

Involvement of an Unusual *mol* Operon in Molybdopterin Cofactor Biosynthesis in *Ralstonia eutropha*

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Abstract

In contrast to its parent strain, transposon Tn5-Mob insertion mutant HB6 of the facultative chemoautotroph *Ralstonia eutropha* was unable to grow organoautotrophically on formate and exhibited no activity of Mo-dependent, membrane-bound formate dehydrogenase (M-FDH) when cultivated mixotrophically on fructose plus formate. The activity of another molybdoenzyme, the soluble, NAD⁺-linked formate dehydrogenase which is the key enzyme of formate utilization in *R. eutropha*, was greatly diminished in the mutant. HB6 also lacked the W-dependent M-FDH activities that were newly discovered in organoautotrophically, lithoautotrophically, or mixotrophically grown wild-type cells. However, an additional W-dependent M-FDH activity, observed in heterotrophically grown stationary-phase cells, was present in the mutant although at a considerably reduced level. Sequence analyses of the complementing chromosomal wild-type and the corresponding mutant DNA fragment revealed the transposon insertion to be located in *moeA*, a gene involved in the biosynthesis of the molybdopterin cofactor (MoCo). Nevertheless, mutant HB6 was able to grow on xanthine as carbon and energy source and with nitrate as nitrogen source. The utilization of these substrates requires the function of the MoCo-containing enzymes xanthine dehydrogenase and assimilatory nitrate reductase, respectively, that were still active in the mutant. A *moeA* deletion mutant exhibited the same phenotype as that of HB6. The *moeA* gene belongs to an unusual *mol* operon consisting of four genes (*moeA*, *moaD*, *moaE*, and *moaF*) and being constitutively expressed at low level. Unlike *MoeA*, the large subunit of molybdopterin

synthase encoded by *moaE* is essential for molybdopterin biosynthesis as was evident by the phenotype of a *moaE* deletion mutant. *MoaF* is a novel gene product which showed no similarity to proteins with known function but was indispensable for reconstituting organoautotrophic growth in HB6. The findings suggest that *MoeA* of *R. eutropha* is differentially involved in the biosynthesis or incorporation of pterin cofactors off/into the various molybdo- and tungstoenzymes.

Introduction

Formate and hydrogen are alternative energy sources for autotrophic growth of the facultatively chemoautotrophic bacterium *Ralstonia eutropha* (formerly *Alcaligenes eutrophus*) H16 (Bowien and Schlegel, 1981). The organism oxidizes formate to CO₂ by means of two types of formate dehydrogenases (FDH): (i) a soluble, NAD⁺-reducing enzyme (S-FDH) and a membrane-bound activity (M-FDH) presumably coupled to the respiratory chain (Friedrich *et al.*, 1979). Assimilation of CO₂ proceeds via the Calvin-Benson-Bassham carbon reduction cycle (Bowien *et al.*, 1990). Hydrogen is oxidized by two different hydrogenases, the structural, accessory, and regulatory genes of which (*hox* regulon) are encoded on megaplasmid pHG1 (Friedrich and Schwartz, 1993; Lenz and Friedrich, 1998). Organoautotrophic growth on formate and formation of active S-FDH are strictly dependent on molybdate and inhibited by tungstate as a biological antagonist of molybdate (Friedebold and Bowien, 1993). Surprisingly, M-FDH-activity has been detected in the presence of both molybdate and/or tungstate in the culture medium, suggesting the unusual occurrence of a W-containing in addition to a Mo-containing M-FDH in this aerobic bacterium (T. Vogt and B. Bowien, unpublished data).

The S-FDH is composed of four nonidentical subunits and contains the molybdenum cofactor (MoCo) molybdopterin guanine dinucleotide (MGD), FMN and a number of [2Fe-2S] and [4Fe-4S] centers as redoxactive components (Friedebold and Bowien, 1993; Friedebold *et al.*, 1995). The enzyme is characterized by a modular structure. It consists of a formate-oxidizing and a NADH-oxidoreductase moiety, the latter showing structural relationships to the corresponding modules in hydrogenases and in complex I of the respiratory chain (Oh and Bowien, 1998). Structural and genetic information on the M-FDH of *R. eutropha* is still lacking. The structural S-FDH genes are part of the *fds* operon, which is controlled by *FdsR* acting as both positive and negative regulator of the operon (Oh and Bowien, 1999). Molybdoenzymes are

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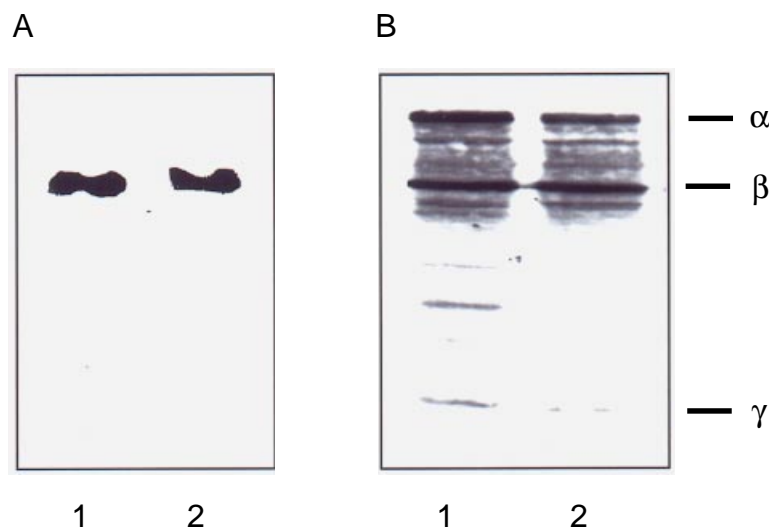


Figure 1. Detection of S-FDH in cell extracts of *R. eutropha* strains HF210 (1) or HB6 (2) grown mixotrophically on fructose plus formate. (A) Activity staining after nondenaturing polyacrylamide gel electrophoresis. (B) Immunostaining with S-FDH antibodies after sodium dodecylsulfate polyacrylamide gel electrophoresis and subsequent Western blotting. The positions of subunits α , β , and γ are indicated (subunit δ did not produce a detectable antigenic reaction). In all cases 20 μ g of extract protein were applied per lane.

not only indispensable for formate metabolism but also for nitrate assimilation, denitrification and xanthine catabolism in *R. eutropha*. Three molybdenum-dependent nitrate reductase activities have been described (Warnecke-Eberz and Friedrich, 1993). *R. eutropha* also produces a Mo-containing xanthine dehydrogenase during growth on xanthine as sole carbon and energy source (J.-I. Oh and B. Bowien, unpublished data).

Molybdoenzymes are ubiquitous throughout all three domains of life. Except for nitrogenase all molybdoenzymes contain a MoCo, a pterin derivative complexing the molybdenum atom (Rajagopalan, 1991; Rajagopalan and Johnson, 1992). Biosynthesis of the active cofactor requires transport of molybdate into the cell, formation of the molybdopterin (MPT) moiety and eventually incorporation of activated molybdate into MPT. In *Escherichia coli* five

MoCo-specific operons, designated *moa*, *mob*, *mod*, *moe* and *mog*, have been identified (Rajagopalan, 1996). Five genes that are involved in the synthesis of the pterin moiety comprise the *moa* locus (Johnson and Rajagopalan, 1987; Rivers *et al.*, 1993). The action of MoeABC leads to the formation of a sulfur-free pterin compound, the so-called precursor Z, the conversion of which to MPT is catalysed by molybdopterin synthase encoded by *moaD* and *moaE*. The small subunit MoeD incorporates the sulfur atoms into precursor Z and is reactivated by MoeB-catalyzed sulfurylation (Pitterle *et al.*, 1993). For MoeA and MogA a function in transfer or activation of molybdate for incorporation into MPT has been suggested (Hasona *et al.*, 1998; Joshi *et al.*, 1996). The Mob proteins are required for the production of MGD (Palmer *et al.*, 1996; Eaves *et al.*, 1997). Recently the crystal structures of the MoeA, MobA and MogA proteins from *E. coli* were solved (Wuebbens *et al.*, 2000; Lake *et al.*, 2000; Stevenson *et al.*, 2000; Liu *et al.*, 2000). X-ray analysis of molybdopterin synthase revealed a strong similarity between its small subunit and ubiquitin (Rudolph *et al.*, 2001).

In the present study the characterization of transposon Tn5-Mob insertional mutant HB6, originally isolated as being deficient in formate utilization, led to the identification of four genes, *moeA*, *moaD*, *moaE* and *moaF*, involved in molybdopterin biosynthesis in *R. eutropha*. The genes appear to form a novel transcriptional unit designated as *mol* operon. The phenotypes of *moeA* and *moaE* deletion mutants indicated a differential involvement of these genes in the formation of active molybdo- and tungstoenzymes in *R. eutropha*.

Results

Phenotypic Properties of Fox⁻ Mutant HB6

Insertional mutagenesis of *R. eutropha* HF210 with transposon Tn5-Mob has been performed to identify genes

Table 1. Specific M-FDH activities in *R. eutropha* strains HF210 (pHG1) and HB6 (pHG1) grown under various conditions in the presence or absence of molybdate or tungstate

	Addition of Metal Specific M-FDH Activity (U/mg)					
	Formate ^c		H ₂ + CO ₂ ^d		Pyruvate ^e	
	HF210	HB6	HF210	HB6	HF210	HB6
None	0	0	0	0	0	0
Mo ^a	0.42	0	0	0	0	0
W ^b	0.35	0	0.49	0	0.84	0.29
Mo ^a + W ^b	0.30	0	0.58	0	0.81	0.28

^a 1.5×10^{-7} M Na₂MoO₄.

^b 1.5×10^{-5} M Na₂WO₄ (final concentration in growth medium).

^c Mixotrophic growth with fructose plus formate.

^d Lithoautotrophic growth with H₂ + CO₂.

^e Growth on pyruvate into the stationary phase.

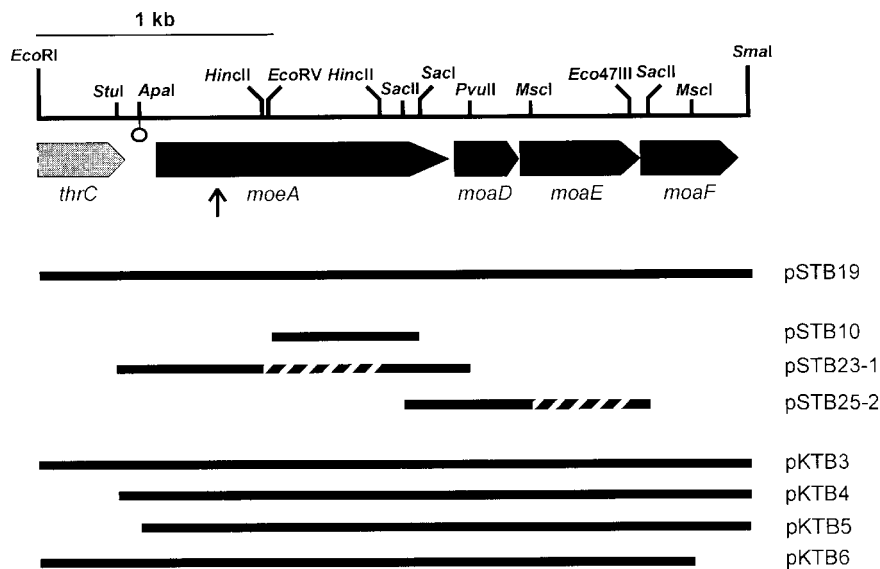


Figure 2. Physical and genetic map of the chromosomal 2690-bp *EcoRI-SmaI* DNA fragment from *R. eutropha* HF39, containing the *mol* operon (*moeA**moaDEF*). The relative orientation of the genes and the positions of cleavage sites for some restriction endonucleases are shown, and the *Tn5*-*Mob* insertion in mutant HB6 (after nucleotide position 763) is marked by an arrow. The position of a stem-loop structure within the intergenic region between the incomplete *thrC* gene and *moeA* is symbolized by a stalked circle. Plasmid pSTB19 carried the completely sequenced fragment. Subclones used for Northern hybridization (pSTB10) and for the construction of in-frame deletions in *moeA* or *moaE* (hatched regions in pSTB23-1 and pSTB25-2, respectively) are also depicted. The pKTB plasmids were employed in phenotypic complementation experiments with HB6; + and - indicate the ability and inability, respectively, of the resulting transconjugants to grow organoautotrophically. The reference bar corresponds to a length of 1 kb.

required for formate metabolism (U. Windhövel and B. Bowien, unpublished data). One of the resulting *Fox*⁻ mutants, HB6, was selected for further studies. Although HB6 was unable to grow on formate or oxalate it retained some capacity to oxidize both substrates as judged by the alkalization of the medium. Oxalate is oxidized in *R. eutropha* via oxalyl-CoA and formyl-CoA to formate (Friedrich *et al.*, 1979). The mutant actually showed only about 15% (0.12 U/mg) of the specific S-FDH activity found in parent strain HF210 (0.80 U/mg) after mixotrophic growth on fructose plus formate, although a nonquantitative activity staining suggested seemingly similar activity levels in wild type and mutant (Figure 1A). Immunoblot analysis indicated that the mutation did not significantly affect the formation (amount, molecular size, and subunit composition) of the S-FDH protein (Figure 1B). In contrast to the parent (0.46 U/mg), M-FDH activity was not detected in the mutant under these conditions. The phenotype of HB6 could not be suppressed by addition of a high molybdate concentration (1 mM) to the growth medium, making it unlikely that the low FDH activities in the mutant were caused by intracellular depletion of molybdate. HB6 was still able to grow on xanthine and to use nitrate as sole nitrogen source. These results suggested that probably an accessory gene function required for the formation of active S- and M-FDH was affected in the mutant.

Since there was preliminary evidence for the occurrence of molybdenum- as well as W-dependent M-FDH activities in *R. eutropha* H16, strains HF210 and HB6 were tested for such activities after growth under different conditions. To enable also lithoautotrophic growth on hydrogen plus CO₂, megaplasmid pHG1 was first transferred into the strains. In fact, mixotrophic cells of

HF210(pHG1) exhibited similar M-FDH activities after growth in the presence of molybdate or tungstate but such cells of the mutant (HB6[pHG1]) lacked both activities (Table 1). In contrast to S-FDH that is exclusively induced by its substrate formate (Oh and Bowien, 1999), M-FDH activity was present in HF210(pHG1) when grown lithoautotrophically in medium supplemented with tungstate. This activity was apparently absent from HB6(pHG1). Moreover, W- but not Mo-dependent M-FDH activities were detected in stationary, i.e. energy-deprived, cells of both strains after heterotrophic growth on pyruvate, although the activity in the mutant was about 60% lower than in the parent (Table 1). The data support the conclusion that in *R. eutropha* at least one Mo- and two W-dependent M-FDH might be produced with a differential involvement of the mutation in HB6.

Identification and Sequence Analysis of the Gene Region Mutationally Affected in HB6

Phenotypic complementation (*Fox*⁺) of mutant HB6 with cosmid clones from a genomic library of *R. eutropha* HF39 (Freter and Bowien, 1994) enabled the isolation of hybrid cosmid pDB1. A chromosomal 18-kb *EcoRI* fragment from HF39 cloned in pDB1 suppressed the mutation. The complementing function encoded on this large fragment was delimited to a 3-kb *EcoRI-SmaI* subfragment (pKTB3). An 25.4-kb *EcoRI* genomic fragment from HB6 carrying the *Tn5*-*Mob* insertion (7.4 kb) was obtained after packaging in λ phage particles. Comparative restriction mapping of the 18- and 25.4-kb fragments located the transposon insertion within a 1-kb *EcoRI-EcoRV* subfragment (Figure 2). The exact insertion site was determined by DNA sequencing.

Sequence analysis revealed the presence of four complete (*moeA*, *moaD*, *moaE*, *moaF*) and one incomplete (*thrC*) open reading frames within the 2960-bp *EcoRI-SmaI* subfragment (Figure 2). The Tn5-Mob insertion was located near the 5' end of *moeA* between nucleotide positions 763 and 764. A significant similarity (39% identity) of the deduced gene product (molecular mass of 43.5 kDa) to MoeA of *E. coli* was found that is known to be required for MoCo biosynthesis (Johnson and Rajagopalan, 1987). The MoCo biosynthesis proteins A1 and A2 of *Pseudomonas aeruginosa* showed the highest similarities (42 and 43 % identity, respectively; Stover *et al.*, 2000). The next two genes, *moaD* (32 bp downstream of *moeA*) and *moaE* (1 bp downstream of *moaD*) encode potential proteins of 9.2 and 18.2 kDa, respectively, and exhibited significant similarities to MoaD and MoaE of *P. aeruginosa* (PA3917 and PA3916; 42 % and 58 % identity, respectively). In *E. coli* MoaD has been shown to represent the small, MoaE the large subunit of MPT synthase (Pitterle *et al.*, 1993). The start codon of the most distal gene overlaps the stop codon of *moaE*. Although the gene product exhibited no similarity to other known proteins involved in MoCo biosynthesis, the gene was designated *moaF* (Figure 2). Significant resemblance of MoaF was, however, observed to several proteins of unknown function, again with the highest similarity to a conserved hypothetical protein of *P. aeruginosa* (PA4383; 53 % identity).

Upstream of *moeA* the 3'-end of the *thrC* gene was identified, its product displaying high similarity to threonine synthases of various organisms, e. g. 67 % identity to ThrC from *Methylobacillus glycogenes* (Motoyama *et al.*, 1994). The DNA segments flanking the analyzed region do not encode genes necessary for either MoCo or molybdo-/tungstoenzyme formation (data not shown). Thus, the sequence data suggested the possible occurrence of a tetracistronic *mol* operon (*moeA moaDEF*) in *R. eutropha* H16.

Transcript Analysis of the *mol* Operon

Northern blot analyses with total RNA isolated from HF210(pHG1) or HB6(pHG1) were performed to study the

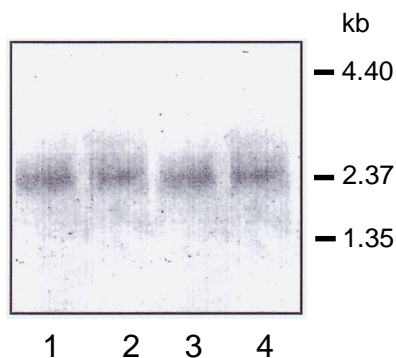


Figure 3. Transcript analysis of the *mol* operon in *R. eutropha* HF210 (pHG1). Autoradiograph of Northern hybridizations of total RNA (20 μ g) isolated from cells grown on (1) fructose + formate, (2) H_2 + CO_2 , (3) pyruvate (log phase), or (4) pyruvate (stationary phase). Numbers indicate the sizes of RNA markers in kilobases (kb).

Table 2. Doubling times (t_d) of *R. eutropha* strains HF210, HB6, and HB7 during growth with various sources of carbon and energy, or nitrogen

Strain	Carbon and Energy Source/Nitrogen Source		
	Formate/ NH_4^+	Xanthine/ NH_4^+	Gluconate/ NO_3^-
HF210	3.5 ^a	2.0	2.2
HB6	n.d. ^b	9.8	2.2
HB7	n.d.	9.8	2.2

^a t_d in h

^b n.d., t_d was not determined as the strains were unable to grow under this condition. Strain HB8 did not grow under any of the conditions tested.

transcriptional organization of the *moe-moa* genes and their activity under different growth conditions. The 0.8-kb *EcoRV-SacI* fragment that comprised the 3'-terminal region of *moeA* downstream of the Tn5-Mob insertion in HB6 was used as gene probe. A weak hybridization signal corresponding to a transcript of about 2.4 kilobases was detected in lithoauto-, mixo- as well as heterotrophically grown cells of HF210(pHG1) (Figure 3). This transcript was apparently absent from HB6(pHG1) (not shown), indicating that the transposon insertion abolished the transcription of all four genes. As the genes encompass a region of 2429 bp they presumably form an operon which is constitutively expressed at low level.

Although no sequence motifs resembling the consensus sequences of σ^{70-} , σ^{54-} , or σ^{32-} -dependent promoters were found within the 172-bp intergenic region downstream of *thrC*, a potential stem-loop structure with a free energy of formation of $\Delta G^\circ = -84.69$ kJ/mol and followed by a short run of thymine bases, typical for Rho factor-independent terminators (Cheng *et al.*, 1991), was identified in this segment (nucleotide positions 435-491; see Figure 2). These findings are also consistent with the existence and constitutive low-level expression of a *mol* operon. Additional complementation experiments in trans confirmed the results of the transcript analyses. Organoautotrophic growth of HB6 was restored in transconjugants harboring pKTB4 or pKTB5, but not in HB6(pKTB6). Plasmid pKTB4 contained all genes of the putative operon including the complete *thrC-moeA* intergenic region, whereas pKTB5 lacked *thrC*. In pKTB6 the *moaF* gene was truncated at its 3' end, indicating that an intact *moaF* is required for the synthesis of MoCo or its incorporation into FDH.

Phenotypic Features of Deletion Mutants HB7 and HB8

In order to verify the roles of *moeA* and *moaE* in MoCo biosynthesis deletion mutants HB7 and HB8, respectively, were constructed. In-frame deletions were introduced into the genes to avoid polar effects on downstream genes. Subsequent growth experiments on substrates, the utilization of which requires MoCo-containing enzymes, showed that HB7, like HB6, was unable to proliferate organoautotrophically. In addition heterotrophic growth on xanthine as carbon and energy source was considerably slower than that of parent strain HF210. However, growth with nitrate as sole nitrogen source was not affected (Table 2). Elevated molybdate concentrations and/or the presence of Na_2S did not restore wild-type growth in both HB6 and HB7. Mutant HB8 failed to grow under all these conditions.

Table 3. Specific activities of the different MoCo-containing enzymes in *R. eutropha* strains HF210, HB6, HB7, and HB8 grown under various conditions

Strain	Specific Activity (U/mg)					
	S-FDH		M-FDH		XDH ^a	NAS ^b
	Formate ^c (Mo) ^g	Formate ^c (Mo) ^g	Formate ^c (W) ^h	Pyruvate ^d (W) ^h	Xanthine ^e (Mo) ^g	Gluconate ^f (Mo) ^g (NO ₃) ⁱ
HF210	0.44	0.38	0.33	0.79	0.30	0.45
HB6	0.08	0.00	0.00	0.29	0.10	0.44
HB7	0.08	0.00	0.00	0.32	0.15	0.40
HB8	0	0	0	0	n.d. ^j	n.d. ^j

^a XDH, xanthine dehydrogenase.

^b NAS, assimilatory nitrate reductase.

^c Mixotrophic conditions with fructose plus formate.

^d Growth on pyruvate into the stationary phase.

^e Growth on xanthine.

^f Growth on gluconate.

^g Growth in the presence of molybdate.

^h Growth in the presence of tungstate.

ⁱ Nitrate as nitrogen source.

^j n.d., not determined, as strain HB8 is unable to grow under these conditions.

Assays of the relevant enzyme activities revealed that S-FDH was strongly reduced in mixotrophic cells of HB6 or HB7 compared to HF210, and neither Mo- nor W-dependent M-FDH were found in these mutants (Table 3). The W-dependent, stationary-phase M-FDH and the xanthine dehydrogenase (XDH) were, however, present, although at significantly reduced levels. As expected from the results of the growth experiments, the activity of the assimilatory nitrate reductase (NAS) was unaffected in HB6 and HB7. Thus, the phenotypes of the *moeA* mutants were virtually identical. None of the enzyme activities were detected in *moeA* mutant HB8.

Discussion

The phenotypic characterization of the formate-negative mutant strain HB6 of *R. eutropha* revealed the occurrence of W-dependent M-FDH activities in addition to the already known Mo-dependent FDH in this aerobic bacterium. Mo- and W-containing FDH isoenzymes have been described for only a few organisms such as *Methanococcus vanielii* (Jones and Stadtman, 1981) and several *Desulfovibrio* species (Almendra *et al.*, 1999) among the anaerobes or the aerobes *Mycobacterium vaccae* (Karzanov *et al.*, 1991) and *Methylobacterium* sp. RXM (Girio *et al.*, 1994). The W-dependent M-FDH activities of *R. eutropha* were found in organo- or lithoautotrophically as well as in heterotrophically grown cells after depletion of the energy and carbon source during the stationary phase. A Mo-dependent M-FDH was only induced in the presence of formate. As the stationary-phase activity was retained in mutant HB6 *R. eutropha* forms at least two different W-dependent M-FDHs. However, the physiological roles of the M-FDH isoenzymes are unclear because tungstate arrests the growth of the organism on formate by

inactivating S-FDH, a strictly Mo-dependent enzyme which is indispensable for provision of reducing equivalents under these conditions (Friedebold and Bowien, 1993). It is currently believed that W-containing enzymes are evolutionary predecessors of their catalytically more efficient Mo-containing counterparts (Almendra *et al.*, 1999; Buc *et al.*, 1999). In fact hyperthermophilic archaea exclusively possess W-dependent enzymes (Kletzin and Adams, 1996).

Mutant HB6 was shown to carry the Tn5-Mob insertion in the *moeA* gene. The inactivation of this gene abolished the formate-induced Mo- and W-dependent M-FDH activities, although the activities of other MoCo-containing enzymes were still present, NAS even at wild-type level. *R. eutropha* possesses an unusual tetracistronic *mol* operon consisting of *moeA*, *moeD*, *moeE*, and *moeF*. While the MPT synthase (MoaDE) catalyzes the last step in the formation of MPT, MoeA is not involved in the early stages of MoCo biosynthesis in *E. coli* since a *moeA* mutant is capable of producing MPT (Johnson and Rajagopalan, 1987). The complete activity loss of all MoCo-dependent enzymes in the *moeA* deletion mutant HB8 indicated that *R. eutropha* contains only one active set of MPT synthase genes. A participation of a MoeF homologue in MoCo biosynthesis has not been reported yet. Thus, MoeF of *R. eutropha* is a novel gene product which is required for the reconstitution of organoautotrophic growth in HB6 and likely to have a function in the formation of an active MoCo.

The *mol* operon is expressed constitutively at low level that is obviously sufficient to supply the MoCo-dependent enzymes of *R. eutropha* with the cofactor. A weak expression has also been found for the *moeA* operon of *Synechococcus* sp. PCC 7942 (Rubio *et al.*, 1998). The expression of genes involved in MoCo biosynthesis in *E. coli* (McNicholas *et al.*, 1998) and *Arthrobacter nicotinovorans* (Menéndez *et al.*, 1997) is correlated to the synthesis of the MoCo-containing enzymes. *Rhodobacter capsulatus* has a gene cluster for MoCo formation that is specific for the biogenesis of dimethylsulfoxide reductase (Solomon *et al.*, 1999). In *Anabaena* sp. PCC 7120 the *moeA* gene reaches its maximal expression only in the presence of nitrate when a high amount of MoCo is needed for the synthesis of nitrate reductase (Ramaswamy *et al.*, 1996). Interestingly, the phenotype of mutant HB6 of *R. eutropha* differs from that of HB8. Despite of the Tn5-Mob insertion in *moeA* the expression of the downstream MPT synthase genes must be sufficient to allow the formation of active MoCo in HB6, presumably at a reduced level. It is possible that there is subpromoter activity originating from the 5'-flanking region of *moeD*. The potential subpromoter, however, would not be strong enough to support adequate expression of *moeF* in HB6. In contrast to the MPT synthase, MoeA is apparently not indispensable for the biogenesis of active MoCo-containing enzymes in *R. eutropha*. A differential effect of the *moeA* disruptions on the activities of these enzymes was observed, including complete abolition (Mo- and W-dependent M-FDHs), reduced activities (one of the W-dependent M-FDHs and XDH), and wild-type level activity (NAS). Although Southern hybridizations did not suggest the presence of extra *moeA* gene copies in the organism (data not shown), the formation

of (an) additional functional MoeA-like protein(s) cannot be ruled out. *Archaeoglobus fulgidus* produces three potential MoeA proteins with mutual identities of only about 30% (Klenk *et al.*, 1997).

Although MoeA proteins have been found in many organisms from all three domains of life, the exact function of the protein in MoCo biosynthesis is still unknown. In *E. coli*, which produces only MGD-containing molybdoenzymes, the phenotype of a *moeA* mutant could not be suppressed by high molybdate concentrations in the culture medium but by sulfate limitation (Johnson and Rajagopalan, 1987). A low sulfate concentration causes the derepression of the *cys* operons, leading to sulfate uptake and reduction to sulfide. Hasona *et al.* (1998) demonstrated that the addition of sulfide to the medium also resulted in the synthesis of active MoCo in such a mutant. MoeA was thus proposed to form a complex with thiosulfate in order to facilitate the complexation of Mo with the pterin backbone. The XDH activity in a *moeA* mutant of *R. capsulatus* was restored to wild-type level by 1 mM molybdate in the growth medium, whereas the activities of the dimethylsulfoxide and nitrate reductases remained undetectable (Leimkühler *et al.*, 1999). XDH contains MPT, the two other enzymes MGD as the cofactor. It was suggested that metal chelation and Mo-MPT incorporation into XDH can be achieved at high molybdate concentrations even in the absence of MoeA, however, the protein is required for the formation of MGD. Purified MoeA from *A. nicotinovorans* was shown to exhibit a molybdate-enhanced ATPase activity and to form filamentous structures which rearranged into amorphous aggregates in the presence of nucleotides. Therefore, *in vivo* the protein may have a structural function by forming a scaffold for the final steps in MoCo biosynthesis (Menéndez *et al.*, 1997).

Several eucaryotic proteins contain a MoeA- together with a MogA-like domain (Kamdar *et al.*, 1994 and 1997; Mendel, 1997; Schwarz *et al.*, 1997). The MoeA domain of gephyrin, a mammalian protein, binds to the cytoskeleton (Kirsch *et al.*, 1991). Interestingly, MoeA from *E. coli* also shows this property (Menéndez *et al.*, 1997). Gephyrin is able to bind MPT and to reconstitute defects in MoCo biosynthesis of bacteria, plants and mammalian cells (Stallmeyer *et al.*, 1999). The plant Cnx1 protein is also associated with the cytoskeleton and transfers molybdenum to the bound pterin derivative (Kuper *et al.*, 2000; Schwarz *et al.*, 2000). These findings led to a model, in which the different domains of the eucaryotic proteins organize and perform the final steps in MoCo biosynthesis. In accordance with their intracellular localization, the proteins could catalyze the incorporation of sulfur and molybdenum at the cytoplasmic side of the membrane in association with a molybdate receptor (Kamdar *et al.*, 1997; Wittle *et al.*, 1999; Schwarz *et al.*, 2000).

In contrast to *moeA* mutants of *E. coli* and *R. capsulatus* that could be rescued by elevated sulfide and molybdate concentrations, respectively (Hasona *et al.*, 1998; Leimkühler *et al.*, 1999), supplementation of the medium with these anions showed no effect on mutant HB7 of *R. eutropha*. As the latter exhibited different activities of MoCo-containing enzymes (see Table 3), this

characteristic phenotype cannot be attributed to a defective MGD synthesis. Our results support the role of MoeA as a skeleton protein arranging the enzymes involved in the final steps of MoCo biosynthesis in a favorable orientation to facilitate the channeling of substrates and products. Surprisingly, Leimkühler and Rajagopalan (2001) observed that precursor Z, MPT synthase and molybdate are sufficient to reconstitute active human sulfite oxidase by incorporating MPT into the apoenzyme. We hypothesize MoeA to act differentially upon the various MoCo-containing enzymes of *R. eutropha* during incorporation of the cofactor. Specific structural changes of the target proteins, caused by the interaction with MoeA, might be necessary or not for proper assembly. The MoaF protein contains four hydrophobic regions which are supposed to interact with the membrane. Such a localization could mediate a contact between a MoeA scaffold and the molybdate uptake system to promote MoCo biosynthesis.

Experimental Procedures

Strains, Plasmids and Growth Conditions

Bacterial strains and plasmids employed in this study are listed in Table 4. Strains of *R. eutropha* were grown under air in nutrient broth or a mineral salts medium as described previously (Kusian *et al.*, 1995). The latter was supplemented with organic substrates at final concentrations of 0.1 or 0.2 % (w/v) for organoautotrophic (formate), heterotrophic (fructose, pyruvate) or mixotrophic (fructose plus formate) cultivation and contained either 1.5×10^{-7} M Na_2MoO_4 or 1.5×10^{-5} M Na_2WO_4 . Lithoautotrophic cultures were incubated under an atmosphere consisting of H_2 , CO_2 and O_2 (8:1:1, v/v) after addition of 0.05 % (w/v) NaHCO_3 to the mineral medium. For mixotrophic growth, the cells were first propagated on 0.1 % (w/v) fructose up to an optical density (OD) of 1 to 2, measured at 436 nm, before 0.2 % (w/v) formate was added and incubation continued for another 9 h. Starvation conditions were obtained by incubating cells that reached the stationary phase after growth on 0.2 % (w/v) pyruvate for additional 12 h. Cultures to be used for RNA isolation were grown in low-phosphate mineral salts medium (Kusian *et al.*, 1995). Strains of *E. coli* were propagated in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989). If indicated, antibiotics were added to the media at the following concentrations: kanamycin at 350 $\mu\text{g/ml}$ in mineral salts medium or 120 $\mu\text{g/ml}$ in nutrient broth, and tetracycline at 20 $\mu\text{g/ml}$ (for *R. eutropha*); ampicillin at 50 $\mu\text{g/ml}$, kanamycin at 50 $\mu\text{g/ml}$, and tetracycline at 20 $\mu\text{g/ml}$ (for *E. coli*).

Nucleic Acid Techniques and Sequence Analysis

DNA isolation, restriction enzyme analysis, agarose gel electrophoresis, and cloning procedures were carried out by use of standard methods (Sambrook *et al.*, 1989). RNA isolations and Northern hybridizations were done as described by Oelmüller *et al.* (1990). DNA probes applied in Northern hybridizations were radioactively labelled with [α - ^{32}P]dCTP (ICN) using a random primer labelling system (Life Technologies). DNA sequencing was performed by the dideoxy chain termination method (Sanger *et al.*, 1977). For radioactive sequencing the SequiTerm Excel cycle

Table 4. Bacterial Strains and Plasmids used in this Study

Strains	Relevant Phenotype or Genotype ^a	Source or Reference
<i>Ralstonia eutropha</i>		
H16	Wild type; Cfx, Hox, Fox; pHG1	DSM 428, ATCC 17699
HF09	Cfx, Hox ⁻ , Fox; pHG1; <i>rpoN</i> ; mutant of H16	Friedrich <i>et al.</i> (1981)
HF39	Sm ^r , Cfx, Hox, Fox; pHG1; Sm ^r mutant of H16	Srivastava <i>et al.</i> (1982)
HF210	Sm ^r , Cfx, Hox ⁻ , Fox; pHG1; pGH1-free mutant of HF39	Kortlücke and Friedrich (1992)
HB6	Sm ^r , Km ^r , Cfx, Hox ⁻ , Fox; pHG1; mutant of HF210 with Tn5-Mob insertion in <i>moeA</i>	This study
HB7	Sm ^r , Cfx, Hox ⁻ , Fox; pHG1; <i>moeA</i> Δ; mutant of HF210	This study
HB8	Sm ^r , Cfx, Hox ⁻ , Fox ⁻ , Nas ⁻ , Xan ⁻ ; pHG1; <i>moeA</i> Δ; mutant of HF210	This study
<i>Escherichia coli</i>		
S17-1	Sm ^r , Mod ⁺ , Res ⁻ , Pro ⁻ ; <i>recA</i> , <i>thi</i> ; integrated RP4 (Tc::Mu-Km::Tn7)	Simon <i>et al.</i> (1983)
XL1-Blue	Tc ^r ; <i>endA1</i> , <i>gyrA96</i> , <i>hsdR1</i> , <i>recA1</i> , <i>relA1</i> , <i>supE44</i> , <i>thi-1</i> , <i>lacF</i> [<i>proAB</i> , <i>lacR</i> , <i>lacZ</i> ΔM15, Tn10]	Bullock <i>et al.</i> (1987)
Plasmids		
pBluescript SK/KS	Ap ^r ; <i>lacPOZ</i>	Stratagene
pBBR1MCS-3	Tc ^r , Mob; <i>lacPOZ</i> ⁻	Kovach <i>et al.</i> (1995)
pMP921	Tc ^r , Mob; Tra ⁻	Freter and Bowien (1994)
pLAFR1	Tc ^r , Mob, Tra ⁻ ; λ- <i>cos</i>	Friedman <i>et al.</i> (1982)
pNHG1	Km ^r , Tc ^r ; <i>sacB</i> , RP4- <i>oriT</i> , ColE1- <i>ori</i>	Jeffke <i>et al.</i> (1999)
pSUP5011	Ap ^r , Km ^r , integrated transposon Tn5-Mob	Simon (1984)
pDB1	pLAFR1::18- + 4-kb <i>EcoRI</i> fragments	This study
pTB1	pMP921::18-kb <i>EcoRI</i> fragment of pDB1	This study
pSTB10	SK::0.68-kb <i>EcoRV</i> - <i>SacI</i> fragment containing the 3' region of <i>moeA</i>	This study
pSTB19	SK::2.96-kb <i>EcoRI</i> - <i>SmaI</i> fragment comprising the <i>mol</i> operon of HF39	This study
pSTB20	SK(<i>EcoRI</i>)::2.64-kb <i>StuI</i> - <i>SmaI</i> fragment from pSTB19	This study
pSTB21	SK::2.57-kb <i>Apal</i> - <i>SmaI</i> fragment from pSTB19	This study
pSTB22	SK(<i>EcoRI</i> - <i>SmaI</i>)::2.75-kb <i>EcoRI</i> - <i>MscI</i> fragment from pSTB19	This study
pSTB23-1	SK(<i>HincII</i>)::1.51-kb <i>StuI</i> - <i>PvuII</i> fragment from pSTB19	This study
pSTB25-2	SK::1.08-kb <i>SacI</i> fragment from pSTB19	This study
pKTB3	pBBR1MCS-3::2.96-kb <i>EcoRI</i> - <i>SmaI</i> (as <i>KpnI</i> - <i>XbaI</i>) fragment from pSTB19	This study
pKTB4	pBBR1MCS-3::2.64-kb <i>StuI</i> - <i>SmaI</i> (as <i>KpnI</i> - <i>XbaI</i>) fragment from pSTB20	This study
pKTB5	pBBR1MCS-3::2.57-kb <i>Apal</i> - <i>SmaI</i> (as <i>KpnI</i> - <i>XbaI</i>) fragment from pSTB21	This study
pKTB6	pBBR1MCS-3::2.75-kb <i>EcoRI</i> - <i>MscI</i> (as <i>KpnI</i> - <i>XbaI</i>) fragment from pSTB22	This study
pT11	pLAFR1::25.5-kb <i>EcoRI</i> fragment of HB6 containing the Tn5-Mob insertion	This study
pTN1	SK::1.25-kb <i>EcoRI</i> - <i>XhoI</i> fragment from pT11, containing the 5' end of <i>moeA</i> and 485 bp of Tn5-Mob	This study
pMDA1	SK(<i>HincII</i>)::1.02-kb <i>StuI</i> - <i>PvuII</i> fragment deletion a 489-bp <i>HincII</i> deletion in <i>moeA</i>	This study
pMDA2	pNHG1::1.02-kb <i>StuI</i> - <i>PvuII</i> fragment (as <i>SacI</i>) from pMDA1	This study
pMDE1	SK::0.73-kb <i>SacI</i> fragment after a 351-bp <i>MscI</i> - <i>Eco47III</i> deletion in <i>moeA</i>	This study
pMDE2	pNHG1::0.73-kb <i>SacI</i> fragment (as <i>SacI</i>) from pMDE1	This study

^a pHG1, megaplasmid of strain H16; Cfx, ability of autotrophic CO₂ fixation; Fox, ability of formate oxidation; Hox, ability of H₂ oxidation; Mob, ability of conjugative plasmid mobilization; Nas, ability of assimilatory nitrate reduction; Xan, ability to grow on xanthine; Tra, ability of conjugative plasmid transfer; Ap^r, ampicillin resistant; Km^r, kanamycin resistant; Sm^r, streptomycin resistant; Tc^r, tetracycline resistant.

sequencing kit (Biozym) employing [α -³⁵S]dATP (ICN) was used. Oligonucleotides were custom synthesized (MWG-Biotech). Nucleotide and deduced amino acid sequences were analyzed by the latest version of the GCG program package of the University of Wisconsin Genetic Computer Group (Devereux *et al.*, 1984). For homology searches in sequence databases the BLAST algorithms were applied (Altschul *et al.*, 1990). Multiple alignments were performed with the program CLUSTAL W (Thompson *et al.*, 1994).

Construction of a Partial Genomic Library and of Plasmids

Genomic DNA from *R. eutropha* HB6 was digested to completion with restriction endonuclease *EcoRI*, ligated to the *EcoRI*-site of cosmid pLAFR1, and subjected to in vitro packaging using the Gigapack II Gold Packaging Kit (Stratagene). The DNA packed in λ phage particles was subsequently transduced into *E. coli* S17-1. Clones containing the Tn5-Mob insertion and harboring a hybrid cosmid were selected on LB agar in the presence of kanamycin and tetracycline.

A 1.5-kb *StuI*-*PvuII* fragment containing the complete *moeA* gene was subcloned into the *HincII* site of pBluescript SK⁺ to initiate the generation of an in-frame deletion in *moeA*. From the resulting plasmid pSTB23-1 a 489-bp *moeA*-internal *HincII* fragment was removed to yield pMDA1. Subsequently, a 916-bp *SacI* fragment from pMDA1 was cloned into the suicide vector pNHG1, producing pMDA2 that was used for gene replacement mutagenesis. The inactivation of *moeA* required the construction of pSTB25-2 carrying a 1-kb *SacI* fragment isolated from pSTB19. Digestion of pSTB25-2 with *Eco47III* and *MscI* deleted a *moeA*-internal 351 bp-fragment. A 623-bp *SacI* fragment from the resulting pMDE1 was finally cloned into pNHG1, yielding pMDE2 which served for allelic exchange.

Conjugative Plasmid Transfer and Mutagenesis

The megaplasmid pHG1 was conjugally transferred into *R. eutropha* strains HF210 and HB6 by agar-spot mating using strain HF09 as the donor. Lithoautotrophically growing transconjugants contained pHG1 as verified by plasmid preparation according to Kado and Liu (1981).

Insertional mutagenesis of *R. eutropha* HF210 by means of transposon Tn5-Mob was done as detailed previously (Windhövel and Bowien, 1990), using the conjugative suicide plasmid pSUP5011 delivered from the mobilizing donor strain *E. coli* S17-1. For gene replacement mutagenesis of *moeA* or *moaE* in *R. eutropha* HF210 plasmids pMDA2 or pMDE2 were transferred from *E. coli* S17-1 to *R. eutropha* HF210 by agar-spot mating. Initially heterogenotes were selected for kanamycin resistance and subsequently homogenotes for sucrose resistance as described (Bömmer *et al.*, 1996). The allelic exchanges in the resulting isogenic deletion mutants were verified by PCR.

Preparation of Cell Extracts and Enzyme Assays

Cells of *R. eutropha* were grown to an OD₄₃₆ of about 2, harvested by centrifugation, and washed once with FDH buffer (20 mM potassium phosphate, pH 7.0, containing 10 mM KNO₃). Disruption of cells was achieved by sonication and debris removed by centrifugation at 13,000 x g for 5 min. The resulting crude extract was separated into a soluble and a membrane fraction by centrifugation at 100,000 x g for 60 min. NAD⁺-dependent S-FDH activity in the soluble fraction was determined by the standard optical test (Friedebold and Bowien, 1993). M-FDH activity in the membrane fraction and xanthine dehydrogenase activity in the soluble extract were measured photometrically at 600 nm with 2,6-dichlorophenolindophenol ($\epsilon_{600} = 16.3 \text{ ml} / \mu\text{mol} \cdot \text{cm}$) as artificial electron acceptor and phenazine methosulfate as redox mediator. The reactions were performed at 30°C under a nitrogen atmosphere. The assay mixtures contained, in a final volume of 1 ml: 12 mM potassium phosphate buffer, pH 7.0, 0.075 mM 2,6-dichlorophenolindophenol, 0.288 mM phenazine methosulfate, and an appropriate amount of enzyme. Reactions were started by addition of 40 mM sodium formate or 6 mM xanthine (dissolved in 2 mM NaOH) after 2 min of equilibration.

The activity of the assimilatory nitrate reductase was followed by the oxidation of reduced benzylviologen ($\epsilon_{600} = 7.78 \text{ ml} / \mu\text{mol} \cdot \text{cm}$) with nitrate under anoxic conditions (Warnecke-Eberz and Friedrich, 1993). Before starting the reaction benzylviologen was reduced by Na₂S₂O₄. The freshly prepared stock solution contained 250 mM Na₂S₂O₄ in 250 mM NaHCO₃. The reaction mixture (final volume of 1 ml) consisted of 30 mM PIPES buffer, pH 7.0, 40 mM KNO₃, 10 mM benzylviologen, and 12.5 mM Na₂S₂O₄. One unit of activity was defined as the amount of enzyme catalyzing the reduction/oxidation of 1 μmol of electron acceptor/donor per min. Protein concentrations were estimated colorimetrically by the method of Bradford (1976).

Gel Electrophoresis and S-FDH Activity Staining

Proteins were resolved by nondenaturing polyacrylamide (6 % [w/v] acrylamide) gel electrophoresis in 300 mM Tris-borate buffer, pH 7.7, containing 20 mM KNO₃. Activity staining of S-FDH was performed according to Friedebold and Bowien (1993) in 75 mM potassium phosphate buffer, pH 7.5, plus 20 mM KNO₃ and 100 mM sodium formate. The reaction mixture also contained 0.18 mM phenazine methosulfate as redox mediator and 0.61 mM nitroblue tetrazolium chloride as artificial electron acceptor. Violet

activity bands appeared after reaction at room temperature in the dark for 5-10 min. The gel was fixed in 7.5 % (w/v) acetic acid for 15 min.

Immunological Techniques

For Western blotting proteins were separated by sodium dodecylsulfate polyacrylamide (14 % [w/v] acrylamide) gel electrophoresis and subsequently transferred electrophoretically onto PVDF membranes (Millipore; Eschborn, Germany; Towbin *et al.*, 1979). Immunostaining of S-FDH was performed using polyclonal antibodies directed against the enzyme (Friedebold *et al.*, 1995) and employing goat-antirabbit IgG conjugated with alkaline phosphatase (Sigma; Deisenhofen, Germany).

Sequence Data

The sequence data reported in this study have been deposited in the EMBL/GenBank/DDBJ Data Libraries under the accession number AJ279073.

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