

The *acnD* Genes of *Shewanella oneidensis* and *Vibrio cholerae* Encode a New Fe/S-Dependent 2-Methylcitrate Dehydratase Enzyme That Requires *prpF* Function In Vivo

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The propionate utilization operons of several bacteria differ from each other in the occurrence of two genes, *acnD* and *prpF*, in place of or in addition to the *prpD* gene encoding an Fe/S-independent 2-methylcitrate dehydratase enzyme. We cloned the *acnD* and *prpF* genes from two organisms, *Shewanella oneidensis* and *Vibrio cholerae*, and found that, together, the AcnD and PrpF proteins restored the ability of a *prpD* mutant strain of *Salmonella enterica* to grow on propionate as a source of carbon and energy. However, neither *acnD* nor *prpF* alone was able to substitute for *prpD*. The AcnD and PrpF proteins were isolated and biochemically analyzed. The AcnD protein required reconstitution of an Fe/S cluster for activity. All detectable AcnD activity was lost after incubation with iron-chelating agents, and no AcnD activity was observed after attempted reconstitution without iron. Nuclear magnetic resonance spectroscopy and *in vitro* activity assay data showed that AcnD dehydrated 2-methylcitrate and citrate to 2-methyl-*cis*-aconitate and *cis*-aconitate, respectively; AcnD also hydrated *cis*-aconitate. However, 2-methylisocitrate and isocitrate were not substrates for AcnD, indicating that AcnD only catalyzes the first half of the aconitase-like dehydration reactions. No aconitase-like activity was found for PrpF. It is hypothesized that, *in vivo*, PrpF is an accessory protein required to prevent oxidative damage of the Fe/S center of active AcnD enzyme or that it may be involved in synthesis or repair of the Fe/S cluster present in AcnD.

First demonstrated in *Yarrowia lipolytica* and several other filamentous fungi and yeast species (26, 32, 33), the 2-methylcitrate (2-MC) cycle was subsequently shown to occur in the bacteria *Salmonella enterica* serovar Typhimurium and *Escherichia coli* (20, 35). Since the finding that prokaryotes can also utilize the 2-MC cycle as their route of propionate catabolism, the 2-MC pathway was also demonstrated in other gram-negative bacteria such as *Ralstonia eutropha* (8) and *Burkholderia sacchari* (7) and in the gram-positive bacterium *Corynebacterium glutamicum* (10). Sequence analysis of the completed genomes of several other bacteria indicates that the 2-MC cycle may be widespread among bacteria; *Vibrio cholerae*, *Shewanella oneidensis*, *Neisseria* spp., and several *Pseudomonas* species contain propionate utilization (*prp*) operons (8, 17) (Fig. 1).

The *prp* operons of *S. enterica* and *E. coli* comprise four genes encoding structural proteins of the 2-MC cycle which have been characterized (Fig. 1A) as follows: *prpB* encodes 2-methylisocitrate (2-MIC) lyase (13, 14, 17), *prpC* encodes 2-MC synthase (17, 18, 35), *prpD* encodes 2-MC dehydratase (9, 17), and *prpE* encodes propionyl-coenzyme A synthetase (19). However, other *prp* operons have a gene organization that differs greatly from that of these two enterics (8, 17) (Fig. 1). Figure 1D shows an operon structure that contains two genes, *acnD* (17) and *prpF*, instead of *prpD*. In *S. enterica*, *prpD* encodes an Fe/S-independent 2-MC dehydratase that gener-

ates 2-methyl-*cis*-aconitate (2-MCA) from 2-MC but will not hydrate 2-MCA into 2-MIC (17). The hydration of 2-MCA is catalyzed by either aconitase AcnA or AcnB (Fig. 2) (17). All currently sequenced *prp* operons that contain an *acnD* ortholog also contain *prpF* and vice versa. The latter is an ortholog of open reading frame 5 (ORF5) of *R. eutropha* (8) and *E. coli ybhH*. The only work on *acnD* and *prpF* reported to date was performed in *R. eutropha* (8). The *R. eutropha prp* operon, represented in Fig. 1C, contains both the *acnD* and *prpF* genes and *prpD*. Brämer et al. recently reported that, in *R. eutropha* HF39, *prpD* was not required for a functional 2-MC cycle, but *acnD* and *prpF* (*acnM* and ORF5 in *R. eutropha*, respectively) functions were needed. However, efforts to demonstrate the conversion of 2-MC to 2-MIC by AcnM-enriched crude cell extracts were inconclusive (8).

In this paper we demonstrate that the *acnD* gene encodes an Fe/S-dependent 2-MC dehydratase enzyme that requires the *prpF* gene product to function *in vivo*. The *acnD* and *prpF* genes from *V. cholerae* and *S. oneidensis* were cloned independently or together and were used to compensate for the lack of the Fe/S-independent 2-MC dehydratase (PrpD) enzyme in *S. enterica* during growth on propionate. The AcnD and PrpF proteins were isolated. AcnD purified in the presence of air was inactive but was reactivated by protocols reported for the reactivation of aconitase (22). Reactivated AcnD had 2-MC dehydratase activity but no measurable 2-MIC dehydratase activity. Even though aconitase-like activity was not observed for PrpF *in vitro*, PrpF was required for the conversion of 2-MC into 2-MCA *in vivo*. Possible roles for the PrpF protein are discussed.

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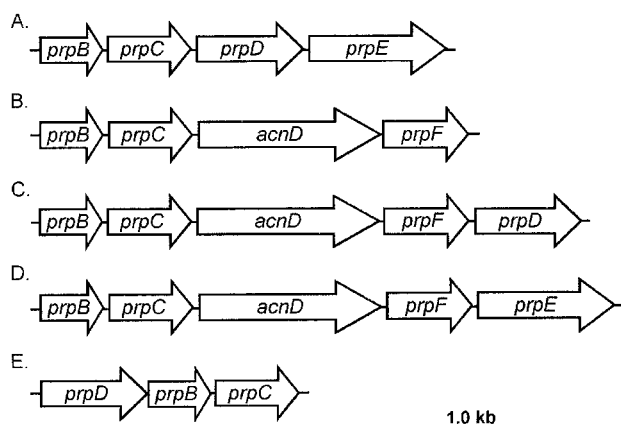


FIG. 1. Structural variations on *prp* operons of several bacteria. (A) *S. enterica* serovar Typhimurium and *E. coli* (*E. coli* contains a 439-nt putative stem-loop region between *prpB* and *prpC*). (B) *R. eutropha* CH34, *S. oneidensis* MR-1, *Neisseria meningitidis*, *Neisseria gonorrhoea* (the *Neisseria* spp. contain a 789-nt ORF of unknown function between *prpC* and *acnD*, which has been designated *yfcA*, and an ORF of 1,200 nt following *prpF* designated *ackA* that shows sequence similarity to propionate kinase [*tdcD*] of *E. coli*), and *B. sacchari* IPT101 (*B. sacchari* contains a putative ORF of 366 nt between *acnD* and *prpF* and two putative ORFs of 372 nt and 198 nt following *prpF*). (C) *R. eutropha* HF39, *Bordetella pertussis*, *Pseudomonas aeruginosa*, and *Pseudomonas putida* KT2440. (D) *V. cholerae*. (E) *prpD2B2C2* operon of *C. glutamicum*. Putative regulators have been excluded from Fig. 1C and D, and the spaces between ORFs are not drawn to scale.

MATERIALS AND METHODS

Chemicals and culture media. Cultures were maintained in Luria-Bertani (LB) broth and solid media. No-carbon E medium supplemented with MgSO_4 (1 mM) and methionine (0.5 mM) was used as minimal medium (5, 11). Propionate and pyruvate were used at concentrations of 30 mM. Antibiotic concentrations in rich media were as follows (in $\mu\text{g/ml}$): ampicillin, 100; kanamycin, 25 (for plasmids, 50 $\mu\text{g/ml}$); tetracycline, 15; and chloramphenicol, 20. Bacterial strains harboring plasmids were grown in minimal media containing ampicillin (50 $\mu\text{g/ml}$) and kanamycin (30 $\mu\text{g/ml}$). Synthetic 2-MC was purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada) as a mixture of stereoisomers; [$2\text{-}^{13}\text{C}$]propionate, 100% deuterium oxide (D_2O), and tetramethylsilane were purchased from Cambridge Isotope Labs (Andover, Mass.). The 2-MIC was a gift from W. W. Cleland. All other chemicals were purchased from Sigma (St. Louis, Mo.) unless otherwise stated. A list of the strains and plasmids used and their genotypes is provided in Table 1.

Recombinant DNA techniques. Restriction and modification enzymes were purchased from Promega (Madison, Wis.) unless otherwise stated and were used according to the manufacturer's specifications. All DNA manipulations were performed in *E. coli* strain DH5 α /F'. Plasmids were transformed into *S. enterica* strains by a quick-electroporation method as follows. Strains were grown to approximately mid-exponential phase, and cells in 1.0 ml of culture were pelleted at $10,000 \times g$ in a Microfuge 18 centrifuge (Beckman Coulter). Cells were

washed once with 100 μl of cold H_2O , resuspended in 100 μl of cold H_2O , and allowed to equilibrate on ice for 5 min. Plasmids were electroporated into the competent cells with a Bio-Rad Gene Pulser (Hercules, Calif.), according to the manufacturer's recommendations.

Construction of plasmids. Plasmids containing *S. oneidensis* or *V. cholerae* genes were constructed by PCR amplification of genomic DNA of *S. oneidensis* MR-1 (a gift from D. Saffarini, University of Wisconsin—Milwaukee) or *V. cholerae* N16961 (a gift from Ron Taylor, Dartmouth Medical School). PCRs typically contained the following in a 100- μl reaction mixture: 1.5 ng of genomic DNA, 50 pmol of each primer (IDT DNA, Coralville, Iowa), and deoxynucleoside triphosphate and HiFi DNA polymerase (Novagen, Madison, Wis.), each at a concentration of 0.2 μM , according to manufacturer's instructions. Reactions were performed under the following conditions: 35 cycles at 95°C for 30 s, at 50°C for 30 s, and at 72°C for 1 min per kb of target DNA. The PCR fragment was purified with a QIAquick PCR purification kit (QIAGEN, Chatsworth, Calif.). The methods of constructing the plasmids used are outlined in Table 2. The primer sequences used in plasmid constructions are available upon request.

Sequence verification of plasmid constructs. All resulting plasmid constructs were sequenced to verify that no mutations were introduced into the genes of interest. PCR sequencing reactions were prepared with Big Dye (Biotechnology Center, University of Wisconsin—Madison). Reactions were purified by means of the CleanSEQ reaction clean-up protocol of Agencourt Bioscience Corporation (Beverly, Mass.) and sequenced at the Biotechnology Center. Searches for sequence similarity were performed by using the BLAST algorithm (1). Protein sequence alignments were conducted with the ClustalW multiple alignment tool (36).

Complementation analysis. Plasmids were introduced into *S. enterica* strains as described above. The resulting strains were grown overnight in LB broth containing appropriate antibiotics. Four microliters of each overnight culture was used to inoculate 200 μl of fresh no-carbon E minimal medium supplemented with propionate (30 mM) and glycerol (1 mM) or acetate (30 mM), the appropriate antibiotic, and various amounts of L-(+)-arabinose (0, 100, or 500 μM). Medium was placed into the wells of a 96-well Falcon (Beckton Dickinson, Franklin Lakes, N.J.) microtiter dish, and the density of the cultures was monitored at 650 nm with a SpectraMAX Plus high-throughput spectrophotometer (Molecular Devices, Sunnyvale, Calif.). The plate chamber in the spectrometer was maintained at 37°C. Absorbance measurements were taken every 15 min for 72 h with agitation (for 780 s) between reads.

Anaerobic growth analysis. For anaerobic growth experiments, LB plates containing 10 mM sodium tetrathionate as a terminal electron acceptor (27) were patched with the strains of interest and introduced into an anoxic environment with Gas-Pak jars (Bethesda Research Laboratories, Gaithersburg, Md.). Plates were incubated at 37°C for 24 h and then transferred into an anaerobic chamber, where they were replica printed onto minimal propionate medium plates supplemented with 10 mM tetrathionate and various concentrations of arabinose. Plates were incubated anaerobically for 3 days at 37°C and growth was assessed.

Purification of *Shewanella* AcnD and PrpF proteins. Plasmids pPRP152 (*S. oneidensis* *prpF*⁺) and pPRP156 (*S. oneidensis* *acnD*⁺) were introduced into *E. coli* BL21(λ DE3) by the CaCl_2 heat shock method described elsewhere (30). Cells (20 ml of an overnight culture) were inoculated into 2 liters of LB broth supplemented with 100 μg of ampicillin/ml and grown with shaking at 37°C. Cells were grown to an A_{600} of approximately 0.6, and the overproduction of proteins was induced with 0.3 mM isopropyl- β -D-thiogalactopyranoside (IPTG). The cultures were harvested 3 h after induction for 10 min at 4°C and $11,700 \times g$. The cell pellets were frozen at -20°C for later use.

Cell pellets were resuspended in 25 ml of 20 mM (pH 7.5) 4-(2-hydroxyethyl)-

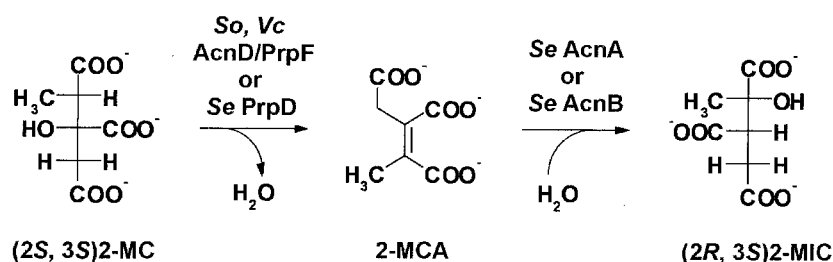


FIG. 2. Conversion of 2-MC to 2-MIC in bacteria. 2-MC and 2-MIC are drawn as Fischer projections; stereochemistry is based on intermediates of the *E. coli* 2-MC cycle (9). *Se*, *S. enterica*; *Vc*, *V. cholerae*; *So*, *S. oneidensis*.

TABLE 1. Strains and plasmids used in this study^a

Strain or plasmid	Genotype	Reference or source
<i>E. coli</i> strains		
BL21(λDE3)	F ⁻ <i>ampT hsdSB</i> (r _B ⁻ m _B ⁺) <i>dcm gal</i> λ(DE3)	New England Biolabs
DH5α/F'	F'/ <i>endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 thi-1 recA1 gyrA</i> (Nal ^r) <i>relA1 Δ(lacZYA-argF)U169 deoR</i> [φ80 <i>dlacΔ(lacZ)M15</i>]	New England Biolabs
JE4570	BL21(λDE3)/pPRP62 (<i>S. enterica prpC</i> ⁺ in pET 15b <i>bla</i> ⁺)	
JE4744	BL21(λDE3)/pPRP67 (<i>S. enterica prpD</i> ⁺ in pET15b <i>bla</i> ⁺)	
<i>S. enterica</i> strains		
TR6583 ^b	<i>metE205 ara-9</i>	K. Sanderson via J. Roth
Derivatives of TR6583 ^c		
JE3056	<i>prpB121::Tn10d</i> (Tc)	18
JE3907	<i>prpC167 zai-6386::Tn10d</i> (Tc) ^d	18
JE3909	<i>prpD169 zai-6386::Tn10d</i> (Tc)	18
JE3946	<i>prpB195 zai-6386::Tn10d</i> (Tc)	18
JE5993	<i>acnA2::cat</i> ⁺ <i>acnB3::kan</i> ⁺	17
JE6501	JE5993/pBAD30 (<i>bla</i> ⁺)	
JE6502	JE5993/pACN9 (<i>S. enterica acnA</i> ⁺ in pBAD30 <i>bla</i> ⁺)	
JE6503	JE5993/pACN10 (<i>S. enterica acnB</i> ⁺ in pBAD30 <i>bla</i> ⁺)	
JE6504	JE5993/pPRP121 (<i>V. cholerae acnD</i> ⁺ <i>prpF</i> ⁺ in pBAD30 <i>bla</i> ⁺)	
JE6506	JE5993/pPRP123 (<i>V. cholerae acnD</i> ⁺ <i>prpF</i> ⁺ in pBAD30 <i>bla</i> ⁺)	
JE7235	JE5993/pPRP138 (<i>S. oneidensis acnD</i> ⁺ in pBAD30 <i>bla</i> ⁺)	
JE7236	JE5993/pPRP140 (<i>S. oneidensis acnD</i> ⁺ <i>prpF</i> ⁺ in pBAD30 <i>bla</i> ⁺)	
JE7357	JE5993/pPRP163 (<i>E. coli ybhJ</i> ⁺ in pBAD30 <i>bla</i> ⁺)	
JE7590	JE5993/pPRP166 (<i>E. coli ybhHII</i> ⁺ in pBAD30 <i>bla</i> ⁺)	
Plasmids		
pBAD30	P _{araBAD} expression vector, <i>bla</i> ⁺	16
pACN9	<i>S. enterica acnA</i> ⁺ in pBAD30 <i>bla</i> ⁺	17
pACN10	<i>S. enterica acnB</i> ⁺ in pBAD30 <i>bla</i> ⁺	17
pPRP12-5.4	<i>S. enterica prpBCD</i> ⁺ in pSU38 <i>kan</i> ⁺	18
pPRP21	<i>S. enterica prpB</i> ⁺ in pBAD30 <i>bla</i> ⁺	18
pPRP36	<i>S. enterica prpD</i> ⁺ in pBAD30 <i>bla</i> ⁺	18
pPRP35	<i>S. enterica prpC</i> ⁺ in pBAD30 <i>bla</i> ⁺	18
pPRP62	<i>S. enterica prpC</i> ⁺ in pET15b <i>bla</i> ⁺	20
pPRP67	<i>S. enterica prpD</i> ⁺ in pET 15b <i>bla</i> ⁺	17
pPRP121	<i>V. cholerae acnD</i> ⁺ in pBAD30 <i>bla</i> ⁺	
pPRP123	<i>V. cholerae acnD</i> ⁺ <i>prpF</i> ⁺ in pBAD30 <i>bla</i> ⁺	
pPRP138	<i>S. oneidensis acnD</i> ⁺ in pBAD30 <i>bla</i> ⁺	
pPRP140	<i>S. oneidensis acnD</i> ⁺ <i>prpF</i> ⁺ in pBAD30 <i>bla</i> ⁺	
pPRP141	<i>S. oneidensis prpB</i> ⁺ <i>prpC</i> ⁺ <i>acnD</i> ⁺ <i>prpF</i> ⁺ in pBAD30 <i>bla</i> ⁺	
pPRP149	<i>S. oneidensis prpB</i> ⁺ in pBAD18Kan <i>kan</i> ⁺	
pPRP150	<i>S. oneidensis prpC</i> ⁺ in pBAD18Kan <i>kan</i> ⁺	
pPRP151	<i>S. oneidensis prpB</i> ⁺ <i>prpC</i> ⁺ in pBAD 18Kan <i>kan</i> ⁺	
pPRP152	<i>S. oneidensis acnD</i> ⁺ in pTYB12 <i>bla</i> ⁺	
pPRP153	<i>S. oneidensis prpF</i> ⁺ in pBAD18Kan <i>kan</i> ⁺	
pPRP154	<i>E. coli ybhH</i> ⁺ in pBAD18Kan <i>kan</i> ⁺	
pPRP155	<i>V. cholerae prpF</i> ⁺ in pBAD18Kan <i>kan</i> ⁺	
pPRP156	<i>S. oneidensis prpF</i> ⁺ in pTYB12 <i>bla</i> ⁺	
pPRP163	<i>E. coli ybhJ</i> ⁺ in pBAD30 <i>bla</i> ⁺	
pPRP166	<i>E. coli ybhHII</i> ⁺ in pBAD30 <i>bla</i> ⁺	

^a All *S. enterica* strains are derivatives of the *S. enterica* serovar Typhimurium LT2 strain. Unless otherwise stated, strains and plasmids were constructed during the course of this work.

^b Formerly SA2979.

^c See Table 3 for additional strains (and their genotypes) used in this work.

^d *Tn10d*(Tc) is an abbreviation of *Tn10DEL 16DEL17* (37).

1-piperazineethanesulfonic acid (buffer A) (HEPES; Fisher Biotech, Itasca, Ill.), containing 100 mM KCl, 0.1% (vol/vol) Triton X-100, and 0.1 mM EDTA. The cell suspensions were broken at 10⁴ kPa in a chilled French pressure cell. Cell debris was removed by centrifugation at 31,000 × *g* for 30 min at 4°C. Crude cell extracts were filtered through a 0.2-μm-pore-size filter and passed through a 5-ml column of chitin beads (New England Biolabs, Beverly, Mass.). The column was washed with buffer A according to the manufacturer's instructions and then quickly washed with 15 ml of buffer A containing 50 mM 1,4-dithio-DL-threitol (DTT; Promega, Madison, Wis.). The column flow was stopped, and the column was kept at 4°C for 96 h. Purified, untagged proteins were eluted off the column with buffer A containing 50 mM DTT and were visualized with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (23). Fractions containing protein were pooled and dialyzed overnight into 20 mM HEPES (pH 7.5), 100 mM KCl, 0.1 mM EDTA, and 5 mM DTT (buffer B). After dialysis for 2 h,

the buffer was changed to buffer B lacking EDTA. The remaining part of the dialysis period was performed with buffer B containing 5% (vol/vol) glycerol. The protein was flash-frozen in liquid nitrogen and stored at -80°C.

Reactivation of purified proteins. Purified AcnD was reactivated according to the method of Kennedy and Beinert (22). All reagents were prepared under strict anaerobic conditions (3, 15). Purified H₆AcnA (17) was reactivated by the same procedure and was used as a positive control in the aconitase assays.

In vitro aconitase assays. Aconitase assays were performed as described (17). Reaction mixtures contained 90 mM Tris (Tris-HCl) buffer (pH 8.0) and 20 mM citrate, isocitrate, 2-MC, 2-MIC, or 2 mM *cis*-aconitate in a 1-ml quartz cuvette. Reactivated enzyme was transferred to the cuvette with a 25-μl Hamilton syringe. No detectable loss of activity was observed during the duration of the assays (2 h). Reactions were monitored for 10 min at 240 nm in a Perkin-Elmer Lambda 40 spectrophotometer (Norwalk, Conn.) equipped with a circulating

TABLE 2. Construction of plasmids used in this work

Plasmid name	Vector backbone	Gene(s) cloned	Method of construction
pPRP117	pDONR201 ^a	<i>V. cholerae acnD</i>	Gateway BP reaction ^b
pPRP121	pBAD30	<i>V. cholerae acnD</i>	Gateway LR reaction
pPRP123	pBAD30	<i>V. cholerae acnD prpF</i>	<i>KpnI</i> , <i>XbaI</i> cloning
pPRP136	pDONR201	<i>S. oneidensis acnD</i> ^c	Gateway BP reaction
pPRP138	pBAD30	<i>S. oneidensis acnD</i>	Gateway LR reaction
pPRP137	pDONR201	<i>S. oneidensis acnD prpF</i>	Gateway BP reaction
pPRP140	pBAD30	<i>S. oneidensis acnD prpF</i>	Gateway LR reaction
pPRP141	pBAD30	<i>S. oneidensis prpB prpC acnD prpF</i>	<i>SacI</i> , <i>XbaI</i> cloning
pPRP149	pBAD18-Kan	<i>S. oneidensis prpB</i>	<i>SacI</i> , <i>XbaI</i> cloning
pPRP150	pBAD18-Kan	<i>S. oneidensis prpC</i>	<i>SacI</i> , <i>XbaI</i> cloning
pPRP151	pBAD18-Kan	<i>S. oneidensis prpB prpC</i>	<i>SacI</i> , <i>XbaI</i> cloning
pPRP152	pTYB12	<i>S. oneidensis prpF</i>	<i>BsmI</i> , <i>XhoI</i> cloning
pPRP153	pBAD18-Kan	<i>S. oneidensis prpF</i>	<i>EcoRI</i> , <i>XbaI</i> cloning
pPRP154	pBAD18-Kan	<i>E. coli</i> ^d <i>ybhH</i>	<i>SacI</i> , <i>XbaI</i> cloning
pPRP155	pBAD18-Kan	<i>V. cholerae prpF</i>	<i>KpnI</i> , <i>XbaI</i> cloning
pPRP156	pTYB12	<i>S. oneidensis acnD</i>	<i>BsmI</i> , <i>XhoI</i> cloning
pPRP163	pBAD30	<i>E. coli ybhJ</i>	<i>SacI</i> , <i>XbaI</i> cloning
pPRP166	pBAD30	<i>E. coli ybhH ybhI ybhJ</i>	<i>SacI</i> , <i>XbaI</i> cloning

^a pDONR201 was purchased from Invitrogen, Carlsbad, Calif.

^b Gateway BP and LR reactions are methods developed by Life Technologies, Invitrogen Corporation.

^c All *S. oneidensis* organisms are strain MR-1.

^d All *E. coli* organisms are strain MG1655.

water bath, which maintained the temperature at 37°C. Specific activities were reported in micromoles per minute per milligram of protein and calculated from the extinction coefficients of 3,600 M⁻¹ cm⁻¹ for the *cis*-aconitate (21) and 4,500 M⁻¹ cm⁻¹ for the 2-methyl-*cis*-aconitate (2).

Requirement of an Fe/S cluster for AcnD activity. To determine if iron was required for AcnD activity, AcnD was reactivated according to the protocol of Kennedy and Beinert (22) with iron excluded from the reactivation mixture. To determine the magnitude of inactivation of AcnD in the presence of iron-chelating agents, reactivated AcnD was incubated with EDTA and ferricyanide in the molar ratios of 1:50:20 (enzyme:EDTA:ferricyanide) as described by Kennedy and Beinert (22). Protein activity was assayed as described above.

H₆PrpC, H₆PrpD, and PrpE proteins. The *S. enterica* H₆PrpC and H₆PrpD proteins were overproduced and purified as previously described (17, 20). The propionyl-coenzyme A synthetase (PrpE) enzyme was provided by V. J. Starai.

¹³C-NMR spectroscopy. Peak assignments were compared to those previously reported (20). Glycerol was present in all protein samples and observed in the spectra due to the natural abundance of ¹³C (17). ¹³C-nuclear magnetic resonance (NMR) spectra were obtained at the National Magnetic Resonance Facility at the University of Wisconsin—Madison by means of a Bruker Instruments DMX-400 Avance console with a 9.4-T wide-bore magnet at 100.6 MHz.

In vitro enzymatic synthesis of [2-¹³C]MC. [2-¹³C]MC was generated in vitro (17) in 0.5-ml reaction mixtures that contained potassium phosphate buffer (pH 7.5; 50 mM), ATP (2.5 mM), MgCl₂ (5 mM), coenzyme A (2.5 mM), [2-¹³C]propionate (2.5 mM), oxaloacetate (5 mM), and homogeneous PrpE and H₆PrpC proteins (75 μg each). To each sample, 0.1 ml of 100% D₂O was added, and the reactions were transferred to 5-mm NMR tubes (Wilmad Glass, Buena, N.J.). A sealed tetramethylsilane capillary was added as an external reference.

Conversion of [2-¹³C]MC to [2-¹³C]MCA. [2-¹³C]MC (~2.5 mM) synthesized as described above was used as substrate for PrpF and reactivated AcnD or for H₆PrpD. Reaction mixtures (0.5 ml) contained [2-¹³C]MC, H₆PrpD, PrpF, or reactivated AcnD (25 μg each) or PrpF and reactivated AcnD (25 μg each). The reaction mixtures were allowed to incubate for 1 h at 37°C and were prepared for ¹³C-NMR analysis as described above.

Other procedures. Protein concentrations were determined from a standard curve generated with bovine serum albumin by the method of Bradford (6) with the Bio-Rad protein reagent. Proteins were separated by SDS-12% PAGE and stained with Coomassie blue (28). Novagen Perfect Protein markers (Madison, Wis.) were used as standards for SDS-PAGE.

RESULTS

***S. oneidensis* and *V. cholerae acnD* and *prpF* gene product functions restore growth of a *S. enterica prpD* mutant strain on propionate.** As pointed out above, *prp* loci from various pro-

karyotes contain two genes (*acnD* and *prpF*) in lieu of *prpD* (Fig. 1). The *acnD* and *prpF* genes from *S. oneidensis* and *V. cholerae* were cloned and used to determine whether they would compensate for the lack of PrpD function during growth of an *S. enterica prpD* mutant strain on propionate. The *acnD* and *prpF* genes were cloned into plasmids under the control of arabinose-inducible promoters either as a pair or individually on compatible plasmids. Plasmids carrying these genes were introduced into *S. enterica* strain JE3909 (*prpD*), and growth on propionate was assessed. Table 3 shows the doubling times of all strains tested. Both *S. oneidensis acnD*⁺*prpF*⁺ and *V. cholerae acnD*⁺*prpF*⁺ constructs complemented strain JE3909 (Fig. 3A). When the *acnD* and *prpF* genes were carried in separate plasmids, however, growth on propionate was observed only when both genes were present in the cell. In all cases when *S. oneidensis* and *V. cholerae* genes were mixed, strain JE3909 was able to grow on propionate (Fig. 3B). This result was not surprising considering that the *S. oneidensis* and *V. cholerae* AcnD and PrpF share 76 and 73% identity, respectively. These results indicate that both *acnD* and *prpF* are required to complement a *prpD* strain of *S. enterica*.

To determine whether PrpF function was required along with AcnD function to complement an *S. enterica prpD* mutant grown on a propionate medium anaerobically, these strains were replica printed onto a propionate medium containing tetrathionate as a terminal electron acceptor. Only the positive control (*prpD*⁺) and strains containing both the *acnD*⁺ and *prpF*⁺ plasmids grew anaerobically on propionate. These data indicate that even under anaerobic conditions, both AcnD and PrpF functions are required to complement an *S. enterica prpD* mutant.

The possibility that the *E. coli ybhJ* and *ybhH* genes could restore the growth of strain JE3909 on propionate was also assessed. The *E. coli YbhJ* protein is an AcnA homolog that shares 22% identity (37% similarity) with *S. oneidensis* AcnD; the YbhH protein shares 34% identity (47% similarity) with *S.*

TABLE 3. Heterologous complementation of *S. enterica prp* mutant strains

Strain	Genotype ^a	Arabinose concentration ^b (μM)	Doubling time ^c (h ± SD)
JE4175	TR6583 (wild type)/pBAD30	500	7.3 ± 0.3
JE6107	JE3909/pPRP36 (<i>S. enterica prpD</i> ⁺)	500	7.6 ± 0.3
JE7280	JE3909/pPRP138 (<i>S. oneidensis acnD</i> ⁺), pPRP153 (<i>S. oneidensis prpF</i> ⁺)	500	7.9 ± 0.3
JE7232	JE3909/pPRP140 (<i>S. oneidensis acnD</i> ⁺ <i>prpF</i> ⁺)	500	8.0 ± 0.5
JE7227	JE3909/pPRP141 (<i>S. oneidensis prpB</i> ⁺ <i>prpC</i> ⁺ <i>acnD</i> ⁺ <i>prpF</i> ⁺)	500	9.2 ± 1.0
JE7282	JE3909/pPRP138 (<i>S. oneidensis acnD</i> ⁺), pPRP155 (<i>V. cholerae prpF</i> ⁺)	500	20.4 ± 1.2
JE7283	JE3909/pPRP121 (<i>V. cholerae acnD</i> ⁺), pPRP153 (<i>S. oneidensis prpF</i> ⁺)	100	11.0 ± 0.5
JE7285	JE3909/pPRP121 (<i>V. cholerae acnD</i> ⁺), pPRP155 (<i>V. cholerae prpF</i> ⁺)	500	25.8 ± 2.0
JE7234	JE3909/pPRP123 (<i>V. cholerae acnD</i> ⁺ <i>prpF</i> ⁺)	100	13.0 ± 0.4
JE6105	JE3909/pBAD30	500	NG
JE7238	JE3056/pPRP12-5.4 (<i>S. enterica prpB</i> ⁺ <i>prpC</i> ⁺ <i>prpD</i> ⁺)	0	15.8 ± 0.5
JE7274	JE3056/pPRP151 (<i>S. oneidensis prpB</i> ⁺ <i>prpC</i> ⁺), pPRP36 (<i>S. oneidensis prpD</i> ⁺)	100	12.7 ± 0.3
JE7275	JE3056/pPRP151 (<i>S. oneidensis prpB</i> ⁺ <i>prpC</i> ⁺), pPRP140 (<i>S. oneidensis acnD</i> ⁺ <i>prpF</i> ⁺)	100	19.2 ± 0.4
JE7277	JE3056/pPRP151 (<i>S. oneidensis prpB</i> ⁺ <i>prpC</i> ⁺), pPRP123 (<i>V. cholerae acnD</i> ⁺ <i>prpF</i> ⁺)	100	14.7 ± 0.5
JE7239	JE3056/pPRP141 (<i>S. oneidensis prpB</i> ⁺ <i>prpC</i> ⁺ <i>acnD</i> ⁺ <i>prpF</i> ⁺)	100	27.0 ± 1.4
JE5585	JE3056/pBAD30	500	NG
JE7242	JE3056/pPRP151 (<i>S. oneidensis prpB</i> ⁺ <i>prpC</i> ⁺)	500	NG
JE5297	JE3946/pPRP21 (<i>S. oneidensis prpB</i> ⁺)	500	9.7 ± 0.3
JE7252	JE3946/pPRP149 (<i>S. oneidensis prpB</i> ⁺)	100	11.3 ± 0.4
JE7253	JE3946/pPRP151 (<i>S. oneidensis prpB</i> ⁺ <i>prpC</i> ⁺)	100	10.5 ± 0.5
JE5296	JE3946/pBAD30	500	NG
JE7270	JE3907/pPRP35 (<i>S. enterica prpC</i> ⁺)	100	11.0 ± 0.2
JE7254	JE3907/pPRP150 (<i>S. oneidensis prpC</i> ⁺)	500	12.6 ± 0.3
JE7255	JE3907/pPRP151 (<i>S. oneidensis prpB</i> ⁺ <i>prpC</i> ⁺)	500	11.6 ± 0.4
JE7251	JE3907/pBAD30	500	NG

^a All plasmids except pPRP12-5.4 (*S. enterica prpBCD*⁺) contain arabinose-inducible promoters. Where two plasmids are indicated, they are of different incompatibility groups. The following strains were used to construct the strains tested: JE3909 [*metE205 ara-9 prpD169 zai-6386::Tn10d(Tc)*], JE3907 [*metE205 ara-9 prpC167 zai-6386::Tn10d(Tc)*], JE3056 [*metE205 ara-9 prpB121::Tn10d(Tc)*], and JE3946 [*metE205 ara-9 prpB195 zai-6386::Tn10d(Tc)*].

^b Each construct was tested in media with 100 and 500 μM arabinose; the highest growth rate was included in this table.

^c JE7230 (JE3909/pPRP138 [*S. oneidensis acnD*⁺]), JE7271 (JE3909/pPRP153 [*S. oneidensis prpF*⁺]), JE7233 (JE3909/pPRP121 [*V. cholerae acnD*⁺]), JE7273 (JE3909/pPRP155 [*V. cholerae prpF*⁺]), JE7523 (JE3909/pPRP166 [*E. coli ybhH*⁺]), JE7356 (JE3909/pPRP163 [*E. coli ybhJ*⁺]), JE7272 (JE3909/pPRP154 [*E. coli ybhH*⁺]), or JE7359 (JE3909/pPRP154 [*E. coli ybhJ*⁺], pPRP163 [*E. coli ybhJ*⁺]) did not grow in a propionate medium supplemented with 500 μM arabinose. NG, no growth observed (doubling times greater than 50 h).

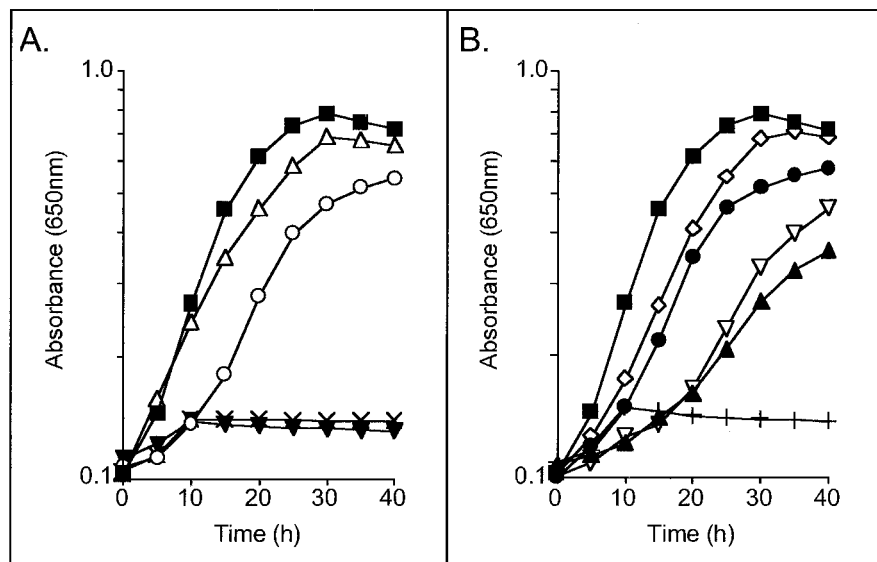


FIG. 3. Heterologous complementation studies. All curves shown are strain JE3909 (*S. enterica prpD*) with plasmids in *trans*. Filled squares, pPRP21 (*S. enterica prpD*⁺); open triangles, pPRP123 (*V. cholerae acnD*⁺ *prpF*⁺); open circles, pPRP140 (*S. oneidensis acnD*⁺ *prpF*⁺); filled inverted triangles, pPRP138 (*S. oneidensis acnD*⁺); X, pPRP153 (*S. oneidensis prpF*⁺); open diamonds, pPRP121 (*V. cholerae acnD*⁺) + pPRP153 (*S. oneidensis prpF*⁺); filled circles, pPRP138 (*S. oneidensis acnD*⁺) + pPRP153 (*S. oneidensis prpF*⁺); open inverted triangles, pPRP138 (*S. oneidensis acnD*⁺) + pPRP155 (*V. cholerae prpF*⁺); filled triangles, pPRP121 (*V. cholerae acnD*⁺) + pPRP155 (*V. cholerae prpF*⁺); plus signs, pPRP163 (*E. coli ybhJ*⁺) + pPRP154 (*E. coli ybhH*⁺).

oneidensis PrpF. The *E. coli* *ybhJ* and *ybhH* genes were cloned individually and as an operon (*ybhHIJ*) under the control of an arabinose-inducible promoter. None of the plasmids tested restored the growth of strain JE3909 on propionate (Table 3). Strain JE3909 failed to grow on propionate when *E. coli* *ybhJ* was introduced with *S. oneidensis* or *V. cholerae* *prpF* or when *E. coli* *ybhH* was added with *S. oneidensis* or *V. cholerae* *acnD*. These data indicated that the *ybhHIJ* operon of *E. coli* did not convert the 2-MC generated by *S. enterica* into 2-MCA or at least did not convert enough to complement the growth phenotype on propionate. The role of the *ybhHIJ* operon in *E. coli* remains unclear.

***S. oneidensis* PrpB (2-MIC lyase) and PrpC (2-MC synthase) restore growth of *S. enterica* *prpB* and *prpC* mutant strains on propionate.** It was of interest to determine whether *S. oneidensis* *prpB* (a 2-MIC lyase ortholog) and *prpC* (a 2-MC synthase ortholog) could complement *S. enterica* *prpB* or *prpC* mutant strains. *S. oneidensis* *prpB* and *prpC* were cloned as a pair into plasmid pBAD18Kan and introduced into strains JE3946 (*prpB*) and JE3907 (*prpC*) (Table 3). The resulting *S. enterica* strains (JE7253 and JE7255, respectively) grew on propionate, suggesting that the same stereoisomer of 2-MC was synthesized in *S. enterica* and *S. oneidensis* and that the PrpB enzymes from both organisms most likely use the same stereoisomer of 2-MIC. It has been shown that *E. coli* only synthesizes the 2*S*,3*S* isomer of 2-MC and generates the 2*R*,3*S* isomer of 2-MIC (9), and because *S. enterica* PrpB and PrpC are greater than 91 and 96% identical, respectively, to the corresponding proteins in *E. coli*, it is inferred that the same stereoisomers of the 2-MC cycle intermediates are produced in these organisms. The same results were obtained when *S. oneidensis* *prpB* or *prpC* were cloned individually and introduced into *S. enterica* JE3946 (JE7252) and JE3907 (JE7254), respectively (Table 3).

AcnD and PrpF do not substitute for TCA cycle aconitase. It was also tested whether AcnD could substitute for *S. enterica* AcnA or AcnB during growth conditions that demanded a functional tricarboxylic acid (TCA) cycle. *S. oneidensis* or *V. cholerae* *acnD* individually or in combination with *prpF* were introduced into strain JE5993 (*acnA acnB*) (17). The resulting strains (JE6504, JE6506, JE7235, and JE7236) failed to grow on pyruvate and various concentrations of arabinose (as inducer). Only the control strains JE6502 (JE5993/pACN9 *S. enterica* *acnA*⁺) and JE6503 (JE5993/pACN10 *S. enterica* *acnB*⁺) grew on pyruvate under the conditions tested. Hence, it was concluded that the *acnD* and *prpF* genes cannot compensate for the lack of aconitase activity required for a functional TCA cycle of *S. enterica*, at least not to the level required for growth on pyruvate. No growth on pyruvate was observed when the *E. coli* *ybhJ* and *ybhH* genes or the complete *ybhHIJ* operon were introduced into strain JE5993.

Purification of AcnD and PrpF proteins. The putative biochemical activity of the AcnD and PrpF proteins was investigated in vitro. For this purpose, the *S. oneidensis* *acnD* and *prpF* genes were cloned and their products produced with an N-terminal chitin-binding tag (plasmids pPRP152 and pPRP156, respectively). Both proteins were purified by chitin affinity chromatography, and the tag was cleaved at the intein site according to the manufacturer's recommendations (New England Biolabs). Each protein was >95% pure as judged by scanning densitometry (Fig. 4). The relative mobility of both

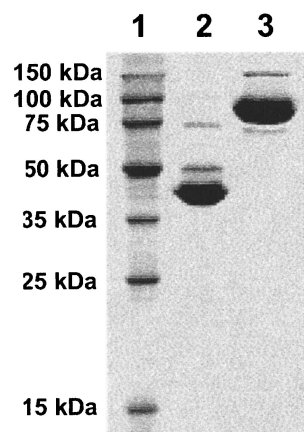


FIG. 4. SDS-PAGE of purified *S. oneidensis* AcnD and PrpF proteins. Lane 1, MW standards (Novagen); lane 2, purified PrpF; lane 3, purified AcnD.

proteins was consistent with their predicted molecular masses, i.e., AcnD was observed at ~94 kDa, and PrpF was observed at ~42 kDa.

Enzymatic activity of AcnD. Purified AcnD and H₆AcnA (17) were reactivated as described in Materials and Methods and assayed spectrophotometrically for activity with various substrates (Table 4). AcnD used citrate, *cis*-aconitate, and 2-MC as substrates but not 2-MIC or isocitrate, indicating that, like PrpD (17), AcnD only catalyzes the first half of the aconitase-like reaction. The specific activity of AcnD for 2-MC was approximately 2.5-fold higher than that for citrate. The specific activity measured with 2-MC as substrate may be an underestimate of AcnD activity since commercially available 2-MC contained a mixture of stereoisomers, some of which may be inhibitory to the enzyme. On the other hand, AcnA used as positive control readily dehydrated 2-MIC, isocitrate, and citrate and hydrated *cis*-aconitate. In agreement with previous work, AcnA did not use 2-MC as substrate (17).

AcnD is an Fe/S 2-MC dehydratase. AcnD activity was only observed after anoxic reactivation with iron, sulfide, and reductant. When reactivation was attempted in the absence of iron, no enzymatic activity was observed. The activity of reconstituted AcnD was lost over time in the presence of air. Also, when ferricyanide and EDTA were added to the enzyme after reactivation, all detectable activity was lost within 15 min of incubation. The primary amino acid sequence of AcnD contains 22 of the 23 residues found at the active site of mitochon-

TABLE 4. Specific activities of AcnD and AcnA^a with different substrates

Substrate	AcnD	AcnA
Citrate	2.9 ± 0.5	26.5 ± 4.0
<i>cis</i> -Aconitate	5.0 ± 0.2	68.5 ± 7.4
Isocitrate	ND ^b	43.4 ± 3.1
2-MC	7.8 ± 0.4	ND
2-MIC	ND	12.7 ± 1.4

^a AcnD and AcnA were reconstituted as stated in Materials and Methods.

^b ND, no detectable activity observed (limits of detection were 0.5 μM/min for *cis*-aconitate conversion and 0.4 μM/min for 2-MCA formation) under the conditions tested.

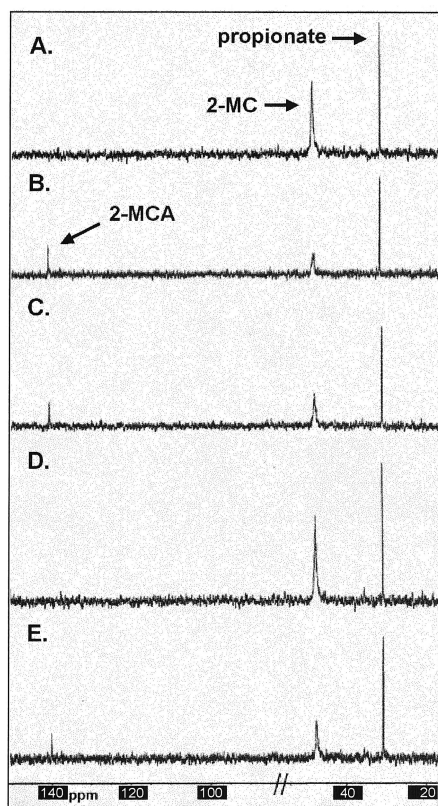


FIG. 5. ^{13}C -NMR spectra of in vitro reactions. The composition of the reaction mixtures is described under Materials and Methods. Reaction A contained the *S. enterica* PrpE and PrpC enzymes; reaction B contained the *S. enterica* PrpE, PrpC, and PrpD enzymes; reaction C contained the *S. enterica* PrpE, PrpC, and reactivated *S. oneidensis* AcnD enzymes; reaction D contained the *S. enterica* PrpE and PrpC enzymes and the *S. oneidensis* PrpF protein; reaction E contained the *S. enterica* PrpE and PrpC enzymes and the *S. oneidensis* AcnD enzyme and PrpF protein. Chemical shifts expressed in parts per million are as follows: $[2-^{13}\text{C}]$ propionate 30.8 ppm; $[2-^{13}\text{C}]$ MC, 47.5 ppm; and $[2-^{13}\text{C}]$ MCA, 141.5 ppm. The portion of the spectra removed contained two glycerol peaks as previously described (17).

drial aconitase (mAcn), including the three cysteine residues that coordinate the 4Fe/4S cluster of mAcn (24). Taken together, these data indicated that in its active form AcnD contains an Fe/S center.

^{13}C -NMR analysis of the AcnD reaction product. $[2-^{13}\text{C}]$ MC was synthesized in vitro as described (17). Reactivated AcnD was added to this reaction, incubated for 1 h, and analyzed by ^{13}C -NMR spectroscopy. Peak assignments were based on those identified previously (17). Excluding two glycerol peaks (glycerol was added to the enzyme as cryoprotectant), only three peaks were observed: for $[2-^{13}\text{C}]$ propionate (30.8 ppm), $[2-^{13}\text{C}]$ MC (47.5 ppm), and $[2-^{13}\text{C}]$ MCA (141.5 ppm) (Fig. 5C). A positive control experiment for $[2-^{13}\text{C}]$ MCA production was performed with H_6PrpD (Fig. 5B). The peaks observed with the H_6PrpD -catalyzed reaction matched exactly the shifts of those seen with the AcnD reaction. The reaction mixture containing only $[2-^{13}\text{C}]$ MC had peaks at 30.8 and 47.5 ppm (Fig. 5A). These data indicated that both AcnD and PrpD had 2-MC dehydratase activity. To test whether the PrpF protein had aconitase-like enzymatic activity, PrpF was added to the

$[2-^{13}\text{C}]$ MC reaction mixture and incubated for 1 h at 37°C. No peak shift or decrease in the 2-MC signal at 47.5 ppm was observed (Fig. 5D). PrpF was also added to the reaction mixture containing reactivated AcnD. No peak shift or decrease in the 2-MCA signal at 141.5 ppm was observed relative to the signal observed in the reaction mixture containing only AcnD (Fig. 5E). These results indicated that PrpF did not convert 2-MCA into 2-MIC or catalyze the conversion of 2-MC into 2-MCA under the assay conditions tested.

Probing for a role for the PrpF protein in propionate metabolism. The possibility that the PrpF protein could catalyze the dehydration of citrate, isocitrate, 2-MC, or 2-MIC or the hydration of *cis*-aconitate was investigated. Even though PrpF does not contain an apparent Fe/S cluster binding motif, anoxic reconstitution of an Fe/S center was attempted. The PrpF protein with or without anoxic iron and sulfide reactivation did not have any detectable amounts of dehydratase or hydratase activities. PrpF was also added in twofold molar excess to AcnD in the *cis*-aconitate hydratase assay. No increase in the AcnD-catalyzed rate or in the overall conversion of substrate was observed.

We also tested whether AcnD required PrpF to catalyze the second half of the aconitase-like reaction, i.e., the conversion of 2-MCA to 2-MIC. PrpF was added to AcnD under anoxic and oxic conditions and was tested in the 2-MIC dehydratase assay. No 2-MIC dehydratase activity was observed.

We also looked into the possibility that PrpF could stabilize AcnD activity in the presence of oxygen. A twofold molar excess PrpF protein was added to anoxic AcnD. The mixture was incubated for 5 min before the seal was removed. Assays were performed, along with the control experiment with a reaction mixture that lacked PrpF protein. No significant differences in AcnD activity were observed over 2 h, and approximately 75% of AcnD (*cis*-aconitate hydratase) activity was lost over this period of time (data not shown). Work is currently being conducted to elucidate the role of PrpF in the 2-MC cycle.

DISCUSSION

This study established, both in vitro and in vivo, the biochemical activity of a new enzyme involved in the 2-MC cycle of several prokaryotes. The genes *acnD* and *prpF* of *S. oneidensis* and *V. cholerae*, when concurrently expressed, compensate for the lack of the Fe/S-independent PrpD enzyme in *S. enterica prpD* mutant strains during growth on propionate. The AcnD protein from *S. oneidensis* was isolated and shown to have a new activity for an enzyme containing an Fe/S center. AcnD catalyzes the dehydration of 2-MC and citrate but does not catalyze the dehydration of 2-MIC or isocitrate (Table 4). ^{13}C -NMR spectroscopy of reactivated AcnD with $[2-^{13}\text{C}]$ MC revealed that AcnD could utilize the 2-MC generated by *S. enterica* PrpC and that the product of the AcnD reaction matched that produced by *S. enterica* PrpD (2-MCA) (Fig. 5). To our knowledge, this is the first report of an Fe/S-dependent 2-MC dehydratase.

To date, the only reported work on an AcnD homolog was performed with the *acnM* gene from *R. eutropha* (*R. eutropha* AcnM shares 83% identity with *S. oneidensis* AcnD) expressed in a crude extract system in *E. coli*. *E. coli* crude extracts

containing *R. eutropha* AcnM protein were found to have *cis*-aconitate hydratase activity, but the data were inconclusive as to whether AcnM could dehydrate 2-MC. It was concluded, however, that AcnM may catalyze the hydration of 2-MCA into 2-MIC (8). In contrast, the data reported in this paper indicate that AcnD (and by extrapolation, AcnM) most likely does not catalyze the hydration of 2-MCA to 2-MIC because the enzyme will not dehydrate 2-MIC, and aconitases are known to catalyze freely reversible reactions. Additional support for this conclusion comes from NMR experiments where no evidence was obtained to indicate that active AcnD protein can convert 2-MC into 2-MIC. The only signal observed in the experiments was that of 2-MCA.

Studies on mammalian aconitases (mAcn) and AcnA and AcnB from both *S. enterica* and *E. coli* have demonstrated that these enzymes will not catalyze the dehydration of 2-MC; however, they will dehydrate 2-MIC and catalyze the full conversion of citrate into *cis*-aconitate into isocitrate (4, 9, 17, 29). The mechanism of aconitases is known to proceed by the binding of *cis*-aconitate in two ways to achieve the *trans* elimination or addition of water across the double bond (29). For this to occur, the substrate (*cis*-aconitate) must rotate 180°. The crystal structure of mAcn bound with 2-MIC allowed the prediction that if 2-MCA were rotated into the analogous 2-MC position, 2-MC would not be able to bind in the active site due to a steric clash of the methyl group with residue Asp165 (25). Interestingly, when aligned with mAcn, AcnD also contains this conserved aspartate residue, along with 21 of the other 22 active site residues of mAcn (24). It has been noted that all sequenced *acnD* homologs contain an Asn residue directly following one of the Cys residues that is likely to coordinate the Fe/S cluster, while in aconitases of the tricarboxylic acid cycle an Ile residue is found at this position (7). Experiments to test whether this residue plays a role in the substrate specificity of the AcnD enzyme are in progress.

It has been reported that horse heart aconitase can catalyze the formation of 2-MC and 2-MIC from 2-MCA (12). These data are in contradiction to the present information on aconitases, but the possibility exists that one enzyme may catalyze the complete conversion of 2-MC to 2-MIC. In *Y. lipolytica*, two enzymes, a 2-MC dehydratase and a 2-MIC dehydratase, have been implicated in its 2-MC cycle; both of these enzymes were found not to contain an Fe/S cluster or to be inactivated by iron-chelating agents (31, 34). To our knowledge, an Fe/S-independent 2-MIC dehydratase has not been identified in any other organism.

Possible roles for PrpF in propionate catabolism. The possibility that PrpF may be an isomerase of one of the intermediates of the 2-MC cycle was considered. However, it is unlikely that PrpF is a 2-MIC isomerase because the *prpB* gene from *S. oneidensis* complemented a *prpB* mutant strain of *S. enterica*, and NMR and spectrophotometric data suggested that AcnD only catalyzed the conversion of (2*S*,3*S*)-MC into 2-MCA. It was also possible that PrpF could be a 2-MC isomerase. However, our data showed that the *S. oneidensis* *prpC*⁺ allele complemented an *S. enterica* *prpC* mutant strain, suggesting that the PrpC protein from *S. oneidensis* generated the same stereoisomer of 2-MC as that generated by the *S. enterica* PrpC enzyme. If the *S. oneidensis* PrpC protein generated a different 2-MC stereoisomer, it would be unlikely that

the *S. enterica* PrpD enzyme would be able to use it as substrate. In support of this hypothesis, Brock et al. showed that the true substrate of the *E. coli* PrpD protein is most likely (2*S*,3*S*)-MC, and a 10-fold decrease in PrpD activity was observed when a racemic mixture of 2-MC stereoisomers was used (9). To further test these ideas, the stereochemical configuration of the reaction product of *S. oneidensis* PrpC must be determined.

Brämer et al. observed that PrpF was weakly similar (24%) to the *pduG* gene product of *S. enterica*, the proposed reactivation factor of diol dehydratase (7). Although this similarity is very weak, PrpF may be involved in AcnD Fe/S cluster formation or repair. As shown above, in vivo, PrpF must accompany AcnD to compensate for the lack of the PrpD enzyme during growth of a *prpD* mutant strain of *S. enterica* on propionate. If PrpF is required to stabilize AcnD in the presence of air, then growth under anoxic conditions should bypass the need for PrpF. However, PrpF was required even under anoxic growth conditions, suggesting that PrpF may not be needed to protect the AcnD Fe/S cluster from oxidation, but it could be involved in the formation, insertion, or stabilization of the Fe/S cluster. At present, the role of PrpF in propionate metabolism remains to be determined.

Bacteria use different strategies to convert 2-MC into 2-MIC. It is interesting that the sequenced *prp* operons of several bacteria contain both *prpD* and the *acnD/prpF* genes. Since these gene products catalyze the same reaction, i.e., the conversion of 2-MC into 2-MCA, they would appear to perform redundant functions in these organisms. Why would these organisms employ this strategy? Perhaps PrpD, the Fe/S-independent 2-MC dehydratase, is needed by these organisms at times when oxygen levels are high in the cell and may be deleterious to AcnD. Then why do these organisms retain the *acnD/prpF* pair of genes? Maybe having both of these ways to convert 2-MC into 2-MCA allows for more efficient growth on carbon sources that require the 2-MC cycle as a route of metabolism. Or perhaps the *acnD/prpF* gene products do carry out the second half of the aconitase-like reaction and convert 2-MCA into 2-MIC, which may not have been apparent in vitro in this study, in addition to the AcnD 2-MC dehydratase activity observed. If these gene products only function to convert 2-MC into 2-MCA, then an enzyme outside of the *prp* operons, most likely one of the aconitases of the cell (most bacteria contain more than one aconitase), must catalyze the conversion of 2-MCA into 2-MIC. It has been described for *Y. lipolytica* that an Fe/S-independent enzyme catalyzes the conversion of 2-MCA into 2-MIC (34). Perhaps some bacteria that utilize the 2-MC cycle also contain a gene encoding an Fe/S-independent 2-MIC dehydratase. Elucidating the function of PrpF both in vivo and in vitro may provide the answers to some of these questions.

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