Sugar-Phosphate Toxicities Attenuate Salmonella Fitness in the Gut

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ABSTRACT  Pathogens are becoming resistant to antimicrobials at an increasing rate, and novel therapeutic strategies are needed. Using Salmonella as a model, we have investigated the induction of sugar-phosphate toxicity as a potential therapeutic modality. The approach entails providing a nutrient while blocking the catabolism of that nutrient, resulting in the accumulation of a toxic intermediate. We hypothesize that this build-up will decrease the fitness of the organism during infection given nutrient availability. We tested this hypothesis using mutants lacking one of seven genes whose mutation is expected to cause the accumulation of a toxic metabolic intermediate. The araD, galE, rhaD, glpD, mtlD, manA, and gaiT mutants were then provided the appropriate sugars, either in vitro or during gastrointestinal infection of mice. All but the glpD mutant had nutrient-dependent growth defects in vitro, suggestive of sugar-phosphate toxicity. During gastrointestinal infection of mice, five mutants had decreased fitness. Providing the appropriate nutrient in the animal’s drinking water was required to cause fitness defects with the rhaD and manA mutants and to enhance the fitness defect of the araD mutant. The galE and mtlD mutants were severely attenuated regardless of the nutrient being provided in the drinking water. Homologs of galE are widespread among bacteria and in humans, rendering the specific target of bacterial pathogens difficult. However, the araD, mtlD, and rhaD genes are not present in humans, appear to be rare in most phyla of bacteria, and are common in several genera of Enterobacteriaceae, making the encoded enzymes potential narrow-spectrum therapeutic targets.

IMPORTANCE  Bacterial pathogens are becoming increasingly resistant to antibiotics. There is an urgent need to identify novel drug targets and therapeutic strategies. In this work we have assembled and characterized a collection of mutations in our model pathogen, Salmonella enterica, that block a variety of sugar utilization pathways in such a way as to cause the accumulation of a toxic sugar-phosphate. Mutations in three genes, rhaD, araD, and mtlD, dramatically decrease the fitness of Salmonella in a mouse model of gastroenteritis, suggesting that RhaD, AraD, and MtlD may be good narrow-spectrum drug targets. The induction of sugar-phosphate toxicities may be a therapeutic strategy that is broadly relevant to other bacterial and fungal pathogens.

KEYWORDS  Salmonella, gastroenteritis, colitis, narrow-spectrum antimicrobial, sugar-phosphate, sugar metabolism
than 40 years ago, with a subsequent decline in the discovery rate (1). In 2017 the World Health Organization (WHO) published Prioritization of Pathogens To Guide Discovery, Research and Development of New Antibiotics for Drug-Resistant Bacterial Infections, Including Tuberculosis, recommending increased research efforts toward identification of novel antimicrobial targets in order to support the development of alternative classes of antibiotics (2). In this work, we explore the induction of sugar-phosphate toxicities as a novel therapeutic modality.

Sugar-phosphates are common intermediates of carbohydrate metabolic pathways (Fig. 1). These intermediates are quickly converted to the subsequent compound in the metabolic pathway by a downstream enzyme in that pathway. However, if that enzyme is inhibited, either by a small molecule or by mutation of the gene encoding it, the sugar-phosphate substrate of that enzyme will not be metabolized and will accumulate, thus leading to wide-ranging toxic effects (3). Consequently, the overall therapeutic strategy is to provide the sugar of interest to the organism while simultaneously inhibiting the appropriate enzyme in that sugar utilization pathway to induce toxic sugar-phosphate accumulation.

The 2017 WHO report recommended continued research and development strategies for novel antibiotics that target “common community bacteria,” explicitly naming resistant Salmonella species and carbapenem-resistant Enterobacteriaceae (CRE) (2). In 2019 the CDC followed with its report on “Antibiotic Resistance Threats in the United States,” considering the CRE an “urgent” priority and the fluoroquinolone-resistant nontyphoidal Salmonella serovars “serious” priorities (4). Salmonella enterica is a Gram-negative, facultative anaerobe that encompasses over 2,500 serovars, many of which cause diseases ranging from self-limiting gastroenteritis to systemic typhoid fever (5, 6). Salmonella enterica is among the top causes of hospitalization and death by foodborne pathogens in the United States and globally (7–9). Symptoms of nontyphoidal Salmonella infection include an inflammatory diarrhea with acute abdominal pain, sometimes with a fever lasting up to a week. Though most cases are self-limiting, more complicated cases can cause bloodstream infections, especially in young children (under 5 years old), the elderly, and the immunocompromised, and these require therapeutic intervention. Oddly, for the vast majority of uncomplicated infections that remain within the intestinal tract, antibiotics are often ineffective or can even worsen symptoms or prolong Salmonella shedding (10–15). This outcome may be because broad-spectrum antibiotics eliminate the colonization resistance provided by the normal microbiota. Such an idea aligns with the pathogenesis of Salmonella, as disruption of the microbiota is of critical importance to Salmonella infection. To disrupt the microbiota, Salmonella uses a pair of type III secretion systems (T3SSs) to promote the invasion of epithelial cells and to provoke a strong inflammatory response (16–21). The inflammatory response disrupts the normal microbiota and causes nutrients and respiratory electron acceptors to accumulate (22–27). Salmonella then thrives on these nutrients, becoming greater than 50% of the gut microbial population (22, 28, 29). In contrast to broad-spectrum antibiotics, a narrow-spectrum therapeutic that exclusively targets Salmonella in the gastrointestinal tract without disrupting the normal microbiota could potentially alleviate the symptoms and shorten the duration of uncomplicated infections as well as reduce the number of infections that progress to the bloodstream.

In this work, we assembled a collection of mutations in seven genes that we hypothesized would cause a sugar-phosphate toxicity and characterized these mutants in vitro and in a mouse model of Salmonella-mediated gastroenteritis. Our findings suggest that the mannitol, rhamnose, and arabinose utilization pathways each contain an enzyme (MtlD, RhaD, and AraD, respectively) that could be inhibited to induce a potent sugar-phosphate toxicity that dramatically attenuates the fitness of Salmonella within the mouse gastrointestinal tract. We then used a bioinformatics approach to determine that these three enzymes would likely provide narrow-spectrum targets for
FIG 1 Metabolic pathways of selected carbohydrates. Each sugar utilization pathway is highlighted with a different color. The genes encoding the enzymes that cause sugar-phosphate accumulation when mutated, and the sugar-phosphates, are in red. TCA, tricarboxylic acid. This figure was adapted from reference 3.
the Enterobacteriaceae, as they are not present in humans and are uncommon among other bacteria.

RESULTS

Identification and mutation of genes predicted to cause sugar-phosphate toxicity in Salmonella. Throughout this work we use genetic mutations to eliminate the activity of the targeted enzymes. This strategy serves as a proxy for small-molecule inhibitors of those enzymes that would be used in real-world therapeutic applications. Most genes within a given sugar utilization pathway are required for utilization of only that sugar, rendering those mutants unable to grow if the sugar of interest is the sole nutrient source (i.e., a galK mutant is galactose negative). We will refer to these as sugar utilization genes. The genes that give rise to sugar-phosphate toxicity when mutated are both negative and sensitive. Such mutants are unable to grow on other nutrients when the sugar of interest is present due to the sugar-phosphate accumulation causing toxicity (i.e., a galE mutant is both galactose negative and galactose sensitive). For brevity, we refer to these as sugar-phosphate genes. Also for simplicity, we are referring to all of the phosphorylated metabolic intermediates in this work as sugar-phosphates, although glycerol-3-P (Gly-3P) and mannitol-1-P (Mtl-1P) are polyol-phosphates, and 6-phosphofructose-aspartate (6-P-F-Asp) is a phosphorylated Amadori product.

We recently reviewed the topic of sugar-phosphate toxicities (3). From that information, we assembled a list of 14 genes of Salmonella either that are known, or that we hypothesize, to cause a sugar-phosphate toxicity when mutated and assuming the appropriate sugar is provided (fraB, araD, galE, gaiT, otsB, pgI, pfkA, pfkB, rhaD, sgrS, fbaB, glpD, mtlD, and manA). Six of these are involved with glucose metabolism and are not discussed further, except for being included in phylogenetic analyses below. Another is fraB, which we have characterized extensively in the past and include here only for comparison (30–34). The remaining seven are characterized in this work (araD, galE, gaiT, rhaD, glpD, mtlD, and manA) (Fig. 1 and 2). Mutations in these seven genes were moved from the collection of Andrews-Polymenis and McClelland (available at BEI Resources) into our wild-type strain using phage P22 transduction. Typically, two types of mutations are available, an insertion of either a chloramphenicol (Camr) or kanamycin (Kanr) resistance gene into a deleted target gene. After moving these mutations into our laboratory strain, we then removed the antibiotic resistance gene using Flp recombinase, leaving a deletion and scar. Therefore, we have a Camr insertion, a Kanr insertion, and a deletion for each of the seven genes of interest, with the exceptions being that we are lacking an araD deletion mutant and a manA::kan insertion mutant.

The induction of sugar-phosphate toxicities in Salmonella leads to growth inhibition. Mutants predicted to suffer sugar-phosphate toxicity were grown in three types of media: (i) nutrient-rich lysogeny broth (LB), plus or minus the sugar of interest; (ii) M9 minimal medium with fructose, plus or minus the sugar of interest; and (iii) M9 containing only the sugar of interest (no additional carbon source). d-Galactose was used as the sugar of interest with the galE and gaiT mutants, l-rhamnose with the rhaD mutants, glycerol with glpD mutants, d-mannitol with mtlD mutants, d-mannose with manA mutants, and l-arabinose with araD mutants. As expected, each mutant grew normally in LB or M9 fructose if the sugar of interest was absent. However, they failed to grow if the sugar of interest was the only carbon source present, i.e., they were all sugar negative (Fig. 2). The addition of the sugar of interest to these media resulted in no growth defect for the glpD mutant and a modest growth defect for the manA mutant that occurred only in M9 medium and not in LB. On the other hand, the galE, gaiT, mtlD, and rhaD deletion mutants had substantial growth defects in both growth media. While we do not have a deletion mutant of araD, both the Camr and Kanr insertion mutants of araD had severe growth defects.

With some genes, different results are obtained with different mutations. Specifically, the Camr insertion mutations in the galE and gaiT genes do not confer growth defects in

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FIG 2 Sugar-phosphate toxicity causes inhibition of growth of \textit{Salmonella} mutants. (A) Genetic organization of sugar utilization loci in \textit{Salmonella enterica} strain ATCC 14028. The genes predicted to cause sugar-phosphate toxicity when mutated are in color. (B) Cultures grown overnight in LB were washed and subcultured 1:100 into LB or M9 plus fructose (5 mM), with or without the indicated sugar at 5 mM. (C) Cultures grown overnight in LB were washed and subcultured 1:100 into M9 plus fructose (5 mM), with or without the indicated sugar at 5 mM, or M9 plus the indicated sugar as the only carbon and energy source. Growth was measured by OD\textsubscript{600} readings every hour for 17 to 20 h. Error bars represent the standard deviation from two independent experiments performed in duplicate (n = 4).

The presence of galactose. The Cam\textsuperscript{r} insertions in the BEI Resources mutant collection are oriented opposite the gene being mutated and are polar. The \textit{galE} and \textit{galT} genes are contained within the same operon, and the Cam\textsuperscript{r} insertions are likely to disrupt expression of the downstream gene, \textit{galK} (Fig. 2). In the absence of \textit{galK}, galactose cannot be phosphorylated and so toxic intermediates do not accumulate in the \textit{galE} and \textit{galT} mutants. The Kan\textsuperscript{r} insertions in the BEI Resources mutant collection are in the opposite orientation from the Cam\textsuperscript{r} insertions, have no transcription terminator, and are not polar. Thus, in some cases downstream genes may be expressed at higher-than-wild-type levels due to the promoter of the Kan\textsuperscript{r} gene. We were unable to obtain a deletion mutant of \textit{araD}. Fortunately, \textit{araD} lies at the end of an operon, and so polarity should not be an issue. Indeed, the \textit{araD}:Cam and \textit{araD}:Kan insertions yield similar results in growth curves with both being inhibited by the presence of arabinose (Fig. 2). Interestingly, the \textit{mtlD}:Cam mutant has a more severe growth defect than either the \textit{mtlD}:Kan or \textit{mtlD} deletion mutant. This defect is likely due to polarity on \textit{mtlR}, a repressor of mannitol utilization (Fig. 2). In the absence of the repressor, the mannitol
phosphotransferase system (PTS) transporter, encoded by mtlA, may have abnormally high expression, leading to increased concentrations of Mtl-1P. We do not suspect that polarity has an impact on the toxicity phenotypes observed with the rhaD mutants, as rhaD lies at the end of an operon and all three mutations confer the same phenotype (Fig. 2).

In contrast to the sugar-phosphate genes, most of the other genes in sugar utilization pathways cause only an inability to utilize a particular sugar when mutated. Such mutants are not intoxicated by the presence of that sugar (i.e., they are sugar negative, not sugar sensitive). We demonstrate this phenotype using a rhaB, mtlA, and araB mutant. These mutants are unable to use rhamnose, mannitol, or arabinose, respectively, but grow like the wild type in the presence of each sugar as long as another carbon and energy source is available (fructose in this case) (Fig. 3).

**Measurement of sugar-phosphate accumulation.** To test the hypothesis that phosphorylated metabolic intermediates are indeed accumulating in these mutants, we performed targeted metabolomics using mass spectrometry (MS) on cell extracts of select sugar-phosphate mutants compared to wild-type controls grown under the same condition. In order to use a quantitative approach, standards of each sugar-phosphate being measured were required to determine the metabolite’s mass-over-charge ratio and the best transition state for quantitation and to build a standard curve. For this reason, we were limited by the commercial availability of compounds and thus examined only a subset. Mutants and wild-type controls were grown in LB supplemented with the appropriate sugar for 4 h, at which point cell pellets were harvested, metabolites were extracted, and liquid chromatographic-mass spectrometric analysis was performed. The results were normalized to total protein concentration. In this experiment, we found that mutants which exhibit growth defects do in fact accumulate the predicted sugar-phosphate compared to their wild-type controls (Fig. 4). More specifically, we find that the mtlD and araD mutants accumulate Mtl-1P and ribulose-5-phosphate (Ru-5P), respectively, compared to wild-type cells in which the sugar-phosphate concentrations are below the limit of detection. Additionally, as expected from

**FIG 3** Sugar utilization mutants do not experience toxicity and are unable to use the sugar of interest as a sole nutrient source. These sugar utilization mutants are those containing mutations in a gene whose enzyme functions upstream of the toxic sugar-phosphate in that pathway. Cultures grown overnight of the indicated mutants and wild type (WT) were washed and subcultured 1:100 into M9 containing fructose (5 mM), the indicated sugar only (5 mM), or both fructose and the indicated sugar (5 mM each). Growth was measured with OD$_{600}$ readings every hour for 20 h. The WT is strain 14028. Error bars represent the standard deviation from one experiment performed in triplicate ($n$ = 3).
previous work we observed the accumulation of 6-phosphofructose-aspartate (6-P-F-Asp) in a fraB mutant (30). In contrast, the manA mutant, which does not have a growth defect in the presence of mannose in LB, predictably does not have a detectable accumulation of mannose-6-P (Man-6P).

Sugar-phosphate toxicity attenuates the fitness of Salmonella in a mouse model of infection. Since sugar-phosphate toxicity inhibits the growth of Salmonella in vitro, we tested the hypothesis that these same mutants would be attenuated during infection of the murine gastrointestinal tract. We employed a competition style of experiment with a streptomycin-treated Swiss Webster mouse model (30). In this model, mice are treated with streptomycin 24 h prior to infection in order to disrupt the normal microbiota and promote Salmonella colonization (35). For infection, the inoculum is prepared as a 1:1 ratio of wild type and a mutant and is delivered via oral gavage at a dose of 1 × 10⁷ CFU. For these experiments, we used strains in which the gene of interest was deleted, and a kanamycin resistance gene was present in a separate, neutral location downstream of pagC (except for the araD mutant, which contained the kanamycin resistance gene within the araD gene) (32, 36). The wild-type strain was marked with a chloramphenicol resistance gene, in the same neutral location downstream of pagC, so that we could distinguish between wild type and mutant using selective medium. Because it was unclear at the start of these experiments which sugars, if any, would be available to Salmonella in the murine gastrointestinal tract, we performed the experiments with or without exogenous sugar (100 mM) provided in the animal’s drinking water. At 4 days postinfection, the mice were euthanized, and each cecum was harvested, homogenized, and dilution plated on selective medium to enumerate recovered CFU for the two different strains (Fig. 5).

The araD mutant was modestly attenuated in the absence of arabinose in the water and severely attenuated in its presence. The galE and mtlD mutants were both severely
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Resistance to sugar-phosphate toxicity can be obtained by mutation of a gene that encodes an enzyme required to generate the sugar-phosphate. These mutations also prevent successful utilization of the sugar as a nutrient but do not allow the accumulation of a sugar-phosphate intermediate (Fig. 3). Thus, some sugar utilization mutants are resistant to sugar-phosphate toxicity. From a therapeutics perspective, it would be ideal if these resistant mutants were also attenuated. To determine if this is the case in mice, we tested mutants deficient in \( rhaB, mtlA, \) and \( araB \) against wild type in the streptomycin-treated Swiss Webster mouse model, as described above. We included the appropriate sugar in the drinking water for each mutant. All three mutants were attenuated, but the attenuation was less severe than that observed with the corresponding sugar-phosphate mutants (\( rhaD, mtlD, \) and \( araD \)) (Fig. 5). This suggests that utilization alone contributes to fitness and that resistance to sugar-phosphate toxicity has a fitness cost.

**Phylogenetic analysis of bacterial groups that may be susceptible to different sugar-phosphate toxicities.** To determine which bacteria may be susceptible to the sugar-phosphate toxicities discussed in this report, we searched bacterial genomes for...
DISCUSSION

In previous work, we characterized the fructose-asparagine (F-Asn) utilization pathway of Salmonella, shown in Fig. 1 (30, 32). This pathway includes a phosphorylated intermediate, 6-P-F-Asp, that is converted by the FraB deglycase to glucose-6-phosphate and aspartate, which then enter central metabolism. When a fraB mutant is grown with F-Asn in the medium, 6-P-F-Asp accumulates and cell growth is inhibited (30) (Fig. 4). Mutants lacking fraB are highly attenuated in mouse models (30, 32). Since F-Asn is
available in mouse chow and in many human foods, it is possible that small-molecule inhibitors of FraB could represent a novel therapeutic for Salmonella-mediated gastroenteritis (34). Inhibitors of FraB would be narrow spectrum as it appears that the F-Asn utilization genes were acquired horizontally by Salmonella (or an earlier ancestor) from the Firmicutes (31). Besides some Firmicutes, only the Salmonella and a few Klebsiella and Citrobacter species can utilize F-Asn (Fig. 6) (31). The discovery of 6-P-F-Asp motivated us to search the literature for other examples, which we reviewed recently (3). We found descriptions of growth inhibition caused by the accumulation of sugar-phosphates dating back to the 1950s (37–39). In this work, we assembled a collection of mutants in Salmonella that might suffer sugar-phosphate toxicity and tested them for sugar-dependent growth inhibition in vitro. Following in vitro characterization, these mutants were studied in mouse models of infection to determine if inducing a sugar-phosphate toxicity might be effective therapeutically.

Our findings suggest that a glpD mutant does not suffer any detectable sugar-phosphate toxicity, as defined by an inhibition of growth in the presence of glycerol. A manA mutant suffers a moderate inhibition of growth in M9 minimal medium containing mannose (and no inhibition in LB containing mannose). However, galE, galT, rhaD, mtlD, and araD mutants all suffer severe sugar-phosphate toxicity, although the severity differs between medium types (Fig. 2). For example, the rhaD mutant appears to be more inhibited in LB than in M9, while the araD and mtlD mutants are more inhibited in M9 than in LB.

Mass spectrometry has been used to demonstrate that UDP-galactose and galactose-1-phosphate (Gal-1P) accumulate in an Escherichia coli galE mutant, while Gal-1P accumulates in an E. coli galT mutant (40). Additionally, Ru-5P has been shown to accumulate in E. coli (37), and Mtl-1P and 6-P-F-Asp have been shown to accumulate in Salmonella (30, 41). Here, we confirmed some of these findings. We demonstrated significant accumulation of Mtl-1P, Ru-5P, and 6-P-F-Asp in the mtlD, araD, and fraB mutants, respectively, compared to wild type (Fig. 4). Unfortunately, we were not able to measure rhamnulose-1-phosphate (Rhu-1P) in a rhaD mutant, as Rhu-1P is not available commercially to use as a standard. Synthesis of Rhu-1P, either chemically or enzymatically, is needed.

To determine if sugar-phosphate toxicities might cause decreased fitness during infection, we competed each sugar-phosphate mutant against wild type in a mouse model of gastroenteritis. Consistent with our in vitro data, the glpD mutant was not attenuated in our mouse model regardless of glycerol in the drinking water (Fig. 5). The manA mutant was attenuated but only when mannose was provided (Fig. 5). The galT mutant, which did display sugar-phosphate toxicity in vitro, is not attenuated in mice (Fig. 5). This finding is somewhat surprising as the accumulation of Gal-1P is known to lead to nucleotide imbalances and growth inhibition in E. coli (40, 42). Inhibition of this mutant by galactose in vitro but not during infection is intriguing. Mutants lacking galE are known to be attenuated in mice (43). Here, we confirm this as the galE mutant was extremely attenuated, regardless of the presence of galactose in the drinking water. However, it is well known that galE mutants of Salmonella, in the absence of galactose, fail to synthesize a complete lipopolysaccharide (LPS) structure which would reduce the mutant’s fitness. However, interestingly, they do obtain enough galactose during infection to elaborate enough full-length LPS to generate an immune response to that LPS (43, 44). In our mouse studies, the attenuation of a galE mutant without galactose may be a result of this deficient LPS rather than sugar-phosphate toxicity. When galactose is provided, the galE mutant may be able to elaborate a more complete LPS, potentially recovering its fitness, but at the expense of sugar-phosphate toxicity being induced and thus again limiting survival. Regardless, GalE is unlikely to make a good therapeutic target as the bacterial enzyme has high structural conservation with human GALE (3).

Based on the data presented here, we believe that RhaD, MtlD, and AraD represent the most promising antimicrobial targets. Mutants lacking these genes are extremely
attenuated in mice when the appropriate sugar is included in the drinking water (Fig. 5). Interestingly, however, the mutants behave differently when sugar is not provided: the rhaD mutant is not attenuated, the araD mutant is moderately attenuated, and the mtlD mutant is extremely attenuated. These results may represent the relative quantities of these sugars available to Salmonella in the gut. Alternatively, the araD and mtlD mutants may have defects beyond sugar-phosphate toxicity that contribute to their attenuation. Consistent with the latter hypothesis, mannitol is a known osmoprotectant and it is likely that the mtlD mutant experiences osmolarity dysregulation in addition to sugar-phosphate toxicity (45, 46). The araD mutant is also susceptible to lysis, but only in the presence of arabinose (47). Overall, the profound effect of some sugars in the drinking water suggests that these sugars are not normally available to Salmonella in the gastrointestinal tract in quantities sufficient to intoxicate the mutants and that provision of the appropriate sugar in drinking water can overcome this deficiency.

The molecular mechanisms by which these sugar-phosphates are toxic are largely unknown, and much more research is needed in this area (3). As with any novel drug target, resistance mechanisms also need to be considered. For sugar-phosphate toxicity, resistance can arise from mutation of genes encoding enzymes required for the formation of the sugar-phosphate. However, these mutants are also sugar utilization mutants and may themselves have decreased fitness. We tested this hypothesis by assessing the phenotypes of an rhaB, an araB, and an mtlA mutant in mice. All three mutants were moderately attenuated (Fig. 5), indicating that acquiring resistance to sugar-phosphate toxicity also reduces fitness. This finding shows that at least part of the phenotype of rhaD, araD, and mtlD mutants in mice is due to a lack of sugar acquisition rather than sugar-phosphate toxicity.

While our study was performed in the clinically significant pathogen Salmonella enterica serovar Typhimurium, we thought it important to determine how widespread RhAD, AraD, and MtlD are in bacteria to consider the specificity of targeting these enzymes. Using bioinformatics, we found that all three are uncommon among most bacteria but are highly prevalent among Cronobacter and some of the genera making up the CRE and ESKAPE (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter species) pathogens, including Escherichia, Klebsiella, Serratia, and Enterobacter (Fig. 6). Some of these organisms, especially Escherichia and Klebsiella, are associated with irritable bowel disease (IBD) (48–52). Cronobacter contaminates powdered baby formula and can cause fatal infections in infants (53). If inhibitors of these enzymes were to be developed, they could be used in combination with the appropriate sugar to reduce the abundance of these organisms in the gut (MtlD inhibitors may not require sugar). Beyond eliminating organisms, an interesting application of sugar-phosphate toxicity could be to “edit” the microbiome. For example, some strains of Enterobacteriaceae carry the pks island that encodes colibactin, which is associated with colon cancer (54–56). Inhibitors of RhAD, AraD, and/or MtlD could be used to remove these organisms from the gastrointestinal tract without causing disruption of the microbiota. The niche could then be filled with appropriate probiotic strains from the same genus that do not carry the pks island. Overall, it is likely that most pathogenic bacteria and fungi encode a metabolic pathway that could be targeted in this manner.

MATERIALS AND METHODS

Strains and media. Strains used in this study are listed in Table 1. Bacteria were routinely grown in lysogeny broth (LB) or on LB agar plates made by adding 1.5% (wt/vol) agar to LB broth (Fisher BioReagents). M9 minimal medium contained 1× M9 salts, 2 mM MgSO4, 0.1 mM CaCl2, 0.01 mM thiamine, and trace elements (57–59).

Construction of mutants. All Salmonella mutants predicted to undergo sugar-phosphate toxicity, except for araD, have been previously created using λ Red mutagenesis in the Andrews-McClelland and McClelland mutant library obtained from BEI Resources (60). Each gene in this collection has been disrupted by deleting all but the first 10 codons and the last 10 codons and inserting a gene encoding either chloramphenicol or kanamycin resistance that was cloned from pCLF3 (CamR) and pCLF4 (KanR) plasmids, respectively. The araD mutants and pagC6:Kan mutant were constructed using lambda Red mutagenesis in our laboratory (61). Briefly, the antibiotic resistance cassette of either pKD3

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**TABLE 1** Wild-type and isogenic sugar-phosphate mutant strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
<th>Source, construction, or reference</th>
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</thead>
<tbody>
<tr>
<td>ATCC 14028 (14028)</td>
<td>Wild-type <em>Salmonella enterica</em> subspecies enterica serovar Typhimurium</td>
<td>American Type Culture Collection (ATCC)</td>
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<tr>
<td>EFC008</td>
<td>transduced from reference 60 into 14028</td>
<td>EFC008 14028 galE1::Cam</td>
</tr>
<tr>
<td>EFB038</td>
<td>transduced from reference 60 into 14028</td>
<td>EFB038 14028 galE1::Kan</td>
</tr>
<tr>
<td>EFB001</td>
<td>Antibiotic cassette in EFC008 was removed using FLP recombinase encoded on pCP20 (61)</td>
<td>EFB001 14028 ΔgalE1</td>
</tr>
<tr>
<td>EFB053</td>
<td>Transduction of I(gipC-STM14_1502)6::Kan from EFB026 into EFB001</td>
<td>EFB053 14028 ΔgalE1 I(gipC-STM14_1502)6::Kan</td>
</tr>
<tr>
<td>EFC009</td>
<td>Original mutation from reference 60 transduced into 14028</td>
<td>EFC009 14028 galT1::Cam</td>
</tr>
<tr>
<td>EFB039</td>
<td>Original mutation from reference 60 transduced into 14028</td>
<td>EFB039 14028 galT1::Kan</td>
</tr>
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<td>EFB002</td>
<td>Antibiotic cassette in EFC009 was removed using FLP recombinase encoded on pCP20 (61)</td>
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<td>Transduction of I(gipC-STM14_1502)6::Kan from EFB026 into EFB002</td>
<td>EFB054 14028 ΔgalT1 I(gipC-STM14_1502)6::Kan</td>
</tr>
<tr>
<td>EFC015</td>
<td>Original mutation from reference 60 transduced into 14028</td>
<td>EFC015 14028 rhaD1::Cam</td>
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<tr>
<td>EFB040</td>
<td>Original mutation from reference 60 transduced into 14028</td>
<td>EFB040 14028 rhaD1::Kan</td>
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<td>EFB003</td>
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<td>Transduction of I(gipC-STM14_1502)6::Kan from EFB026 into EFB003</td>
<td>EFB055 14028 ΔrhaD1 I(gipC-STM14_1502)6::Kan</td>
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<tr>
<td>EFB006</td>
<td>Original mutation from reference 60 transduced into 14028</td>
<td>EFB006 14028 gplD1::Cam</td>
</tr>
<tr>
<td>EFB041</td>
<td>Antibiotic cassette in EFB041 was removed using FLP recombinase encoded on pCP20 (61)</td>
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<td>EFB009</td>
<td>Transduction of I(gipC-STM14_1502)6::Kan from EFB026 into EFB009</td>
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<td>EFB056</td>
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<td>EFB042</td>
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<td>EFB004</td>
<td>Antibiotic cassette in EFC019 was removed using FLP recombinase encoded on pCP20 (61)</td>
<td>EFB004 14028 ΔmtlA1</td>
</tr>
<tr>
<td>EFB057</td>
<td>Transduction of I(gipC-STM14_1502)6::Kan from EFB026 into EFB004</td>
<td>EFB057 14028 ΔmtlA1 I(gipC-STM14_1502)6::Kan</td>
</tr>
<tr>
<td>EFC021</td>
<td>Original mutation from reference 60 transduced into 14028</td>
<td>EFC021 14028 manA1::Cam</td>
</tr>
<tr>
<td>EFB005</td>
<td>Antibiotic cassette in EFC021 was removed using FLP recombinase encoded on pCP20 (61)</td>
<td>EFB005 14028 ΔmanA1</td>
</tr>
<tr>
<td>EFB058</td>
<td>Transduction of I(gipC-STM14_1502)6::Kan from EFB026 into EFB005</td>
<td>EFB058 14028 ΔmanA1 I(gipC-STM14_1502)6::Kan</td>
</tr>
<tr>
<td>EFC037</td>
<td>Lambda Red mutation of araD made using PCR primers BA3522 and BA3523 to amplify the Kan(^r) gene from pKD4.</td>
<td>EFC037 14028 araD6::Kan</td>
</tr>
<tr>
<td>EFC039</td>
<td>Lambda Red mutation of araD made using PCR primers BA3522 and BA3523 to amplify the Cam(^r) gene from pKD3.</td>
<td>EFC039 14028 araD6::Cam</td>
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<tr>
<td>HMB206</td>
<td>Lambda Red mutation downstream of pagC made using PCR primers BA3849 and BA3850 to amplify the Kan(^r) gene from pKD4</td>
<td>HMB206 14028 fnaB80::Kan</td>
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<tr>
<td>JLD1214</td>
<td>Original mutation from reference 60 transduced into 14028</td>
<td>JLD1214 14028 I(gipC-STM14_1502)6::Cam</td>
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<tr>
<td>EFB026</td>
<td>Lambda Red mutation downstream of pagC made using PCR primers BA3849 and BA3850 to amplify the Kan(^r) gene from pKD4</td>
<td>EFB026 14028 I(gipC-STM14_1502)6::Kan</td>
</tr>
<tr>
<td>EFB051</td>
<td>Transduction of I(gipC-STM14_1502)6::Kan from EFB026 into 14028</td>
<td>EFB051 14028 I(gipC-STM14_1502)6::Kan</td>
</tr>
<tr>
<td>EFB063</td>
<td>Original mutation from reference 60 transduced into 14028</td>
<td>EFB063 14028 rhaB1::Kan</td>
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<tr>
<td>EFB037</td>
<td>Transduction of I(gipC-STM14_1502)6::Kan from EFB026 into 14028</td>
<td>EFB037 14028 araD1::Kan</td>
</tr>
<tr>
<td>EFB036</td>
<td>Original mutation from reference 60 transduced into 14028</td>
<td>EFB036 14028 mtlA1::Cam</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Plasmids</th>
<th>Genotype or description</th>
<th>Source, construction, or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKD4</td>
<td>FRT(^r)-cam-FRT oriR6K (Ampr)</td>
<td>61</td>
</tr>
<tr>
<td>pKD3</td>
<td>FRT(^r)-kan-FRT oriR6K (Ampr)</td>
<td>61</td>
</tr>
<tr>
<td>pCP20</td>
<td>cI857 XPR flp pSC101 oriTS (Ampr Cam(^r))</td>
<td>68</td>
</tr>
</tbody>
</table>

\(^{a}\)FRT, FLP recombinase target.
of interest by selection on LB-ampicillin (LB-Amp) at 30°C. Single colonies were streaked onto LB and incubated at 42°C to allow for loss of the antibiotic cassette and curing of the plasmid. Individual colonies were confirmed to lack the antibiotic resistance gene and pCP20 by patching on LB-Amp (pCP20) and LB-Kan or LB-Cam. PCR was used to verify the deletions.

**Growth assays.** Growth of strains was assessed using clear, flat-bottom, 96-well plates. Bacterial cultures that had been grown overnight were centrifuged at 18,000 × g and resuspended in an equal volume of sterile water. Two microtiter of bacteria was then inoculated into a well containing 198 μL of the indicated medium. A Breathe-Easy membrane film (Diversified Biotech) was placed over the 96-well plate. Growth was measured using a SpectraMax M5 (Molecular Devices) microplate reader and the SoftMax Pro 6.1 software, taking hourly readings of optical density at 600 nm (OD600) over a course of 17 to 20 h at 37°C.

**Quantitative mass spectrometry for sugar-phosphates.** Cell pellets for mass spectrometry (MS) were prepared by inoculating 5 mL of LB containing the indicated sugar at 5 mM with 50 μL of overnight culture in a glass tube and incubating at 37°C for 4 h. After 4 h, the entire culture was poured into a 15-mL conical tube and centrifuged for 10 min at 10,000 × g at 4°C. The majority of the supernatant was poured off, leaving about 1 mL in which to resuspend the pellet and transfer it to a 1.5-mL microcentrifuge tube. The bacteria were centrifuged for 1 min at 18,000 × g. All supernatant was pipetted off, and cell pellet was immediately frozen at −80°C. For each measurement, 100 μL of *Salmonella* cell pellets (equivalent to 5-mL samples of cells) was used. Two hundred microtiter of liquid chromatography (LC)-grade water was added to result in a final volume of 300 μL, which was split into three aliquots for subsequent use as analytical replicates for each analysis. The sugar-phosphate was extracted from each 100-μL aliquot, by adding 250 μL of chilled LC-grade water and 500 μL of chilled methanol (Fisher Optima LC/MS grade; Fisher Scientific) with 20 nmol [13C]fructose-asparagine ([13C]F-Asn) added as internal standard. ([13C]F-Asn was used as internal standard due to the lower cost and simplicity of the synthesis of [13C]F-Asn compared to the 13C heavy atom-labeled sugar-phosphates. The cell suspension was then vortexed for 2 min to facilitate pellet disruption and metabotive extraction. Samples were subjected to 10 cycles of ultrasonication (30 s each with 30-s intervals in between) using a Bioruptor Pico (Diagenode). Following cell lysis, samples were centrifuged at 16,000 × g for 15 min at 4°C, and the supernatants were transferred to new tubes and dried under vacuum (SpeedVac concentrator; Thermo Scientific). Before mass spectrometry analysis, the dried pellets were resuspended in 50 μL water- acetoni-trile, 98%:2%, with 0.1% (vol/vol) formic acid (LC-MS grade; Thermo Scientific) and filtered using a 0.2-μm polytetrafluoroethylene (PTFE) filter (Thermo Scientific). The supernatant of the flowthrough fraction was then injected for liquid chromatographic-mass spectrometric analysis. For the standard curve analysis, different amounts of standard sugar-phosphates (0, 20, 80, 160, 200, 400, or 600 nmol) were spiked into the extraction solvent and were prepared as described above. Ten microtiter of each sample was introduced into an Ultimate 3000 ultraperformance LC (Thermo Scientific) system with an HSS T3, C18 column (Waters) by 15 min at 4°C. The majority of the supernatant was filtered using a 0.2-μm PTFE filter (Thermo Scientific). The supernatant of the flowthrough fraction was then injected for liquid chromatographic-mass spectrometric analysis. For the standard curve analysis, different amounts of standard sugar-phosphates (0, 20, 80, 160, 200, 400, or 600 nmol) were spiked into the extraction solvent and were prepared as described above. Ten microtiter of each sample was introduced into an Ultimate 3000 ultraperformance LC (Thermo Scientific) system with an HSS T3, C18 column (Waters; 2.1 μm by 100 mm, 1.8 μm) and coupled into a triple quadrupole mass spectrometer (Thermo Scientific). Mobile phases were buffer A, 0.1% (vol/vol) formic acid (LC-MS grade; Thermo Scientific) in water, and buffer B, 0.1% (vol/vol) formic acid in acetonitrile. A gradient separation started with 2% B for 3 min at a flow rate of 100 μL/min and was then followed by a gradient: 3 to 4 min, 2 to 10% B; 4 to 7 min, 10 to 98% B; 7 to 7.5 min, 98% B; 7.5 to 7.6 min, 98 to 2% B; 7.6 to 10 min, 2% B. The mass spectrometer was operated in positive-ion electrospray ionization mode (ESI+). The gradient was stabilized with a capillary voltage of 4 kV, source temperature of 100°C, desolvation temperature of 350°C, sheath gas flow of 12 L/min, and auxiliary gas flow of 13 L/min. The gas flow rate for the collision cell was 0.15 L/min. A multiple-reaction monitoring (MRM) mode was used for sugar phosphate analysis. Skyline (v2.02; MacCoss Lab, Department of Genome Sciences, University of Washington, Seattle, WA, USA) was used for calculating the peak area of each transition. Transition conditions, including retention time, precursor ion, product ion, and collision energy, are described in Fig. 4B.

**Animal experiments.** The fitness of *Salmonella* mutants was compared to that of the wild type in the streptomycin-treated Swiss Webster mouse model (30). The mice were treated by oral gavage with 20 mg of streptomycin (35). Twenty-four hours later, the mice were inoculated with a 1:1 ratio of wild-type and mutant bacteria. The inoculum titer was determined on LB-Kan and LB-Cam to determine the input quantities of the two strains being used. Total inoculum dose was 1 × 107 CFU. Where indicated, mice were provided the sugar of interest in their drinking water (100 mM). At 4 days postinfection, mice were euthanized and each cecum was harvested. Cecum samples were weighed, homogenized, and dilution plated on LB-Kan and LB-Cam for enumeration of CFU.

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**TABLE 2 PCR primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BA3522</td>
<td>GAGAAACACGTAGTGAGATCCTAAAGCCTGAATGTTAGCCTGGACTGCTTC</td>
<td>Forward primer for araD lambda Red mutagenesis</td>
</tr>
<tr>
<td>BA3523</td>
<td>TTTAGAGGCATTACTGCCCGTAATAGGCTTTTGCGCCGTCATATGAATATCCTCCTTAG</td>
<td>Reverse primer for araD lambda Red mutagenesis</td>
</tr>
<tr>
<td>BA3849</td>
<td>CGAAGGCGGTCAACAATCTGTGACATTTGTGATTAATGTGTAGGCTGGAGCTGCTTC</td>
<td>Forward primer for pagC intergenic region lambda Red mutagenesis</td>
</tr>
<tr>
<td>BA3850</td>
<td>CTCTTCTACCCGTAAGCCTACGTCGTAGCCTGTCTTTTCTCTCATATGAATATCCTCCTTCTTAG</td>
<td>Reverse primer for pagC intergenic region lambda Red mutagenesis</td>
</tr>
</tbody>
</table>
Phylogenetic and genomic analyses. With the exception of genes related to fructose-asparagine, the genes described below that, when mutated, result in toxic sugar-phosphate intermediates were searched in the AnnoTree v1.2 database with default parameters (e = 20). The genes, the sugar-phosphate that accumulates, and the enzyme EC number were as follows: pgp (glucose-6-phosphate; EC 5.3.1.9), pfaA (fructose-6-phosphate; EC 2.7.1.11), frbA (6-phosphofructose aspartate; accession no. WP_010098908.1), galE (UDP-galactose; EC 5.1.3.2), galT (galactose-1-phosphate; EC 2.7.7.12), frux (fructose-1-phosphate; EC 2.7.1.56), otb8 (trehalose-6-phosphate; EC 3.1.3.12), gldE (maltose-1-phosphate; EC 2.4.99.16), gldP (sn-glycerol-3-phosphate; EC 1.1.5.3), araD (ribulose-5-phosphate; EC 5.1.3.4), manA (mannose-6-phosphate; EC 5.3.1.8), and radD (rhamnulose-1-phosphate; EC 4.1.2.19). For frbA (6-phosphofructose aspartate), we used previously determined cutoffs (BLAST e = 20) to differentiate homologs from fructoselysine-6-P-deglyceraldehyde (frbA) (31, 63). The AnnoTree v1.2 database was downloaded and analyzed on 18 February 2018 (http://annotree.uwaterloo.ca/app/downloads.html) (63). The maximum likelihood phylogenetic tree was downloaded from AnnoTree v1.2, with methods for tree generation detailed in reference 64. Briefly, this phylogenetic tree was constructed using 120 ubiquitous single-copy marker genes (bac120 marker set) from 30,238 bacterial genomes that were obtained from GTDB r95. The relative abundance of genes in the genomes for each phylogeny was overlaid onto the phylogenetic tree and visualized in iTOL 5.7 (65). For selected gut-relevant genera within the Enterobacteriaceae, the percentage of the sugar genes in genomes from each genus was calculated. The number of genomes in the genus is derived from the parameters that are used for searches in the AnnoTree v1.2 database (63). The heatmap was created using R Studio and visualized using Adobe Illustrator (66).

Animal assurance. All animal work was performed using protocols approved by our Institutional Animal Care and Use Committee (IACUC; OSU 2009A0035) and in accordance with the relevant guidelines set forth in the Guide for the Care and Use of Laboratory Animals (67).

SUPPLEMENTAL MATERIAL

Supplemental material is available only online.

SUPPLEMENTAL FILE 1, XLSX file, 0.02 MB.

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