PAGE, and the proteins were stained with Coomassie blue. (A and B) Sections of 2D gels with separated proteins from *M. maripaludis* JJ; (C) section of a 2D gel with separated proteins of *M. maripaludis* JB14 grown in medium containing 10 μM selenite. Arrows indicate the proteins that were subjected to N-terminal sequencing.

trace metals are known to greatly influence formate utilization in vivo (11). A possible explanation for the observed phenotype of JB14, therefore, is that the responsible enzyme formate dehydrogenase (FDH) is selenium dependent.

To address the question whether the activity of this enzyme could be responsible for the observed selenium dependence of *M. maripaludis* JJ and JB14 on formate, cell extracts were separated by nondenaturing PAGE, and the gels were analyzed by FDH activity staining (Fig. 6A). It was found that the level of FDH activity in the *M. maripaludis* wild type was lower under selenium starvation compared to the situation in selenium-adequate cells (Fig. 6A, lanes 1 to 4). There was no qualitative difference in the observed results whether formate or H_2/CO_2 served as the carbon and energy source. In extracts from JB14, no FDH activity could be detected with this method (Fig. 6A, lanes 5 and 6).

Next, the level of FDH was analyzed immunologically. The same extracts as those used for the activity staining were probed with antibodies specific for the FDH of *Methanobacterium formicicum* (26). A protein of ca. 72 kDa (Fig. 6B) was the major reacting protein species that probably represents the large subunit (FdhA) of this enzyme. The size corresponds well with that of the largest of the ^{75}Se-labeled proteins of *M. maripaludis* (see Fig. 3 and reference 19). This finding supports the conclusion that FdhA of this organism is a selenoprotein. The intensity of the signal decreased when the wild type was

TABLE 1. Proteins of *M. maripaludis* JJ synthesized in a selenium-dependent manner[a]

Se status of the culture	Protein (spot no.)	Sequence (accession no.)	Identity and/or function
With Se added	VhuA (1)	GKVTIEPLSRLEGHGK (NP248187)	Subunit of SeCys-containing hydrogenase; methanogenesis
Without Se	VhcA (2)	TKLSIEPVTRVEGHGK (S16727)	Subunit of Se-free hydrogenase; methanogenesis
	Hmd (3)	MKVAILGAGCYRTHAAS (Q50840)	H_2-forming Hmd; methanogenesis
	Ftr (4)	MELNGVIEDTFA (G64339)	Ftr; methanogenesis

[a] Protein spots were excised from 2D gels and analyzed by N-terminal sequencing. The respective proteins were identified by comparison of the resulting sequence and protein sequences in the databases by using the BLAST tool (accession numbers of the best matching sequences are given in parentheses. The spot numbers in parentheses indicate the spot numbers given in Fig. 4.

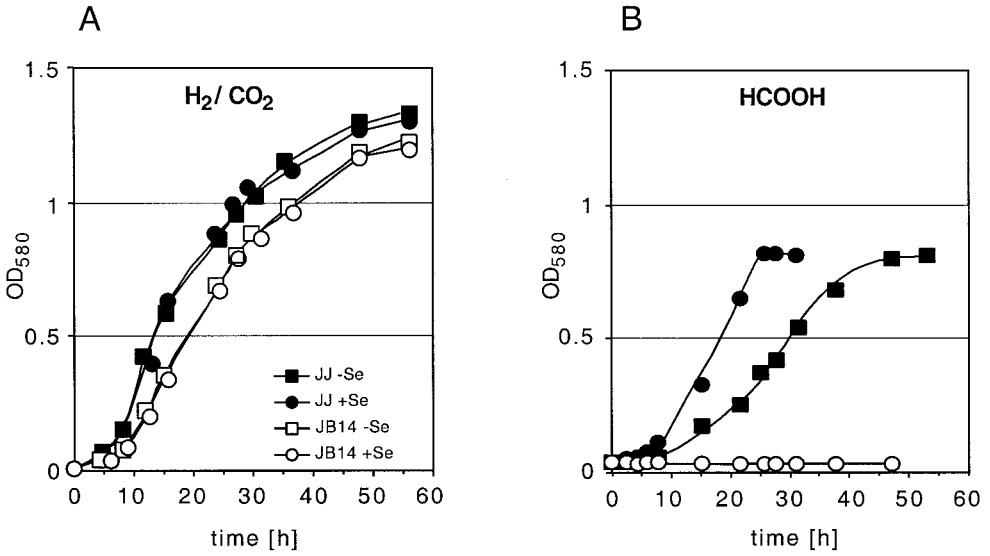

FIG. 5. Selenium dependence of growth. (A) Growth of *M. maripaludis* JJ (solid symbols) and JB14 (open symbols) cultivated on H_2/CO_2 with (circles) or without (squares) selenite (10 μM). (B) Same as panel A, but with cultivation on formate.

cultivated without supplemented selenium, regardless of whether formate or H_2/CO_2 served as the carbon and energy source (Fig. 6B, second and fourth lanes). When the signal from the 72-kDa protein decreased, an additional protein of 75 to 80 kDa was recognized by the antiserum (Fig. 6B, asterisk). In extracts from JB14, only the larger protein was detected by the antiserum (Fig. 6B, fifth and sixth lanes).

The function of the 75- to 80-kDa protein that interacts with the α-FDH serum is not clear at present; the existence of a selenium-independent subunit of FDH that could substitute for the selenium-dependent one seems unlikely because no FDH activity was detectable in the *selB* mutant and no growth was observed with formate as a substrate.

DISCUSSION

The development of genetic systems for methanogenic *Archaea* opens the many unique functional and physiological features of these organisms for a causal analysis (4, 7, 17). By employing the genetic tools derived for *M. maripaludis* by Whitman and coworkers (29), we addressed three issues connected with the metabolic role of the trace element selenium.

The first concerns the in vivo role of a protein (MJ0495 of *M. jannaschii*) previously characterized as the putative archaeal homolog of the bacterial translation factor SelB (21). It shares with SelB the capacity to bind guanosine nucleotides and selenocysteylated $tRNA^{Sec}$ but, according to in vitro data, lacks the ability to bind to the archaeal SECIS element which, like the eukaryal one, is located in the 3′-untranslated region of the selenoprotein mRNA (19). The disruption of the respective gene now proves that its function is indispensable for selenoprotein synthesis since it leads to the inability of the mutant to synthesize the set of selenoproteins detectable by our procedure. Formation of selenylated tRNAs is not affected significantly, indicating that the same pathway may function as in *Bacteria* and *Eukarya* (28, 35). MJ0495 can be annotated now as the true homolog of SelB.

A second issue addressed here concerns the fact that many organisms that are able to synthesize selenoproteins possess the genetic capacity for the formation of a selenium-free cysteine-containing backup system. Predominant examples are the hydrogenases of *M. voltae* (12) and the formylmethanofuran dehydrogenases of *Methanopyrus kandleri* (34). In both cases, synthesis of the backup system is repressed when adequate amounts of selenium are present in the medium. The same pattern of regulation of the hydrogenases is displayed by the wild-type strain of *M. maripaludis*; in contrast, however, when the *selB* gene is inactivated as in mutant JB14, this repression by high levels of selenium is relieved. Clearly, free selenium itself cannot be the compound active in regulation but rather a derivative synthesized in its presence. Plausible

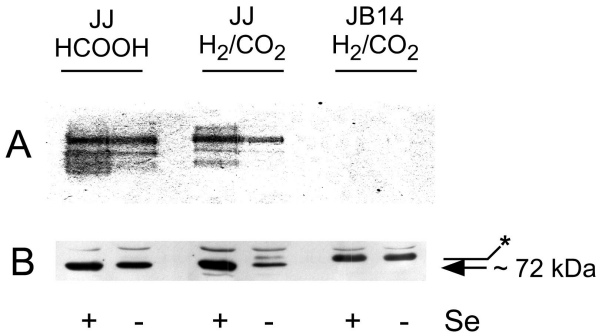

FIG. 6. Selenium dependence of the formation of FDH by *M. maripaludis*. (A) FDH activity staining of extracts from *M. maripaludis* JJ and JB14 after nondenaturing PAGE. Cells were grown on H_2/CO_2 or formate and with (+Se) or without (−Se) selenite (10 μM). (B) Immunoblotting analysis of FDH. The extracts in panel A were separated by SDS-PAGE, blotted onto a nitrocellulose membrane, and probed with antibodies directed against FDH from *Methanobacterium formicicum*. The arrow at 72 kDa indicates the position of the putative FdhA selenoprotein (see Fig. 3); the asterisk indicates a protein of 75 to 80 kDa, the synthesis of which is stimulated by selenium starvation (see the text for details).

candidates might be selenocysteyl-tRNA^{Sec} or some selenoprotein. In *E. coli*, a complex of selenocysteyl-tRNA^{Sec} and SelB regulates the expression of the *selAB* operon from *E. coli* at the translational level (M. Thanbichler and A. Böck, unpublished results).

In *M. voltae*, reduction of the selenium supply led to significantly reduced growth of this organism (4), whereas *M. maripaludis*, even when deficient in *selB*, still grows at almost wild-type rates under the same conditions. The capabilities to compensate for the loss of selenoproteins, therefore, must be highly efficient in *M. maripaludis*.

Synthesis of both Hmd and Ftr increased upon *selB* disruption or selenium depletion. The physiological role of this phenomenon is unclear at present. It might reside in an augmentation of the flux through reactions thermodynamically less favorable when the selenoproteins are absent. Sulfur variants are known to generally display a grossly decreased kinetic efficiency (3, 5, 8). This decreased kinetic efficiency would then have to be compensated for by increasing the amount of the respective proteins to retain competitive substrate turnover. Synthesizing selenoproteins thus could be a strategy to avoid unnecessary protein synthesis.

Finally, it was surprising to find that inactivation of the *selB* gene prevented growth at the expense of formate but not of H_2/CO_2. The dependence could be correlated with the selenium-dependent synthesis of an FDH. Apparently, no cysteine form of this enzyme can be formed by *M. maripaludis* JJ. The genetic capacity for the synthesis of the Se or S forms of FDH, therefore, varies greatly among methanogens. *M. maripaludis* is able to solely form the Se form, *M. vannielii* has the ability to synthesize both forms (14), and *Methanobacterium formicicum* can only generate the S form (26). These findings may be due to the chemical microenvironment in which these organisms exist.

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