

Function and Regulation of Isoforms of Carbon Monoxide Dehydrogenase/Acetyl Coenzyme A Synthase in *Methanosarcina acetivorans*

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Conversion of acetate to methane (acetoclastic methanogenesis) is an ecologically important process carried out exclusively by methanogenic archaea. An important enzyme for this process as well as for methanogenic growth on carbon monoxide is the five-subunit archaeal CO dehydrogenase/acetyl coenzyme A (CoA) synthase multienzyme complex (CODH/ACS) catalyzing both CO oxidation/CO₂ reduction and cleavage/synthesis of acetyl-CoA. *Methanosarcina acetivorans* C2A contains two very similar copies of a six-gene operon (*cdh* genes) encoding two isoforms of CODH/ACS (Cdh1 and Cdh2) and a single CdhA subunit, CdhA3. To address the role of the CODH/ACS system in *M. acetivorans*, mutational as well as promoter/reporter gene fusion analyses were conducted. Phenotypic characterization of *cdh* disruption mutants (three single and double mutants, as well as the triple mutant) revealed a strict requirement of either Cdh1 or Cdh2 for acetotrophic or carboxidotrophic growth, as well as for autotrophy, which demonstrated that both isoforms are *bona fide* CODH/ACS. While expression of the Cdh2-encoding genes was generally higher than that of genes encoding Cdh1, both appeared to be regulated differentially in response to growth phase and to changing substrate conditions. While dispensable for growth, CdhA3 clearly affected expression of *cdh1*, suggesting that it functions in signal perception and transduction rather than in catabolism. The data obtained argue for a functional hierarchy and regulatory cross talk of the CODH/ACS isoforms.

Methanogenic archaea play a pivotal role in the global carbon cycle because they catalyze the terminal step in anaerobic biomass degradation in the absence of exogenous electron acceptors. While most methanogens are very limited in the range of the substrates they can convert to methane and can use only H₂+CO₂ or formate, which is reduced to methane via the pathway of hydrogenotrophic methanogenesis, *Methanosarcina* species are more versatile in their energy metabolism and can use, beside H₂+CO₂, methylated compounds and acetate as growth substrates (9, 46). Acetate is first activated to acetyl coenzyme A (acetyl-CoA) (12) and subsequently cleaved into a methyl group, an enzyme-bound carbonyl, and free CoA by the archaeal five-subunit carbon monoxide dehydrogenase/acetyl-CoA synthase multienzyme complex (CODH/ACS), also designated acetyl-CoA decarbonylase synthase (ACDS) (16, 17). The methyl group is transferred by CODH/ACS to the C1-transferring cofactor tetrahydrosarcinapterin and subsequently reduced to methane (13). The reducing equivalents are derived from the oxidation of the carbonyl group to CO₂, but how the electrons are transferred is currently unclear.

Of the few methanogens for which carbon monoxide (CO)-dependent growth could be demonstrated (32), *M. acetivorans* is unique, as it does not produce H₂, due to the absence of hydrogenase activity. Instead, it produces, beside methane, substantial amounts of formate and acetate (37), as well as small amounts of methyl sulfides from CO (31, 33). Previous genetic analysis suggests that acetate formation from CO proceeds via acetyl-CoA and, thus, via a pathway analogous to the reductive acetyl-CoA (the Wood-Ljungdahl) pathway, the central energy-conserving pathway of acetogenic bacteria (37). Key to this pathway is again CODH/ACS, combining a methyl group formed in the CO₂ reduction pathway, CO and CoA. Acetyl-CoA is subsequently con-

verted via acetyl-phosphate to acetate, a process that is catalyzed by phosphotransacetylase and acetate kinase, which allows energy conservation by substrate-level phosphorylation. Autotrophic carbon assimilation in methanogens also proceeds via the reductive acetyl-CoA pathway, involving the archaeal five-subunit CODH/ACS multienzyme complex (17, 24).

M. acetivorans contains genes for five catalytic subunits of CODH (15). The genes (*ma1309* and *ma3282*), encoding two orthologs of the homodimeric CODH, first characterized in anaerobic CO-oxidizing bacteria (22), were shown not to be required for growth on any substrate; however, a mutant lacking both isoforms grew more slowly on CO than the wild type, indicating a role in carboxidotrophic growth of the organism (39). Furthermore, two archaeal five-subunit CODH/ACS isoforms (encoded by *cdh* genes), termed Cdh1 (MA1016-MA1011) and Cdh2 (MA3860-MA3865) (39), which are encoded in putative transcriptional units (Fig. 1), and a single gene encoding a CdhA subunit, referred to as CdhA3 (MA4399) (Fig. 1), are also present in *M. acetivorans*. So far, no function has been assigned to CdhA3.

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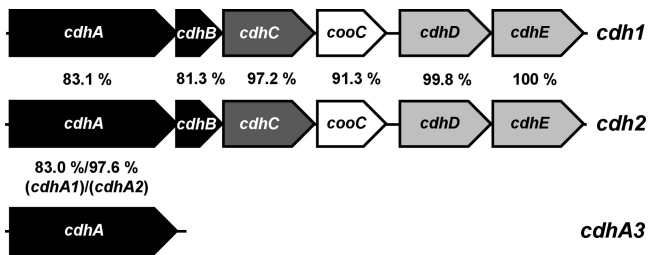


FIG 1 Comparison of the *cdh* isogenes in *M. acetivorans*. The two putative operons encoding CODH/ACS, designated *cdh1* (*ma1016-ma1011*) and *cdh2* (*ma3860-ma3865*), as well as *cdhA3* (*ma4399*), are shown. Genes for CODH subunits are in black, those for ACS are in dark gray, those for corrinoid-containing methyltransferase are in light gray, and those for the *cooC* (encoding the putative nickel insertase) are in white; percentages are amino acid identities between the respective deduced Cdh subunits.

Proteome analyses showed that both isoforms of the five-subunit enzyme are abundant in *M. acetivorans* when acetate or CO serves as the sole source of energy (26, 39), but whether the two isoforms have discrete functions is not clear. Qualitative analysis of CODH/ACS-encoding transcripts of *Methanosarcina mazei* Gö1 led to the conclusion that one CODH/ACS isoform may be involved in carbon fixation, while the other may be responsible for acetate catabolism (11). *Methanosarcina thermophila*, an acetotrophic methanogen, was shown to contain only one isoform of CODH/ACS, which therefore has to function in both anabolic acetyl-CoA synthesis and catabolic acetyl-CoA cleavage (18).

In this study, we addressed the function and regulation of the CODH/ACS-encoding genes in *M. acetivorans*. Phenotypic analyses of mutants carrying deletions in the *Cdh*-encoding genes show that autotrophic growth, as well as utilization of either acetate or CO, requires the presence of at least one CODH/ACS. This finding unequivocally demonstrates that both isoforms are *bona fide* CODH/ACS capable of playing both anabolic and catabolic roles. Furthermore, we show by analyzing promoter/reporter gene fusions in different genetic backgrounds, under different growth conditions, and in different growth phases that the *Cdh1* and *Cdh2* encoding genes are differentially regulated, particularly during the shift from one energy substrate to another, which suggests discrete but overlapping physiological functions of the two isoforms. Surprisingly, our data point to a role for *CdhA3* in the substrate-dependent signal transduction pathways involved in transcriptional regulation of the *Cdh*-encoding genes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* was grown under standard conditions (48). *M. acetivorans* strains, described in Table 1, were grown in HS medium as described previously (44). Either methanol (125 mM), sodium acetate (120 mM), or CO (150 kPa) served as the energy source for growth. Adaptation of *M. acetivorans* to CO was carried out as described previously (37). When they had been growing on a substrate for at least 15 generations, cultures were considered fully adapted. For a shift from methanol to acetate as the growth substrate, cells of a culture grown on methanol were either directly transferred (10% inoculum) into medium containing only acetate as the energy source (growth curves of *cdh* mutants) or harvested by centrifugation, washed twice in substrate-free medium to avoid carryover of methanol, and transferred to acetate-containing medium (reporter gene expression analysis). When required for methylotrophic growth of *M. acetivorans* mutants, the medium was supplemented either with 40 mM sodium acetate or 100 mM

pyruvate. Solid medium contained 1.5% (wt/vol) Bacto agar (4). For selection of *M. acetivorans* strains carrying the puromycin transacetylase gene (*pac*), puromycin (CalBiochem, San Diego, CA) was added from sterile, anaerobic stocks at a final concentration of 2 µg/ml. The purine analog 8-aza-2,6-diaminopurine (8-ADP; Sigma, St. Louis, MO) was added from sterile, anaerobic stocks at a final concentration of 20 µg/ml for selection against the hypoxanthine phosphoribosyl transferase gene (*hpt*). Growth of *M. acetivorans* was monitored by assessing optical density at 578 nm (OD₅₇₈) using a spectrophotometer (Genesys 10vis; Thermo, Dreieich, Germany). Cell titer and size were also determined by phase-contrast microscopy using a Thoma chamber and micrometer-engraved oculars.

Molecular methods, plasmid construction, and transformation. Standard molecular methods were used for manipulation of plasmid DNA from *E. coli* (3). Plasmids used are presented in Table 1. All plasmids in this study are nonreplicating in *Methanosarcina*. Genomic DNA from *M. acetivorans* was isolated using a modified cetyl trimethylammonium bromide-NaCl method (30). Genomic DNA of *M. acetivorans* was used as the template for PCR or subjected to Southern hybridization using a digoxigenin (DIG) system (Roche) (34) to verify the genotypes of *M. acetivorans cdh* mutants. For Southern hybridization chromosomal DNA of *M. acetivorans* was isolated, restricted with EcoRV, separated electrophoretically, transferred onto a nylon membrane, and treated with one DIG-labeled DNA fragment as a probe. The probe was generated by PCR using *ocdhA-SB/for* and *ocdhA-SB/rev* (see Table S1 in the supplemental material), which corresponds to approximately 540 bp of *cdhA1* but hybridizes to each of the three *cdhA* genes. DNA sequences of all cloning intermediates employing PCR were confirmed by sequencing at SRD Biotech (Bad Homburg) using the BigDye terminator cycle sequencing protocol (Applied Biosystems, Foster City, CA). *E. coli* was transformed by electroporation (14). Liposome-mediated transformation was used for *Methanosarcina* species as described previously (29), with modifications (4).

Construction of *M. acetivorans cdh* mutants. Approximately 1-kb regions flanking the first (*ma1016* and *ma3860*) and last (*ma1011* and *ma3865*) genes of the two *cdh* operons, or flanking *cdhA3* (*ma4399*), were amplified by PCR and cloned into pJK301 (19). Thus, gene disruption constructs were created in which the *Frt-pac-hpt-Frt* cassette was flanked by *M. acetivorans* DNA for homologous recombination. The resulting plasmids pKoCD1 (to delete *cdh1*, *ma1016* to *ma1011*), pKoCD2 (to delete *cdh2*, *ma3860* to *ma3865*), and pKoCD3 (to delete *cdhA3*, *ma4399*) (Table 1) were linearized using *AscI* and transferred into *M. acetivorans* WWM1 (Table 1). There, a double homologous recombination event replaced the respective target gene or operon with the *Frt-pac-hpt-Frt* cassette, selected for by puromycin. Subsequent transformation of the respective mutant strain with pMR55 (38), and thus, transient synthesis of FLP recombinase led to elimination of the region flanked by the *Frt* sites, selected for by 8-ADP. In this way, unmarked chromosomal *cdh* lesions were created with only a single *Frt* site remaining. The resulting mutants were MCD1 (*cdh1* deleted), MCD2 (*cdh2* deleted), and MCD3 (*cdhA3* deleted). The same procedure was applied to the *cdh* single mutants to generate *cdh* double mutants: pKoCD1 was used to create MCD21 from MCD2 and to create MCD31 from MCD3 (Table 1); the latter strain was used to create MCD32 using pKoCD2; the double mutant MCD21 was used to create the triple mutant MCD213 using pKoCD3 (Table 1).

Promoter/reporter gene fusion analysis. To analyze expression from the promoter regions preceding the three *cdhA* genes of *M. acetivorans* approximately 1 kb of the genomic region upstream of the respective gene (*ma1016* for *cdhA1*; *ma3860* for *cdhA2*; *ma4399* for *cdhA3*) was amplified by PCR. At the 3' end of the fragment, an *NdeI* site was introduced which overlaps the predicted translation start codon of the corresponding *cdhA* gene; the respective DNA fragment was cloned into pMR51, thereby creating a transcriptional/translational fusion of the respective *cdhA* promoter region and *uidA* with its translational start codon superimposed on that of the corresponding *cdhA* gene. Thus, β-glucuronidase activity in these strains can be used as a measure of *cdhA* transcription and transla-

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype, description, and/or construction ^a	Reference
Strains		
<i>E. coli</i>		
DH10B	General cloning	Invitrogen
WM1788	<i>pir</i> ⁺ for replication of R6K derivatives	20
<i>M. acetivorans</i>		
C2A	Wild type	43
WWM1	C2A, Δhpt	34
MCD1	WWM1, $\Delta cdh1::frt$	This study
MCD2	WWM1, $\Delta cdh2::frt$	This study
MCD3	WWM1, $\Delta cdhA3::frt$	This study
MCD31	MCD3, $\Delta cdh1::frt$	This study
MCD32	MCD3, $\Delta cdh2::frt$	This study
MCD21	MCD2, $\Delta cdh1::frt$	This study
MCD213	MCD21, $\Delta cdhA3::frt$	This study
P1016	C2A, $\Delta hpt::cdhA1p-uidA$	This study
P3860	C2A, $\Delta hpt::cdhA2p-uidA$	This study
P4399	C2A, $\Delta hpt::cdhA3p-uidA$	This study
P1016D2	MCD2, $\Delta hpt::cdhA1p-uidA$ (integration at Δhpt locus using pKY1016)	This study
P1016D3	P1016, $\Delta cdhA3::frt$	This study
P3860D1	P3860, $\Delta cdh1::frt$	This study
P3860D3	P3860, $\Delta cdhA3::frt$	This study
Plasmids		
pJK301	pBluescript based; contains <i>Frt-pac-hpt-Frt</i> to construct disruption cassette	49
pMP44	Vector for markerless deletion of genes in <i>M. acetivorans</i> ; <i>oriR6K</i> ; encodes <i>hpt</i>	34
pUP1016	<i>ma1016</i> upstream PCR (using primers 5-up1016 and 3-up1016) cloned into pJK301 via <i>XhoI/HindIII</i>	This study
pKoCD1	<i>ma1011</i> downstream PCR fragment (using primers 5-do1011 and 3-do1011) cloned into pUP1016 via <i>BamHI/SpeI</i>	This study
pUP3860	<i>ma3860</i> upstream PCR (using primers 5-up3860 and 3-up3860) cloned into pJK301 via <i>XhoI/HindIII</i>	This study
pKoCD2	<i>ma3865</i> downstream PCR fragment (using primers 5-do3865 and 3-do3865) cloned into pUP3860 via <i>BamHI/SpeI</i>	This study
pUP4399	<i>ma4399</i> upstream PCR (using primers 5-up4399 and 3-up4399) cloned into pJK301 via <i>XhoI/HindIII</i>	This study
pKoCD3	<i>ma4399</i> downstream PCR fragment (using primers 5-do4399 and 3-do4399) cloned into pUP4399 via <i>BamHI/SpeI</i>	This study
pMR55	Encodes <i>Flp</i> recombinase	49
pMR51	Vector for insertion of <i>uidA</i> fusions into <i>hpt</i> locus of <i>M. acetivorans</i> ; <i>oriR6K</i>	6
pP1016	<i>cdhA1p</i> PCR (using primers oP1016for and oP1016rev) cloned into pMR51 5' of <i>uidA</i> via <i>NdeI/NheI</i>	This study
pP3860	<i>cdhA2p</i> PCR (using primers oP3860for and oP3860rev) cloned into pMR51 5' of <i>uidA</i> via <i>NdeI/NheI</i>	This study
pP4399	<i>cdhA3p</i> PCR (using primers oP4399for and oP3860rev) cloned into pMR51 5' of <i>uidA</i> via <i>NdeI/NheI</i>	This study
pKY1016	<i>AflIII/ApaI</i> fragment (containing <i>cdhA1p-uidA</i> fusion) of pP1016 blunted, cloned into <i>NruI</i> site of pMP44	This study

^a Details of construction and plasmid maps are available upon request; the oligonucleotides used are listed in Table S1 in the supplemental material.

tion initiation. These reporter gene fusions were inserted into the *hpt* locus of *M. acetivorans* C2A via the markerless insertion procedure (34). The resulting strains were designated P1016 (to assess *cdhA1* expression), P3860 (to assess *cdhA2* expression), and P4399 (to assess *cdhA3* expression) (Table 1). Reporter strains carrying deletions of the *cdh* loci were created by deleting the respective operon or gene in either P1016 or P3860 (Table 1) or by inserting a reporter gene fusion into the *hpt* locus of a *cdh* mutant (strain P1016D2) (Table 1).

Batch cultures (300 ml) were grown in 1-liter Müller-Krempel bottles and analyzed for growth phase-dependent β -glucuronidase activity. The cultures used were fully adapted to methanol, acetate, or CO, or methanol-adapted cultures were shifted to acetate (see above). Samples were taken during the whole course of growth and analyzed for β -glucuronidase activity. Briefly, cells were harvested by centrifugation and osmotically lysed by addition of 50 mM Tris HCl buffer, pH 8.0, containing 1 mM dithiothreitol, 0.1 μ g/ml DNase I, and 0.1 μ g/ml RNase A. The lysate was cleared by centrifugation, and the specific activity of β -glucuronidase was determined by monitoring cleavage of *p*-nitrophenol-glucuronide at 415 nm as described previously (34). Protein concentration was determined by the method of Bradford (7) using bovine serum albumin as the standard.

For single-point, steady-state determination of β -glucuronidase activity, 10-ml cultures (5 ml for growth on CO) (five experimental replicates) were grown in Balch tubes, and the cells in the parallel cultures were harvested at exactly the same optical density in the early exponential growth phase (OD_{578} , 0.2 to 0.25 for CO and acetate and 0.4 for methanol). Samples were processed as described above.

Determination of CODH activity. All manipulations to determine CODH activity were carried out under strictly anaerobic conditions using gas-tight vials or inside an anaerobic glove box (Coy, Grass Lake, MI) containing N_2-H_2 (96:4 [vol/vol]). Crude extract of *M. acetivorans* was prepared from cultures at early exponential growth phase at the same optical densities as for β -glucuronidase determination (see above). Cells were harvested by centrifugation and lysed in assay buffer (50 mM potassium phosphate, pH 7.2; 2 mM dithiothreitol) containing 0.1 μ g/ml DNase I and 0.1 μ g/ml RNase A, for 30 min. The lysate was cleared by centrifugation at 6,000 \times g, and the supernatant was used for enzymatic assays. CODH activity was determined by monitoring CO-dependent reduction of methylviologen (MV) at 603 nm ($\epsilon_{603} = 11.3 \text{ mM}^{-1} \text{ cm}^{-1}$) as described previously (35), except that 8 mM MV was used and sodium dithionite and 2-mercaptoethanol were omitted. Nonspecific MV reduction activity was measured independently by omitting an electron donor

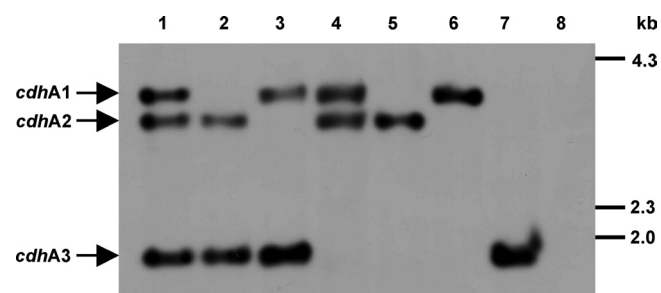


FIG 2 Verification of the genotypes of *cdh* mutants via Southern hybridization. Restricted genomic DNA was probed with a DIG-labeled DNA fragment hybridizing with all three *cdhA* alleles. Lanes: 1, wild type (WWM1); 2, MCD1; 3, MCD2; 4, MCD3; 5, MCD31; 6, MCD32; 7, MCD21; 8, MCD213. Size standards are shown on the right.

and was used for correction of the specific CODH activity. Protein concentration was determined as described above.

RESULTS

Genotypic and phenotypic analysis of *M. acetivorans* *cdh* mutants. In order to assess the *in vivo* role of the two CODH/ACS isoforms, encoded by *ma1016-ma1011* (Cdh1) and *ma3860-ma3865* (Cdh2), as well as of the single CdhA3 subunit, encoded by *ma4399*, the encoding genes were deleted from the *M. acetivorans* chromosome by using the markerless deletion/disruption technique (38). Of the seven strains constructed, MCD1 lacks the entire *cdh1* region (i.e., *ma1016* through *ma1011*), MCD2 lacks the entire *cdh2* region (i.e., *ma3860* through *ma3865*), and MCD3 lacks *cdhA3* (*ma4399*). The double mutants and the triple mutant were derived from these strains (see Materials and Methods), and their genotypes were verified by Southern hybridization. The sizes and pattern of the chromosomal fragments hybridizing with the probe (Fig. 2) are fully consistent with *in silico* predictions (3,729, 3,336, and 1,783 bp for the *cdh1*, *cdh2*, and *cdhA3* loci, respectively), which confirmed the nature of the seven *cdh* mutants.

The consequences for *M. acetivorans* of the loss of one, two, or all three *cdh* loci were assessed by quantifying growth of the mutant strains with either methanol, acetate, or CO as the sole source of energy (Fig. 3 and 4; also, see Table S2 in the supplemental material). Growth on methanol was not affected in the *cdh* mutants, as long as either *cdh1* or *cdh2* was present (Fig. 3A and 4A). The same was observed when acetate was the growth substrate: neither the growth rate nor the final optical density reached by the mutants differed significantly from that of the wild type (Fig. 3C and 4C; also, see Table S2) when the cultures had been adapted to this substrate for at least 15 generations and provided that one of the *cdh* operons was still present on the chromosome. However, when shifted from methanol to acetate by inoculation of a methanol-grown culture into acetate-containing medium, the strains lacking *cdh2* and still containing *cdh1*, irrespective of whether *cdhA3* was also absent (strains MCD2 and MCD32), displayed a lag phase prolonged by approximately 10 days compared to the strains containing *cdh2* (Fig. 3B and 4B). The transient decrease in optical density during the adaptation period resulted from both a decrease in cell titer, probably due to cell lysis, and a decrease in average cell size, probably resulting from reductive cell division, as evidenced by microscopy (data not shown). These data suggest that Cdh2 plays a more important role during the shift from methylo-trophic to acetate conditions than Cdh1, but also that

loss of Cdh2 is fully compensated during continuous growth on acetate. However, the data allow no firm conclusion as to how the fitness of the mutants lacking Cdh2 for growth on acetate is improved. When strains were grown on CO, loss of Cdh1 (strains MCD1 and MCD31) had no obvious effect on growth (Fig. 3D and 4D), but the absence of Cdh2 (strain MCD2) resulted in a significantly reduced growth rate compared to the wild type (Fig. 3D), indicating that under this condition also, Cdh2 plays a more important role than Cdh1. While loss of CdhA3 alone had no adverse effect on either methylo-trophic or acetate growth, strikingly, the same mutation appeared to enable *M. acetivorans* to grow faster than the wild type on CO (strain MCD3) (Fig. 3D) and to compensate for loss of Cdh2 (strain MCD32) (Fig. 4D). These data strongly indicate that in the wild type, CdhA3 exerts an effect on carboxidotrophic growth by somehow negatively affecting Cdh1.

Both putative *cdh* operons encode *bona fide* CODH/ACS isoforms in *M. acetivorans*. Regardless of the growth substrate and of the presence or absence of CdhA3, either Cdh1 or Cdh2 had to be present to enable the mutant to grow autotrophically. When both *cdh* operons had been deleted (*cdh1 cdh2* double mutant MCD21 and *cdh* triple mutant MCD213), methylo-trophic growth of the respective strains was strictly dependent on the presence of acetate or pyruvate in the medium (data not shown). Growth on methanol in the presence of 40 mM acetate was significantly slower in MCD21 and MCD213 than in the wild type (see Fig. S1 and Table S2 in the supplemental material), which may indicate that acetyl-CoA is not generated from exogenous acetate as efficiently as it is endogenously through the Wood-Ljungdahl pathway. Thus, either uptake or activation of acetate (via acetate kinase and phosphotransacetylase) could represent a growth-limiting step in these two strains. When MCD21 and MCD213 were shifted from a medium containing methanol plus acetate to a medium containing only acetate, no growth was observed (scored after 6 months of incubation [data not shown]). Also, MCD21 and MCD213 could not be adapted to grow with CO as the sole energy source (data not shown). Together with the phenotypes of the other *cdh* mutants, the data unequivocally demonstrate that in *M. acetivorans* both *cdh1* and *cdh2* encode *bona fide* CODH/ACS isoforms, one of which is required and sufficient for both catabolic acetyl-CoA cleavage (during acetate growth) and anabolic acetyl-CoA synthesis (during CO₂ fixation via the Wood-Ljungdahl pathway). The fact that the strains lacking both *cdh1* and *cdh2* could not be adapted to grow with CO indicates an important role for the CODH/ACS system under this condition, either via its acetyl-CoA-forming activity (which leads to acetate formation) or via its CO oxidizing activity, or both.

Differential expression of the *cdh* genes during growth. Although principally able to complement each other, the growth phenotypes of the *cdh* mutants (see above) and previously reported differential abundances of subunits (26, 39) suggest discrete functions of the two CODH/ACS isoforms during growth on acetate and on CO. To assess the contribution of the isoforms to utilization of these substrates in the wild type, expression of the operons/genes encoding the CODH/ACS isoforms and CdhA3 was analyzed during growth in batch cultures. Since expression of highly homologous genes (Fig. 1) is not easily analyzed by PCR-based approaches, we chose reporter gene fusion analysis (see Materials and Methods). No significant β -glucuronidase activity (background level, approximately 0.5 mU/mg) could be measured

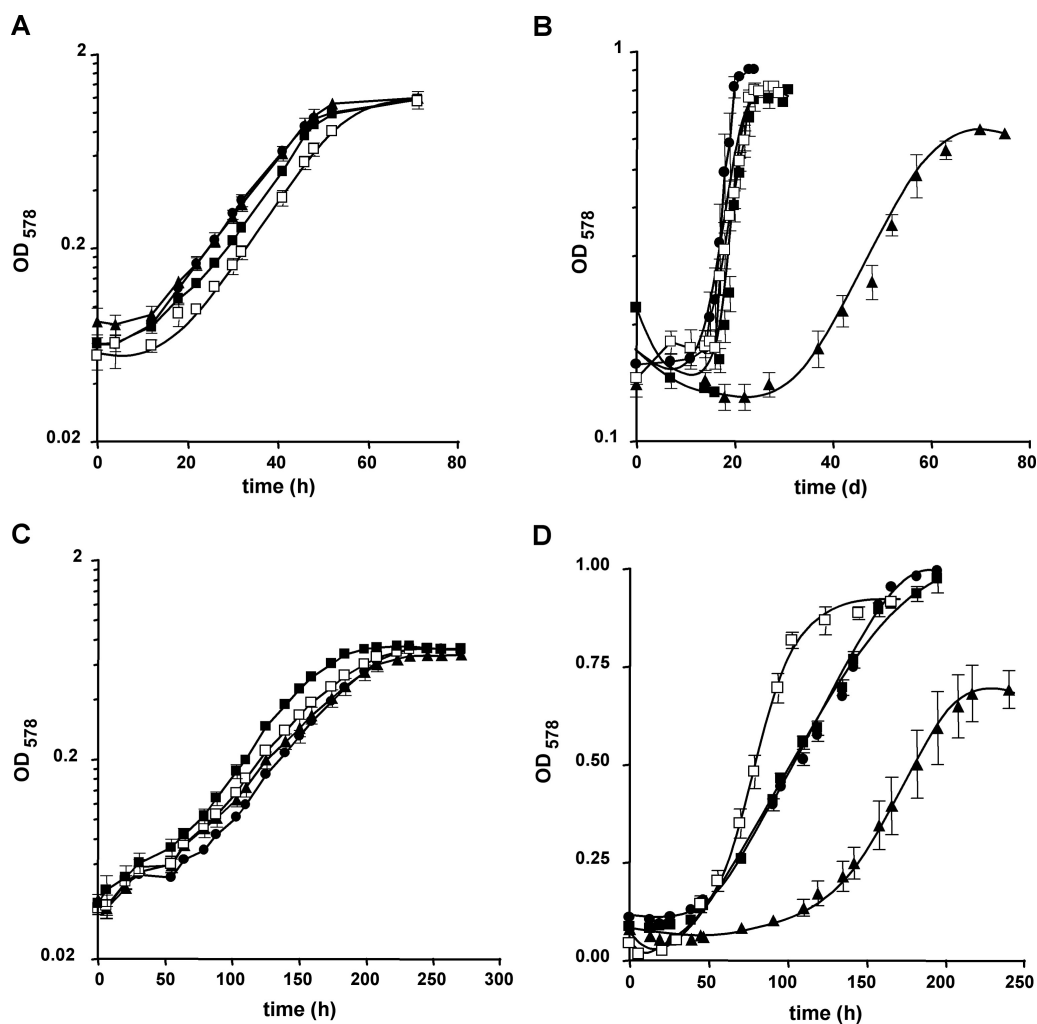


FIG 3 Substrate-dependent growth of *M. acetivorans* *cdh* single mutants. Growth of the strains in the presence of methanol (A), during the shift from methanol to acetate (B; the zero time point indicates the transfer of the culture), in the presence of acetate (C), and in the presence of CO (D) was monitored by measuring the OD₅₇₈. Filled squares, wild type (WWM1); filled circles, MCD1; filled triangles, MCD2; open squares, MCD3. Values are averages and standard deviations from at least three independent cultures; the experiments were qualitatively reproduced at least once. Doubling times and final optical densities are listed in Table S2 in the supplemental material.

in the strain carrying the *cdhA3p-uidA* fusion (P4399) under any of the conditions tested. Since the promoters for the *cdhA* genes have not been precisely mapped, we therefore only assumed that all required regulatory sequences were present within the 1-kb region upstream of the coding region. Thus, a plausible explanation for this observation is that the promoter region chosen did not contain all required expression signals, which could obviate *uidA* expression. Alternatively, the gene may not be expressed at a level sufficient to be detected with the method used here. Strain P4399 was therefore not analyzed further.

Methanol-dependent expression from the *cdhA1* (strain P1016) and the *cdhA2* (strain P3860) promoters was relatively low and remained constant throughout the different phases of growth during batch cultivation (Fig. 5A), which is in accordance with proteomic analyses (26, 39) and the anabolic role of CODH/ACS during methylotrophic growth. Under acetate conditions (Fig. 5C), both *cdhA* genes were expressed at a much higher level, and their expression changed with the growth phases. While *cdhA2* expression was lowest after dilution into fresh medium, it

increased linearly during exponential growth, stagnating only in late-exponential growth phase and declining in stationary phase. On the other hand, *cdhA1* expression increased only modestly (threefold) early in the exponential growth phase but was otherwise expressed at a rather constant level. When methanol-grown cells were shifted to medium containing only acetate as the sole source of energy (Fig. 5B) expression of both *cdh* promoters stagnated at the level found during growth on methanol for the whole adaptation period, which is typically 12 to 21 days (Fig. 3B and 4B). The number of washed cells pregrown on methanol and used for inoculation was rather high, to ensure that β -glucuronidase activity was significantly above the detection limit of the assay; notably, the time required for adapting to the new substrate was not affected by this measure (compare Fig. 3B, 4B, and 5B). Only when the cells started to grow did both *cdhA* promoters commence expression, which displayed a pattern similar to that of acetate-adapted cells. A remarkable difference, however, was the levels of expression, which were at least two- to threefold increased in both reporter strains. The higher levels of *cdh* expression could

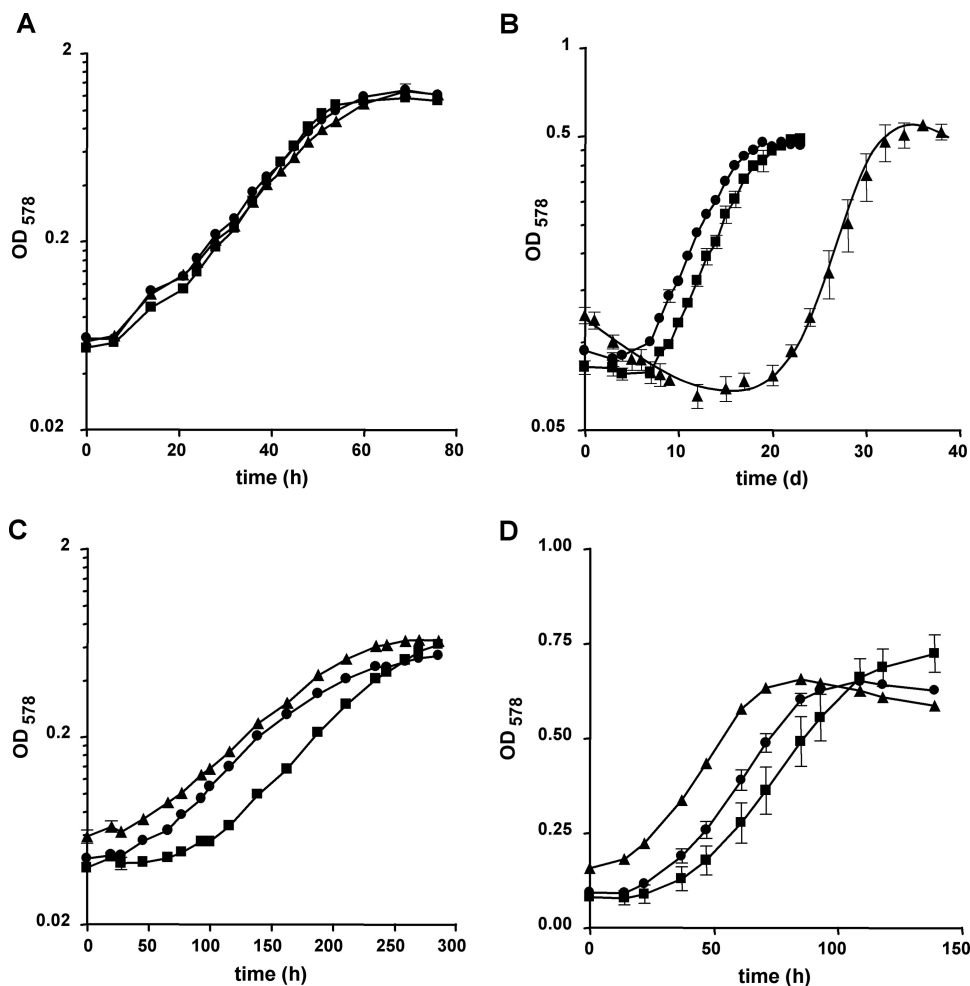


FIG 4 Substrate-dependent growth of *M. acetivorans* *cdh* double mutants. Growth of the strains in the presence of methanol (A), during the shift from methanol to acetate (B; the zero time point indicates the transfer of the culture), in the presence of acetate (C), and in the presence of CO (D) was monitored by measuring OD₅₇₈. Squares, wild type (WWM1); circles, MCD31; triangles, MCD32. Values are averages and standard deviations from at least three independent cultures; the experiments were qualitatively reproduced at least once. Doubling times and final optical densities are listed in Table S2 in the supplemental material.

mean that larger amounts of CODH/ACS may be required during the substrate shift than under adapted conditions. Based on the levels of expression both during substrate shift and during growth on acetate after adaptation, it appears that Cdh2 is mainly responsible for acetyl-CoA cleavage during aceticlastic growth and that Cdh1 plays only a supplementary role. This conclusion is fully consistent with the observed growth behavior of the *cdh2* mutants when shifted from methanol to acetate, where lack of Cdh2 leads to a dramatically prolonged lag phase (Fig. 3B and 4B).

Interestingly, neither of the *cdhA* genes was as highly expressed during growth on CO (Fig. 5D) as during aceticlastic growth. Expression of *cdhA2* increased during early exponential growth and declined somewhat during late exponential growth before a second peak in expression occurred at the beginning of stationary phase (Fig. 5D). Thus, expression of the Cdh2-encoding genes is dynamic and is probably influenced by various parameters, which change during batch cultivation. On the other hand, expression of *cdhA1* remained at a rather constant level during carboxidotrophic growth, increasing only somewhat as the cells entered stationary growth phase.

CO oxidation in *M. acetivorans* is mainly catalyzed by the CODH/ACS system. Based on the reporter gene expression data

one would expect lower CODH activity in *M. acetivorans* during growth on CO than during growth on acetate. The phenotypes of the *cdh* mutants further indicate that loss of one Cdh Isoform can be compensated and that CdhA3 plays a role in this process. Substrate-dependent CODH activity was therefore analyzed by determining CO-dependent methylviologen reduction (see Materials and Methods) in the wild type and in the *cdh* mutants. As can be seen from Table 2, CODH activity in the wild type is lowest when the strain is grown on methanol, consistent with the anabolic role of the enzyme under this condition. The CODH activities of most *cdh* mutants were similar to that of the wild type under this condition, except that the *cdh2* mutant MCD2 exhibited approximately 50% of the wild-type CODH activity. Notably, and fully consistent with the previous results, this reduction of CODH activity was compensated to wild-type levels when CdhA3 was also absent (strain MCD32). When both Cdh1 and Cdh2 were lacking (strains MCD21 and MCD213) CODH activity dropped to approximately 5% of that of the wild type, corroborating the notion that CODH/ACS is the major CO-oxidizing activity under this condition. The remaining CODH activity is probably that of the homodimeric CODH isoforms (MA1309 and MA3282). How-

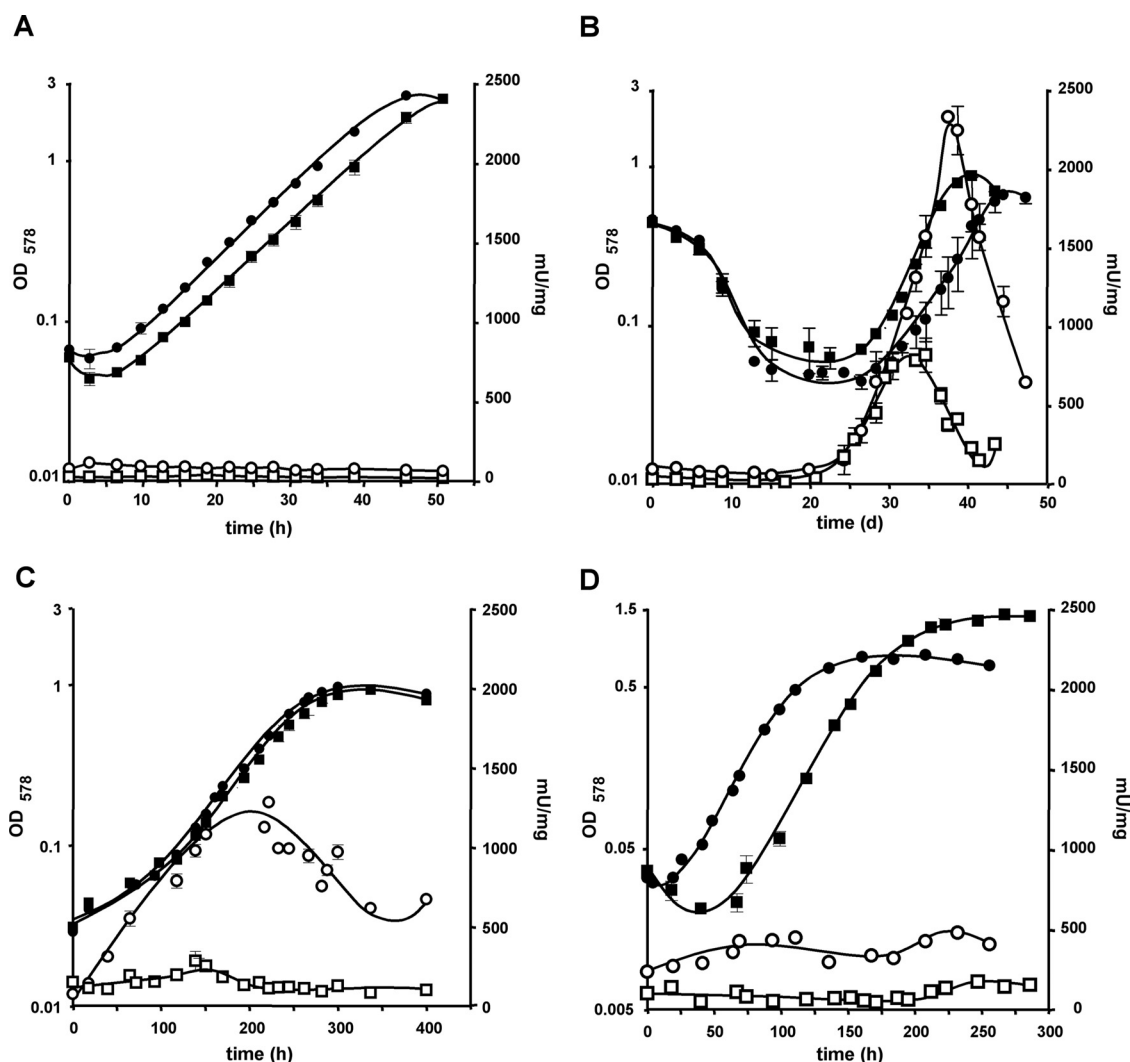


FIG 5 Growth- and substrate-dependent *cdhAp-uidA* expression in *M. acetivorans*. Cultures were grown on methanol (A), shifted from methanol to acetate (B), grown on acetate (C), or grown on CO (D); OD₅₇₈ (filled symbols) indicates growth; β-glucuronidase activity (open symbols) indicates expression from the specific promoters. Squares, P1016 (*cdhA1p-uidA*); circles, P3860 (*cdhA2p-uidA*).

ever, this activity is apparently not sufficient to enable the strains to grow on acetate or CO. CODH activity in the wild type was approximately 7- to 10-fold higher when CO or acetate served as the growth substrate than when methanol was the substrate

TABLE 2 Specific CODH activity of the *cdh* mutants

Strain	Specific activity on ^a :		
	Methanol	CO	Acetate
WWM1	446 ± 19	3,363 ± 275	5,807 ± 794
MCD1	492 ± 73	2,349 ± 65	2,497 ± 478
MCD2	177 ± 43	2,278 ± 328	3,318 ± 356
MCD3	539 ± 78	4,705 ± 259	4,083 ± 627
MCD31	395 ± 6	4,026 ± 560	3,231 ± 279
MCD32	481 ± 14	4,759 ± 1,223	17,931 ± 2,718
MCD21	25.3 ± 6.1	ND	ND
MCD213	28.8 ± 8.4	ND	ND

^a CO-dependent methylviologen reduction (nmol min⁻¹ mg⁻¹) in crude extracts of strains fully adapted to the respective growth substrates. Values are averages ± standard deviations from at least two independent experiments conducted three times in parallel. ND, not detectable (the strain cannot grow on the substrate).

(Table 2), consistent with the catabolic role of this enzyme during carboxidotrophic and acetate growth. Interestingly, the difference in CODH activity between CO- and acetate-grown cells was not as high as expected from the *cdhA* gene expression analyses, indicating that transcription and enzyme activity do not correlate directly but that the latter is influenced by posttranscriptional events acting on the Cdh system.

CODH activity during acetate and carboxidotrophic growth was reduced in the *cdh1* mutant (strain MCD1) compared to the wild type, which suggests that Cdh1 contributes significantly to overall CODH activity in *M. acetivorans*. However, this contribution is not sufficient to affect growth on acetate or CO negatively when it is lacking (Fig. 3). A reduction in CODH activity was also observed when Cdh2 was absent (strain MCD2), except that the reduction in CODH activity was more pronounced during growth on acetate, again arguing for a more prominent role of Cdh2 under acetate than carboxidotrophic conditions. As MCD1 and MCD2 contained similar levels of CODH activity during growth on CO, it seemed unlikely that it was the reason for

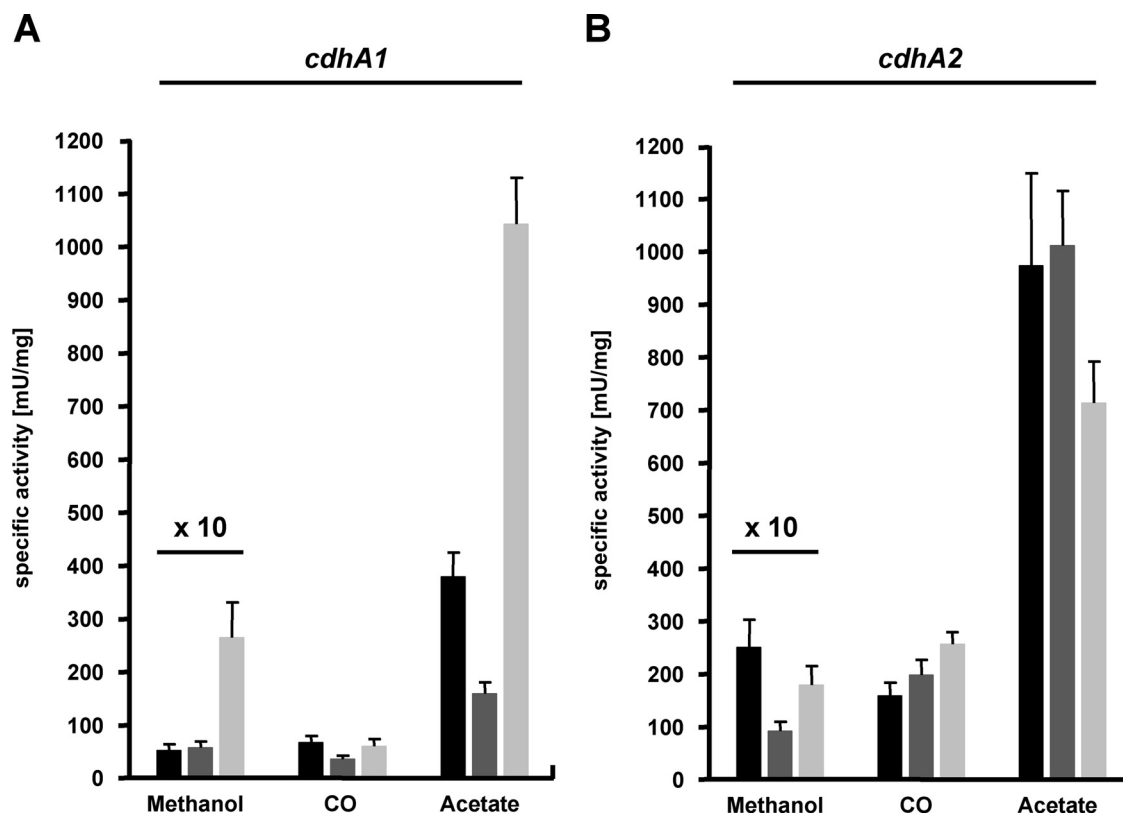


FIG 6 Substrate-dependent *cdhAp-uidA* expression in *cdh* mutants of *M. acetivorans*. Cultures were grown on methanol, acetate, or CO; the *cdhA1p-uidA* fusion (left) was analyzed in the wild-type background (P1016, black bars), in the absence of *cdh2* (P1016D2, dark gray bars), and in the absence of *cdhA3* (P1016D3, light gray bars); the *cdhA2p-uidA* fusion (right) was analyzed in the wild-type background (P3860, black bars), in the absence of *cdh1* (P3860D1, dark gray bars), and in the absence of *cdhA3* (P3860D3, light gray bars). For clarity, all values obtained from cultivation on methanol were multiplied by a factor of 10.

the slower growth of the latter under this condition. It also became apparent that the CODH activities of the strains MCD1 and MCD2 combined were larger than that of the wild type under any condition tested, i.e., that removing one of the Cdh isoform led to an increase in the activity of the other. CODH activity of the strain lacking CdhA3 (MCD3) was similar during acetate and carboxidotrophic growth and significantly higher than that of the wild type growing on CO, which could explain why this mutant grew faster than its parental strain on this substrate (Fig. 3D). The mutant lacking both Cdh2 and CdhA3 (strain MCD32) contained a somewhat higher CODH activity than the wild type during growth on CO but significantly more than the Cdh2-deficient strain (MCD2), which probably allowed compensation of the growth defect seen in the latter strain (compare Fig. 3D and 4D). Again, this finding strongly suggests that CdhA3 exerts—directly or indirectly—a negative effect on the activity of Cdh1. This notion is emphasized by the finding that when MCD32 is grown on acetate, its CODH activity is increased approximately threefold compared to that of MCD2 or the wild type (Table 2).

CdhA3 influences expression of *cdh* isogenes. The CODH activities observed in the *cdh* mutants could not be plausibly explained by simple subtraction of isoform-specific activity from overall CODH activity. Instead, either different substrate-dependent catalytic efficiencies or different abundances of the isoforms, depending both on the growth substrate and on the mutant background, seemed to be the basis for the observations made. To investigate whether the two isoforms and/or CdhA3 influence ex-

pression of the other respective *cdh* genes, strains carrying, beside the reporter gene fusion, lesions of *cdh1*, *cdh2*, or *cdhA3* were constructed (Table 1). We did not succeed in deleting *cdh2* in strain P1016. Instead, an isogenic strain was constructed by inserting the *cdhA1p-uidA* fusion (Table 1) into the chromosomal *hpt* locus of the *cdh2* mutant MCD2. Reporter activity in the respective single mutants during early exponential growth phase at one particular optical density was analyzed. The β -glucuronidase activities in the “wild-type” strains P1016 and P3860 (Fig. 6) qualitatively corresponded to the *cdh* expression analyses during the course of growth (Fig. 5). The generally somewhat lower β -glucuronidase activities in this steady-state analysis might be due to the different cultivation scale (see Materials and Methods). While loss of Cdh1 barely affected expression of *cdhA2* (Fig. 6B), the absence of Cdh2 resulted in an approximately 50% decrease of *cdhA1* expression when CO or acetate served as the growth substrate (Fig. 6A). Furthermore, loss of *cdhA3* did not affect *cdhA1* expression during growth on CO but resulted in an almost-threefold increase in *cdhA1* expression during growth on acetate (Fig. 6A). Conversely, loss of *cdhA3* reduced expression of *cdhA2* under the same conditions, if only by approximately 15% (Fig. 6B). Thus, CdhA3 exerts a negative effect on *cdh1* transcription but a positive effect on *cdh2* transcription, both of which appear to be acetate specific.

DISCUSSION

Methanosarcina species metabolize a broader spectrum of substrates than other methanogenic archaea. Beside the amount of

genetic inventory such a more generalistic lifestyle requires, *Methanosarcina* species are also known to often encode multiple homologs for numerous metabolic functions, which makes their genomes the largest of the *Archaea* (8, 15, 28). The selective advantage which retaining isoenzymes confers is not known for most cases, but it was shown that isoenzyme genes encoding, for example, catabolic methyltransferases are regulated in a sophisticated fashion (5, 6). In this study, we addressed the function and regulation of CODH/ACS isoforms present in *M. acetivorans*.

Function of the Cdh isoforms in *M. acetivorans*. The genome of *M. acetivorans* C2A encodes, like those of *Methanosarcina barkeri* and *M. mazei*, two isoforms of CODH/ACS in putative transcriptional units. Unique to *M. acetivorans* is the presence of a single *cdhA3* gene. Previously, analysis of CODH/ACS-encoding transcripts in *M. mazei*, a close relative of *M. acetivorans*, led to the proposal that the two isoforms may play distinct metabolic roles, one more active in synthesizing acetyl-CoA for biosynthetic purposes and the other more active in cleaving acetyl-CoA during energy metabolism (11). The genome of *M. thermophila* encodes only one CODH/ACS that has to be active in both directions, acetyl-CoA synthesis and cleavage (18). Here, we provide unambiguous experimental evidence that each Cdh isoform of *M. acetivorans* is capable of both catabolic and anabolic functions, i.e., that both Cdh isoforms are *bona fide* CODH/ACS, by showing that (i) one isoform is required and sufficient for acetoclastic and carboxidotrophic growth and that (ii) lack of both isoforms renders the respective mutant auxotrophic for acetate. Still, both the mutational and gene expression analyses argue that both isoforms do not share equal functionality but that Cdh2 is the major isoform because it is expressed at a higher level than Cdh1 under all tested conditions, and consequently, its loss results in more severe growth defects. It therefore appears that in *M. acetivorans*, Cdh1 plays an auxiliary role that is beneficial under certain physiological conditions.

Regulation of the CODH/ACS system in *M. acetivorans*. We confirmed for *M. acetivorans* the findings of previous analyses, which showed that transcription of CODH/ACS-encoding genes is strongly regulated by the growth substrate. The resulting expression levels are consistent with the enzyme's catabolic role during acetoclastic growth and its anabolic role during methylotrophic growth (2, 45). The *cdhABCDE* transcript of *M. thermophila* contains a 371-nucleotide 5' untranslated (UTR) leader sequence, which is involved in regulating transcription elongation into the *cdhA* coding region in a growth substrate-dependent fashion (1). The corresponding sequence of the putative *cdhA2* 5' UTR in *M. acetivorans* is very similar to the one in *M. thermophila* and could, therefore, be one means of regulating transcription in *M. acetivorans*. Noteworthy, the reporter gene fusion constructs used here contain all transcription signals, including the promoters on the DNA and the resulting 5' UTR on the mRNA. The observation that expression of the *cdhA* genes is significantly higher during growth on acetate than during growth on CO may be partly explained by the different amounts of carbon channeled through CODH/ACS for energy conservation and biosynthesis. During growth of *M. acetivorans* on CO, 8 to 14% of the carbon is metabolized via acetyl-CoA, and thus CODH/ACS, for acetate and cell mass formation (37). During growth of *Methanosarcina* species on acetate, 98 to 99% of the carbon is metabolized to methane and CO₂ via CODH/ACS (42, 47). Also, very little free energy is available from acetoclastic methanogenesis, necessitating a high sub-

strate turnover, and thus high levels of CODH/ACS, to conserve enough energy for growth (10).

Although expression levels of *cdhA1* and *cdhA2* in the wild type are at least 4-fold lower during growth on CO than during growth on acetate, the overall CODH activity differs only 2-fold at most between the two substrates, which implies growth substrate-dependent regulation of the CODH/ACS system beyond that of transcription. Carboxidotrophic methanogenesis in *M. acetivorans* is relatively slow (33), and regulating CODH/ACS not only on the transcriptional level may be advantageous for the organism. To achieve this, CODH/ACS activity or the half-lives of the enzymes could be directly affected by cellular effectors, like the substrates or products, other small molecules, or other enzymes. Such signals could also affect transcript stability or efficiency of translation. However, nothing is known about posttranscriptional gene regulation in methanogens. Still, this level of regulation is obviously also influenced by the genetic context. For example, the acetate-dependent growth defect observed in the *cdh2* mutant (MCD2) is fully compensated, probably by Cdh1, after prolonged cultivation on this substrate. In contrast to what was expected, *cdh1* expression in this strain was not increased but decreased, which suggests posttranscriptional processes leading to increased CODH/ACS activity responsible for this compensation. However, pleiotropic mutations at some second site(s) that improve the fitness of the mutants on acetate cannot be ruled out.

Our analysis further indicates that there are genetic interactions between the *cdh* coding loci or direct interactions between the proteins themselves. The fact that CODH activities of the *cdh1* and *cdh2* mutants combined are greater than that of the wild type under any conditions tested strongly suggests a regulatory cross talk within the CODH/ACS system, directly effecting either enzyme activity or gene expression. The fact that absence of Cdh2 results in an approximately 50% decrease of *cdhA1* expression during growth on CO or acetate argues for the latter scenario. However, how this effect could be brought about cannot be plausibly explained at present, but such changes in expression of a gene upon disruption of isogenes have been observed in other organisms (21, 40).

CdhA3 seems to be involved in regulation of the CODH/ACS system of *M. acetivorans*. This study further revealed that CdhA3 affects, to various degrees and in different directions, regulation of the CODH/ACS system on both the transcriptional and posttranscriptional levels. Although we were not able to determine expression of *cdhA3* using reporter gene fusions, microarray analyses showed that *cdhA3* is expressed (27). While deletion of *cdhA3* leads to a slight decrease of *cdhA2* expression, transcription of *cdhA1* is significantly increased, but only under acetoclastic conditions. Under carboxidotrophic conditions, *cdhA2* expression is increased when CdhA3 is absent, which may explain the improved growth under this condition. While the data presented clearly indicate that CdhA3 affects transcription, it is very unlikely that CdhA3 directly acts as a regulator of *cdh* gene expression, because CdhA3 harbors no motif suggestive of binding to DNA or RNA and no other CODH is known to interact with nucleic acids. Instead, CdhA3 could be part of a catabolite responsive signal transduction pathway. The proteobacterium *Ralstonia eutropha* (also termed *Wautersia eutropha* and *Cupriavidus necator*) contains, beside two catabolic hydrogenases, a regulatory hydrogenase (HoxBC) forming a complex with the histidine kinase HoxJ, thereby relaying H₂ status to the response regulator HoxA, which

activates expression of hydrogenase genes (25). Methanogenic CODH/ACS multienzyme complexes exchange the carbonyl of acetyl-CoA with exogenous CO (36). It is therefore feasible that CdhA3 may sense CO released during acetyl-CoA cleavage, thus integrating both CO and acetate as metabolic signals. Interestingly, the ORFs (*ma1017* and *ma3866*, respectively) upstream of *cdhA1* (*ma1016*) and downstream of *cdhE2* (*ma3865*) encode putative helix-turn-helix motif-containing proteins, often characterized by binding of nucleic acid, which could, therefore, be involved in transcriptional regulation of the CODH/ACS system of *M. acetivorans*. Despite the fact that *M. acetivorans* does not encode homologs of CooA or RcoM (23, 41), the only CO sensors known thus far, *M. acetivorans* is the most CO tolerant among the methanogens investigated in this respect (32) and specifically alters its protein inventory in response to CO (26, 39). Probably not coincidentally, it is also the only *Methanosarcina* species (with a published genome sequence) encoding CdhA3 (see Fig. S2 in the supplemental material). *M. acetivorans*, thus, appears to have evolved a unique system to sense CO, to acclimate to this toxic gas, and to regulate the pathways of its utilization.

Beside this very hypothetical role in a potential signal transduction pathway it is also feasible that CdhA3 may affect CODH/ACS more directly. The fact that CODH activity during growth on acetate of the *cdh2 cdhA3* double mutant (MCD32) is almost 3-fold higher than that of the wild type and more than 5-fold higher than that of the *cdh2* mutant suggests that CdhA3 may influence the enzyme's activity, for example by direct binding or by binding of an effector. Clearly, further investigations toward the role of CdhA3 are warranted to resolve this interesting issue, and the *cdh* mutants are uniquely suited for these analyses.

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