In Vitro Conversion of Propionate to Pyruvate by *Salmonella enterica* Enzymes: 2-Methylcitrate Dehydratase (PrpD) and Aconitase Enzymes Catalyze the Conversion of 2-Methylcitrate to 2-Methylisocitrate†

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ABSTRACT: *Salmonella enterica* serovar Typhimurium LT2 catabolizes propionate through the 2-methylcitric acid cycle, but the identity of the enzymes catalyzing the conversion of 2-methylcitrate into 2-methylisocitrate is unclear. This work shows that the *prpD* gene of the *prpBCDE* operon of this bacterium encodes a protein with 2-methylcitrate dehydratase enzyme activity. Homogeneous PrpD enzyme did not contain an iron−sulfur center, displayed no requirements for metal cations or reducing agents for activity, and did not catalyze the hydration of 2-methyl-*cis*-aconitate to 2-methylisocitrate. It was concluded that the gene encoding the 2-methyl-*cis*-aconitate hydratase enzyme is encoded outside the *prpBCDE* operon. Computer analysis of bacterial genome databases identified the presence of orthologues of the *acnA* gene (encodes aconitase A) in a number of putative *prp* operons. Homogeneous AcnA protein of *S. enterica* had strong aconitase activity and catalyzed the hydration of the 2-methyl-*cis*-aconitate to yield 2-methylisocitrate. The purification of this enzyme allows the complete reconstitution of the 2-methylcitric acid cycle in vitro using homogeneous preparations of the PrpE, PrpC, PrpD, AcnA, and PrpB enzymes. However, inactivation of the *acnA* gene did not block growth of *S. enterica* on propionate as carbon and energy source. The existence of a redundant aconitase activity (encoded by *acnB*) was postulated to be responsible for the lack of a phenotype in *acnA* mutant strains. Consistent with this hypothesis, homogeneous AcnB protein of *S. enterica* also had strong aconitase activity and catalyzed the conversion of 2-methyl-*cis*-aconitate into 2-methylisocitrate. To address the involvement of AcnB in propionate catabolism, an *acnA* and *acnB* double mutant was constructed, and this mutant strain cannot grow on propionate even when supplemented with glutamate. The phenotype of this double mutant indicates that the aconitase enzymes are required for the 2-methylcitric acid cycle during propionate catabolism.

In *Salmonella enterica*, propionate is oxidized to pyruvate via the 2-methylcitric acid cycle (Figure 1). In this bacterium, functions encoded by the *prpBCDE* operon are required to catalyze the reactions in this pathway (*1*, *2*). The first two steps of the pathway convert propionate to 2-methylcitrate by the sequential action of the propionyl-CoA synthetase (PrpE)[1] and 2-methylcitrate synthase (PrpC) enzymes (*2*, *3*). The last step of the methylcitric acid cycle, i.e., the cleavage of 2-methylisocitrate into pyruvate and succinate, is catalyzed by the 2-methylisocitrate lyase (PrpB) enzyme (T. L. Grimek and J. C. Escalante-Semerena, unpublished results). The work reported herein was undertaken because the enzyme(s) responsible for the conversion of 2-methylcitrate to 2-methylisocitrate and the role of the PrpD protein in this pathway were unknown.

Previous studies showed that 2-methylcitrate accumulated in *prpD* mutant strains of *S. enterica* during growth on propionate (*2*), suggesting the involvement of the PrpD protein in the conversion of 2-methylcitrate to 2-methylisocitrate. This reaction is reminiscent of the citrate to isocitrate conversion catalyzed by aconitase. Interestingly, the PrpD protein shows no homology to proteins of known function (*1*) including aconitases, suggesting that PrpD is either a novel aconitase, or it is a new enzyme that catalyzes the dehydration of 2-methylcitrate to 2-methyl-*cis*-aconitate, or the hydration of the latter to 2-methylisocitrate. If PrpD catalyzed only one of the steps in the conversion of 2-methylcitrate to 2-methylisocitrate, one would have to conclude that the enzyme catalyzing the remaining hydration step would be encoded by a gene outside of the *prpBCDE* operon.

In this report, results of in vitro experiments addressing the role of the PrpD protein in propionate catabolism are presented. These experiments were performed with homogeneous preparations of PrpB, PrpC, PrpD, and PrpE enzymes, and the breakdown of [2-^{13}C]propionate was

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[1] Abbreviations: Ap, ampicillin; ATP, adenosine triphosphate; AMP, adenosine monophosphate; Cm, chloramphenicol; CoA, coenzyme A; D_2O, deuterated water; DTT, 1,4-dithio-DL-threitol; EDTA, ethylenediaminetetracetic acid; Gm, gentamycin; GSH, glutathione; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); IPTG, isopropyl-*β*-D-thiogalactoside; Kan, kanamycin; NMR, nuclear magnetic resonance; PMSF, phenylmethanesulfonyl fluoride; ppm, parts per million; TCEP-HCl, tris(2-carboxyethyl)phosphine hydrochloride; TMS, tetramethylsilane; Tris, tris(hydroxymethyl)aminomethane; P-CoA, propionyl-CoA; UNK, unknown; MCA, methyl-*cis*-aconitate; MC, methylcitrate; PYR, pyruvate; MIC, methylisocitrate.

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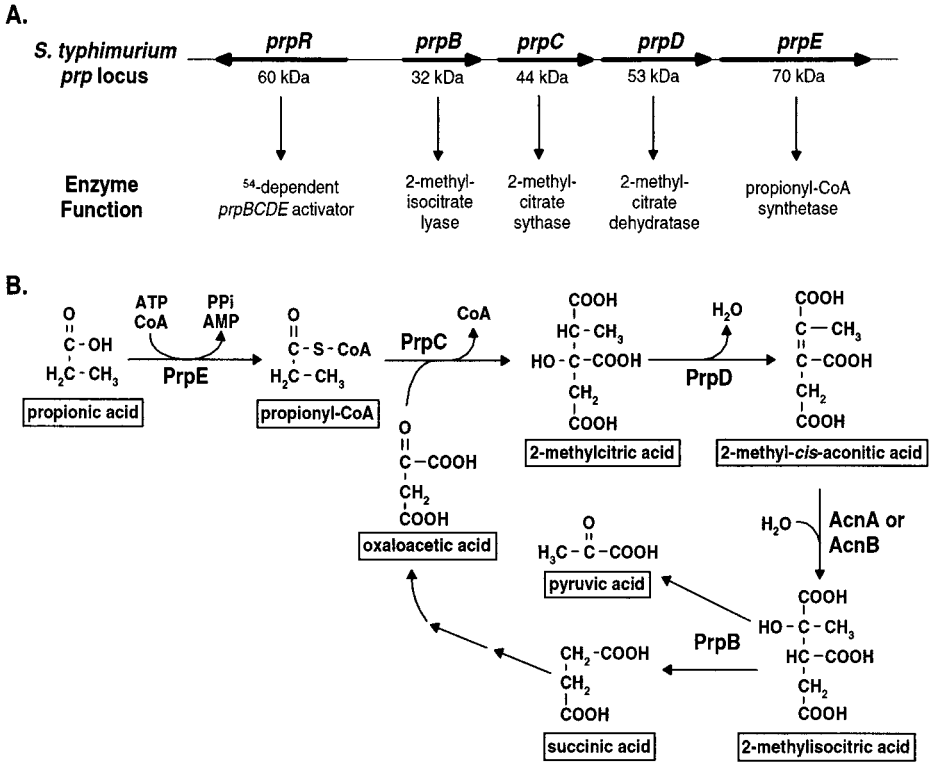

FIGURE 1: The *prp* locus and the 2-methylcitric acid cycle of *S. enterica*. Panel A is a schematic representation of the *prpRBCDE* locus. The enzymatic activity and size of each protein (monomer) is shown. Panel B diagrams the 2-methylcitric acid cycle in *S. enterica*. The catabolic functions of Prp enzymes and the aconitases, AcnA and AcnB, are indicated.

monitored by ^{13}C NMR spectroscopy. The data showed that the PrpD protein dehydrated 2-methylcitrate to 2-methyl-*cis*-aconitate but did not convert the latter to 2-methylisocitrate, i.e., PrpD did not have aconitase-like activity. On the basis of information obtained by computer analysis of genome databases, it was hypothesized that the aconitase A (AcnA) enzyme was responsible for the hydration of 2-methyl-*cis*-aconitate to 2-methylisocitrate. This idea was further supported by previous reports of this activity being associated with eucaryotic aconitases (*4*, *5*). Experiments with homogeneous preparations of enzymes established that both the AcnA and the AcnB proteins from *S. enterica* had 2-methyl-*cis*-aconitate hydratase activity.

It is clear that in *S. enterica* the conversion of 2-methylcitrate to 2-methylisocitrate is performed by two separate enzymes. Genetic studies suggest that AcnB is the primary enzyme that catalyzes the hydration of 2-methyl-*cis*-aconitate during growth of the cell on propionate. The existence of a 2-methyl-*cis*-aconitate-specific hydratase in this bacterium is currently being explored.

EXPERIMENTAL PROCEDURES

Culture Media and Growth Conditions. Nutrient broth (NB) at 0.8% (w/v) containing 85 mM NaCl (*6*) was routinely used as rich medium. *Escherichia coli* cultures were maintained Luria-Bertani (LB) broth. No-carbon E (NCE) medium supplemented with 1 mM $MgSO_4$ and 0.5 mM methionine was used as minimal medium (*6*, *7*). The final concentrations of compounds provided in culture medium were as follows: glucose 10 mM; glutamate, 5 mM; propionate, 30 mM; succinate, 30 mM. For anaerobic respiration experiments, strains were incubated on agar plates supplemented with tetrathionate as an electron acceptor (*8*). An anaerobic atmosphere was maintained using a plate chamber with a gas pack (Becton Dickinson & Co, Cockeysville, MD). Antibiotic concentrations in rich medium were (in μg/mL): ampicillin, 50 (100 for plasmids); chloramphenicol, 20 (50 for plasmids); kanamycin, 25 (50 for plasmids). Antibiotic concentrations for plasmids in minimal medium were (in μg/mL): ampicillin, 50; kanamycin, 25. All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. A list of strains and plasmids used and their genotypes is provided in Table 1.

Recombinant DNA and Genetic Techniques. Transductions involving phage P22 HT105 *int-201* were performed as described elsewhere (*9*−*11*). Restriction and modification enzymes were purchased from Promega Corporation (Madison, WI) unless stated otherwise and were used according to manufacturer's instructions. All DNA manipulations were performed in *E. coli* DH5α/F′. Plasmids were introduced into *E. coli* by $CaCl_2$ heat-shock as described elsewhere (*12*). Plasmids transferred to *S. enterica* were first introduced into recombination-deficient *S. enterica* strain JR501 by transformation (*13*). Plasmids from strain JR501 were introduced into other *S. enterica* strains by transformation as described elsewhere (*14*).

Plasmid Constructions. (a) Plasmid pPRP67. Plasmid pPRP24 was digested with *Nde*I and *Bam*HI to remove a 1.5-kb fragment containing *prpD*$^+$ with a *Nde*I-methionine start site (*1*). This fragment was cloned into vector pET-15b (Novagen, Madison, WI) cut with the same enzymes. The resulting plasmid was 7.2 kb (*bla*$^+$, Ap^r), directed the synthesis of a histidine-tagged PrpD protein, and was called pPRP67.

(b) Plasmid pACN1. The *acnA* gene (with a ribosome binding site) was PCR-amplified from strain TR6583 and

Table 1: Strain List

strain or plasmid	genotype	ref or source[a]
	E. coli	
BL21(λDE3)	F⁻ *ompT hsdS*$_B$ (r_B^- m_B^+) *dcm gal* λ(DE3)	New England Biolabs
DH5α/F′	F′/*endA1 hsdR17* (r_k^- m_k^+) *supE44 thi-1 recA1 gyrA* (Na1^r) *rel* A1 Δ(*lacZYA-argF*)*U 169 deoR* [ϕ80d*lac*Δ(*lacZ*)*M15*]	New England Biolabs
ER2566	F⁻ λ⁻ *fhu*A2 [*lon*] *ompT lacZ::T7 geneI gal sulA11* Δ(*mcr*C−*mrr*)*114::IS10 R*(*mcr-73*::miniTn*10*)*2 R*(*zgb-210*::Tn*10*)*1* (Tet^s) *end*A1 [*dcm*]	New England Biolabs
JE3969	BL21(λDE3)/ pPRP24 (T7 *rpo*$^+$, *prpD*$^+$, *bla*$^+$)	(*1*)
JE4570	BL21(λDE3)/ pPRP62 (T7 *rpo*$^+$, *prpC*$^+$, *bla*$^+$)	this study
JE4744	BL21(λDE3)/ pPRP67 (T7 *rpo*$^+$, *prpD*$^+$, *bla*$^+$)	this study
JE4813	ER2566/ pPRP68 (T7 *rpo*$^+$, *prpE*$^+$, *bla*$^+$)	this study
JE5167	DH5α/ pACN1 (*acnA*$^+$, *bla*$^+$)	this study
JE5169	DH5α/ pACN2 (*acnB*$^+$, *bla*$^+$)	this study
JE5172	DH5α/ pACN5 (*acnA1*::*kan*$^+$, *bla*$^+$)	this study
JE5179	DH5α/ pACN7 (*acnA1*::*kan*$^+$, *cat*$^+$)	this study
JE5181	DH5α/ pACN9 (*acnA*$^+$, *bla*$^+$)	this study
JE5183	DH5α/ pACN10 (*acnB*$^+$, *bla*$^+$)	this study
JE5177	DH5α/ pCO19 (*gen*$^+$)	Gary Roberts
JE5253	ER2566/ pACN11 (T7 *rpo*$^+$, *acnA*$^+$, *bla*$^+$)	this study
JE5254	DH5α/ pACN13 (*acnB*$^+$, *bla*$^+$)	this study
JE5265	ER2566/ pACN12 (T7 *rpo*$^+$, *acnA*$^+$, *bla*$^+$)	this study
JE5644	DH5α/ pACN14 (*acnB3*::*kan*$^+$, *cat*$^+$)	this study
JE6042	DH5α/ pACN15 (*acnA2*::*cat*$^+$, *bla*$^+$)	this study
	S. enterica	
JR501	*hsdSA29 hsdSB121 hsdL6 metA22 metE551 trpC2 ilυ-452 rpsL120 xyl-404 galE719 H1-b H2-en,n,x* (Fels2$^-$) *fla*−66 *nml*$^-$	(*14*)
TR6583	*metE205 ara*-9	K. Sanderson via J. Roth
	derivatives of TR6583	
JE2996	*polA71::*Mu*dJ*	(*49*)
JE4088	*polA72 zig-6420::*Tn*10d*(Tc)	lab collection
JE5216	*acnA1::kan*$^+$	this study
JE5991	*acnA2::cat*$^+$	this study
JE5992	*acnB3::kan*$^+$	this study
JE5993	*acnA2::cat*$^+$ *acnB3::kan*$^+$	this study

cloned. Primers were designed from sequence information from the *S. enterica* genome project (see below) and the *E. coli* genome (*15*). The primers contained restriction sites for later cloning steps: primer site 5′-TAACGGTACCCTGTCATTTAAG-3′ for the 5′ end of *acnA* with *Kpn*I, and primer site 5′-GCATTATCTAGATACTAAAGCG-3′ for the 3′ end of *acnA* with *Xba*I. For the PCR reactions, DNA template was prepared by mixing 10 μL of NB-grown overnight cultures of TR6583 with 90 μL of water, heating the cell suspensions at 100 °C for 5 min, and centrifuging them for 2 min at 15000*g* in a Marathon 16 K/M microcentrifuge (Fisher Scientific, Itasca, IL). PCR reactions were prepared using one-tenth the volume of boiled template, 50 pmol of each primer, 0.2 mM of each dNTP, and Pfu polymerase (Stratagene, La Jolla, CA) according to manufacturer's instructions. Reactions were performed using the following conditions: 30 cycles at 94 °C for 90 s, 50 °C for 30 s, and 72 °C for 150 s. The PCR fragment was purified using the QIAquick PCR Purification kit (Qiagen, Chatsworth, CA). The purified PCR fragment was cloned by A-tailing the DNA and ligating into pGEM-T Easy Vector system (Promega, Madison, WI) according to manufacturer's instructions. The final plasmid was 5.6 kb (*bla*$^+$, Ap^r), encoded *acnA* and was called pACN1.

(c) Plasmid pACN2. The *acnB* gene (with a ribosome binding site) was PCR-amplified from strain TR6583 and cloned. Primers were designed from sequence information from the *S. enterica* genome project and the *E. coli* genome (*15*). The primers contained restriction sites for later cloning steps: primer site 5′-TTATGACAATGAGCTCGAGGA-3′ for the 5′ end of *acnA* with *Sac*I and primer site 5′-AAAATACGCAACATCTAGAAAAG-3′ for the 3′ end of *acnA* with *Xba*I. The *acnB* gene was PCR amplified and cloned into pGEM-T using the same method as described above for pACN1. The final plasmid was 5.6 kb (*bla*$^+$, Ap^r), encoded *acnB*, and was called pACN2.

(d) Plasmid pACN7. Plasmid pACN1 was digested with *BamH*I and *Cla*I to remove a 600-bp fragment of *acnA*, and the restriction site overhangs were blunt-ended with DNA polymerase Klenow fragment. A 1.3-kb kanamycin cassette was removed from plasmid pUC4K (Amersham Pharmacia Biotech, Piscataway, NJ) with *Pst*I, blunt-ended with Klenow, and ligated into the remaining *acnA* sequence on pACN1. The new plasmid was 6.3 kb (*bla*$^+$, Ap^r; *kan*$^+$, Kan^r) and was called pACN5. The 3.3 kb *acnA1::kan*$^+$ construction was removed from pACN5 with *Kpn*I and *Xba*I and cloned into gene-replacement vector pMAK705 (*16*) cut with the same enzymes. The new plasmid was 10 kb (*cat*$^+$, Cm^r; *kan*$^+$, Kan^r), encoded *acnA1::kan*$^+$, and was called pACN7.

(e) Plasmid pACN9. Plasmid pACN1 was digested with *Kpn*I and *Xba*I to remove the *acnA* gene, which was ligated into pBAD30 (*17*) cut with the same enzymes. The resulting plasmid was 7.5 kb (*acnA*$^+$, *bla*$^+$, Ap^r) and was called pACN9.

(f) Plasmid pACN10. Plasmid pACN2 was digested with *Sac*I and *Xba*I to remove the *acnB* gene, which was ligated into pBAD30 (*17*) cut with the same enzymes. The resulting

plasmid was 7.5 kb (*acnB*$^+$, *bla*$^+$, Ap^r) and was called pACN10.

(g) Plasmid pACN11. The *acnA* gene was PCR-amplified from strain TR6583 and cloned. Primers were designed from sequence information as described above. The designed primers are as follows: primer 5′-CATTTAAGGAGGACATATGTCG-3′ for the 5′ end of *acnA* with *Nde*I site at methionine start codon, and primer 5′-GCATTATCTCGAGACTAAAGCG-3′ for the 3′ end of *acnA* with *Xho*I site. The *acnA* gene was PCR-amplified and purified as described for plasmid pACN1. The PCR fragment was digested with *Nde*I and *Xho*I and cloned into vector pET-15b (Novagen) cut with the same enzymes. The resulting plasmid was 8.0 kb (*bla*$^+$, Ap^r), directed the synthesis of a histidine-tagged AcnA protein (H_6AcnA), and was called pACN11.

(h) Plasmid pACN12. The *acnB* gene was PCR amplified from strain TR6583 and cloned. Primers were designed from sequence information as described above. The designed primers are as follows: primer 5′-GGAGATATCGCATATGCTAGAAG-3′ for the 5′ end of *acnB* with *Nde*I site at methionine start codon, and primer 5′- AACCGCAGTCTGGAAAATCA-3′ for the 3′ end of *acnB*. The *acnB* gene was PCR-amplified and purified as described for plasmid pACN1. The PCR product was digested with *Nde*I and cloned into pTYB2 (New England Biolabs, Beverly, MA) to construct a C-terminal chitin fusion. The resulting plasmid, called pACN13, overexpressed an AcnB-fusion but did not allow purification of the AcnB protein. To construct an N-terminal histidine-tag of *acnB*, a 130-bp *Nde*I−*Eco*RI fragment from pACN13 was ligated into *Nde*I−*Eco*RI cut pACN2. This plasmid was digested with *Nco*I, Klenow-blunted, and then digested with *Nde*I to remove a 2.6-kb fragment containing the *acnB* gene. This fragment was cloned into vector pET16b (Novagen) digested with *Nde*I and *Bam*HI (blunted). The resulting plasmid was 8.3 kb (*bla*$^+$, Ap^r), directed the synthesis of a decahistidine-tagged AcnB protein (H_{10}AcnB), and was called pACN12.

(i) Plasmid pACN14. Plasmid pACN2 was digested with *Eco*RI and the restriction site overhangs were blunt-ended with DNA polymerase Klenow fragment. A 1.6-kb gentamycin cassette was removed from plasmid pCO19 with *Pvu*II and ligated into the *acnB* gene on pACN2. The new plasmid was 7.2 kb (*bla*$^+$, Ap^r; *gent*$^+$, Gm^r) and was called pACN8. The gentamycin cassette worked poorly and was replaced with a kanamycin cassette. To replace the cassette, plasmid pACN8 with digested *Kpn*I and *Bam*HI, which removed the gentamycin fragement and 2.2 kb of *acnB* sequence. The 1.3-kb kanamycin cassette was removed from plasmid pUC4K with *Pst*I, blunt-ended with Klenow, and ligated into the remaining *acnB* sequence on pACN8. The new plasmid was 7.3 kb (*cat*$^+$, Cm^r; *kan*$^+$, Kan^r), encoded a 1.7 kb *acnB3::kan*$^+$ construction and was called pACN14.

(j) Plasmid pACN15. Plasmid pACN1 was digested with *Bam*HI and *Cla*I to remove a 600-bp fragment of *acnA*, and the restriction site overhangs were blunt-ended with DNA polymerase Klenow fragment. A 4.0-kb chloramphenicol cassette was removed from plasmid pHP45Ω-Cm (*18*) with *Bam*HI, blunt-ended with Klenow, and ligated into the remaining *acnA* sequence on pACN1. The new plasmid was 9.0 kb (*bla*$^+$, Ap^r; *cat*$^+$, Cm^r), encoded *acnA2::cat*$^+$, and was called pACN15.

Construction of acnA1::*kan*$^+$ *and acnB3*::*kan*$^+$ *Chromosomal Insertions*. The *acnA1*::*kan*$^+$ and *acnB3*::*kan*$^+$ insertions were constructed by gene replacement using plasmids pACN7 and pACN14 as previously described (*3*).

Construction of an acnA2::*cat*$^+$ *Chromosomal Insertion*. Plasmid pACN15 (*acnA2*::*cat*$^+$; *bla*$^+$, Ap^r) was introduced into strain JE4088 (polA^{ts}) by transformation at 30 °C. The resulting strain was grown in a 125-mL Klett flask to Klett 40 in LB Cm Ap media at 30 °C. Cells were dilution plated onto prewarmed LB Cm plates and incubated at 44 °C. Ten putative cointegrates were selected and grown individually at 30 °C in LB Cm media. After 24 h, cointegrates were subcultured into fresh LB Cm broth and grown an additional 24 h at 30 °C. Bacteriophage P22 lysate was prepared on a pool of all 10 cointegrates, and strain JE2996 (*polA71*::MudJ) was transduced with this lysate. Transductants were isolated on LB Cm plates and were screened for ampicillin resistance. Phage lysate was prepared on ampicillin sensitive strains and was crossed into TR6583 to construct an *acnA2*::*cat* mutant. The insertion was verified by genetic linkage, PCR, and sequencing.

Sequence Analysis. Sequence comparisons were performed with BLAST 2.0 (*19*). Homologues of *acnA*, *acnB*, and *ybhH* were identified with sequence information from *E. coli* (*15*). Primers for *S. enterica acnA* and *acnB* were designed from preliminary sequence data from the Washington University Genome Sequencing Center website at http://genome.wustl.edu/gsc/index.shtml. The *S. enterica prpBCDE* operon sequence (GenBank accession U51879) was used to identify *prp* homologues in other bacteria (*1*). *Salmonella typhi* and *Bordetella pertussis* preliminary sequence data were obtained from the Sanger Center website at http://www.sanger.ac.uk. *Pseudomonas putida* KT2440 preliminary sequence data were obtained from the Institute for Genomic Research (TIGR) website at http://www.tigr.org. Sequence information from *Bacillus subtilis*, *E. coli*, *Neisseria meningitidis* MC58, *Pseudomonas aeruginosa* PAO1, and *Vibrio cholerae* was obtained from published sources (*15*, *20*−*23*).

Biochemical Characterization of the PrpD, AcnA, and AcnB Proteins. (a) Overexpression of prpD. Plasmid pPRP67 was introduced into *E. coli* BL21(λDE3) by transformation, and the resulting strain, JE4744, was used to overexpress and purify PrpD. For overexpression, 20 mL of an LB-grown overnight culture of JE4744 was used to inoculate 2 L of LB broth with Ap. The culture was grown with shaking at 30 °C to an absorbance of 0.5−0.6 at 600 nm, isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.3 mM, and the culture was incubated 3 h with shaking at 30 °C. Cells were harvested in 250-mL Nalgene polypropylene copolymer bottles (Fisher Scientific) bottles by centrifugation for 10 min at 10500*g*, 4 °C with a SS34 rotor in a RC-5B refrigerated centrifuge (DuPont, Wilmington, DE). Cell pellets were stored at 4 °C no longer than 16 h before use.

(b) Purification of PrpD. Cell pellets were resuspended in 200 mL of 20 mM Tris-HCl buffer, pH 7.9, with 0.5 M NaCl and 0.04 M imidazole. The cells were centrifuged in 250-mL bottles as described above, and the cell pellets were resuspended in 20 mL of the same buffer. The cell suspension was broken using a chilled French pressure cell at 10^4 kPa. Cell debris was removed by centrifugation in 40-mL Nalgene polypropylene copolymer Oakridge tubes (Fisher Scientific)

at 31000*g* for 30 min at 4 °C. Crude, cell-free extracts were treated with deoxyribonucleotidase to remove chromosomal DNA, and PrpD was purified on His·Bind resin (Novagen, Madison, WI) according to manufacturer's instructions. Following purification, the enzyme was dialyzed using SnakeSkin 10 000 molecular weight cutoff Dialysis Membrane (Pierce Chemical Co, Rockford, IL) at 4 °C against 1 L of 50 mM *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) buffer (HEPES), pH 7.5, with 5 mM ethylenediaminetetraacetic acid (EDTA) and 0.1 M KCl. The dialysis buffer was replaced after 2 h (without EDTA) and again at 4 h (5 mM 1,4-dithio-DL-threitol (DTT) and 20% glycerol (v/v) added. The protein was dialyzed overnight, flash-frozen in liquid nitrogen, and stored at −80 °C. Approximately 75 mg of PrpD was obtained per liter of culture.

(c) Overexpression and Purification of the AcnA Protein. Plasmid pACN11 was introduced into *E. coli* strain ER2566 by transformation, and the resulting strain, JE5253, was used to overexpress and purify AcnA. During this purification, the manufacturer's instructions were modified due to poor solubility of H_6AcnA and its low affinity for the His·Bind resin. For overexpression, 5 mL of an overnight culture of JE5253 was used to inoculate 0.25 L of LB broth with Ap. The culture was grown at 20 °C with shaking, to an absorbance of 0.5−0.6 at 600 nm. IPTG was added to a final concentration of 0.5 mM, and the culture was incubated another 15 h with shaking at 20 °C. Cells were harvested as described above and washed with 200 mL of 20 mM Tris-HCl buffer, pH 8.0, with 0.5 M NaCl and 1 mM imidazole. Cells were broken immediately with a French press, and insoluble material removed as described for H_6PrpD. Cell-free extracts were treated with EDTA and potassium ferricyanide to remove all metal ions as described (*24*). The treated extract was immediately dialyzed at 4 °C into 2 L of 20 mM Tris-HCl buffer, pH 8.0, with 0.5 M NaCl and 1 mM imidazole. The buffer was changed at 2 h, and dialysis was continued overnight. The dialyzed extract was mixed with a 20-mL suspension of His·Bind resin (5 mL of resin) equilibrated in dialysis buffer. This mixture was allowed to incubate 15 h at 4 °C to allow H_6AcnA to bind to the resin. The resin mix was poured into a small column and washed with 50 mL of the same buffer. H_6AcnA protein was eluted with a 100-mL gradient from 1 to 300 mM imidazole. Following purification, the enzyme was dialyzed at 4 °C against 1 L of 50 mM Tris-HCl buffer, pH 8, with 5 mM EDTA and 0.1 M NaCl. The dialysis buffer was replaced after 2 h (without EDTA) and again at 4 h [20% glycerol (v/v) added]. The protein was dialyzed overnight, flash-frozen in liquid nitrogen, and stored at −80 °C. Approximately 20 mg of H_6AcnA protein was obtained per liter of culture.

(d) Overexpression of acnB, *and Purification of the H_{10}AcnB Protein*. Plasmid pACN12 was introduced into *E. coli* strain ER2566 by transformation, and the resulting strain, JE5265, was used to overexpress and purify H_{10}AcnB. H_{10}AcnB was purified using the same method as AcnA except PMSF added during the purification to 0.2 mM and metal ions were not removed with EDTA and potassium ferricyanide. Following purification, the enzyme was dialyzed and stored under the same conditions as H_6AcnA. Approximately 120 mg of H_{10}AcnB protein was obtained per liter of culture.

(e) Reactivation of H_6AcnA and H_{10}AcnB. Apo-H_6AcnA was reconstituted using the method described by Kennedy and Beinert (*24*). H_{10}AcnB was reactivated using the method described by Kennedy et al. (*25*). All reagents were prepared under strict anoxic conditions as described (*26*, *27*). Reactivation of Apo-H_6AcnA and H_{10}AcnB was verified using the activity assay described below.

Enzyme Assays. (a) Aconitase Assays. This assay was based on the aconitase assay described by Kennedy et al. (*25*). Aconitase assays (1.0 mL) contained 0.09 M Tris-HCl buffer, pH 8.0 (at room temperature), and 20 mM DL-isocitrate (sodium salt form). Assays were performed at room temperature and were started by the addition of reactivated H_6AcnA or H_{10}AcnB. Reactions were monitored for 10 min at 240 nm on a Lambda Bio-40 spectrophotometer (Perkin-Elmer, Norwalk, CT). Specific activities were calculated from the extinction coefficient of 3600 M^{-1} cm^{-1} for the *cis*-aconitate (*25*), and they are reported as μmol min^{-1} (mg of protein)$^{-1}$.

(b) 2-Methylcitrate Dehydratase Assays. 2-Methylcitrate dehydratase assays (1.0 mL) contained 90 mM Tris-HCl buffer, pH 8.0 (at room temperature), and 5 mM 2-methylcitrate. For these assays, buffer and H_6PrpD enzyme were preincubated in quartz cuvettes at 37 °C in a Lambda 6 spectrophotometer (Perkin-Elmer) equipped with a circulating water-jacket. After 5 min, assays were started with substrate and the reaction was monitored for 10 min at 240 nm. Specific activities were calculated from the extinction coefficient of 4500 M^{-1} cm^{-1} for the 2-methyl-*cis*-aconitate (*28*), and they are reported as μmol min^{-1} (mg of protein)$^{-1}$. Synthetic 2-methylcitrate was purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada), as a mixture of four stereoisomers.

(c) 2-Methylcitrate Synthase and Propionyl-CoA Synthetase Assays. These assays were performed as previously described (*2*, *3*).

^{13}C NMR Spectroscopy. Peak assignments were based on results from previously reported experiments (*2*). To confirm the identity of the ^{13}C NMR peaks, proton-coupled spectra were obtained in all experiments described below. Although these spectra are not shown, they are available upon request. Glycerol was present in all protein samples and was observed in the spectra due to natural abundance ^{13}C NMR. For the sake of clarity, the region of the spectrum containing the glycerol peaks was cropped out in all of the spectra shown.

In Vitro Synthesis of [2-^{13}C]Propionyl-CoA. The following were combined in a 2-mL reaction mixture: [2-^{13}C]propionate, 6 μmol; HS-CoA, 5 μmol; ATP, 5 μmol; $MgCl_2$, 10 μmol; PrpE, 30 μg; phosphate buffer, 100 μmol, pH 7.5. The reaction was incubated for 60 min at 37 °C to allow formation of [2-^{13}C]propionyl-CoA. PrpE was purified using a chitin affinity tag as previously described (Horswill and Escalante-Semerena, unpublished results). CoA solutions were prepared fresh for each experiment.

In Vitro Synthesis of [2-^{13}C]Methylcitrate. After 30 min, 5 μmol of oxaloacetate and 30 μg PrpC were added to the reaction mixture. The mixture was incubated for an additional 30 min to allow complete conversion to [2-^{13}C]methylcitrate. H_6PrpC was purified as previously described (*2*). Oxaloacetate solutions were prepared fresh for each experiment.

In Vitro Synthesis of [2-^{13}C]Methyl-cis-aconitate. To synthesize 2-methyl-*cis*-aconitate, 30 μg of H_6PrpD protein

was added to the reaction mixture, which was incubated for 30 min at 37 °C.

In Vitro Synthesis of [2-^{13}C]Methylisocitrate. Apo-H_6AcnA or H_{10}AcnB was reactivated as described above immediately prior to its use. To synthesize 2-methylisocitrate, 30 μg of reactivated H_6AcnA or H_{10}AcnB protein was added to the reaction, and the mixture was allowed to incubate for 1 h at 37 °C.

Cleavage of [2-^{13}C]Methylisocitrate into [2-^{13}C]Pyruvate and Succinate. For the final step of the pathway, 30 μg of H_6PrpB was added to the reaction, and the mixture was incubated for 30 min at 37 °C for complete cleavage. Homogeneous H_6PrpB protein was kindly provided by T. Grimek (Grimek and Escalante-Semerena, unpublished results).

Preparation of Samples for ^{13}C NMR. The mixture was transferred into a NMR tube (10 mm, Wilmad Glass, Buena, NJ), and 0.3 mL of 100% D_2O was added. The concentration of D_2O in samples ranged from 10 to 15%. A sealed tetramethylsilane (TMS) capillary was prepared and added as an external reference. [2-^{13}C]Propionate, D_2O, and TMS were purchased from Cambridge Isotope Labs (Andover, MA).

^{13}C NMR Spectra Acquisition. ^{13}C NMR spectra were acquired at 100.6 MHz with a deuterium lock on a Bruker Instruments DMX-400 Avance console with a 9.4 T wide-bore magnet (Nuclear Magnetic Resonance Facility at the University of Wisconsin-Madison). Decoupled and coupled (gated-decoupling) spectra were obtained at a 90° pulse angle with a relaxation time of 5 s, and the spectra were Fourier transformed with 5 Hz line-broadening. Quantitative spectra (inverse-gated) were obtained at a 90° pulse angle with a relaxation time of 30 s. Generally, 400 scans were obtained for decoupled spectra and 800−2000 scans for coupled and quantitative spectra. Chemical shifts presented in this work were relative to TMS which was set to 0.0 ppm.

Other Procedures. Protein concentrations were determined by the method of Bradford (*29*) using the BioRad protein reagent (BioRad, Hercules, CA). A standard curve was generated for protein determinations with bovine serum albumin. Proteins were separated by SDS−PAGE (*30*) using 12% polyacrylamide gels and were visualized with Coomassie Blue (*31*). Midrange standards (14−150 kDa) were used for SDS−PAGE (Novagen). UV−vis spectroscopy was performed on a computer-controlled Lambda 6 or a Lambda Bio-40 spectrophotometer (Perkin-Elmer) equipped with temperature control.

RESULTS

PrpD Has 2-Methylcitrate Dehydratase Activity. It was previously observed that [2-^{13}C]methylcitrate accumulated in *prpD* missense mutant strains during growth of *S. enterica* on [2-^{13}C]propionate (*2*). This finding suggested that 2-methylcitrate is the substrate for the PrpD enzyme. Previous results indicated that 2-methylcitrate was further catabolized in *S. enterica* to 2-methylisocitrate via a 2-methyl-*cis*-aconitate intermediate. It was concluded that the PrpD protein was required for the conversion of 2-methylcitrate to 2-methylisocitrate, but it was unclear which of the two reactions needed for this conversion was catalyzed by PrpD (*2*). To address these questions, the *prpD* gene was overexpressed and its gene product was isolated with an N-terminal hexahistidine tag to facilitate its purification by nickel affinity chromatography (Figure 2, lane 3).

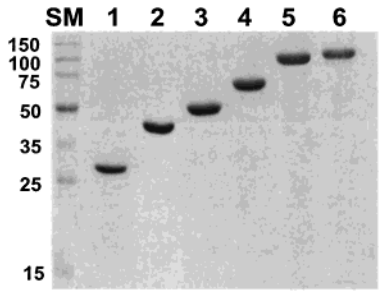

FIGURE 2: SDS−PAGE of *S. enterica* 2-methylcitric acid cycle enzymes. Approximately 2 μg of each protein was loaded per lane. Lane SM, size marker; lane 1, H_6PrpB; lane 2, H_6PrpC; lane 3, H_6PrpD; lane 4, PrpE; lane 5, H_6AcnA; lane 6, H_{10}AcnB.

To test the activity of H_6PrpD, we synthesized [2-^{13}C]-methylcitrate using pure preparations of the PrpE and H_6PrpC enzymes and monitored the reactions with ^{13}C NMR (Figure 3). Initially, PrpE was used to convert [2-^{13}C]-propionate (30.8 ppm) to [2-^{13}C]propionyl-CoA (37.2 ppm). Since CoA was limiting, complete conversion to propionyl-CoA was not observed. An unknown peak at 28.9 ppm was observed (Figure 3, panel A), and it was tentatively identified as propionyl-AMP, a proposed intermediate of the reaction catalyzed by PrpE, the propionyl-CoA synthetase. H_6PrpC and oxaloacetate were added to convert [2-^{13}C]propionyl-CoA (37.2 ppm) to [2-^{13}C]methylcitrate (47.5 ppm). After the reaction was completed, H_6PrpD was added to the sample and activity was monitored again by ^{13}C NMR. Approximately 50% of the [2-^{13}C]methylcitrate was converted to [2-^{13}C]methyl-*cis*-aconitate (141.5 ppm) during a 30 min incubation at 37 °C (Figure 3, panel C). Proton-coupled spectra verified the labeled carbon [2-^{13}C]methyl-*cis*-aconitate had no attached hydrogens as expected (data not shown). This observation provided the first evidence that PrpD had 2-methylcitrate dehydratase activity.

The [2-^{13}C]methylcitrate/[2-^{13}C]methyl-*cis*-aconitate ratio for the PrpD reaction was also obtained under quantitative ^{13}C NMR conditions. [2-^{13}C]Methylcitrate was synthesized with PrpE and H_6PrpC protein at pH 7.5 as described under Experimental Procedures. H_6PrpD protein was added to the mixture, and the reaction was allowed to equilibrate for 15 h at room temperature. ^{13}C NMR spectra were obtained without nuclear overhauser enhancement (NOE) to allow accurate peak integration. The [2-^{13}C]methylcitrate/[2-^{13}C]-methyl-*cis*-aconitate ratio was 1:1.08, indicating 52% of the 2-methyl-*cis*-aconitate was present at equilibrium under these reaction conditions. The chemical shifts of 47.5 ppm for [2-^{13}C]methylcitrate and 141.5 ppm for [2-^{13}C]methyl-*cis*-aconitate were slightly different than the 48.8 and 140.5 ppm values previously reported (*2*). The difference was due to the presence of excess Mg^{2+} in the samples for the propionyl-CoA synthetase reaction. Tricarboxylic acids are known to chelate Mg^{2+} ions, resulting in altered chemical shifts in the ^{13}C NMR spectrum. The chemical shifts previously observed were readily obtained after the addition of EDTA to remove the Mg^{2+} ions (data not shown).

Does PrpD Catalyze the Hydration of 2-Methyl-cis-aconitate? Although PrpD did not synthesize 2-methylisocitrate during our initial ^{13}C NMR experiments, it was possible that the conditions used were unfavorable for PrpD to

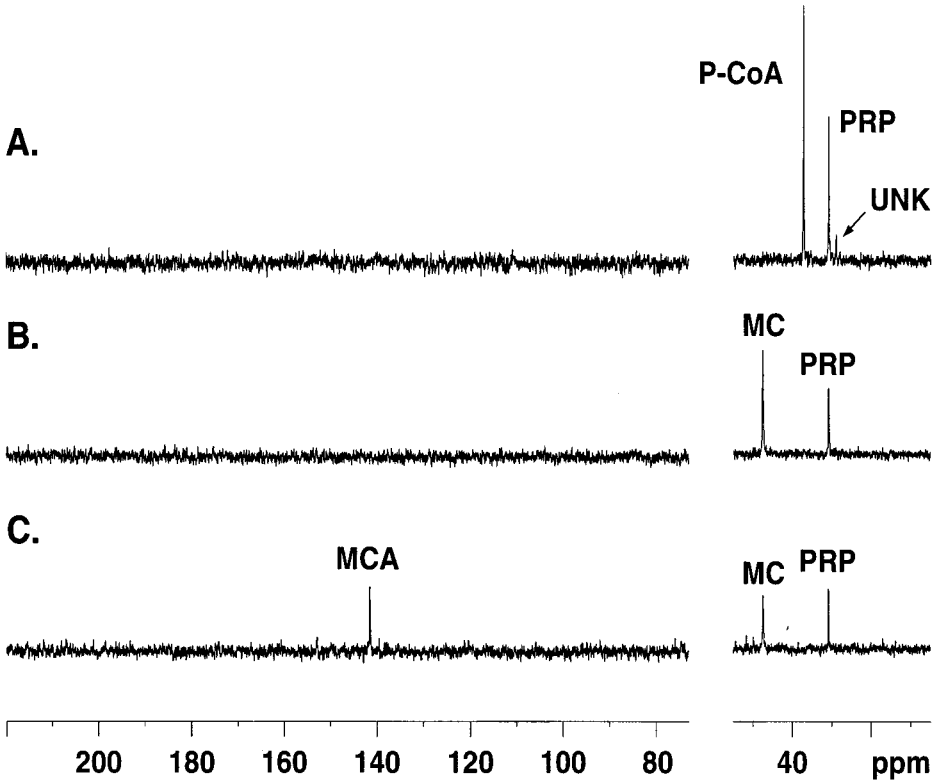

FIGURE 3: The conversion of [2-^{13}C]propionate into [2-^{13}C]methyl-*cis*-aconitate by PrpE, H_6PrpC, and H_6PrpD monitored by ^{13}C NMR. The removed portion of the spectra (60−73 ppm) contained only glycerol peaks present in the protein preparations at 62.8 and 72.3 ppm. (A) Conversion of [2-^{13}C]propionate (PRP, 30.8 ppm) to [2-^{13}C]propionyl-CoA (P-CoA, 37.2 ppm) with PrpE; UNK indicates unknown tentatively identified as propionyl-AMP; (B) conversion to [2-^{13}C]methylcitrate (MC, 47.5 ppm) with H_6PrpC; (C) conversion to [2-^{13}C]-methyl-*cis*-aconitate (MCA, 141.5 ppm) by H_6PrpD.

catalyze the reaction. To displace the equilibrium toward 2-methylisocitrate, homogeneous 2-methylisocitrate lyase, H_6PrpB, was added to the reaction mixture. The addition of excess H_6PrpB protein did not result in the conversion of [2-^{13}C]methyl-*cis*-aconitate to [2-^{13}C]pyruvate (data not shown). It was also considered that PrpD required an iron−sulfur center for activity like an aconitase enzymes, although the PrpD amino acid sequence did not have an iron−sulfur center motif (*1*). To test this idea, PrpD was treated with DTT and $(NH_4)_2Fe(SO_4)_2$ to reactivate the iron−sulfur center as described for aconitases (*25*) and tested for 2-methyl-*cis*-aconitate hydratase activity using ^{13}C NMR spectroscopy to monitor the reaction. Treated H_6PrpD protein converted approximately 50% of the 2-methylcitrate to 2-methyl-*cis*-aconitate and no signal for 2-methylisocitrate was detected. The addition of H_6PrpB to this reaction mixture had no effect on the spectrum (data not shown). H_6PrpD was also treated under conditions to reconstitute iron−sulfur centers of apo proteins (*24*). Again, no change in the spectrum was observed when treated H_6PrpD enzyme was used in the experiment (data not shown). Further, UV/visible spectra of concentrated H_6PrpD did not reveal the expected diagnostic of the presence of an iron−sulfur cluster (*32*).

H_6PrpD enzyme was also tested for 2-methylcitrate dehydratase activity using an in vitro UV assay (see Experimental Procedures). The assay used authentic 2-methylcitrate, and H_6PrpD activity was monitored by the formation of 2-methyl-*cis*-aconitate at 240 nm. Using this assay, H_6PrpD had a specific activity of 2.8 μmol min^{-1} (mg of protein)$^{-1}$. This value may be an underestimate of the dehydration rate of the substrate if the enzyme uses as substrate only one of the four 2-methylcitrate stereoisomers present in the mixture or if any of the stereoisomers inhibits enzyme activity.

Structurally similar substrates such as citrate were tested with H_6PrpD. Citrate was a poor substrate (specific activity of 0.2 μmol min^{-1} mg^{-1}), and isocitrate was not a substrate for H_6PrpD. However, H_6PrpD hydrated *cis*-aconitate at a rate of 2.6 μmol min^{-1} (mg of protein)$^{-1}$, a specific activity that compared well to the value measured with 2-methylcitrate. H_6PrpD showed no requirement for reducing agents or divalent metal cations. Taken together, these results led to the conclusion that H_6PrpD had 2-methylcitrate dehydratase activity, and this enzyme did not require an iron−sulfur center for activity.

What Enzyme Catalyzes the Hydration of 2-Methyl-cis-aconitate? If PrpD did not hydrate 2-methyl-*cis*-aconitate, a gene outside of the *prp* operon in *S. enterica* must encode the missing hydratase enzyme. Aconitases from mammals and the fungi *Yarrowia* (formerly *Saccharomycopis) lipolytica* have been shown to catalyze the hydration of 2-methyl-*cis*-aconitate, which suggested the *S. enterica* aconitase(s) may do the same (*4*, *5*, *33*). *E. coli* has two aconitase genes, *acnA* and *acnB* (*34*, *35*), and sequence comparisons to the partial genome database of *S. enterica* identified homologues for both genes (see Experimental Procedures). There is no evidence in *E. coli* or *S. enterica* to suggest these enzymes play a role in propionate catabolism. However, sequence analysis of other procaryotic genomes suggested the *acnA* function might be involved in propionate breakdown. This gene was found in operons of *prp* gene homologues in *B. pertussis*, *N. meningitidis*, *P. aeruginosa*, *P. putida*, and *V. cholerae* (Figure 4). The *acnA* homologue found in the *prp*

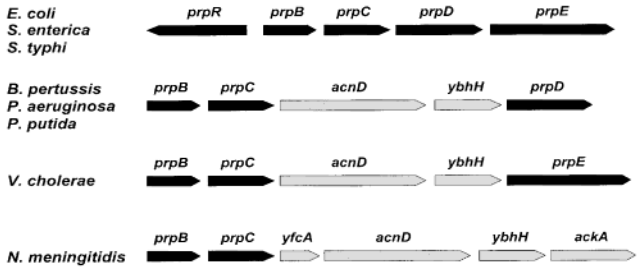

FIGURE 4: A comparison of *prp* operons from a number of bacteria. The operons are grouped by similarities in gene organization, although minor differences exist between each operon in a group. ORFS were not drawn exactly to scale due to differences in gene sizes between each species. Predicted ORFs from *S. typhi*, *B. pertussis*, and *P. putida* KT2440 are all unfinished genomes and could be subject to change (see Experimental Procedures). Sequence information from *E. coli*, *N. meningitidis* MC58, *P. aeruginosa* PAO1, and *V. cholerae* was obtained from published sources (*15*, *19*, *21*, *22*). ORFs were named after the *S. enterica* gene designations except *ybhH*, *yfcA*, and *ackA* (acetate kinase), which were named first in *E. coli* (*15*). The putative *ackA* gene of *N. meningitidis* also shows sequence similarity to the propionate kinase *tcdD* of *E. coli* (*15*).

operons of these procaryotes was named *acnD* because *B. pertussis*, *P. aeruginosa*, and *P. putida* have additional *acnA* homologues elsewhere in their genomes. The *S. enterica* aconitase A protein is only 17% identical to aconitase B (*acnB*), a fact that allows the *acnA* homologues to be distinguished from *acnB* homologues (*34*). The Prp proteins and AcnA of these bacteria were at least 70% identical to the *S. enterica* enzymes, suggesting that they catalyze the same reactions. This finding strongly suggested the AcnA protein of *S. enterica* was involved in propionate breakdown. To address this question, the aconitase A protein of *S. enterica* was biochemically and genetically characterized.

H_6AcnA Has 2-Methyl-cis-aconitate Hydratase Activity. *S. enterica* H_6AcnA protein was purified to homogeneity by nickel affinity chromatography (see Experimental Procedures, Figure 2, lane 5). The protein was isolated in its apo form to >95% homogeneity. Before use, apo-H_6AcnA was reactivated anoxically. The specific activity of reactivated H_6AcnA was very reproducible and ranged from 57 to 63 μmol min^{-1} (mg of protein)$^{-1}$. The specific activity of the *S. enterica* H_6AcnA was very similar to the one reported for *E. coli* AcnA [59 μmol min^{-1} (mg of protein)$^{-1}$] (*36*). This was interpreted to mean that the histidine tag had no deleterious effect on the activity of the enzyme.

Results of ^{13}C NMR experiments designed to test if H_6AcnA had 2-methyl-*cis*-aconitate hydratase activity are shown in Figure 5, panel A. Under the conditions used, H_6AcnA converted [2-^{13}C]methyl-*cis*-aconitate (141.5 ppm) into [2-^{13}C]methylisocitrate (76.8 ppm). Proton-coupled spectra verified the labeled carbon [2-^{13}C]methylisocitrate had no attached hydrogens as expected (data not shown). Formation of 2-methylisocitrate was further verified by the addition of H_6PrpB to the assay mixture (Figure 5, panel B). H_6PrpB cleaved [2-^{13}C]methylisocitrate (76.8 ppm) into [2-^{13}C]pyruvate (205.4 ppm) and succinate as expected (Grimek and Escalante-Semerena, unpublished results). Together, these experiments demonstrated that H_6AcnA could catalyze the hydration of 2-methyl-*cis*-aconitate. Further, the demonstration that H_6AcnA catalyzed this reaction allowed the reconstitution of the 2-methylcitric acid cycle in vitro, that is, [2-^{13}C]propionate was oxidized to [2-^{13}C]pyruvate using homogeneous preparations of the PrpE, H_6PrpC, H_6PrpD, H_6AcnA, and H_6PrpB enzymes.

Quantitative ^{13}C NMR experiments were performed with H_6AcnA to determine the ratio of 2-methyl-*cis*-aconitate/2-methylisocitrate at equilibrium. The data showed a ratio of 1:1.37, i.e., 58% of 2-methyl-*cis*-aconitate was converted to 2-methylisocitrate. This result compared favorably to experiments with beef liver aconitase, where 57% of 2-methyl-*cis*-aconitate was converted to 2-methylisocitrate at pH 7.5 (*4*). In contrast, the 2-methyl-*cis*-aconitate hydratase of *Y. lipolytica* converted 92% of the aconitate to 2-methylisocitrate at pH 7.0 (*28*).

Surprisingly, we found that [2-^{13}C]methylcitrate produced by H_6PrpC was a substrate, albeit a very poor one, for H_6AcnA (data not shown). This result was in contrast with previous findings with eucaryotic aconitases, for which 2-methylcitrate was not a substrate at all (*4*, *5*). The [2-^{13}C]-methylisocitrate made by H_6AcnA was readily converted into [2-^{13}C]pyruvate and succinate by H_6PrpB (data not shown).

In Vivo Assessment of the Requirement for Aconitase A during Propionate Catabolism. To construct an *acnA* mutation, the gene was PCR amplified and cloned. The *acnA* gene was inactivated by inserting kanamycin and chloramphenicol cassettes into the *BamH*I and *Cla*I sites of *acnA*, removing 650 bp from the middle of the gene. The *acnA1*::*kan*$^+$ element was moved onto the chromosome using the gene-replacement vector pMAK705 (*16*). The *acnA2*::*cat*$^+$ element was moved onto the chromosome using a temperature-sensitive DNA polymerase I mutant (see Materials and Methods). Putative *acnA1*::*kan*$^+$ and *acnA2*::*cat*$^+$ mutant strains were identified by the increased size of the PCR-amplified product. Comparisons to the *E. coli* genome predicted that in *S. enterica* the *acnA* gene would be co-transducible with the *cobA* gene by phage P22 (*15*). Indeed, the *acnA1*::*kan*$^+$ and *acnA2*::*cat*$^+$ elements were approximately 30% co-transducible with *cobA*, confirming the presence of a chromosomal mutation. The location of the *acnA1*::*kan*$^+$ and *acnA2*::*cat*$^+$ elements were verified by determining the sequence flanking the chromosomal insertion. The bona fide *acnA* mutants were used to test the predicted involvement of the AcnA protein in propionate catabolism. The *acnA* mutant strains JE5216 and JE5991 were tested for growth deficiencies on propionate and other carbon sources including acetate, succinate, glycerol, and glucose. Under the growth conditions tested, no phenotypes were observed when compared to an *acnA*$^+$ strain (TR6583). This result indicated that either AcnA was not required for growth on propionate or that alternative aconitase activities (e.g., AcnB) compensated for the lack of AcnA in the mutant. Growth behavior of the *S. enterica acnA* mutant strains on acetate and glucose was consistent with the one reported for *E. coli acnA* mutants (*35*).

H_{10}AcnB Has 2-Methyl-cis-aconitate Hydratase Activity. The phenotype of an *acnA* mutation in *S. enterica* suggested that AcnB may also catalyze the hydration of 2-methyl-*cis*-aconitate. To address this question, the *S. enterica* H_{10}AcnB protein was purified to homogeneity by nickel affinity chromatography (see Experimental Procedures, Figure 2). Before use, AcnB was reactivated anoxically. The specific activity of reactivated H_{10}AcnB was reproducible and ranged

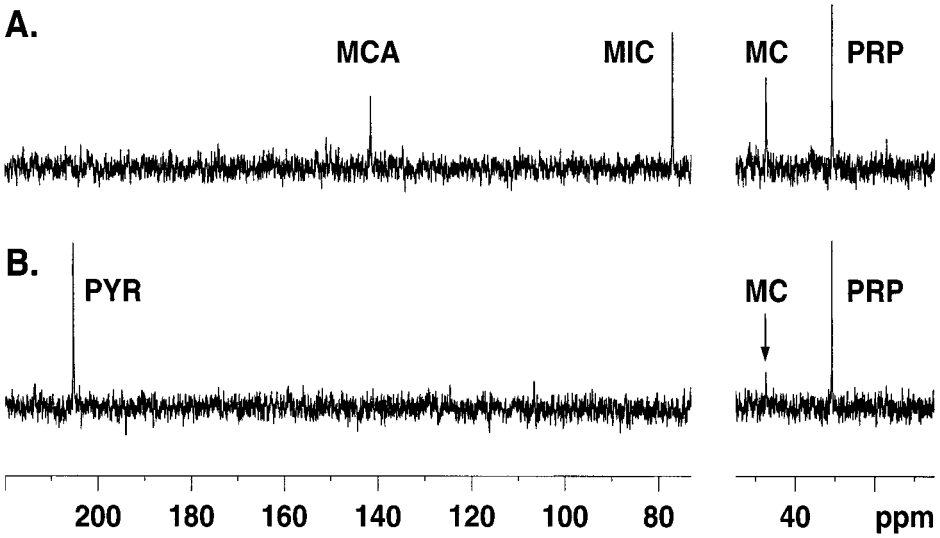

FIGURE 5: The conversion of [2-^{13}C]methyl-*cis*-aconitate to [2-^{13}C]pyruvate by H_6AcnA and H_6PrpB monitored by ^{13}C NMR. The removed portion of the spectra (60−73 ppm) contained only glycerol peaks present in the protein preparations at 62.8 and 72.3 ppm. (A) Conversion of [2-^{13}C]methyl-*cis*-aconitate (MCA) to [2-^{13}C]methylisocitrate (MIC) by H_6AcnA; (B) cleavage of [2-^{13}C]methylisocitrate into [2-^{13}C]-pyruvate (PYR) and succinate by H_6PrpB.

from 27 to 30 μmol min^{-1} (mg of protein)$^{-1}$. This value is close to that reported for *E. coli* AcnB protein [38 μmol min^{-1} (mg of protein)$^{-1}$] (*34*). This result was interpreted to mean that the histidine tag had little, if any, deleterious effect on the activity of the enzyme.

The H_{10}AcnB was tested as a 2-methyl-*cis*-aconitate hydratase using the same method as H_6AcnA (see Experimental Procedures). H_{10}AcnB was added to a mixture containing [2-^{13}C]methyl-*cis*-aconitate, and the course of the reaction was monitored by ^{13}C NMR. After 1 h of incubation, a fraction of the [2-^{13}C]methyl-*cis*-aconitate was converted to [2-^{13}C]methylisocitrate (data not shown). As expected, the addition of H_6PrpB to the mixture cleaved [2-^{13}C]methylisocitrate into [2-^{13}C]pyruvate and succinate (data not shown). These results demonstrated that H_{10}AcnB had 2-methyl-*cis*-aconitate hydratase activity and can substitute for AcnA in the 2-methylcitric acid cycle of *S. enterica*.

In Vivo Assessment of the Requirement for Aconitase A and B during Propionate Catabolism. Since AcnB protein can hydrate 2-methyl-*cis*-aconitate, the requirements of AcnB during growth on propionate were tested. For these experiments, an *acnB* single mutant and an *acnA acnB* double mutant were constructed. To construct an *acnB* mutation, the gene was PCR amplified and cloned. The *acnB* gene was inactivated by inserting a kanamycin cassette into the *Eco*RI and *Kpn*I sites of *acnB*, removing 2.2 kb from the middle of the gene. The *acnB3*::*kan*$^+$ element was moved onto the chromosome as described (*3*, *16*). The location of the *acnB3*::*kan*$^+$ element was verified by PCR and sequencing. To test the involvement of the aconitases in propionate catabolism, the growth phenotypes of the *acnB* single mutant (JE5992) and the *acnA acnB* double mutant (JE5993) strains were tested. The *acnB* mutant strain did not grow on propionate unless glutamate was added to the medium, and the addition of glutamate did not restore growth to the level of an *acnB*$^+$ strain (TR6583). This result indicated that AcnB was the primary aconitase for growth on propionate. In addition, the *acnB* mutant strain did not grow on acetate and citrate and only slight improvements were observed when glutamate was added. The *acnA acnB* double mutant strain did not grow on propionate even when supplemented with glutamate. This result indicated that both aconitases were required for aerobic growth on propionate. Further, the *acnA acnB* double mutant strain was unable to respire propionate under anaerobic growth conditions suggesting that oxygen did not play any role in the observed propionate phenotype. Growth behavior of the *S. enterica acnB* mutant and *acnA acnB* double mutant strains on acetate and glucose were consistent with those reported for the corresponding *E. coli* mutants (*37*). To demonstrate the aconitase mutations did not affect flanking genes on the chromosome, complementing plasmids for *acnA* and *acnB* were constructed (see Experimental Procedures). These plasmids complemented the aerobic phenotypes of an *acnA acnB* double mutant when induced with 1 mM L-arabinose (data not shown).

DISCUSSION

The 2-methylcitric acid cycle responsible for the conversion of propionate to pyruvate in *S. enterica* was reconstituted using homogeneous preparations of five enzymes. To the best of our knowledge, this is the first report of the complete reconstitution of this pathway. Results from experiments reported in this paper demonstrate that the PrpD protein has 2-methylcitrate dehydratase activity. It is also shown that the aconitase A and B enzymes can catalyze the hydration of 2-methyl-*cis*-aconitate.

PrpD Characterization. It was not surprising to find out that PrpD could only catalyze one of the two reactions typically catalyzed by aconitases, because PrpD (54 kDa) is significantly smaller than aconitases (~100 kDa). The fact that PrpD could only dehydrate 2-methylcitrate to 2-methyl-*cis*-aconitate was consistent with the enzymology of the 2-methylcitric acid cycle in *Y. lipolytica*, a fungus in which separate enzymes catalyze the dehydration and hydration steps of this pathway (*28*, *33*). Many properties of the 2-methylcitrate dehydratase identified in *Y. lipolytica* are consistent with those of PrpD (*33*). For example, isocitrate was not a substrate for PrpD or the enzyme from *Y. lipolytica*, and neither enzyme demonstrated requirements for metal cations or

reducing agents (*33*). Unlike the *Y. lipolytica* 2-methylcitrate dehydratase, PrpD used citrate or *cis*-aconitate as substrate (*33*). Surprisingly, neither the fungal or bacterial enzymes were iron−sulfur proteins, even though most enzymes of the hydro-lyase family do have iron−sulfur centers (*38*).

The PrpD reaction mechanism may be similar to the fumarase reaction catalyzed by *E. coli* FumC or other class II fumarases (*39*, *40*). These enzymes catalyze the reversible hydration of fumarate to L-malate and display no requirements for metal cations or reducing agents. In contrast, the class I fumarases are labile, iron−sulfur proteins (*38*, *40*). The related enzyme properties of PrpD and class II fumarases suggests PrpD may proceed through a similar reaction mechanism, although these two different enzymes share no apparent sequence similarity. Further characterization of the PrpD enzyme is necessary to clarify this issue.

Significance of the prpD Sequence. The DNA sequence of the *prpD* gene and the predicted amino acid sequence of its gene product show no similarity to known genes or their encoded proteins. This observation is surprising considering the large number of dehydratases with substrates similar to 2-methylcitrate. One explanation may be that many dehydratases contain iron−sulfur centers (*38*), although there are dehydratases such as D-(−)-tartrate dehydratase from *Rhodobacter* (formerly *Rhodopseudomonas*) *sphaeroides* and fumarase C from *E. coli* that are not iron−sulfur enzymes (*39*, *41*). PrpD shows striking sequence similarity to a number of hypothetical proteins such as YqiQ from *Bacillus subtilis* (GenBank accession 1709059), PDH1 from *Saccharomyces cerevisiae* (GenBank accession 6325258), and others (*1*). Among the organisms with *prpD* homologues, only *E. coli* and *S. cerevisiae* have been shown to catabolize propionate via the 2-methylcitric acid cycle (*42*−*44*). The uniqueness of the PrpD sequence could be used to search for the 2-methylcitric acid cycle in genomes of organisms, although some procaryotes with *prp* operons do not have the *prpD* gene (Figure 4). All of the other enzymes in this pathway show sequence similarity to enzymes that catalyze related reactions.

Requirements for Aconitase during Growth on Propionate. It is intriguing that in several procaryotes, but not in *S. enterica*, a homologue of the *acnA* gene (herein referred to as *acnD*, Figure 4) is found within putative *prp* operons. The absence of the *acnA* gene in the *prpBCDE* operon of *S. enterica* suggests AcnA may have other functions in this bacterium, and thus their syntheses cannot be restricted to growth conditions where propionate is available in the environment. Additional functions of AcnA are currently unknown. Surprisingly, the *acnB* gene, or a homologue of it, has not been found in any putative *prp* operon, although it is next to the *prp* operon in *P. putida*. In studies with *E. coli* aconitases, AcnA was more oxygen-stable than AcnB (*32*). The presence of the *acnA* homologues, but not *acnB*, in putative *prp* operons may reflect a general requirement for propionate catabolism during oxygen stress.

Further Support for the Involvement of Aconitase or Another Iron−Sulfur Center-Containing Hydratase. Glutathione (GSH) is required for the synthesis of active aconitase (*45*). In *S. enterica*, *gshA* mutants grow poorly on propionate as a sole carbon and energy source (*46*). This effect of the lack of GSH on propionate catabolism is consistent with the proposed involvement of aconitases in the breakdown of propionate. It is also possible, however, that the lack of GSH affects the activity of an as-yet-unidentified 2-methyl-*cis*-aconitate specific hydratase.

Does S. enterica have a specific 2-methyl-cis-aconitate hydratase enzyme? The apparent similarities between the 2-methylcitric acid cycles of *S. enterica* and *Y. lipolytica* suggests that like *Y. lipolytica*, *S. enterica* may have a specific 2-methyl-*cis*-aconitate hydratase (*28*). However, the growth phentoypes of a *S. enterica acnA acnB* double mutant indicate aconitases are essential for growth on propionate. Considering this result, it seems unlikely that a specific 2-methyl-*cis*-aconitate hydratase exists in *S. enterica*. It is possible that the pleotrophic growth phenotypes of the *S. enterica acnA acnB* double mutant is masking the 2-methyl-*cis*-aconitate hydratase activity. Similar pleotrophic phenotypes were observed for *E. coli* aconitase double mutants (*37*). We suspect that genetic approaches for the identification of the gene encoding this putative hydratase would be unsuccessful given the documented activity of AcnA and AcnB and the phenotypes of aconitase mutants. Different approaches are needed to investigate the existence of this enzyme in *S. enterica.*

Function of the Putative YbhH Enzyme. Also of interest is the *ybhH* gene (Figure 4) found in *acnD*-containing *prp* operons. It is possible that the YbhH protein has unknown 2-methyl-*cis*-aconitate hydratase activity. Although it seems redundant to have AcnD in addition to a specific 2-methyl-*cis*-aconitate hydratase in the same operon, there may be advantages to this redundancy. For example, sequence analysis suggests that YbhH may not have an iron−sulfur center, which could allow propionate breakdown in iron limiting or highly oxidizing environments. This situation would be similar to the presence of three fumarases in *E. coli* (*47*). FumA and FumB are labile, iron−sulfur proteins expressed under aerobic and anaerobic conditions, respectively (*40*), while FumC lacks an iron−sulfur center and is induced by oxygen stress (*39*, *48*). It was surprising that the sequence analysis in *S. enterica*, *S. typhi*, *and S. paratyphi* failed to identify a YbhH homologue. If this putative enzyme were the 2-methyl-*cis*-aconitate hydratase, the lack of this activity would suggest *S. enterica* catalyzes the reaction solely through AcnA and AcnB. It was surprising that, in *E. coli*, *ybhH* was located in a three-gene operon with *ybhJ*, which is predicted to encode a third aconitase (AcnC) activity (*15*, *32*). The *ybhJ* gene is also missing in *S. enterica*.

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REFERENCES

1. Horswill, A. R., and Escalante-Semerena, J. C. (1997) *J. Bacteriol. 179*, 928−940.

2. Horswill, A. R., and Escalante-Semerena, J. C. (1999) *J. Bacteriol. 181*, 5615−5623.
3. Horswill, A. R., and Escalante-Semerena, J. C. (1999) *Microbiology 145*, 1381−1388.
4. Schloss, J. V., Emptage, M. H., and Cleland, W. W. (1984) *Biochemistry 23*, 4572−4580.
5. Beach, R. L., Aogaichi, T., and Plaut, G. W. E. (1977) *J. Biol. Chem. 252*, 2702−2709.
6. Davis, R. W., Botstein, D., and Roth, J. R. (1980) *A Manual for Genetic Engineering: Advanced Bacterial Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
7. Berkowitz, D., Hushon, J. M., Whitfield, H. J., Roth, J., and Ames, B. N. (1968) *J. Bacteriol. 96*, 215−220.
8. Bobik, T. A., Havemann, G. D., Busch, R. J., Williams, D. S., and Aldrich, H. C. (1999) *J. Bacteriol. 181*, 5967−5975.
9. Chan, R. K., Botstein, D., Watanabe, T., and Ogata, Y. (1972) *Virology 50*, 883−898.
10. Schmieger, H. (1971) *Mol. Gen. Genet. 100*, 378−381.
11. Schmieger, H., and Bakhaus, H. (1973) *Mol. Gen. Genet. 120*, 181−190.
12. Seidman, C. G., Struhl, K., Sheen, J., and Jessen, T. (1997) in *Current Protocols in Molecular Biology* (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., Eds.) pp 1.8−1.8.10, Wiley-Interscience, New York.
13. Tsai, S. P., Hartin, R. J., and Ryu, J. (1989) *J. Gen. Microbiol. 135*, 2561−2567.
14. Ryu, J.-I., and Hartin, R. J. (1990) *Biotechniques 8*, 43−45.
15. Blattner, F. R., Plunkett III, G., Bloch, C. A., Perna, N. T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J. D., Rode, C. K., Mayhew, G. F., Gregor, J., Davis, N. W., Kirkpatrick, H. A., Goeden, M. A., Rose, D. J., Mau, B., and Shao, Y. (1997) *Science 277*, 1453−1474.
16. Hamilton, C. M., Aldea, M., Washburn, B. K., Babitzke, P., and Kushner, S. R. (1989) *J. Bacteriol. 171*, 4617−4622.
17. Guzman, L.-M., Belin, D., Carson, M. J., and Beckwith, J. (1995) *J. Bacteriol. 177*, 4121−4130.
18. Fellay, R., Frey, J., and Krisch, H. (1987) *Gene 52*, 147−154.
19. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Miller, W., and Lipmann, D. J. (1997) *Nucleic Acids Res. 25*, 3389−3402.
20. Heidelberg, J. F., Eisen, J. A., Nelson, W. C., Clayton, R. A., Gwinn, M. L., Dodson, R. J., Haft, D. H., Hickey, E. K., Peterson, J. D., Umayam, L., Gill, S. R., Nelson, K. E., Read, T. D., Tettelin, H., Richardson, D., Ermolaeva, M. D., Vamathevan, J., Bass, S., Qin, H., Dragoi, I., Sellers, P., McDonald, L., Utterback, T., Fleishmann, R. D., Nierman, W. C., and White, O. (2000) *Nature 406*, 477−483.
21. Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., Bertero, M. G., Bessieres, P., Bolotin, A., Borchert, S., Borriss, R., Boursier, L., Brans, A., Braun, M., Brignell, S. C., Bron, S., Brouillet, S., Bruschi, C. V., Caldwell, B., Capuano, V., Carter, N. M., Choi, S.-K., Codani, J.-J., Connerton, I. F., Cummings, N. J., Daniel, R. A., Denizot, F., Devine, K. M., Düsterhöft, A., Ehrlich, S. D., Emmerson, P. T., Entian, K. D., Errington, J., Fabret, C., Ferrari, E., Foulger, D., Fritz, C., Fujita, M., Fuma, S., Harwood, C. R., Hénaut, A., Hilbert, H., Holsappel, S., Hosono, S., Hullo, M.-F., Itaya, M., Jones, L., Joris, B., Karamata, D., Kasahara, Y., Klaerr-Blanchard, M., Klein, C., Kobayashi, Y., Koetter, P., Koningstein, G., Krogh, S., Kumano, M., Kurita, K., Lapidus, A., Lardinois, S., Lauber, J., Lazarevic, V., Lee, S.-M., Levine, A., Liu, H., Masuda, S., Mauël, C., Médigue, C., Medina, N., Mellado, R. P., Mizuno, M., Moestl, D., Nakai, S., Noback, M., Noone, D., O'Reilly, M., Ogawa, K., Ogiwara, A., Oudega, B., Park, S.-H., Parro, V., Pohl, T. M., Portetelle, D., Porwollick, S., Prescott, A. M., Prtesecan, E., Pujic, P., Purnelle, B., Rapoport, G., Rey, M., Reynolds, S., Rieger, M., Rivolta, C., Rocha, E., Roche, B., Rose, M., Sadaie, Y., Sato, T., Scanlan, E., Schleich, S., et al. (1997) *Nature 390*, 249−256.
22. Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrener, P., Hickey, M. J., Brinkman, F. S., Hufnagle, W. O., Kowalik, D. J., Lagrou, M., Garber, R. L., Goltry, L., Tolentino, E., Westbrock-Wadman, S., Yuan, Y., Brody, L. L., Coulter, S. N., Folger, K. R., Kas, A., Larbig, K., Lim, R., Smith, K., Spencer, D., Wong, G. K., Wu, Z., and Paulsen, I. T. (2000) *Nature 406*, 959−964.
23. Tettelin, H., Saunders, N. J., Heidelberg, J., Jeffries, A. C., Nelson, K. E., Eisen, J. A., Ketchum, K. A., Hood, D. W., Peden, J. F., Dodson, R. J., Nelson, W. C., Gwinn, M. L., DeBoy, R., Peterson, J. D., Hickey, E. K., Haft, D. H., Salzberg, S. L., White, O., Fleischmann, R. D., Dougherty, B. A., Mason, T., Ciecko, A., Parksey, D. S., Blair, E., Cittone, H., Clark, E. B., Cotton, M. D., Utterback, T. R., Khouri, H., Qin, H., Vamathevan, J., Gill, J., Scarlato, V., Masignani, V., Pizza, M., Grandi, G., Sun, L., Smith, H. O., Fraser, C. M., Moxon, E. R., Rappuoli, R., and Venter, J. C. (2000) *Science 287*, 1809−1815.
24. Kennedy, M. C., and Beinert, H. (1988) *J Biol Chem 263*, 8194−8198.
25. Kennedy, M. C., Emptage, M. H., Dreyer, J. L., and Beinert, H. (1983) *J. Biol. Chem. 258*, 11098−11105.
26. Gunsalus, R. P., Tandon, S. M., and Wolfe, R. S. (1980) *Anal. Biochem. 101*, 327−331.
27. Balch, W. E., and Wolfe, R. S. (1976) *Appl. Environ. Microbiol. 32*, 781−791.
28. Aoki, H., Uchiyama, H., Umetsu, H., and Tabuchi, T. (1995) *Biosci. Biotech. Biochem. 59*, 1825−1828.
29. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−255.
30. Laemmli, U. K. (1970) *Nature 227*, 680−685.
31. Sasse, J. (1991) in *Current Protocols in Molecular Biology* (Ausubel, F. A., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., Eds.) pp 10.6.1−10.6.8, Wiley-Interscience, New York.
32. Jordan, P. A., Tang, Y., Bradbury, A. J., Thomson, A. J., and Guest, J. R. (1999) *Biochem. J. 344*, 739−746.
33. Tabuchi, T., Aoki, H., Uchiyama, H., and Nakahara, T. (1981) *Agric. Biol. Chem. 45*, 2823−2829.
34. Bradbury, A. J., Gruer, M. J., Rudd, K. E., and Guest, J. R. (1996) *Microbiology 142*, 389−400.
35. Gruer, M. J., and Guest, J. R. (1994) *Microbiology 140*, 2531−41.
36. Bennett, B., Gruer, M. J., Guest, J. R., and Thomson, A. J. (1995) *Eur. J. Biochem. 233*, 317−326.
37. Gruer, M. J., Bradbury, A. J., and Guest, J. R. (1997) *Microbiology 143*, 1837−1846.
38. Flint, D. H., and Allen, R. M. (1996) *Chem. Rev. 96*, 2315−2334.
39. Weaver, T. M., Levitt, D. G., Donnelly, M. I., Stevens, P. P., and Banaszak, L. J. (1995) *Nat. Struct. Biol. 2*, 654−662.
40. Weaver, T., and Banaszak, L. (1996) *Biochemistry 35*, 13955−13965.
41. Rode, H., and Giffhorn, F. (1982) *J. Bacteriol. 150*, 1061−1068.
42. London, R. E., Allen, D. L., Gabel, S. A., and DeRose, E. F. (1999) *J. Bacteriol. 181*, 3562−3570.
43. Pronk, J. T., van der Linden-Beuman, A., Verduyn, C., Scheffers, W. A., and van Dijken, J. P. (1994) *Microbiology 140*, 717−722.
44. Textor, S., Wendisch, V. F., De Graaf, A. A., Müller, U., Linder, M. I., Linder, D., and Buckel, W. (1997) *Arch. Microbiol. 168*, 428−436.
45. Gardner, P. R., and Fridovich, I. (1993) *Arch. Biochem. Biophys. 301*, 98−102.
46. Rondon, M. R., Kazmierczak, R., and Escalante-Semerena, J. C. (1995) *J. Bacteriol. 177*, 5434−5439.
47. Guest, J. R., Miles, J. S., Roberts, R. E., and Woods, S. A. (1985) *J. Gen. Microbiol. 131*, 2971−2984.
48. Liochev, S. I., and Fridovich, I. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 5892−5896.
49. Rondon, M. R., Horswill, A. R., and Escalante-Semerena, J. C. (1995) *J. Bacteriol. 177*, 7119−7124.

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