Studies of Propionate Toxicity in Salmonella enterica Identify 2-Methylcitrate as a Potent Inhibitor of Cell Growth*

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Abstract

Methylococcus capsulatus was found to inhibit aconitase and citrate synthase (gltA) genes. These results suggested that citrate synthase activity was the source of the increased sensitivity to propionate observed in the absence of the 2-methylcitric acid cycle. DNA sequencing of the wild-type and mutant glutA alleles revealed that the ATG start codon of the wild-type gene was converted to the rare GTG start codon in the revertant strain. This result suggested that lower levels of this enzyme were present in the mutant. Consistent with this change, cell-free extracts of the propionate-resistant strain contained 12-fold less citrate synthase activity. This was interpreted to mean that, in the wild-type strain, high levels of citrate synthase were the source of a toxic metabolite. In vitro experiments performed with homogeneous citrate synthase enzyme indicated that this enzyme was capable of synthesizing 2-methylcitrate from propionyl-CoA and oxaloacetate. This result lent further support to the in vivo data, which suggested that citrate synthase was the source of a toxic metabolite.

Short chain fatty acids (SCFAs) are common by-products of bacterial fermentations and are produced in abundance in the gastrointestinal tracts of mammals. Although SCFAs are a good source of carbon and energy for procaryotes, they also are a hazard because they inhibit cell growth. The growth inhibitory properties of SCFAs have made them useful as preservatives in the food industry. Propionate (a three-carbon SCFA) is one of the most abundant fermentation byproducts, and it is extensively used in the food industry to protect baked goods against microbial contamination.

Enteric bacteria such as Salmonella spp. are exposed to high concentrations of propionate in their environments. In the gas-

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1 The abbreviations used are: SCFA, short chain fatty acid; Ap, ampicillin; H6, hexahistidine tag; NB, nutrient broth; PCR, polymerase chain reaction; Tn10d(Tc), Tn10DEL16DEL17; Tc, tetracycline; MsdJ, MsdI1734; kb, kilobase(s).

2-methylcitrate accumulation (22).

The ability of microorganisms to metabolize SCFAs may be the primary line of defense against the negative effects of these compounds on cell growth (2, 4). Enteric bacteria such as Salmonella enterica serovar Typhimurium LT2 and E. coli catalyze propionate via the 2-methylcitric acid cycle (23, 24). However, when this pathway was blocked in Salmonella, sensitivity to propionate increased dramatically (25), suggesting that intermediates of the 2-methylcitric acid cycle may have a more profound negative effect on cell growth than propionate itself. This idea would be consistent with reports in the literature where 2-threo-α-methylisocitrate was found to inhibit bovine heart NADP isocitrate dehydrogenase (26) and synthetic 2-methylcitrate was found to inhibit aconitase and citrate synthase activities (27).

This paper reports the isolation and characterization of a...
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derivative of a prpC mutant (lacks methylcitrate synthase activity) of S. enterica serovar Typhimurium LT2 with increased resistance to propionate. The data indicate that by lowering the level of citrate synthase in the prpC mutant, the negative effect of propionate was drastically relieved. The data strongly suggest that 2-methylcitrate is a toxic catabolite or the precursor of an as yet unidentified toxic compound.

EXPERIMENTAL PROCEDURES

Culture Media and Growth Conditions

Nutrient broth (NB) at 0.8% (w/v) containing 85 mM NaCl (28) was routinely used as rich medium. E. coli cultures were maintained in Luria-Bertani (LB) broth. Non-carbon E medium supplemented with 1 mM MgSO4, and 0.5 mM methionine was used as a minimal medium (26, 29). The final concentrations of compounds provided in culture medium were as follows: 50 mM acetate, 20 mM citrate, 11 mM glucose, 5 mM glutamate, 30 mM glyceral, 30 mM malate, 30 mM propionate, and 50 mM succinate. Antibiotic concentrations in rich medium were 100 μg/ml ampicillin, 50 μg/ml kanamycin, and 20 μg/ml tetracycline. Antibiotic concentrations for plasmids in minimal medium were 50 μg/ml ampicillin and 50 μg/ml kanamycin. All chemicals were purchased from Sigma unless otherwise stated. A list of strains and plasmids used and their genotypes is provided in Table I.

Growth Curves

Overnight cultures of strains grown in NB were subcultured 1:100 (v/v) in duplicate into 5 ml of minimal medium with appropriate supplements. Cultures were incubated in 18 × 150-mm tubes at 37 °C with shaking, and cell growth was monitored at 650 nm with a Spectronic 20D spectrophotometer furnished with a red filter (Milton Roy Co., Rochester, NY).

Genetic Crosses

Transductions involving phage P22 HT105 int-201 (30, 31) were performed as described (28). Transductants were freed of phage as described (32).

Recombinant DNA Techniques

Restriction and modification enzymes were purchased from Promega Corp. (Madison, WI) unless otherwise stated and were used according to the manufacturer's instructions. All DNA manipulations were performed in E. coli DH5α/F′. Plasmids were transferred into E. coli or S. enterica serovar Typhimurium LT2 by CaCl2 heat shock as described (33). Plasmids transferred into Salmonella were first transferred into recombination-deficient or S. enterica serovar Typhimurium LT2 strain JR501 (34). Plasmids from strain JR501 were transferred into other Salmonella strains as described (35).

Isolation of Strains Resistant to Propionate

Ten tubes containing 2 ml of NB broth each were inoculated with isolated colonies of strain JE2170 (prpC114::MudJ) and grown overnight at 37 °C with shaking. Cells in a 1-ml sample from each culture were pelleted in 1.5 ml microcentrifuge tubes for 2 min at 15,000 × g in a MarathonTM 16KM microcentrifuge (Fisher). The spent medium supernatant was removed, and cells were resuspended in 100 μl of sterile saline and plated on minimal medium supplemented with succinate, propionate, and kanamycin. After 4 days of incubation at 37 °C, revertants were observed. These colonies were picked and streaked for isolation on the same medium. One revertant in particular grew better than the others and was selected for further characterization. A phage P22 lysate was prepared on this strain and used to transduce strain JE2170 to growth on succinate in the presence of propionate. The reconstructed strain was freed of phage and saved as strain JE3777. As shown below, the mutation responsible for the observed resistance to propionate toxicity in this strain changes the start codon of the gltA gene from ATG (Met) to GTG (Met). Because the mutation did not change the amino acid sequence of the GltA protein, the mutant allele is hereafter referred to as gltA<i>CTG</i>.

Mapping the gltA<i>CTG</i> Mutation

Unlike its parent strain, strain JE3777 (prpC114::MudJ gltA<i>CTG</i>) failed to utilize acetate as carbon and energy source. This growth phenotype was used to isolate a Tn10DEL16DEL17 element (hereafter referred to as Tn10DEL(Tc)) near gltA<i>CTG</i>. Briefly, phage P22 was grown on a pool of strains (approximately 100,000) each assumed to contain one transposition-defective Tn10DEL(Tc) element (36), and a P22 lysate was prepared. This lysate was used to transduce strain JE3777 to tetracycline resistance. Tetracycline-resistant (Tc) transductants that regained the ability to grow on acetate were freed of phage and analyzed further. A Tn10DEL(Tc) element cloned to gltA<i>CTG</i> (>90% transductible by P22) was isolated, moved back into strain JE3777, and saved as strain JE3845 (prpC114::MudJ gltA<i>CTG</i> zbg-6391::Tn10DEL(Tc)).

Determination of DNA Sequence Flanking the zbg-6391::Tn10DEL(Tc) Element

Arbitrary primer PCR was used to identify a DNA sequence flanking the linked insertion (37, 38). Chromosomal DNA of strain JE3845 was prepared using the MasterPureTM genomic DNA isolation kit (Epicentre, Madison, WI). Two 50-μl reactions were prepared for the first round of PCR using 1 μg of DNA template, 50 pmol of each primer, 0.2 μM of each dNTP (Promega), and Vent® Expand DNA polymerase (New England Biolabs, Beverly, MA) in a GeneAmp® PCR System 2400 (PerkinElmer Life Sciences). Primers used were as follows: Tn10-L, 5′-CTCATGCGGTGGACAAA-3′; Tn10-R, 5′-ACCTTTGTTGCACCAACAGTT-3′; and ARB1, 5′-GGCCACCGTCACGAATTGATTCNWNWNNNNGAT-3′. The first PCR round used the following conditions: 5 cycles at 94 °C for 30 s, 30 °C for 30 s, and 72 °C for 2 min. Then, 20 cycles were added: 30 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1 min. PCR amplification was verified by agarose gel electrophoresis (39) using 2% gels. Amplified DNA was purified using the QiAquick® PCR purification kit (Qiagen, Chatsworth, CA).

PCR sequencing reactions were prepared using the ABI PRISM® dye terminator cycle sequencing kit (PerkinElmer Life Sciences) according to the manufacturer's instructions. Reactions were purified in AutoSeq® G-50 columns (Amersham Pharmacia Biotech), dried in a SpeedVac® concentrator (Savant Instruments, Farmingdale, NY), and sequenced at the Biotechnology Center of the University of Wisconsin, Madison.

Sequenceing of the gltA Gene and the gltA<i>CTG</i> Mutation

A primer for the 3′ end of the gltA gene (5′-CGGAGGCTGAGATA-GAGAAAAATA-3′) was designed from the sequence obtained using the Tn10-L side of the zbg-6391::Tn10DEL(Tc) element in JE3845 (see above). A primer for the 5′ end of gltA (5′-GATCCAGATGATGTTCAGTTT-3′) was designed based on the reported sequence (40). The gltA gene was sequenced in its entirety by PCR amplifying the gene from strain TR6583 (gltA′) and directly sequencing the entire PCR fragment by primer walking. DNA template for the PCR reactions was prepared by mixing 10 μl of NB overnight cultures of strain TR6583 with 90 μl of water, heating the cell suspensions to 100 °C for 5 min, and centrifuging them for 1 min at 15,000 × g in a Marathon 16KM microcentrifuge (Fisher). PCR reactions were prepared using one-tenth the volume of boiled template, 50 pmol of each primer, 0.2 μM of each dNTP, and Pfu polymerase (Stratagene, La Jolla, CA) according to the 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 90 s. The PCR reactions were verified and purified, and the DNA was sequenced as described above. Sequences of all of the primers used for primer walking can be provided upon request. Each side of the gltA gene was sequenced at least two times. The DNA sequence was compiled and submitted to GenBank® (accession number AF056043). The gltA<i>CTG</i> mutation in strain JE3777 is identified by sequencing the entire gltA gene.

Plasmid Constructions

Plasmid pPRP68—The prpE gene was PCR-amplified as described above using an NdeI primer for the 5′ end (5′-CAGGAGGACCATAT-GTCTTTAGC-3′) and a 3′ end primer (5′-TTCTTCGATCGCCTGGC-3′). The PCR fragment was purified as described above, digested with NdeI, and cloned into pTVB2 (New England Biolabs, Beverly, MA) cut with the same enzyme. The plasmid pPRP68 (9.4 kb, Ap′). PrpE protein purified using this plasmid has an additional C-terminal glycine residue.

Plasmid pGLTA1—The gltA gene was PCR-amplified as described above (using same primers) and cloned to make plasmid pGLTA1 (4.3 kb, Ap′). The PCR fragment was cloned by A-tailing the DNA and ligating into pGEM™-T vector using the pGEM®-T vector sys-
tem kit (Promega) according to the manufacturer’s instructions. Plasmid pGLTA2—Plasmid pGLTA1 was digested with enzymes KpnI and XbaI (sites in primers) to remove the gltA gene as a 1.3-kb fragment. This piece was cloned into plasmid pBAD30 (41) with the same enzymes to generate plasmid pGLTA2 (2.2 kb, Ap+). The PCR-amplified fragment was subcloned into the pCR-ampil (5′-GAGACCGCATATGGCT-GATACA-3′) of the PCR primer 5′-GATACA-3′ and a BamHI primer for the 3′ end (5′-CATCCGTC-CCAAATTACG-G3′). The PCR fragment was purified using the QIAquick® PCR purification kit (Qiagen), digested with Ndel and BamHI, and cloned into pET-15b (Novagen, Madison, WI) cut with the same enzymes. The resulting plasmid was named pGLTA5 (7 kb, Ap+). The GltA protein encoded by plasmid pGLTA3 contained a hexahistidine tag at its N terminus (H6-GltA).

Purification of the Propionyl-CoA Synthetase (PrpE) Enzyme

Overexpression of prpE—Plasmid pPRP68 (encodes PrpE) was transformed into E. coli ER2566, and the resulting strain, JE4813, was used to overexpress and purify PrpE protein. To overexpress prpE, 20 ml of an LB overnight culture of JE4813 was used to inoculate 1 liter of SOC broth (42, 43) containing ampicillin. The culture was grown at 20 °C to overexpress and purify PrpE protein. To overexpress prpE, 20 ml of an LB overnight culture of JE4813 was used to inoculate 1 liter of SOC broth (42, 43) containing ampicillin. The culture was grown at 20 °C to overexpress and purify PrpE protein.

Purification of PrpE—Cells overexpressing PrpE were resuspended in 100 ml of 200 mM HEPES, pH 7.5, containing 0.5 mM KCl, 1 mM EDTA, and 0.1% Triton X-100. Cells were centrifuged in 250-ml bottles as described above and resuspended in 20 ml of the same buffer. Cells were broken by French press at 1.03 x 107 kPa using a chilled pressure cell. Cell debris was removed by centrifugation in 50-ml Oakridge tubes at 31,000 g for 30 min at 4 °C using a SS34 rotor (DuPont). PrpE protein was purified on chitin beads (New England Biolabs) according to the manufacturer’s instructions. Following purification, the enzyme was dialyzed at 4 °C against 1 liter of 50 mM HEPES buffer, pH 7.5, containing 1 mM EDTA, and 0.25 mM Tris-2-carboxyethylphosphine hydrochloride. The dialysis buffer was replaced after 2 h and again after 4 h (20% glycerol (v/v) added to the buffer). The PrpE protein was dialyzed overnight and stored at −80 °C in this buffer.

Propionyl-CoA Synthetase Assays—The propionyl-CoA synthetase activity was monitored using a myokinase, pyruvate kinase, and lactate dehydrogenase coupling assay (44). Standard assays (0.8 ml of reaction mixture volume) contained 50 mM HEPES buffer, pH 7.5, 0.1 mM KCl, 5 mM glycerol (v/v), 0.2 mM 5,5′-dithiobis-(2-nitrobenzoic acid), 0.1 mM oxaloacetate, and 0.1 mM acetyl-CoA. To assay for 2-methylcitrate synthase activity, the same conditions were used except propionyl-CoA was substituted for acetyl-CoA. For these assays, buffer and substrates were preincubated in 1.5-ml methacrylate cuvettes (Fisher) at 37 °C in this buffer.

Biochemical Characterization of the Citrate Synthase (GltA) Enzyme

Preparation of Cell-free Extracts—Five ml of NB overnight cultures of strains TR6583, JE2170, and JE3777 were subcultured into 500 ml of minimal medium supplemented with succinate and glutamate. The cultures were grown at 37 °C with shaking for 24 h. Cells were pelleted in 250 ml of Nalgene® polypropylene copolymer bottles (Fisher) by centrifugation for 10 min at 10,500 x g at 4 °C using a GSA rotor in a RC-5B refrigerated centrifuge (DuPont). Cells were resuspended in 100 ml of 50 mM HEPES, pH 7.5, containing 100 mM KCl, 1 mM EDTA, and 0.2 mM of the protease inhibitor phenylmethylsulfonyl fluoride. Cells were centrifuged in 250-ml bottles as described above and resuspended in 5 ml of the same buffer. Cell suspensions were kept on ice by recirculation with a stir bar and a 4 °C recirculating bath (Fisher). Cell debris was removed by centrifugation in 50-ml Nalgene® polypropylene copolymer Oakridge tubes (Fisher) at 31,000 x g for 30 min at 4 °C with a SS34 rotor (DuPont). Supernatants were dialyzed at 4 °C in SnakeSkin® 10,000 molecular weight cut off dialysis membrane (Fisher) against 1 liter of 50 mM HEPES, pH 7.5, containing 100 mM KCl, 1 mM EDTA, and 0.2 mM phenylmethylsulfonyl fluoride. The dialysis buffer was replaced after 2 and 4 h and then allowed to dialyze an additional 16 h. Dialyzed cell-free extracts were tested for citrate synthase activity as described below.

Detection of [14C,2-14C]2-Methylcitrate

H6-GltA-dependent 2-methylcitrate synthesis was tested using [C-2,14C]propionyl-CoA. [C-2,14C]Propionyl-CoA was synthesized from [C-2,14C]propionate (0.02 μmol), ATP (0.12 μmol), MgSO4 (0.3 μmol) in 50 mM HEPES buffer, pH 7.5, containing 100 mM KCl, 5% glycerol (v/v), and 2 μg of PrpE protein (prepared as described in Ref. 47). The final volume was 100 μl. This reaction mixture was incubated for 1 h at 37 °C. The PrpE reaction mixture was divided into three 33-μl samples. Oxaloacetate (0.05 μmol) was added to two of these samples, and 2-methylcitrate was synthesized on the addition of 1.2 μmol of 2-methylcitrate (5). The third sample was used as a no-addition control. The three reactions were incubated at 37 °C for 3 h. Five-μl samples were removed after 1 and 3 h of incubation. The samples were diluted 4-fold with reaction buffer containing 3 mM HCl. Five μl of the acid-treated samples was spotted onto a silica gel TLC plate with fluorescence (Whatman Ltd., Maidstone, Kent, UK) and air-dried for 30 min. The TLC plate was developed with a chloroform:methanol (3:2) mobile phase for about 30 min. The plates were dried in a flow hood for 1 h before exposing the TLC plate overnight to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA).

Inhibition of Aconitase A (AcnA) and Aconitase B (AcnB)

Overexpression and Purification—The AcnA and AcnB genes were overexpressed, and the AcnA and AcnB proteins were purified as previously described.2

2 A. R. Horswill and J. C. Escalante-Semerena, unpublished results.
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TABLE I

| Strain or plasmid | Genotype | Reference or source |
|-------------------|----------|---------------------|
| E. coli DH5α/F' | F′kan-dA1 hsdR17 (rK− mK+) supE44 thi-1 recA1 gyrA (NalR') relA1 ΔlacZYA-argF169 deor [80lacZΔM15] | New England Biolabs |
| ER2566 | F−lacZΔM15 [λ] F′ [λ]− phiA2 [lon] ompT tacZ-T7 gene1 gal ual11 Δmcrl-mer114::IS10 Rcnr-73−miniTn102 Rscbl-210−Tn10(1 TetR) endA1 [dem] | New England Biolabs |
| JE4813 | ER2566/pPRP68 (T7 rpo−, prpE−, bla+) | This study |
| JE4905 | ER2566/pGLTA3 (T7 rpo−, gltA+, bla−) | This study |
| S. typhimurium DM2370 | purF2085 gltA1182: MudJ | Ref. 40 |
| JE501 | hisdS29 hisdB121 hisdL6 metA22 metE551 trpC120 thy-1 (F'F− F'− F−) 50% (Fus2') f1a−66 narm1 | Ref. 35 |
| TR6583 (formerly SA2929) | metE205 ara-9 | K. Sanderson via J. Roth |
| Derivatives of TR6583 | | |
| JE2170 | prpC114: MudJ | Ref. 25 |
| JE3777 | prpC114: MudJ gltA<sup>C<sup>TRG</sup></sup> | This study |
| JE3845 | prpC114: MudJ gltA<sup>C<sup>TRG</sup></sup> zbg-6391::Tn10d(Tc) | This study |
| JE3884 | gltA1182: MudJ | This study |
| JE3907 | prpD196 zai-6386::Tn10d(Tc) | Ref. 54 |
| JE3947 | prpD196 zai-6386::Tn10d(Tc) | Ref. 54 |
| JE4271 | pBAD30 (bla−) | This study |
| JE4274 | gltA1182::MudJ/prpP35 (prpC<sup>C</sup>− bla−) | This study |
| JE4550 | prpD196 zai-6386::Tn10d(Tc) gltA1182: MudJ | This study |
| JE4556 | prpD196 zai-6386::Tn10d(Tc) gltA1182: MudJ | This study |
| Plasmid genotypes | | |
| pGLTA1 | gltA cloned into pGEM-7 (Promega), bla<sup>+</sup> | This study |
| pGLTA2 | gltA cloned into pBAD30, (41) bla<sup>+</sup> | This study |
| pGLTA3 | gltA cloned into pET-15b (Novagen), bla<sup>+</sup> | This study |
| pPRP68 | prpE cloned into pTYB2 (New England Biolabs), bla<sup>+</sup> | This study |

2-Methylcitrate Inhibition—Aconitase activity was monitored as previously described. In inhibition studies, 2-methylcitrate was added to final concentrations ranging from 0.5 to 5.0 mM. Synthetic 2-methylcitrate was purchased from C/D/N Isotopes (Pointe-Claire, Quebec, Canada) as a mixture of four stereoisomers.

Other Procedures

Protein concentrations were determined by the method of Bradford (49) using the Bio-Rad protein reagent. A standard curve was generated for protein determinations with bovine serum albumin. Proteins were separated by SDS-polyacrylamide gel electrophoresis (50) using 12% polyacrylamide gels and were visualized with Coomassie Blue (51). Mid-range standards (14–97.4 kDa) were used for SDS-polyacrylamide gel electrophoresis (Promega). UV-visible spectroscopy was performed in a computer-controlled Lambda Bio-6 spectrophotometer (PerkinElmer Life Sciences).

RESULTS

Isolation and Characterization of a Propionate-resistant Strain—A derivative of strain JE2170 (prpC114::MudJ) with improved growth on succinate in the presence of propionate was isolated. The new strain (strain JE3777; Table I) showed a slower growth rate (8.0 h doubling<sup>−1</sup>) than the prpC<sup>C</sup>− (3.3 h doubling<sup>−1</sup>) strain TR6583 on minimal medium supplemented with succinate and propionate (Fig. 1B). In addition, strain JE3777 stayed in lag phase for ~30 h, a lag that was 10-fold longer than the one measured for strain TR6583. The effect of the mutation in strain JE3777 was not dependent on the use of succinate as a carbon and energy source. The same relief of propionate toxicity was observed when malate substituted for succinate. The mutation responsible for the propionate resistance of strain JE3777 did not improve the growth of this strain when citrate or glycerol substituted for succinate in the presence of propionate (data not shown).

Further phenotypic characterization of strain JE3777 revealed that this strain was unable to grow on acetate as a carbon and energy source (data not shown). Genetic crosses established that the inability of strain JE3777 to grow on acetate correlated with the inheritance of the mutation responsible for the resistance to propionate, not with the presence of the insertion in prpC (data not shown). The acetate phenotype was used to isolate Tn10d(Tc) elements near the mutation, causing resistance to propionate. Comparisons of the nucleotide sequences of the regions flanking element zbg-6391::Tn10d(Tc) (90% cotransducible with the propionate resistance mutation) with those in the data bases located this element near the gltA gene (encodes citrate synthase). On the basis of these results, the mutation responsible for the resistance to propionate was postulated to be an allele of gltA. The known inability of gltA null mutants to grow on acetate supported this idea (52).

Resistance to Propionate Is Afforded by Lower Levels of Citrate Synthase (GltA) Enzyme—To identify the nature of the mutation responsible for the newly acquired resistance to propionate of strain JE3777, the alleles of gltA in strains TR6583 (gltA<sup>C</sup>−) and JE3777 (unknown gltA allele) were sequenced. In both cases, the gltA gene was amplified with a proofreading DNA polymerase, followed by sequencing of both strands by primer walking. The nucleotide sequence of the wild-type gltA gene from strain TR6583 was deposited into GenBank<sup>TM</sup> (ac-
Accumulation of 2-Methylcitrate Is Deleterious to Cell Growth—It should be noted that the null allele of gltA (i.e. gltA1182::MudJ) failed to relieve the propionate toxicity when introduced into a prpD point mutant (strain JE3947). Growth of the gltA prpD double mutant strain (JE4550) was strongly inhibited by propionate in the medium (Fig. 2). It was concluded that the inhibitor of cell growth was 2-methylcitrate or a derivative of it, because prpD mutants are known to accumulate 2-methylcitrate when the medium is supplemented with propionate (23).

Correlation between High Levels of Citrate Synthase and Propionate Toxicity—To establish a direct correlation between the level of GltA and the toxic effects of propionate, gltA was placed under the control of an inducible promoter. Plasmid pGLTA2 (P araBAD ::gltA) was used for this purpose, because it carried the wild-type gltA allele under the control of the arabinose-inducible P araBAD promoter (41). Plasmid pGLTA2 complemented the glutamate auxotrophy of strain JE3884 (gltA1182::MudJ) on minimal medium supplemented with glucose but lacking arabinose (data not shown). This suggested that residual levels of GltA enzyme were synthesized in the absence of arabinose and that such levels were sufficient to satisfy the requirement of the cell for glutamate. The presence of arabinose in the medium, however, was required to complement the phenotype of strain JE3884 (data not shown). This result was consistent with a requirement for higher levels of GltA when the cell was growing on acetate. The presence of arabinose in the medium also restored propionate toxicity in strain JE3777 (carries allele gltA prpC) and in the prpC − gltA − double mutant strain JE4556 carrying plasmid pGLTA2 (data not shown). It was concluded that high levels of citrate synthase were deleterious to a prpC mutant lacking 2-methylcitrate synthase activity when the cell was growing in medium containing propionate and succinate.

GltA Catalyzes the Synthesis of 2-Methylcitrate from Propionyl-CoA and Oxaloacetate—All of the in vivo data strongly suggested that GltA activity was deleterious to cell growth, probably because this enzyme was synthesizing 2-methylcitrate from propionyl-CoA and oxaloacetate. To directly address this possibility, the gltA + gene was overexpressed using plasmid pGLTA3. Homogeneous H213GltA protein was obtained using nickel affinity chromatography (Fig. 3). The H213GltA protein had a specific activity of 24 μmol of product/min/mg of protein, using acetyl-CoA as substrate in the dithiothreitol-(2-nitrobenzoic acid) system.
acid) assay (45). This assay, however, detected only a trace of 2-methylcitrate synthase activity when propionyl-CoA substituted for acetyl-CoA in the reaction mixture (data not shown).

To increase the sensitivity of the assay, [C-2,14C]propionyl-CoA was synthesized (see “Experimental Procedures”) and used to assess the 2-methylcitrate synthase activity of GltA. [C-2,14C]Propionyl-CoA was synthesized using the propionyl-CoA synthetase (PrpE) enzyme (47). The prpE gene was overexpressed with a C-terminal chitin-binding protein fusion using vector pTYB2 (New England Biolabs). Homogenous PrpE protein was obtained using a chitin affinity resin. An additional C-terminal glycine residue was added to PrpE to allow removal of the glutamate auxotrophy of a strain (JE4274) with prpC overexpressed in trans (pPRP35); the solid circles represent the growth without arabinose; and the filled circles represent the growth with 1 mM arabinose.

Homogeneous PrpC (2-methylcitrate synthase) enzyme was used as a positive control for the synthesis of [C-2,14C]2-methylcitrate from [C-2,14C]propionyl-CoA and oxaloacetate. Hydrochloric acid was added to reaction mixtures (final concentration, 1 N) containing GltA or PrpC to stop the reaction, and products and reactants in each reaction mixture were separated using thin layer chromatography (Fig. 4). Under the conditions used, [C-2,14C]2-methylcitrate displayed a relative mobility (Rf) of 0.63, as determined by the PrpC control reaction. In the GltA reaction, three 14C-labeled compounds were present on the TLC plate (Fig. 4). Control experiments with authentic standards established that the compound near the origin was [C-2,14C]propionyl-CoA (data not shown). A 14C-labeled compound with the same specific activity of 10 mol of product/min/mg of protein, using propionate as substrate in a coupled assay (44). [C-2,14C]Propionate was completely converted to [C-2,14C]propionyl-CoA.

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2-Methylcitrate Synthase (PrpC) Can Substitute for GltA during Glutamate Biosynthesis—The ability of PrpC to compensate for the lack of GltA in gltA mutants was assessed. Mutant strains lacking citrate synthase activity are glutamate auxotrophs when growing on glucose (52). Surprisingly, GltA activity was not required for growth on propionate as a carbon and energy source, suggesting that PrpC was able to synthesize citrate from acetyl-CoA and oxaloacetate (data not shown). This observation was supported by the plate phenotypes shown in Fig. 2. Taken together, these observations suggested that PrpC had sufficient citrate synthase activity to substitute for GltA during glutamate production in the cell. Consistent with this idea, plasmid pPRP35 (P\textsubscript{araBAD}\textsuperscript{prpC}'; Table I) (54) complemented the glutamate auxotrophy of a gltA mutant on minimal medium supplemented with glucose even in the absence of arabinose in the medium (data not shown). This plasmid also complemented the acetate phenotype of gltA mutants, but the medium had to be supplemented with 1 mM arabinose (Fig. 5), indicating that a higher level of citrate synthase activity was needed during growth on acetate.

DISCUSSION

Insights into why propionate is toxic to cells were obtained through the genetic and biochemical analyses of mutants of S. enterica serovar Typhimurium LT22 lacking the 2-methylcitric acid cycle and citrate synthase.

2-Methylcitrate or a Derivative of It Is a Potent Inhibitor of Cell Growth—The data lead to the conclusion that cells that catabolize propionate via the 2-methylcitric acid cycle are at risk because 2-methylcitrate, or a derivative of it, is a potent inhibitor of cell growth. In prpC mutants (which lack 2-methylcitrate synthase), the accumulation of 2-methylcitrate appears to enhance when the cell grows on carbon and energy sources that increase the intracellular level of oxaloacetate, such as succinate or malate. This is not surprising because oxaloacetate is the cosubstrate needed for the synthesis of 2-methylcitrate. Despite the lack of PrpC, cells growing on succinate and propionate still synthesize 2-methylcitrate. The data show that this compound is synthesized by the Krebs cycle enzyme citrate synthase (GltA). GltA catalyzes a reaction very similar to that of PrpC, except that it uses acetyl-CoA as a
substrate instead of propionyl-CoA in the condensation with oxaloacetate that yields citrate. Eucaryotic citrate synthases are known to use propionyl-CoA as a substrate at 1–2% the rate of acetyl-CoA (55, 56). The E. coli GltA enzyme is even less efficient, using propionyl-CoA at less than 0.1% the rate of acetyl-CoA (20). Even though the citrate synthase of S. enterica is not very proficient at making 2-methylcitrate, it is clear that the low levels made by this enzyme are sufficient to arrest cell growth.

It is important to consider the stereochemistry of 2-methylcitrate when thinking about its cytotoxic effects. 2-Methylcitrate has two chiral carbons; thus there are four different stereoisomers of this compound. The stereochemistry of the 2-methylcitrate product synthesized by GltA has not been determined, and neither has the one for the product of the PrpC reaction. In Yarrowia lipolytica (formerly Candida or Saccharomyces lipolyticans), the stereochemistry of the 2-methylcitric acid cycle has been investigated by several groups (57–59). Regardless of whether the products of the GltA and PrpC reactions are stereoisomerically different, it is clear that both products exert strong negative effects on cell growth. One probable target for 2-methylcitrate inhibition isaconitase. Mammalian aconitase (unspecified source) is inhibited noncompetitively with one diastereoisomer pair of 2-methylcitrate (27). However, 2-methylcitrate does not inhibit beef heart aconitase (26). In this work, pure preparations of aconitase A and B were not inhibited by synthetic 2-methylcitrate. A better understanding of the effects of 2-methylcitrate or its derivative(s) requires the identification of the target(s) affected by this metabolite.

The results of studies on the negative effects of propionyl-CoA on cell function (19–21) could be explained as an accumulation of 2-methylcitrate, especially if the 2-methylcitric acid cycle were functional in the bacteria where propionyl-CoA was found to inhibit. In the absence of this pathway, however, it is possible that if the ratio of propionyl-CoA to other important acyl-CoA intermediates was increased too much, the cell may find itself with insufficient acetyl-CoA and succinyl-CoA levels to function properly. Experiments aimed at learning how 2-methylcitrate inhibits growth are in progress.

Is the Propionate Catabolic Pathway in S. enterica a Detoxification Pathway?—The propionate catabolic pathway may have evolved to solve the problem of high levels of propionyl-CoA accumulating in the cell. Given the composition of the environments occupied by S. enterica, it is possible that this bacterium may be under constant pressure to maintain the level of propionyl-CoA low enough to avoid the negative effects caused by its accumulation. Propionyl-CoA levels may increase in response to factors such as (i) propionate in the environment (4, 5, 8, 9); (ii) breakdown of odd chain fatty acids (60); (iii) the anaerobic catabolism of threonine (61); or (iv) the catabolism of 1,2-propanediol, a product of rhamnose and fucose fermentation (48, 62). In this bacterium, the 2-methylcitric acid cycle may function as a detoxification pathway for propionyl-CoA. It appears that in wild-type S. enterica, the deleterious synthesis of 2-methylcitrate by GltA is avoided probably by the low Km of the PrpC enzyme for propionyl-CoA (48 μM) (23). During propionate breakdown, the level of 2-methylcitrate at any given point must be kept low by the enzymes acting downstream of 2-methylcitrate, i.e., PrpD and PrpB. The biochemical characterization of the PrpD and PrpB enzymes is needed to investigate this hypothesis.

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