An Engineered Methanogenic Pathway Derived from the Domains *Bacteria* and *Archaea*

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ABSTRACT **A plasmid-based expression system wherein *mekB* was fused to a constitutive *Methanosarcina acetivorans* promoter was used to express MekB, a broad-specificity esterase from *Pseudomonas veronii*, in *M. acetivorans*. The engineered strain had 80-fold greater esterase activity than wild-type *M. acetivorans*. Methyl acetate and methyl propionate esters served as the sole carbon and energy sources, resulting in robust growth and methane formation, with consumption of >97% of the substrates. Methanol was undetectable at the end of growth with methyl acetate, whereas acetate accumulated, a result consistent with methanol as the more favorable substrate. Acetate was consumed, and growth continued after a period of adaptation. Similar results were obtained with methyl propionate, except propionate was not metabolized.**

IMPORTANCE **The fragile interactions of multispecies food chains converting complex biomass to methane are easily disrupted, a major impediment to efficient and reliable conversion of renewable biomass as an alternative to fossil fuels. The hybrid pathway, derived by combining catabolic pathways from a methanogen of the domain *Archaea* and a strictly aerobic species of the domain *Bacteria*, catalyzes the complete conversion of an industrial solvent that is also a naturally occurring compound to methane and carbon dioxide. The engineered pathway expands the exceptionally narrow range of substrates utilized by methanogens, exemplifying the simplification of food chains leading to the more-efficient conversion of complex biomass to methane.**

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Methane-producing microbes, methanogens, constitute the largest described group within the domain *Archaea* and are among the most ancient of extant life forms. The phylogenetic diversity of methanogens is immense, consistent with the extremes of anaerobic habitats in which they proliferate. Methanogens grow at temperatures ranging from 0 to 110°C (1, 2), salinities ranging from those in freshwater to those in hypersaline (3, 4), pHs ranging from 4.5 to 9.5 (5, 6), and anaerobic environments ranging from deep sea hydrothermal vents to the intestinal tracts of humans (7, 8). This remarkable diversity is in stark contrast to the few simple substrates, primarily one-carbon substrates, utilized for growth and methanogenesis. Most described species reduce CO_2 to methane with H_2, formate, or carbon monoxide (CO_2 reduction pathway). A few species convert the methyl groups of acetate (aceticlastic pathway) or the one-carbon substrate methanol, methylamines, or methyl sulfide (methylotrophic pathway) to methane (4). In nature, methanogens are terminal organisms of anaerobic microbial food chains where other microbes, primarily from the domain *Bacteria*, break down complex biomass to supply simple substrates for methanogens. In true symbiotic fashion, microbes acting on the complex biomass are also dependent on methanogens to remove products of their thermodynamically unfavorable reactions (9). The process occurs in diverse anaerobic environments and is a vital link in the global carbon cycle, producing nearly 1 billion metric tons of methane each year (10). Here, anaerobic microbial food chains have evolved to efficiently coordinate their metabolism in natural environments where biomass is most often limiting. Conversion of organic wastes and renewable biomass to methane is a viable alternative to the use of fossil fuels (11); however, in large-scale reactors receiving high loading rates of biomass, the fragile community interactions are easily disrupted, hindering efficient implementation of this technology. A potential route to overcoming this impediment is the engineering of methanogens with hybrid pathways incorporating enzymes from the domain *Bacteria*, thereby simplifying microbial food chains. Although genetic exchange systems have been available for nearly a decade (12), this technology has not been applied to successful engineering of novel methanogenic pathways.

We set out to broaden the substrate range of *Methanosarcina acetivorans* C2A through the rational design of a pathway that allows growth and production of methane with nonnative substrates. *M. acetivorans* C2A (13) is a particularly good candidate, as it employs all three methanogenic pathways (14–18) and a robust genetic exchange system is available (19). We chose the methyl esters of acetate (methyl acetate [MeAc]) and propionate (methyl propionate [MePr]), both of which are widely used industrial solvents and also produced biologically (20, 21). Furthermore, both compounds are relatively hydrophobic and likely to be passively transported across the cytoplasmic membrane. Importantly,

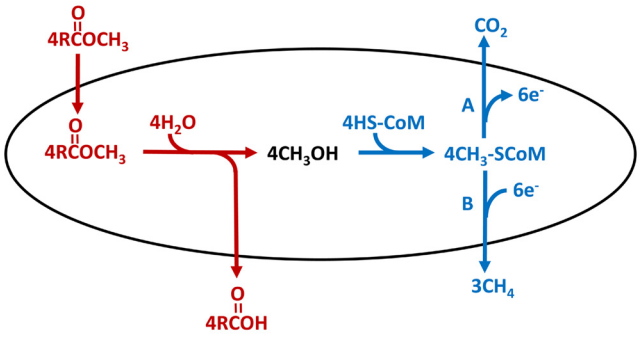

FIG 1 Design of a methylotrophic pathway for metabolism of methyl esters by *Methanosarcina acetivorans* C2A. Reactions in red are from the domain *Bacteria* and those in blue from the domain *Archaea*. (A) Oxidation of one methyl group of methanol to CO_2 by a reversal of the CO_2 reduction pathway of methanogens. (B) Reduction of three methyl groups to methane by the electrons generated from section A. R represents CH_3 or CH_3CH_2 (methyl acetate or methyl propionate).

methanogens are not documented to metabolize either compound. Recently, the aerobic isolate *Pseudomonas veronii* MEK700 was shown to utilize an esterase (MekB) with broad substrate specificity in the aerobic metabolism of 2-butanone (22). MekB was shown to hydrolyze a variety of esters, including MeAc and MePr. Thus, the metabolic pathway shown in Fig. 1, whereby the expression of *mekB* within *M. acetivorans* C2A would confer growth with methyl esters when combined with the methylotrophic pathway of methanogenesis, was designed.

A plasmid-based expression system was used to express MekB within wild-type *M. acetivorans* C2A. The *mekB* gene was PCR amplified from pJOE5358.1 (22) and fused to the promoter (P_{tbp}) for the gene encoding the TATA binding protein of *M. acetivorans* (23). The DNA fragment containing P_{tbp}-*mekB* was then cloned into the XhoI/BamHI sites of the *Escherichia coli-M. acetivorans* shuttle vector pWM321 to form pDL203. Plasmids were transformed into *M. acetivorans* C2A using a liposome-mediated protocol (19). Transformants were selected by plating cells on solid high-salt (HS) medium (19) containing 125 mM methanol and 2 μg/ml puromycin. The resultant strains, C2A(pWM321) and C2A(pDL203), were grown with HS medium containing the indicated growth substrate. Cell lysates from methanol- or acetate-grown C2A(pWM321) had low but detectable esterase activity (~25 units/mg protein), whereas the activities for lysates from methanol- or acetate-grown C2A(pDL203) were approximately 80-fold higher (~2,000 units/mg protein). Activity was measured as previously described (22), with units defined as nmol of 4-nitrophenyl acetate hydrolyzed/min. Protein was determined as previously described (24), with bovine serum albumin as the standard. These data reveal that wild-type *M. acetivorans* lacks a highly active esterase and that MekB is expressed in an active form in C2A(pDL203). Expression of MekB did not adversely affect growth of C2A(pDL203) with methanol or acetate (data not shown).

The ability of C2A(pWM321) and C2A(pDL203) to utilize MeAc or MePr as the sole carbon and energy sources was examined (Fig. 2). MeAc, MePr, methanol, and methane were measured using a Shimadzu gas chromatograph (GC-14A) equipped with a flame ionization detector (FID) and a thermal conductivity detector. A silico steel 100/120 ShinCarbon-ST column (Restek) with He as the carrier gas was used at a constant temperature of 100°C for determination of methane. A glass 80/100 Porapak QS column (Alltech) with He as the carrier gas was used at 150°C to measure methanol, MeAc, and MePr. Acetate and propionate were detected by high-performance liquid chromatography using an Aminex HPX-87H column (Bio-Rad). Strains C2A(pWM321) and C2A(pDL203) were grown in HS medium supplemented with 150 mM morpholinepropanesulfonic acid (MOPS; pH 7.5) to increase the buffering capacity. Medium containing either substrate was left uninoculated (abiotic control) or inoculated with C2A(pWM321) or C2A(pDL203). Approximately 10% of the MeAc and 20% of the MePr were abiotically hydrolyzed to methanol and acetate or propionate in the uninoculated controls during the time course of the experiment. The decrease of MeAc or MePr in C2A(pWM321) cultures was nearly identical to the level for the uninoculated abiotic controls, indicating no hydrolysis of the esters by C2A(pWM321), a result consistent with the intrinsically low esterase activity in the native strain. However, a low level of growth, which could have been due to abiotically produced methanol, was observed for the C2A(pWM321) culture.

In contrast to C2A(pWM321), the C2A(pDL203) culture consumed >97% of the MeAc or MePr initially added, resulting in robust growth and methane production (Fig. 2). Approximately 50% of the MeAc was hydrolyzed within 60 h and 95% within 74 h, at which time growth was only half maximal (optical density at 600 nm [OD_{600}] = 0.41), consistent with the idea that MekB has high specific activity with MeAc. Rapid hydrolysis of MeAc by C2A(pDL203) resulted in accumulation of methanol and acetate to similar levels in the medium (230 μmol and 270 μmol, respectively) before any significant growth occurred (50 h; OD_{600} = 0.09). Methanol was subsequently consumed during exponential growth, concomitant with accumulation of methane (Fig. 2) and CO_2 (data not shown). At the end of growth, methanol levels were below the detection limit, whereas acetate was not significantly metabolized, consistent with methanol as a more energetically favorable substrate. The generation time for growth with MeAc was 10.7 ± 0.2 h (Fig. 2), compared to 10.2 ± 0.2 h for growth with methanol (data not shown). The total amount of products at the end of growth (acetate, methane, and CO_2) accounted for 89% of the carbon in the MeAc consumed, with the balance assumed to be cell carbon. Accumulated acetate was consumed after a lag period (data not shown), characteristic of the diauxic growth parameters for this species (23). Unlike the metabolism of MeAc, during metabolism of MePr, hydrolysis and propionate accumulation paralleled growth and methane production (Fig. 2). Further, methanol never accumulated in the medium, suggesting that the methanol liberated from hydrolysis of MePr by MekB in the cytosol was immediately consumed. It took approximately 80 h to hydrolyze 50% of the MePr added and approximately 115 h to hydrolyze 97%, consistent with the idea that MekB has lower esterase activity with MePr. The generation time for growth with MePr was 18.5 ± 0.2 h (Fig. 2), compared to 10.2 ± 0.2 h for growth with methanol, consistent with the idea that hydrolysis is the limiting factor. The total amount of products at the end of growth (propionate, methane, and CO_2) accounted for 88% of the carbon in the MePr consumed, with the balance assumed to be cell carbon. Propionate was not significantly metabolized, even after prolonged incubation (data not shown). Cell lysates from MeAc- and MePr-grown C2A(pDL203) had esterase activity (~1,500 to 3,000 units/mg protein) comparable to the activity determined

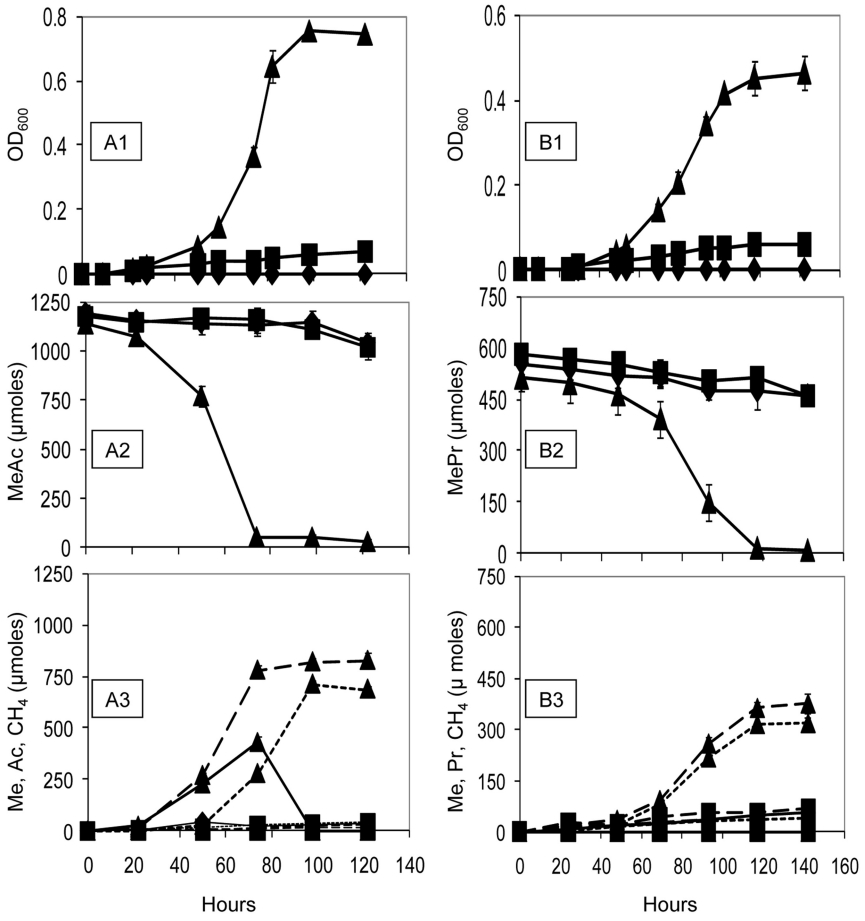

FIG 2 Comparison of rates of methyl ester metabolism by *Methanosarcina acetivorans* C2A containing pWM321 or pDL203. Growth, substrate concentration, and product formation were monitored during metabolism of 100 mM MeAc (A1 to A3) or 50 mM MePr (B1 to B3) by strain C2A(pWM321) (■) and strain C2A(pDL203) (▲), compared to the levels for uninoculated abiotic controls (♦). (A3 and B3) Methanol (solid line), acetate or propionate (dashed line), and methane (dotted line). The reported data represent the means of results from triplicate experiments.

with methanol- and acetate-grown cells, suggesting that MeAc and MePr do not affect the expression or activity of MekB. These data are consistent with the pathway illustrated in Fig. 1, whereby expression of active MekB in C2A(pDL203) confers the ability to hydrolyze the methyl esters with the liberated methanol metabolized through the methylotrophic pathway.

Metabolism of MeAc and MePr by C2A(pDL203) through the methylotrophic pathway was further supported by molar growth yields when C2A(pDL203) was cultured with either MeAc (4.93 ± 0.05 g [dry weight]/mol methane) or MePr (5.33 ± 0.30 g [dry weight]/mol methane), that were similar to those observed for methanol-grown cultures (5.23 ± 0.05 g [dry weight]/mol methane) but not acetate-grown cultures (2.68 ± 0.06 g [dry weight]/mol methane). Metabolism of MeAc and MePr by C2A (pDL203) was further examined with resting cell suspensions (Table 1). *M. acetivorans* strains were grown in HS medium containing the indicated substrates to mid-exponential phase (OD_{550} = 0.4 to 0.6). Cells were harvested by centrifugation at 5,000 × *g* for 10 min in an anaerobic chamber (Coy Manufacturing, Inc.), washed with substrate-free HS medium, and resuspended in HS medium containing 150 mM MOPS (pH 7.5) to give a final OD_{550} of 3 to 4. Cell suspensions (1 ml) were anaerobically aliquoted into 10-ml serum bottles containing 1 atm N_2 and sealed with butyl rubber stoppers. Substrates were added from anoxic stock solutions to give a final concentration of 50 mM and the bottles incubated at 37°C. Gas samples were withdrawn every 30 min for 6 h for measurement of methane. Cells of C2A(pDL203) grown with either methanol, MeAc, or MePr supported substantial rates of methane production when supplied with methanol but did not produce significant amounts when supplied with acetate (Table 1). However, acetate-grown C2A (pDL203) produced methane at comparable initial rates with both acetate and MeAc but did not produce significant amounts when supplied with methanol or MePr, confirming that acetate liberated from MeAc was metabolized through the aceticlastic pathway.

Implications. The results demonstrate the heterologous expression of a catabolic enzyme from an aerobic species of the domain *Bacteria* in a strictly anaerobic methanogen from the domain *Archaea* that confers robust metabolism of substrates more complex than previously reported for any methanogen. This work

TABLE 1 Rates of methane production by *M. acetivorans* cell suspensions

Strain	Growth substrate	Specific activity with substrate added (nmol methane/min/mg [dry wt] cells)[a]			
		Methanol	Acetate	MeAc	MePr
C2A(pWM321)	Methanol	135 ± 7	BDL	BDL	BDL
C2A(pWM321)	Acetate	BDL	48 ± 4	BDL	BDL
C2A(pDL203)	Methanol	166 ± 2	BDL	155 ± 2	86 ± 2
C2A(pDL203)	Acetate	BDL	92 ± 3	103 ± 3	BDL
C2A(pDL203)	MeAc	210 ± 18	BDL	183 ± 25	60 ± 7
C2A(pDL203)	MePr	158 ± 3	BDL	130 ± 17	123 ± 17

[a] MeAc, methyl acetate; MePr, methyl propionate; BDL, below the detection limit of 1 nmol methane/min/mg (dry weight) of cells.

inaugurates the engineering of metabolic pathways expanding the narrow range of simple substrates for methanogens, leading to the simplification of anaerobic microbial food chains converting complex biomass to methane.

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