

In *Salmonella enterica*, 2-Methylcitrate Blocks Gluconeogenesis[∇]

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Strains of *Salmonella enterica* serovar Typhimurium LT2 lacking a functional 2-methylcitric acid cycle (2-MCC) display increased sensitivity to propionate. Previous work from our group indicated that this sensitivity to propionate is in part due to the production of 2-methylcitrate (2-MC) by the Krebs cycle enzyme citrate synthase (GltA). Here we report *in vivo* and *in vitro* data which show that a target of the 2-MC isomer produced by GltA (2-MC^{GltA}) is fructose-1,6-bisphosphatase (FBPase), a key enzyme in gluconeogenesis. Lack of growth due to inhibition of FBPase by 2-MC^{GltA} was overcome by increasing the level of FBPase or by micromolar amounts of glucose in the medium. We isolated an *fbp* allele encoding a single amino acid substitution in FBPase (S123F), which allowed a strain lacking a functional 2-MCC to grow in the presence of propionate. We show that the 2-MC^{GltA} and the 2-MC isomer synthesized by the 2-MC synthase (PrpC; 2-MC^{PrpC}) are not equally toxic to the cell, with 2-MC^{GltA} being significantly more toxic than 2-MC^{PrpC}. This difference in 2-MC toxicity is likely due to the fact that as a *si*-citrate synthase, GltA may produce multiple isomers of 2-MC, which we propose are not substrates for the 2-MC dehydratase (PrpD) enzyme, accumulate inside the cell, and have deleterious effects on FBPase activity. Our findings may help explain human inborn errors in propionate metabolism.

Humans have used fermentation as an effective method of preservation for a wide variety of foods (41). Today, the weak short-chain fatty acids (SCFAs) produced by fermentation, such as acetic, propionic, butyric, and lactic acids, are widely used as food preservatives and in pre- and postharvest agricultural processes (34, 38, 45). Propionate, one of the most abundant SCFAs found in the environment (12), is widely used as a preservative of baked goods in the food industry (38).

While SCFAs such as propionate are extensively used as food preservatives, our understanding of how microbial growth is prevented by them is incomplete. Early studies argued that growth inhibition either was caused by dissipation of the proton motive force (4, 48) or was due to decreases in intracellular pH (15, 48) or the intracellular accumulation of the propionate anion (46, 47). More recently, the global effects of SCFAs on gene expression (1, 43, 44) and protein synthesis (8, 37, 52, 56) were reported, revealing wide-ranging effects on gene expression in response to propionate in the environment (43). Evidence also suggests that central metabolic processes may be inhibited by SCFAs or their catabolites. An overview of the effects of propionate on the cell can be seen in Fig. 1.

Propionyl coenzyme A (Pr-CoA), an intermediate in propionate metabolism, was shown to inhibit pyruvate dehydrogenase in *Rhodobacter sphaeroides* (40) and *Aspergillus niger* (10) and competitively inhibit citrate synthase in *Escherichia coli* (39). 2-Methylcitrate (2-MC), the product of the condensation of oxaloacetate (OAA) and Pr-CoA, was shown to inhibit growth of *Salmonella enterica*, but the mechanism of action remained unclear (28) (Fig. 1). With such broad negative effects exerted by propionate or its catabolites, the best strategy

for microbes to deal with SCFAs such as propionate is to efficiently catabolize them into central metabolites (Fig. 1).

S. enterica, like many other enteric bacteria, is exposed to high levels of propionate in human digestive tracts with total SCFA levels varying from 20 to 300 mM and propionate reaching levels as high as 23.1 mmol/kg (9, 17). To cope with such high concentrations of propionate, this bacterium and other enterobacteria like *E. coli* utilize the 2-methylcitric acid cycle (2-MCC) to convert propionate to pyruvate (31, 53). In *S. enterica*, the *prpBCDE* operon encodes most of the 2-MCC enzymes (30). These genes encode a 2-methylisocitrate lyase (PrpB), a 2-methylcitrate synthase (PrpC), a 2-methylcitrate dehydratase (PrpD), and a propionyl coenzyme A (CoA) synthetase (PrpE) (Fig. 1). Early work with *S. enterica* showed that insertion elements placed within the *prpBCDE* operon greatly increased the sensitivity of *S. enterica* to propionate (23). Strains carrying insertions in *prpE*, however, were still able to grow on propionate and were not sensitive to propionate because acetyl-CoA synthetase (Acs) compensates for the lack of PrpE (32).

The goal of the studies reported here was to identify a target of 2-MC in *S. enterica*. Our *in vivo* and *in vitro* data support the conclusion that 2-MC inhibits fructose-1,6-bisphosphatase (FBPase), a key enzyme of gluconeogenesis. The inhibition of FBPase blocks the synthesis of glucose, with the concomitant broad negative effects on cell function. We show that while both the 2-MC synthase (PrpC) and citrate synthase (GltA) enzymes synthesize 2-MC, the 2-MC made by GltA (2-MC^{GltA}) is more toxic to the cell than the 2-MC made by PrpC (2-MC^{PrpC}), and we suggest that the reason for this toxicity is due to the difference in stereochemistry of the GltA and PrpC reaction products.

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MATERIALS AND METHODS

Chemicals and culture media. All chemicals and enzymes were purchased from Sigma unless otherwise stated, except 2-MC, which was purchased from

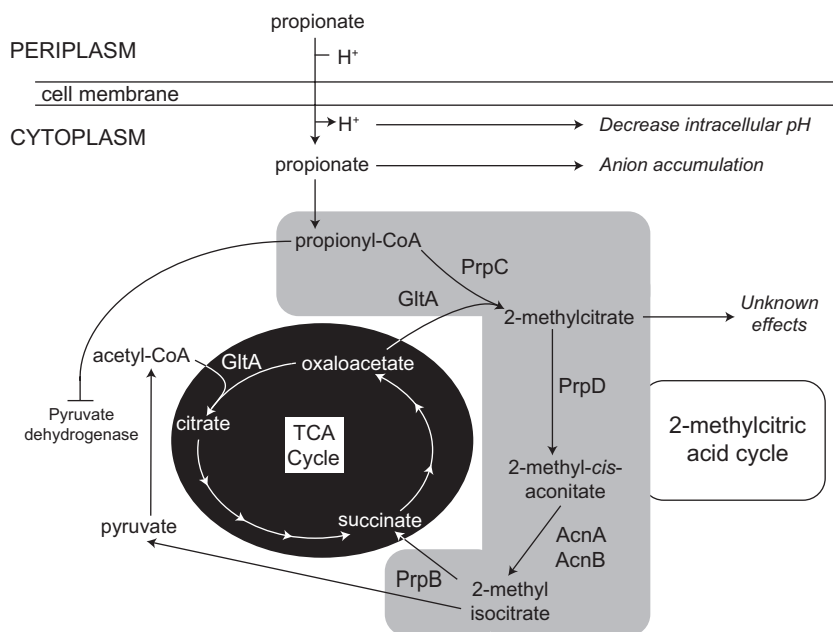


FIG. 1. Overview of propionate metabolism and toxicity in *Salmonella*.

CDN Isotopes (Pointe-Claire, Quebec, Canada). Bacterial cultures were grown in lysogeny broth (LB) (6, 7) for DNA manipulations and in nutrient broth (NB; Difco) for overnight cultures used as inocula. LB medium containing 1.5% Bacto Agar (Difco) was used as solid agar medium where indicated. No-carbon essential (NCE) medium (5) was used as minimal medium supplemented with $MgSO_4$ (1 mM), methionine (0.5 mM), and trace minerals (2, 19). Additional supplements were added as indicated. When used, antibiotics were added to the culture medium at the concentrations in parentheses: ampicillin (100 μ g/ml), kanamycin (50 μ g/ml), and chloramphenicol (25 μ g/ml).

Construction of plasmids and strains. Strains and plasmids used in this study are shown in Table 1. All DNA-modifying enzymes were purchased from Fermentas unless otherwise stated. Restriction endonuclease *SacI* was purchased from Promega. All cloning was done in $CaCl_2$ competent *Escherichia coli* DH5 α /F' (New England Biolabs) using established protocols (33). Plasmids were mobilized into *S. enterica* strains as follows. Overnight cultures grown from an isolated colony were diluted 1:100 into LB medium supplemented with appropriate antibiotics. Cultures were grown to approximate mid-log phase (optical density at 650 nm [OD_{650}] = 0.6 to 0.8), and 1.5 ml of cell culture was harvested by centrifugation at 18,000 \times g in a Beckman Coulter Microfuge 18 centrifuge. Cells were washed three times in 1 ml of ice-cold sterile water and resuspended in 100 μ l of water. Plasmids were electroporated into the cells using a Bio-Rad Gene Pulser following manufacturer's instructions.

Plasmid pFBP2. The *fbp* gene of *S. enterica* was amplified using primers 5'-TAC GGT CGA ATT CCT CCA ATC AAT-3' and 5'-CAA TGG CGT CTA GAT GCG TTA TTC-3'. The resulting fragment (~1 kb) was resolved in a 1% agarose gel, extracted from the gel using the QIAquick gel extraction kit (Qiagen), cut with the restriction endonucleases *EcoRI* and *XbaI*, and ligated with T4 DNA ligase into vector pBAD30 (22) cut with the same enzymes. The resulting plasmid, pFBP2, was transformed into *E. coli* DH5 α /F', and cells were plated on LB agar supplemented with ampicillin.

Plasmid pAMN1. The *amn* gene of *S. enterica* was amplified using primers 5'-ATC GAA TGA GCT CCC TCA CCT GTG AAC GCT-3' and 5'-TAC GCC TCT AGA GCT CCT GTC CAG CAG CAG-3'. The resulting DNA fragment, ~1.5 kb, was resolved and isolated as described above and cut with restriction endonucleases *SacI* and *XbaI*. The resulting DNA fragment was ligated with T4 DNA ligase into vector pBAD30 and transformed into *E. coli* DH5 α /F'. Cells were plated on LB agar supplemented with ampicillin.

Phage P22-dependent transductions. Transductions were performed using the high-frequency-of-transduction, generalized transducing phage strain P22 HT105 *int-201* according to established protocols (14, 19, 49, 50).

Isolation of an *fbp* allele encoding a variant enzyme that allows a 2-MC synthase (*prpC*) strain to grow in the presence of propionate. Localized chemical mutagenesis was performed as previously described (27). Briefly, bacteriophage

was grown on strain JE10713 that contained a *cat*⁺ cassette replacing *iolR* (formerly STM4417) (36) located near the *fbp* gene. Bacteriophage P22 was concentrated at 39,000 \times g for 2 h at 4°C in a Beckman Coulter Avanti J-25I centrifuge using a JA-25.50 rotor. Hydroxylamine mutagenesis was monitored by a plaque assay (19) using strain TR6583 as an indicator until a 2-log decrease in the titer of PFU was observed. Recipient strain JE2170 (*prpC114::MudJ*) was transduced to chloramphenicol resistance using mutagenized bacteriophage as donor. Recombinants were replica printed onto NCE minimal medium plates containing succinate (30 mM) and propionate (10 mM) as carbon and energy sources, kanamycin, and chloramphenicol. Any colonies that grew on minimal medium were restested to confirm the phenotype and reconstructed by bacteriophage P22-mediated transduction, and the *fbp* allele was sequenced. For the purpose of sequencing *fbp*, the latter was PCR amplified from each strain using primers 5'-TAC GGT CGA ATT CCT CCA ATC AAT-3' and 5'-CAA TGG CGT CTA GAT GCG TTA TTC-3'. The amplified *fbp* fragment was purified by gel extraction using the Qiagen PCR purification kit and sequenced with non-radioactive BigDye protocols (ABI Prism) with the above primers and internal primers 5'-CCG GTC ACC GAA GAA GAT-3' and 5'-CTT TTT CCG GGA AAC GCA-3'. Sequencing reaction mixtures were purified using the CleanSEQ reaction cleanup procedure (Agencourt Biotechnology) and resolved at the UW-Madison Biotechnology Center.

Preparation and use of cell extracts. Cell extracts were prepared as follows. Overnight cultures (100 ml) were grown in LB supplemented with kanamycin and chloramphenicol. Cells were harvested by centrifugation in a Beckman Coulter Avanti J-25I centrifuge using a JLA 16.250 rotor at 10,000 \times g for 15 min at 4°C. Cells were resuspended in 0.5 ml of HEPES buffer (50 mM, pH 7.5) containing KCl (100 mM). Cells were broken by sonication (50 s, 50% duty, 0.5-s pulses, setting 5) with a 500 sonic dismembrator (Fisher Scientific). Cell debris was removed by centrifugation at 16,100 \times g for 25 min in an Eppendorf 5415 D centrifuge at 4°C. Supernatants were then filtered through a 0.45- μ m filter (Thermo Fisher Scientific). Filtered cell extracts were dialyzed for 1 h at 4°C against 1 liter of potassium phosphate buffer (10 mM, pH 7.5) containing dithiothreitol (DTT; 1 mM), EDTA (1 mM), and glycerol (20%, vol/vol).

Dialyzed cell extracts were used in reaction mixtures that contained Tris-HCl buffer (100 mM, pH 7.5, at 37°C) containing $MgCl_2$ (10 mM), $(NH_4)_2SO_4$ (2 mM), EDTA (50 μ M), β -mercaptoethanol (5 mM), glucose-6-phosphate dehydrogenase (192 U/mg; 2.5 μ g/ml), phosphoglucoisomerase (701 U/mg; 2.5 μ g/ml), NADP⁺ (0.2 mM), and fructose-1,6-bisphosphate (35 μ M). Reaction mixtures were preincubated at 37°C for 5 min before the addition of 500 μ g of cell extract protein to start the reaction. Reactions were performed in triplicate and were monitored using a Perkin-Elmer Lambda 40 UV/Vis spectrometer at 340 nm; temperature was maintained using a circulating water bath set at 37°C. Data

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype ^a	Source or reference
Bacterial strains		
<i>E. coli</i>		
BL21(λDE3)	F ⁻ <i>ompT hsdS_B(r_B⁻ m_B⁺) dcm gal</i> (DE3)	New England Biolabs
DH5α/F'	F'/endA1 <i>hsdR17(r_K⁻ m_K⁺) supE44 thi-1 recA1 gyrA (Nal^r) relA1 Δ(lacZYA-argF)U169 deoR [φ80dlacD(lacZ)M15]</i>	New England Biolabs
<i>S. enterica</i>		
TR6583	<i>metE205 ara-9</i>	K. Sanderson via J. Roth
Derivatives of TR6583		
JE2170	<i>prpC114::MudJ</i>	23
JE4175	pBAD30	Lab collection
JE4199	<i>prpC114::MudJ/pPRP35</i>	54
JE4440	<i>prpC114::MudJ/pBAD30</i>	54
JE8431	Δ <i>prpC134::cat</i> ⁺	
JE8571	Δ <i>prpC254</i>	
JE10609	<i>prpC114::MudJ/pFBP2</i>	
JE10713	<i>iolR1::cat</i> ⁺	Lab collection
JE10831	<i>prpC114::MudJ ΔiolR1::cat</i> ⁺	
JE11237	<i>prpC114::MudJ ΔiolR1::cat</i> ⁺ <i>fbp121</i>	
JE12230	Δ <i>prpC254 gltA1182::MudJ</i>	
JE12239	Δ <i>prpC254/pBAD30</i>	
JE12240	Δ <i>prpC254/pGLTA2</i>	
JE12241	Δ <i>prpC254 gltA1182::MudJ/pBAD30</i>	
JE12242	Δ <i>prpC254 gltA1182::MudJ/pGLTA2</i>	
JE12243	Δ <i>prpC254 gltA1182::MudJ/pPRP35</i>	
JE12246	Δ <i>prpC254/pPRP35</i>	
JE12516	<i>prpC114::MudJ/pAMN1</i>	
JE12597	pAMN1	
JE11377	<i>prpD126::cat</i> ⁺ <i>gltA1182::MudJ</i>	
JE11392	<i>prpD126::cat</i> ⁺ <i>gltA1182::MudJ/pPRP35</i>	
JE11393	<i>prpD126::cat</i> ⁺ <i>gltA1182::MudJ/pGLTA2</i>	
JE11394	<i>prpD126::cat</i> ⁺ <i>gltA1182::MudJ/pBAD30</i>	
JE11397	<i>prpC114::MudJ/pGLTA2</i>	
Plasmids		
pBAD30	Cloning vector; pACYC184 origin of replication, <i>bla</i> ⁺ (Amp ^r); expression of the gene of interest under the control of the arabinose-inducible P _{araBAD} promoter	22
pFBP2	<i>fbp</i> ⁺ cloned into pBAD30	
pGLTA2	<i>gltA</i> ⁺ cloned into pBAD30	28
pPRP35	<i>prpC</i> ⁺ cloned into pBAD30	54
pAMN1	<i>amn</i> ⁺ cloned into pBAD30	

^a MudJ is an abbreviation of MudI1734 (13).

collection and analysis were performed with Perkin-Elmer UV Kinlab software. An extinction coefficient of 6.22 mM⁻¹ cm⁻¹ was used for NADPH (20).

In-frame deletion of *prpC*. An in-frame insertion of a chloramphenicol resistance cassette in the *prpC* gene was constructed using the phage λ Red recombinase as described previously (18). Primers 5'-ATG ACA GAC ACG ACG ATC CTG CAA AAC AAC ACG CAT GTC ATT AAG CCT AAA GTG TAG GCT GGA GCT GCT TC-3' and 5'-TTA GCA ACG ATC GTC TAT CGA GAC AAA CGG ACG ATC TTC CGG CCC GGT ATA CAT ATG AAT ATC CTC CTT AG-3' were used to amplify the *cat*⁺ gene and to direct the insertion to the *prpC* gene, resulting in strain JE8431. The insertion was resolved utilizing methods described elsewhere (18); the resulting chloramphenicol-sensitive (Cm^s) strain was JE8571.

Growth behavior analyses. We analyzed the growth behavior of strains of interest in liquid NCE medium. Carbon sources utilized were glycerol (10 mM), glucose (0.3 mM), or succinate (30 mM, pH 7.0 at 25°C) with or without propionate (pH 7.0 at 25°C). Strains were grown from single colonies overnight in NB medium supplemented with antibiotic. Fresh medium (196 μl) was inoculated with ~8 × 10⁶ CFU and grown in 96-well microtiter plates (Becton Dickinson). Growth was monitored in an ELx808 plate reader (Bio-Tek Instruments) at 37°C. Absorbance readings were taken every 15 min at 630 nm with 850 s of shaking between readings; each culture was grown in triplicate. Data were graphed using Prism v4.0 software (GraphPad Software).

RESULTS

2-Methylcitrate causes glucose auxotrophy in *S. enterica*.

Previous work by others demonstrated that active FBPase was stabilized when citrate was bound to it (25). We hypothesized that if FBPase bound citrate, it might also bind 2-MC, and in so doing, 2-MC might prevent citrate binding, rendering the enzyme unstable. If this idea were true, then *S. enterica* strains that accumulate 2-MC should have a defect in gluconeogenesis and thus require exogenous glucose to grow. To test this possibility, we grew strain JE2170 (*prpC114::MudJ*) on succinate (30 mM) and propionate (30 mM), with or without the addition of micromolar amounts of glucose (0.3 mM) to the medium. We predicted that if the accumulation of 2-MC in the cell blocked gluconeogenesis, the addition of glucose would restore growth. In Fig. 2 we show that, while a *prpC*⁺ strain (TR6583) grew in medium containing succinate plus propionate as carbon and energy sources, a *prpC* strain (JE2170) did not. However, the addition of glucose to the medium restored

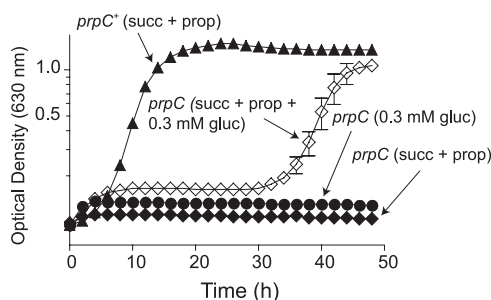


FIG. 2. Glucose circumvents the problem caused by the presence of propionate in the medium. Growth curves were determined in NCE medium. Succinate and propionate were each at 30 mM. A *prpC*⁺ strain (closed triangles) grew on succinate plus propionate medium, while a *prpC* strain (closed diamonds) did not. However, the addition of glucose (0.3 mM) to the medium allowed the *prpC* strain to grow (open diamonds). The *prpC* strain did not grow when 0.3 mM glucose was the sole carbon and energy source (closed circles).

growth of strain JE2170 on succinate plus propionate, albeit after a 30-h lag. The fact that strain JE2170 grew to full density when given small concentrations of glucose suggested that propionate toxicity was, among other things, negatively affecting gluconeogenesis.

Increased levels of FBPAse relieve propionate toxicity. To investigate whether 2-MC destabilized FBPAse, the *fbp*⁺ gene was cloned into pBAD30 and the resulting plasmid (pFBP2) was introduced into strain JE2170, yielding strain JE10609. We posited that induction of *fbp*⁺ could provide enough FBPAse to alleviate the negative effect of 2-MC. Induction of *fbp*⁺ expression restored growth of strain JE10609 when low concentrations of propionate (≤ 5 mM) were present in the medium (Fig. 3) but did not restore growth at higher concentrations of propionate (data not shown). This result was not unexpected, since most likely cells could not produce enough FBPAse to match or surpass the intracellular level of 2-MC.

As a control, we expressed a protein that was not involved in gluconeogenesis or propionate metabolism, with the expectation that expression of such a gene should not improve growth

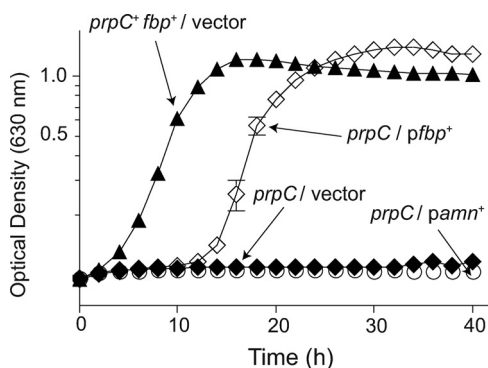


FIG. 3. Increased levels of FBPAse restore growth of a *prpC* strain in the presence of propionate. Growth curves were determined in NCE medium supplemented with arabinose (10 mM). Carbon/energy sources were succinate (30 mM) and propionate (5 mM). Cells with functional *prpC* and *fbp* genes (JE4175, closed triangles) grew, while *prpC* cells carrying the cloning vector (closed diamonds) or expressing *amn* (AMP nucleosidase, open circles) did not.

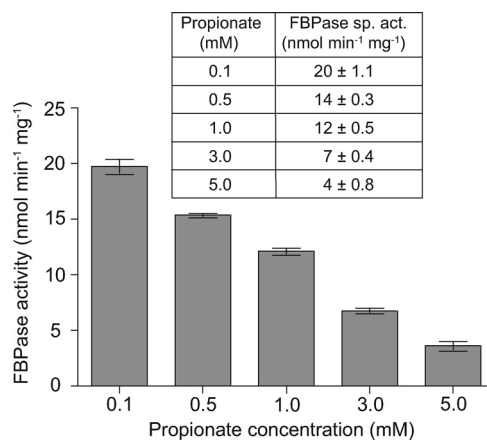


FIG. 4. FBPAse activity in cell extracts grown in the presence of propionate. Cell extracts of strain JE10609 (*prpC/pfbp*⁺) were prepared from overnight cultures grown in NCE minimal medium supplemented with succinate (30 mM) plus propionate varying from 0.1 mM to 5 mM. Specific activities were measured using 100 μ g of cell extract and are reported as nmol min⁻¹ mg⁻¹.

of strain JE2170 on succinate plus propionate. In this case, we used the *amn* gene (encoding AMP nucleosidase), yielding strain JE12516. Expression of *amn*⁺ from plasmid pAMN1 did not restore growth on succinate plus propionate (5 mM) (Fig. 3). Additionally, expression of pAMN1 in a wild-type (*prp*⁺) background (JE12597) did not result in a decrease in growth, indicating that the lack of growth of JE12516 was not due to increased levels of Amn in the cell (data not shown). These results were consistent with the argument that higher levels of FBPAse alleviate the negative effect of 2-MC.

To determine whether FBPAse activity was directly affected by the presence of propionate in the medium, cell extracts were prepared from overnight cultures of JE10609 grown in various concentrations of propionate. The specific activity of FBPAse in each culture was determined, and the results showed a marked decrease in FBPAse activity as a function of increasing propionate concentration in the medium (Fig. 4). These results were consistent with those presented in Fig. 2, where we showed that overexpression of FBPAse overcame the negative effects of 2-MC.

A single amino acid variant of Fbp makes *S. enterica* resistant to propionate. To further understand the role of FBPAse in propionate toxicity, localized chemical mutagenesis of *fbp* was performed in an attempt to isolate FBPAse variants resistant to 2-MC. Hydroxylamine-mutagenized phage P22 grown on strain JE10713 (*iolR1::cat*⁺) was used as donor to transduce strain JE2170 to chloramphenicol resistance. Cm^r transductants were screened for strains that could grow on succinate plus propionate. Strains with the latter phenotype were used to grow P22, and the resulting lysate was used to transduce strain JE2170 to chloramphenicol resistance, again screening for growth on succinate plus propionate. Analysis of the *fbp* gene in the reconstructed strain (JE11237) identified one mutation, which resulted in an S123F substitution. Growth curve analysis of strain JE11237 compared to its isogenic pair (*fbp*⁺, JE10831) indicated that strain JE11237 tolerated the presence of propionate, even at high concentrations (Fig. 5). While cultures of strain JE11237 reached full density when grown in the presence of as much as 30 mM propionate, strain JE10831 could

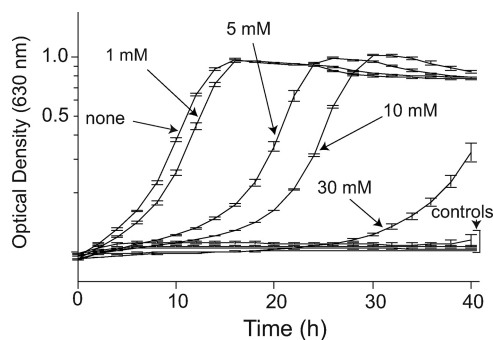


FIG. 5. A single amino acid substitution in FBPase confers increased resistance to propionate. Growth curves were determined in NCE minimal medium; carbon and energy sources were succinate (30 mM) and propionate ranging from 30 mM to 1 mM. Strain JE11237 (*prpC iolR fbp121* [Fbp(S123F)]) grew in the presence of as much as 30 mM propionate. The control isogenic strain JE10831 (*prpC iolR fbp*⁺) was grown under the same conditions as strain JE11237. A no-propionate, succinate-only control is shown to indicate growth rates for the strains when propionate was absent. No significant difference was observed when the strains were grown on succinate.

not grow in the presence of even 1 mM propionate over the course of the experiment (Fig. 5).

Biochemical analysis of FBPase activities was performed using cell extracts of strains JE10831 and JE11237. The Fbp^{S123F} variant protein was active, but its specific activity was only about 10% of that of the wild-type enzyme ($39 \pm 3 \text{ nmol min}^{-1} \text{ mg}^{-1}$ for JE10831 versus $4 \pm 0.3 \text{ nmol min}^{-1} \text{ mg}^{-1}$ for JE11237).

2-Methylcitrate synthesized by GltA (2-MC^{GltA}) is more toxic than the 2-methylcitrate synthesized by PrpC (2-MC^{PrpC}). Experiments were undertaken to determine if there was a connection between the effect of 2-MC^{GltA} and the lack of growth of a *prpC* strain on succinate plus propionate. Previous work from our laboratory showed that GltA can condense Pr-CoA and OAA to yield 2-MC and that decreased levels of GltA alleviated propionate toxicity (28). At the time, it was unclear whether the observed effect was due to a buildup of 2-MC or whether 2-MC made by GltA was the problem.

We took a genetic approach to distinguish between these possibilities. For this purpose an in-frame deletion of *prpC* ($\Delta prpC$, strain JE8571) was constructed to allow for the expression of *prpD*, a downstream gene in the propionate operon and the subsequent biochemical step in the 2-MC cycle. If PrpD used 2-MC^{GltA} as substrate, overproduction of GltA should have no effect on the growth of a $\Delta prpC$ strain on succinate plus propionate because the strain would have a functional 2-MC cycle that would convert 2-MC^{GltA} to pyruvate and succinate (29, 31). If PrpD could not use 2-MC^{GltA}, then a $\Delta prpC$ strain would not grow, suggesting that 2-MC^{GltA} was probably toxic. Additionally, propionate would not be toxic to a $\Delta prpC gltA$ strain (JE12230) because 2-MC could not be synthesized.

Plasmids that carried either *prpC*⁺ or *gltA*⁺ were introduced into the above-mentioned strains, and the growth behavior of the resulting strains was assessed in medium containing glycerol (10 mM) as carbon and energy source. We used glycerol instead of succinate because the effect of propionate on growth of *prp* strains is less severe when glycerol is the source of

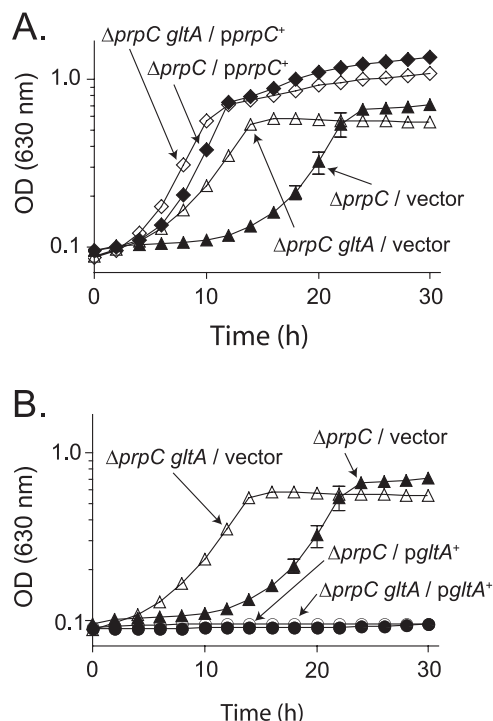


FIG. 6. Overexpression of *gltA* is more toxic than overexpression of *prpC*. Growth curves were determined in NCE medium supplemented with L-(+)-arabinose (5 mM) and glutamate (5 mM). Carbon sources were glycerol (10 mM) and propionate (30 mM). Strains containing plasmid pPRP35 or pGLTA2 are shown in panels A and B, respectively. A strain carrying a chromosomal in-frame deletion of *prpC* ($\Delta prpC$) and an empty cloning vector (closed triangles) and a $\Delta prpC gltA$ strain carrying an empty vector (open triangles) were used as controls and are shown in both sets of graphs. The $\Delta prpC/pPRP35$ and the $\Delta prpC gltA/pPRP35$ strains are displayed as closed and open diamonds, respectively. The $\Delta prpC/pGLTA2$ and the $\Delta prpC gltA/pGLTA2$ strains are shown as closed and open circles, respectively.

carbon and energy. While *prp* strains do not grow on succinate plus propionate, they do grow on glycerol plus propionate, albeit with a distinct lag (23).

When grown on glycerol plus propionate, a $\Delta prpC$ strain harboring a vector control (JE12239) showed a significant lag in growth, while a $\Delta prpC gltA$ (JE12241) strain displayed a much less severe lag (Fig. 6A). The decrease in lag of the JE12241 culture was attributed to the absence of 2-MC synthesis in this strain, most likely because in the absence of 2-MC FBPase would not be destabilized, thus allowing the cells to make glucose and grow. As expected, the introduction of plasmid pPRP35 (*prpC*⁺) into strain JE12239 (resulting in strain JE12246) and into strain JE12241 (resulting in strain JE12243) lessened the lag to an even greater extent and allowed cells to grow to a higher final density (Fig. 6A). Conversely, in strains JE12240 ($\Delta prpC/pGLTA2 gltA$ ⁺) and JE12242 ($\Delta prpC gltA/pGLTA2 gltA$ ⁺) the presence of plasmid pGLTA2 prevented growth (Fig. 6B), consistent with the idea that PrpD cannot utilize 2-MC^{GltA}. These results suggested that while 2-MC made by either PrpC or GltA was inhibitory, 2-MC^{GltA} was a more powerful inhibitor.

Experiments were performed to block detoxification of 2-MC by PrpD and to determine the relative toxicity of

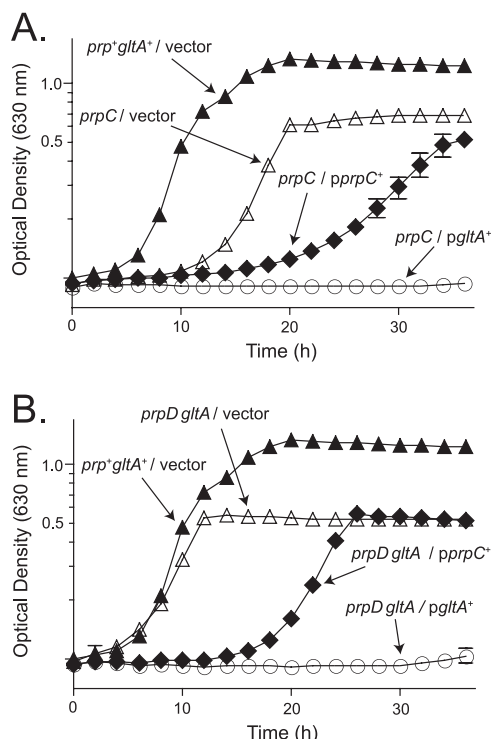


FIG. 7. 2-MC^{GltA} is more toxic than 2-MC^{PrpC}. Growth curves were determined in NCE medium supplemented with L-(+)-arabinose (5 mM) and glutamate (5 mM). Glycerol (10 mM) and propionate (30 mM) were used as carbon and energy sources. Data in panel A show that an insertion in *prpC* has polar effects on the expression of *prpC* and *prpD*. (A) Overexpression plasmid pPRP35 (*prpC*⁺) results in a defect in growth of the strain (closed diamonds) compared to the vector-only control (open triangles), while overexpression of plasmid pGLTA2 (*gltA*⁺) blocks growth (open circles). (B) A *prpD gltA* strain had a growth rate comparable to a wild-type strain (open triangles versus closed triangles). While overexpression of plasmid pPRP35 resulted in a growth defect (closed diamonds), overexpression of plasmid pGLTA2 resulted in a much more severe defect in growth (open circles).

2-MC^{PrpC} and 2-MC^{GltA} accumulation. Plasmids pPRP35 and pGLTA2 were introduced into the strain harboring a polar insertion in *prpC* (JE2170), yielding strains JE4199 and JE11397, respectively. When grown on glycerol plus propionate (30 mM), strain JE4199 showed a significant defect in growth compared to the vector control (Fig. 7A). However, strain JE11397 showed an even more severe defect under the same conditions (Fig. 7A).

We constructed derivatives of a *prpD gltA* strain (JE11377) that carried plasmid pPRP35 (*prpC*⁺; JE11392) or pGLTA2 (*gltA*⁺; JE11393). When grown in minimal medium with glycerol plus propionate as carbon sources, the control strain JE11394 (vector) grew at almost the same rate as JE4175, the wild-type strain carrying the empty cloning vector (Fig. 7B, open versus closed triangles). Introduction of plasmid pPRP35 resulted in a significant defect in growth, while introduction of pGLTA2 blocked growth within the time limits of the experiment (Fig. 7B). These results showed that PrpD did not catabolize 2-MC^{GltA} and that 2-MC^{GltA} was more toxic than 2-MC^{PrpC}.

DISCUSSION

Here we report data supporting conclusions from previous work which suggested that 2-MC, not propionate or propionyl-CoA, is a powerful inhibitor of cell growth. The data reported herein are consistent with the conclusion that 2-MC synthesized by citrate synthase inhibits FBPase, a key enzyme of gluconeogenesis.

FBPase is a target for 2-MC inhibition. FBPase is a gluconeogenesis-specific enzyme whose activity is regulated by activators and inhibitors, both directly and allosterically. These activators and inhibitors interconvert the FBPase homotetramer between its active R state and inactive T state (25, 57). Specific allosteric inhibitors of FBPase include AMP (51), glucose 6-phosphate (26), and 5-amino-4-imidazole carboxamide ribotide (AICAR) (21). Activators include phosphoenolpyruvate (PEP), citrate, and sulfate ions (24, 25). The fact that 2-MC inhibits FBPase is not surprising since citrate functions allosterically to stabilize the active R state of FBPase, preventing the conversion of the tetramer to an inactive form (25). While the structural similarities between 2-MC and citrate may allow 2-MC to bind to FBPase, the stereochemical properties of 2-MC may ultimately inhibit FBPase activity. In-depth mechanistic studies are needed to determine if this is the case.

That the S123F substitution in FBPase allowed a $\Delta prpC$ strain of *S. enterica* to grow in the presence of propionate was a surprising result because residue S123 is neither near the citrate-binding site nor near the active site of the enzyme. At present we do not understand how the S123F substitution might prevent the inhibitory effects of 2-MC, but we suspect that it might not be a direct effect on citrate binding or substrate binding. Residue S123 does appear to be on the C-1-C-2/C-3-C-4 interfaces of the FBPase tetramer and could possibly affect the ability of FBPase to interconvert between the active R state and inactive T state. The FBPase^{S123F} protein had only 10% of the activity of the wild-type FBPase and was less stable. Here again, additional studies are needed to understand the effect of the S123F substitution on the activity of FBPase and how it might relieve the inhibitory effects of 2-MC.

Not all 2-MCs are equally inhibitory. The data show that the severity of the 2-MC toxicity correlates with its source. It is known that citrate synthase (GltA) synthesizes 2-MC and that a buildup of 2-MC in the cell inhibits growth (28). The experiments shown here indicate that the 2-MC synthesized by GltA (2-MC^{GltA}) has stronger inhibitory effects than the 2-MC synthesized by PrpC (2-MC^{PrpC}). One explanation for this observation is that the *S. enterica* GltA enzyme is a *si*-citrate synthase, which synthesizes three different isomers of 2-MC (2*S*,3*S*; 2*S*,3*R*; and 2*R*,3*S*) when propionyl-CoA and oxaloacetate serve as substrates (55). PrpC, on the other hand, synthesizes a single 2-MC isomer (2*S*,3*S*) (11). This fact helps us understand why a strain containing an in-frame deletion of *prpC* fails to grow when *gltA* is overexpressed but can grow when *prpC* is overexpressed (Fig. 5). PrpD, the 2-MC dehydratase, is also a stereospecific enzyme, utilizing only the (2*S*,3*S*)-2-MC isomer (11). The (2*S*,3*R*) and the (2*R*,3*S*) isomers of 2-MC produced by GltA could build up to levels that become inhibitory to the cell. If the 2-MC^{GltA} isomers accumulate inside the cell, it would provide a rationale for why one or more of the 2-MC^{GltA} isomers is a stronger inhibitor of cell growth than 2-MC^{PrpC}.

What is the meaning of the observed lag? We can only speculate on this issue. At present, we suspect that the observed lag may reflect on additional negative effects of 2-MC^{GltA} on as-yet-undefined processes. Because the cell eventually recovers from these effects (as long as the glucose is provided), we suggest that, whatever the response the cell mounts to solve other problems caused by 2-MC^{GltA}, it takes time. We have isolated additional propionate-resistant strains with lesions in loci other than *fbp* whose function appears to be targets of 2-MC^{GltA}. We are currently trying to establish the identity of these loci.

Our results may shed light on human inborn errors in propionate metabolism. In humans, propionate is catabolized via the methylmalonyl-CoA mutase pathway, not via the 2-methylcitric acid cycle. In the former pathway, propionyl-CoA is converted to succinyl-CoA by carboxylation, isomerization, and a C skeleton rearrangement. The product of the pathway, succinyl-CoA, is an intermediate of the tricarboxylic acid (TCA) cycle and is readily metabolized by the cell. Mutations affecting the carboxylase function of the methylmalonyl-CoA pathway lead to propionic acidemia, an often-fatal metabolic disorder. Interestingly, reports in the literature show that patients with a deficiency in propionyl-CoA carboxylase accumulate 2-MC in their urine and cerebrospinal and amniotic fluids (3, 16, 35, 42). Conclusions drawn from our work suggest that in patients deficient in propionyl-CoA carboxylase, 2-MC is synthesized by the citrate synthase enzyme, leading to broad negative effects and eventually cell death.

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