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REVIEW

Sugar-Phosphate Toxicities

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SUMMARY Accumulation of phosphorylated intermediates during cellular metabolism can have wide-ranging toxic effects on many organisms, including humans and the pathogens that infect them. These toxicities can be induced by feeding an upstream metabolite (a sugar, for instance) while simultaneously blocking the appropriate metabolic pathway with either a mutation or an enzyme inhibitor. Here, we survey the toxicities that can arise in the metabolism of glucose, galactose, fructose, fructose-asparagine, glycerol, trehalose, maltose, mannose, mannitol, arabinose, and rhamnose. Select enzymes in these metabolic pathways may serve as novel therapeutic targets. Some are conserved broadly among prokaryotes and eukaryotes (e.g., glucose and galactose) and are therefore unlikely to be viable drug targets. However, others are found only in bacteria (e.g., fructose-asparagine, rhamnose, and arabinose), and one is found in fungi but not in humans (trehalose). We discuss what is known about the mechanisms of toxicity and how resistance is achieved in order to identify the prospects and challenges associated with targeted exploitation of these pervasive metabolic vulnerabilities.

KEYWORDS antimicrobials, drug targets, fructose-asparagine, sugar phosphate, toxicity

INTRODUCTION

With pathogens becoming increasingly resistant to antibiotics, new approaches are necessary to counter this threat. One strategy to induce bacterial toxicity is by simultaneously inhibiting an enzyme while feeding an appropriate nutrient/metabolite to the organism in order to cause an accumulation of a toxic intermediate. Here, we review 11 metabolic pathways that can be disrupted to cause the accumulation of what we refer to broadly as sugar phosphates, although two are more accurately referred to as polyol-phosphates (*sn*-glycerol-3P [Gly-3P] and mannitol-1P [Mtl-1P]), and one is a phosphorylated Amadori compound (6-phospho-fructose-aspartate [6P-F-Asp]). Despite initial reports of sugar-phosphate toxicities 7 decades ago, little is known about the mechanisms by which cells are poisoned and how they recover. By reviewing the advances and knowledge gaps, we expect to motivate studies that will further our understanding of the biocomplexity of sugar phosphate-mediated bacterial/fungal vulnerabilities and inspire drug discovery. We first provide a broad overview of the mainstay and the organizational structure of the review.

Metabolic disruptions caused by accumulation of a specific sugar phosphate must be considered from the perspective of toxicity and the associated cellular response. With respect to toxicity: What is the immediate consequence of a metabolite build-up? Does it inhibit another enzyme, or cause depletion of a downstream metabolite derived from it? Are there ripple effects? What are the "metabolite" pointers that reflect a state of heightened stress? In terms of the response to the toxin's build-up: How is the accrued metabolite enzymatically detoxified? Since all stress and environmental alterations lead to changes in gene expression, how do these responses help accomplish metabolic rebalancing, stress alleviation, and cellular homeostasis reset?

Answering the above-mentioned questions with each sugar phosphate helps pinpoint similarities and variances among the 11 different sugar-phosphate toxicities. For example, in galactose-mediated toxicity of *Escherichia coli* mutants lacking galactose-1-phosphate-uridylyltransferase (*galT*, GalT) and fructose-mediated toxicity in humans deficient in aldolase B, part of the toxicity appears to be related to phosphate starvation or ATP depletion (1, 2). Other similarities include DNA damage and osmotic imbalance in the case of trehalose-6-phosphate (Tre-6P) and maltose-1-phosphate (Mal-1P) toxicities and cell wall defects associated with galactose- and mannose-mediated toxicities. However, despite unifying themes, there appear to be unique aspects to each sugar-phosphate toxicity. For example, the mystery of why glycerol was unable to rescue a *galT* mutant was solved when results from an omics study showed that galactose-1-phosphate (Gal-1P), which accumulates in the *galT* mutant, represses glycerol utilization genes. Instead of the typical glycerol-3-phosphate inducer, Gal-1P was postulated to act as an

anti-inducer to cause transcriptional reprogramming. Better understanding of these distinct and not easily predicted mechanisms is key to enhancing the toxicities, avoiding resistance mechanisms, or finding combinations of toxicities that are synergistic. A central objective of this review is to identify the key unanswered questions in each sugar-phosphate toxicity.

In this review, each sugar will be covered separately. There are subsections for background, evidence of sugar-phosphate accumulation, mechanism of toxicity, and topics for further investigation. Individual organisms known to experience a particular sugar-phosphate toxicity are considered independently. However, there is more information about some sugar-phosphate toxicities (i.e., glucose-/galactose-phosphate) than others, inevitably resulting in variable coverage for the different sugar phosphates and with specific organisms described in depth for one sugar phosphate but not another. In some instances, we describe in detail past experiments mainly to showcase the fact that the severity of some of these toxicities depends on specific growth conditions. For example, galactose-mediated toxicities are much more severe in rich than in minimal media (3–7), trehalose-mediated toxicities are temperature sensitive in many fungi (8), and the presence of glucose alleviates arabinose- and glycerol-mediated toxicities in *E. coli* but has no effect on fructose-asparagine-mediated (F-Asn) toxicity in *Salmonella enterica* (9–11).

Relevant metabolic pathways are shown in Fig. 1, with spotlights on the different sugar phosphates and the enzymes that would need to be inhibited to generate a toxic intermediate, as well. A list of key enzymes and their presence in a selection of eukaryotes is described in Table 1. While our emphasis is on bacterial studies, we have highlighted important parallels to fungal pathways that also provide interesting therapeutic targets, and to other eukaryotic pathways that will need to be considered when designing antimicrobials for use in humans or livestock.

GLUCOSE

Background

***Escherichia coli* and *Salmonella enterica*.** Glucose is transported into *E. coli* and *S. enterica* via two different phosphoenolpyruvate (PEP)-dependent sugar phosphotransferase systems (PTS). The primary route exploits $EIICB^{Glc}$ encoded by *ptsG*, with additional contributions from the *manXYZ*-encoded $EIIABCD^{Man}$ transporter. In both cases, import is coupled to phosphorylation of glucose to generate glucose-6-phosphate (Glc-6P) (Fig. 1). Glc-6P, thus formed, can either be isomerized to fructose-6-phosphate (Fru-6P) by phosphoglucose isomerase (*pgi*; PGI), or it can be oxidized to 6-phosphoglucono-1,5-δ-lactone by glucose-6-phosphate dehydrogenase (*zwf*; G6PD). While Fru-6P enters the glycolytic pathway, the lactone is further catabolized via either the pentose phosphate (PP) or the Entner-Doudoroff (ED) pathway.

Within glycolysis, three different toxic sugar phosphates can be induced: Glc-6P, Fru-6P, or fructose 1,6-bisphosphate (Fru-1,6BP) (Fig. 1). A mutant lacking *pgi* accumulates Glc-6P and grows slowly on glucose, while a *pgi zwf* double mutant does not grow at all (12, 13). This double mutant grows well on alternative carbon sources, including gluconate, mannitol, and glycerol. However, when glucose is also present, the double mutant can no longer grow on glycerol but it continues to grow on gluconate and mannitol (13). This finding suggests that the accumulation of Glc-6P, during growth on glucose, might inhibit a gluconeogenic enzyme required for growth on glycerol. Indeed, fructose 1,6-bisphosphatase (*fbp;* FBP) activity is inhibited by Glc-6P *in vitro* (13).

When a phosphofructokinase (*pfkA*; PfkA) mutant is fed glucose, the glycolytic intermediate Fru-6P is believed to accumulate (14). Likewise, the accumulation of Fru-1,6BP occurs in a type II fructose 1,6-bisphosphate aldolase mutant (*fbaA*, FbaA [15]; *fbaA* was previously known as *fda*). Surprisingly, this *fbaA* mutant could grow on glycerol, suggesting the existence of a backup route to support gluconeogenesis (15, 16). Indeed, *E. coli* has a second type I (metal-independent) fructose 1,6-bisphosphate aldolase encoded by *fbaB* (FbaB).

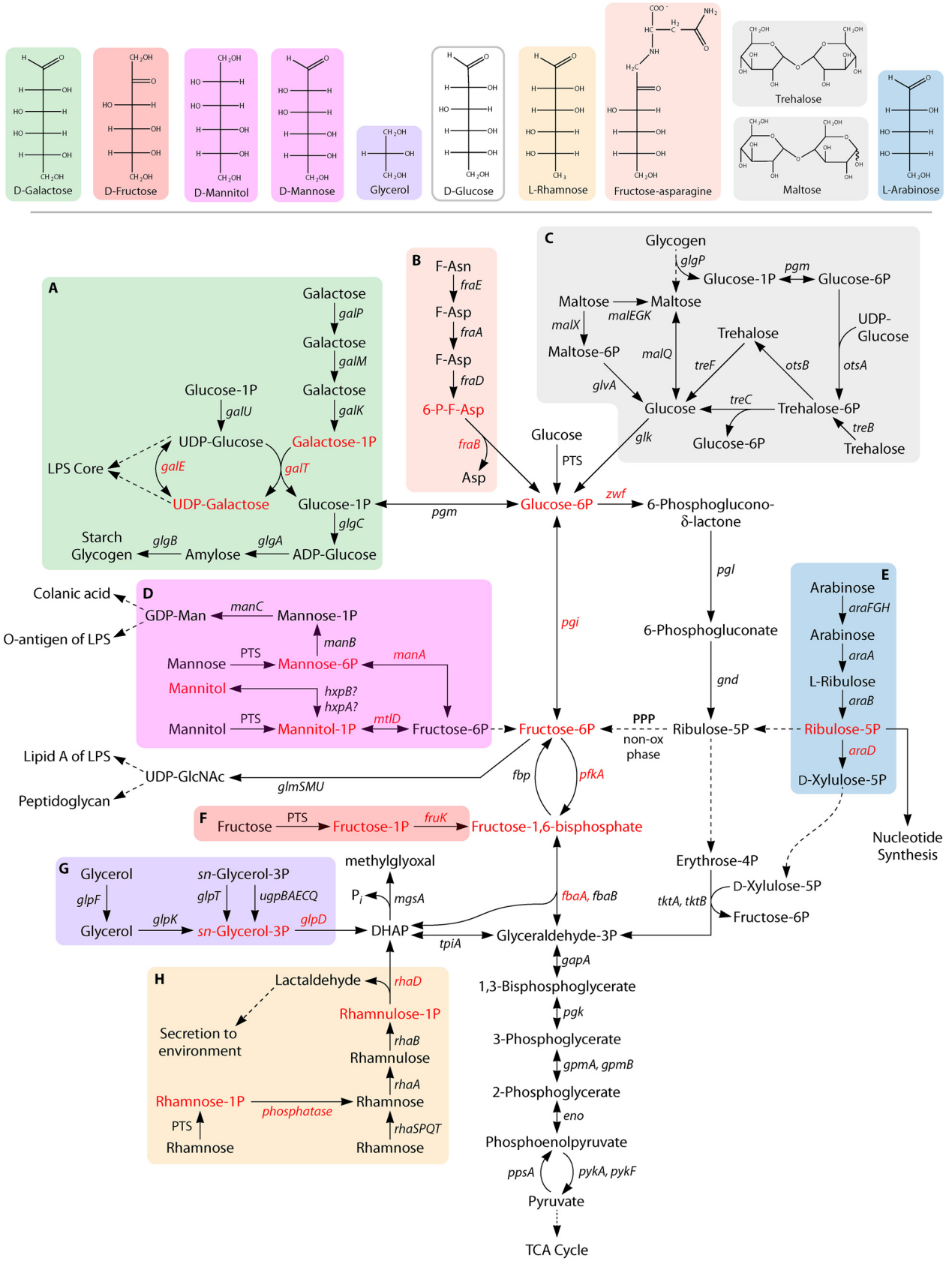

FIG 1 Metabolic pathways for microbial utilization of select carbohydrates. Genes whose mutation/dysfunction cause accumulations of toxic phosphosugars and the respective built-up phosphosugar are highlighted in red. Although the pathways are based on those present in *E. coli*, there

(Continued on next page)

Interestingly, *fbaB* is not expressed in the presence of glucose and serves as a backup during glycerol metabolism (17).

In the presence of glucose, the three glycolytic mutants that experience phosphosugar stress—*pgi*, *pfkA*, and *fbaA*—all respond with an RNase E-dependent degradation of the *ptsG* transcript, which encodes the EIICB^{Glc} component of the glucose PTS (14, 18). A common thread in these responses is to prevent further uptake of glucose, which would otherwise exacerbate the stress. No other mutations within the glycolytic pathway, however, engender either the phosphosugar stress or the *ptsG* degradation response (14). This response is controlled by SgrR (Fig. 2) (19–22). SgrR responds to glucose-phosphate stress (by an unknown mechanism) and activates the adjacent, divergently transcribed *sgrS* gene that encodes a small regulatory RNA (20, 23). This regulation appears to be specific to toxic glycolytic intermediates as accumulation of toxic arabinose- or galactose-derived metabolic intermediates does not result in increased expression of *sgrS* (23).

Pseudomonas fluorescens. Carbon metabolism in *Pseudomonas fluorescens* differs from that in *E. coli* because of the absence of PfkA. Thus, glucose is metabolized via the ED and PP pathways. Fructose is imported through a PTS system and then metabolized by the ED pathway. During growth on fructose, a mutant lacking *zwf-1* and *zwf-2*, the genes encoding the first step of the ED pathway, has impaired growth and presumably accumulates Glc-6P and Fru-6P, although the concentrations of these compounds were not measured (24). Interestingly, *P. fluorescens* uses Fru-6P as the precursor to alginate, an extracellular polysaccharide. Although alginate production alleviates phosphosugar stress by depleting Fru-6P, the payoffs are not as straightforward because the levels of Fru-6P need to be buffered carefully. *P. fluorescens* encodes a phosphatase of the YigL family named Spp that preferentially uses as substrates L-ribulose-5-phosphate (Ru-5P) > Fru-6P > Glc-6P (25). (The functions of YigL homologs are described in more detail in the section "Mechanism of Toxicity" [for *E. coli*] below.) The *spp* gene was found to be essential in alginate-producing strains grown on fructose but not essential in alginate-negative strains, an observation that could be rationalized by the ability of Spp to offset toxic, if temporary, Fru-6P build-up during alginate synthesis. This scenario illustrates the requirement for tight regulation of intracellular Fru-6P levels: although Fru-6P is being shunted toward alginate, no build-up can be tolerated, and Spp provides that safeguard.

Bacillus subtilis. In *Bacillus subtilis*, a double mutant lacking *zwf* and *pgi* is glucose sensitive in minimal medium, a finding indicative of toxicity associated with build-up of glucose-1-phosphate (Glc-1P) and Glc-6P, with Glc-1P being implicated as the toxic metabolite (26).

Evidence for Sugar-Phosphate Accumulation

Escherichia coli. Within 10 min of growth on glucose, a *pgi* mutant of *E. coli* experiences a 9-fold increase in Glc-6P concentration, as determined by an enzymatic assay (14). There have been no direct measurements for Fru-6P in a *pfkA* mutant. However, because the addition of Fru-6P to a *pgi pfkA* double mutant, but not to a *pgi* single mutant, caused the destabilization of *ptsG* mRNA, it was concluded that Fru-6P accumulation induces a sugar-phosphate stress response (14). The levels of Fru-1,6BP were measured using an enzymatic assay and found to increase 7- to 20-fold in a temperature-sensitive *fbaA* mutant that was grown on glucose at the nonpermissive temperature (15).

Bacillus subtilis. In *B. subtilis*, a double mutant lacking *zwf* and *pgi* is glucose-sensitive in minimal medium (26). The mutant accumulates Glc-6P and Glc-1P, as determined by enzymatic assays, and lyses. Interestingly, it is not the Glc-6P but rather Glc-1P that is the toxin because lysis is prevented by introduction of a third mutation in phosphoglucomutase (*pgm* in *E. coli*; *pgcA* in *B. subtilis*; PGM), the enzyme that converts Glc-6P to Glc-1P. Lysis is prevented by the addition of rapidly (e.g., fructose, gluconate, glucosamine, glycerol, malate, or mannose) but not slowly (e.g., citrate, succinate, or L-α-glycerophosphate) metabolizable carbon sources (26).

FIG 1 Legend (Continued)
are variations and exceptions in other bacteria. For example, the pathway shown in panel B is found in *S. enterica* but not in *E. coli*. In *B. subtilis*, the *pgm* gene and Glc-1P would be highlighted in red. No toxicities have been found in *E. coli* or *S. enterica* pathways shown in panel C, but the *M. tuberculosis* pathways do have toxicities (see Fig. 5). Structures for various sugars are depicted in the top panel and color coordinated with their degradation pathway in the bottom panel.

TABLE 1 Relevant sugar utilization pathways and their toxic phosphosugar intermediates

Pathway	Gene name[a]	Gene product	Toxic intermediate	Enzyme presence[e] in:				Suppressor mutations[b]	Bacteria studied[c]	Other organism(s)[d]	Key references
				Humans	*C. albicans*	*C. neoformans*	*A. nidulans*				
L-Arabinose	*araD* EC 5.1.3.4	L-Ribulose-5-phosphate-4-epimerase	L-Ribulose-5-phosphate (Ru-5P)	No	No	No	No	*araB*, EC 2.7.1.16, arabinose efflux pumps	*E. coli*		10, 178, 226
Fructose	*fruK*, EC 2.7.1.56	1-Phosphofructokinase	Fructose-1-phosphate (Fru-1P)	No	No	No	No	Unknown	*E. coli*, *A. aerogenes* (*E. aerogenes*)	Humans	134, 138, 227
Fructose-asparagine	*fraB*	Deglycase	6-Phospho-fructose-aspartate (6P-F-Asp)	No	No	No	No	Unknown	*S. enterica*		118, 228
Galactose	*galT*, EC 2.7.7.12	Galactose-1-phosphate uridylyltransferase	Galactose-1-phosphate (Gal-1P)	Yes, GALT	Yes, GAL7	Yes, CNB03650	Yes, AN6182.2	*galK*, EC 2.7.1.6, *glpP*	*E. coli*, *S. enterica*	Humans	60, 227
	galE, EC 5.1.3.2	UDP-glucose-4-epimerase	Uridine diphosphate galactose (UDP-Gal)	Yes, GALE	Yes, GAL10	Yes, CNA06760	Yes, AN4727.2	*galK*, EC 2.7.1.6	*E. coli*, *S. enterica*	Humans	1
Glucose	*pgi*, EC 5.3.1.9	Phosphoglucose isomerase	Glucose-6-phosphate (Glu-6P)	Yes, GPI	Yes, PGI1	Yes, CNB04050	Yes, AN6037.2	*pitA*	*E. coli*, *S. enterica*, *B. subtilis*[f]		14, 21, 33, 229
	pfkA, EC 2.7.1.11	6-Phosphofructokinase	Fructose-6-phosphate (Fru-6P)	Yes, HK1	Yes, HXK1	Yes, CNB02660	Yes, AN2638.2	*pitA*	*E. coli*, *S. enterica*		
	fbaA, EC 4.1.2.13	Fructose-bisphosphate aldolase	Fructose-1,6-bisphosphate (Fru-1,6BP)	Yes, ALDOB	Yes, FBA1	Yes, CNB00300	Yes, AN1888.2	*pitA*	*E. coli*, *S. enterica*		14, 24, 33
	zwf, EC 1.1.1.49	Glucose-6-phosphate-1-dehydrogenase	Fructose-6-phosphate (Fru-6P)	Yes, G6PD	Yes, ZWF1	Yes, CNG03280	Yes, AN2981.2	*pitA*	*E. coli*, *S. enterica*, *P. fluorescens*[g]		
Glycerol	*glpD*, EC 1.1.5.3	Glycerol-3-phosphate dehydrogenase	*sn*-Glycerol-3-phosphate (Gly-3P)	Yes, GPD2	Yes, GUT2	Yes, CNC04310	Yes, AN1396.2	*glpF*, *glpK*, EC 2.7.1.30	*E. coli*, *S. enterica*, *Mycobacterium*		9
Maltose	*glgE*, EC 2.4.99.16	α-1,4-Glucan:maltose-1-phosphate	Maltose-1-phosphate (Mal-1P)	No	No	No	No	*treS*, EC 2.7.1.175, *pep2*	*Mycobacterium*		157
Mannitol	*mtlD*, EC 1.1.1.17	Mannitol-1-phosphate 5-dehydrogenase	Mannitol, mannitol-1-phosphate (Mtl-1P)	No	No	No	Yes, AN5975.2	Unknown	*E. coli*, *S. enterica*, *S. aureus*, *A. baylii*	Fungi, plants	187, 194
Mannose	*manA*, EC 5.3.1.8	Mannose-6-phosphate isomerase	Mannose-6-phosphate (Man-6P)	Yes, MPI	Yes, PMI1	Yes, CNI02370	Yes, PMI	Unknown	*E. coli*, *S. enterica*, *B. subtilis*	*S. cerevisiae*, *A. fumigatus*, human tumor cells, mice, honeybees	52, 208, 210, 211, 230
Rhamnose		Rhamnose-1-phosphate phosphatase	L-Rhamnose-1-phosphate (Rha-1P)					PTS transporter	*A. pyridinolis*		224
	rhaD, EC 4.1.2.19	Rhamnulose-1-phosphate aldolase	L-Rhamnulose-1-phosphate (Rhu-1P)	No	No	No	No	PTS transporter	*E. coli*, *S. enterica*		
Trehalose	*otsB*, EC 3.1.3.12	Trehalose-6-phosphate phosphatase	Trehalose-6-phosphate (Tre-6P)	No	Yes, TPS2	Yes, CNB02610	Yes, AN4262.2	*otsA*, EC 2.4.1.15, trehalose PTS	*Mycobacterium*	*S. cerevisiae*, *C. albicans*, *C. neoformans*, *A. nidulans*, *A. fumigatus*, *C. elegans*	152

[a]Gene name of the enzyme that produces the phosphosugar intermediate. Mutation of this gene causes build-up of the phosphosugar and results in the toxic phenotype.
[b]Secondary mutations that alleviate the toxic phenotype.
[c]Bacterial organisms in which sugar-phosphate toxicity has been observed.
[d]Other organisms relevant to the sugar phosphate described.
[e]These columns indicate the presence or absence of the particular EC numbered enzyme in a particular organism.
[f]Glu-1P accumulates in *B. subtilis*.
[g]Fructose was provided to a *P. fluorescens zwf-1 zwf-2* mutant to accumulate the corresponding intermediate.

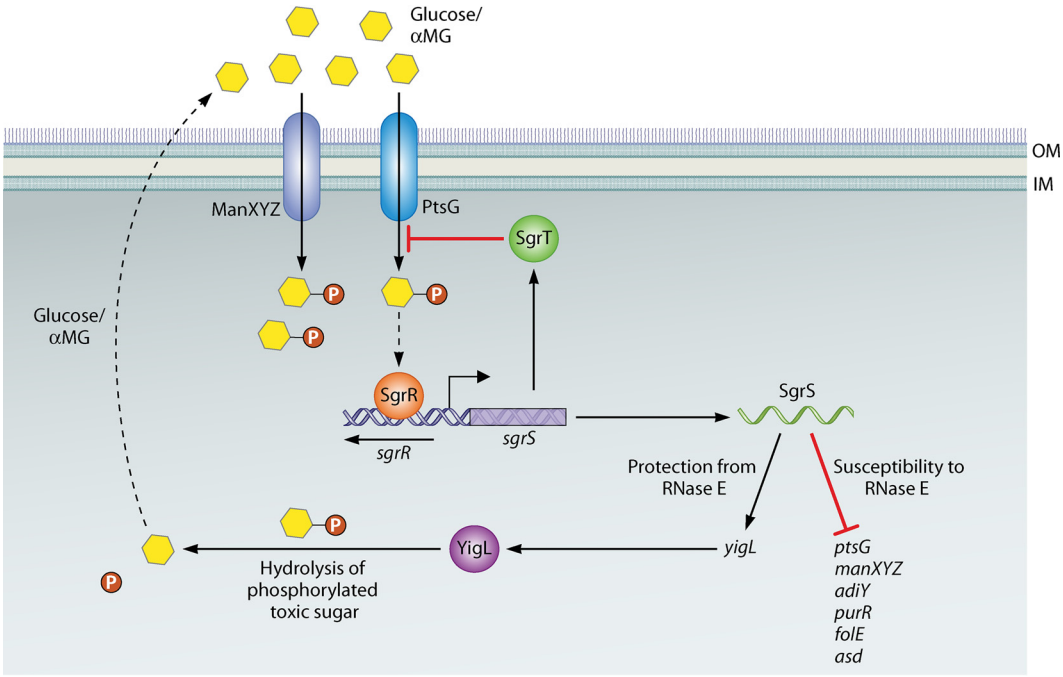

FIG 2 *sgrR/sgrS/sgrT* response to sugar-phosphate stress in *E. coli*. Glucose and α-methyl-glucoside (αMG) (yellow hexagons) are primarily transported by the phosphoenolpyruvate (PEP)-dependent transporter PtsG with some contribution from ManXYZ. Glucose-phosphate stress is triggered by accumulation of glucose-6-phosphate or phosphorylated αMG (yellow hexagon with attached phosphate group). During the phosphosugar stress response, SgrR induces expression of *sgrS*. SgrS is a noncoding RNA that base pairs with target mRNAs to either allow or protect these mRNAs from RNase E degradation, thus engendering opposite translation outcomes (upregulation, *yigL*; downregulation, *ptsG*). YigL belongs to the family of HAD-like phosphatases that can hydrolyze the phosphate group from the intoxicating sugar phosphate. SgrS also produces SgrT, which inhibits the activity of PtsG, but not ManXYZ, curtailing further transport of glucose or αMG into the cell.

Mechanism of Toxicity

Escherichia coli. Different strategies have been exploited to ascertain whether growth retardation and cell death in the instances described above arises from either poisoning due to the toxicity of the accumulated metabolite (or its derivatives) or energy depletion from the glycolytic block at the steps catalyzed by PGI, PfkA, or FbaA. We consider below two examples which highlight the importance of the physiological context while considering these two distinct mechanistic underpinnings.

In the first case study, we consider Glc-6P, Fru-6P, or mannose-6-phosphate (Man-6P) provided to cells transformed with a plasmid for the expression of UhpT (hexose-6-phosphate:phosphate antiporter; *uhpT*), a transporter that ensures efficient uptake of sugar phosphates (27). The idea is to reenact a phosphosugar stress in the absence of genetic alterations. Addition of the glycolytic sugar phosphates Glc-6P, Fru-6P, or Man-6P caused an increase in methylglyoxal concentration and cell killing, but the nonglycolytic sugar phosphates arabinose-5-phosphate (Ara-5P) or galactose-1-phosphate (Gal-1P) cause growth inhibition without methylglyoxal accumulation or cell killing (27). In another study, exogenous addition of Fru-6P causes *ptsG* transcript degradation in a *pgi pfkA mlc* triple mutant (in which *mlc* was deleted to increase *ptsG* transcription) (14), a finding that supports the notion that sugar-phosphate supplements elicit the same gene expression changes as those from genetic disruptions.

Straining the glycolytic capacity by adding upstream glycolytic intermediates diverts the midpath intermediate dihydroxyacetone phosphate (DHAP) to methylglyoxal via the action of methylglyoxal synthase (*mgsA*; MgsA) (Fig. 1). All organisms have low concentrations of methylglyoxal, but this highly reactive dicarbonyl metabolite, which is capable of generating advanced glycation products, is kept in check by detoxification pathways (28, 29). At first glance, the methylglyoxal shunt would appear to be "a suicidal, rather than a survival, strategy" (28). Yet, when glycolytic intermediates are at high intracellular concentrations (even

if artificially imposed), glyceraldehyde-3-phosphate dehydrogenase (*gapA*; GAPDH) is unable to oxidize glyceraldehyde-3-phosphate (GAP) due to a phosphate limitation (28). At this juncture, the conversion of DHAP to methylglyoxal and phosphate affords two benefits: a phosphate-rescue mechanism to sustain glycolysis and a safety valve to deplete built-up sugar phosphates. However, this relief mechanism has a narrow utility range: at low concentrations (0.08 mM), methylglyoxal is not toxic and offsets sugar-phosphate toxicity; however, when the methylglyoxal concentration exceeds the cell's detoxification capabilities (0.3 mM), one toxin has merely been replaced with another and there is loss of cell viability (30). For nonglycolytic sugar phosphates, it was postulated that the mechanism of growth inhibition might involve a salting-out effect due to the sequestration of intracellular water by the high concentrations of sugar phosphates ($>$10 mM) (27).

In the second paradigm, we consider α-methyl-glucoside (αMG), a nonmetabolizable analog of glucose that is used to trigger glucose-phosphate stress. αMG is efficiently transported and phosphorylated as if it were glucose but is metabolized no further, thus mirroring built-up Glc-6P (31, 32). The presence of αMG has little to no effect on wild-type (WT) cells, but it does lead to degradation of *ptsG* transcripts, the same regulatory response observed when *pgi*, *pfkA*, or *fbaA* mutants are exposed to glucose. Although αMG does not inhibit the growth of wild-type cells, it does inhibit the growth of a *sgrS* mutant, which fails to shut down further αMG uptake (see below). Supplementation of growth media with glycolytic intermediates allows the *sgrS* mutant to grow in the presence of αMG (33). Glc-6P, Fru-6P, or Fru-1,6BP, which are transported into the cell via the UhpT transporter, rescue the toxicity with Glc-6P being the most adept. While it is easy to rationalize how these supplements simply bypass the glycolytic blockade caused by the respective *pgi* or *pfkA* mutation, what is the barrier that is being bypassed in the case of αMG toxicity to a *sgrS* mutant?

Since Glc-6P restores growth, it appears that only the glucose transporter, PtsG, was bypassed. We speculate on the underlying mechanism. In contrast to the positive effects of glycolytic intermediates, providing exogenous pyruvate (the end product of glycolysis) to an *sgrS* mutant in the presence of αMG causes cell lysis by an unknown mechanism (33). This unexpected effect was postulated to be mediated by an imbalance in the PEP/pyruvate ratio, an idea bolstered by the finding that lysis is prevented by overexpression of PpsA, which converts pyruvate to PEP (33). Together, these findings suggest that the mechanism of αMG toxicity is a depletion of PEP, rather than toxicity related directly to the accumulated αMG-6P. A low PEP/pyruvate ratio, reflective of metabolically active cells, keeps the PTS proteins in a dephosphorylated state that is not primed for PTS-mediated sugar uptake. Even though the *sgrS* mutation is expected to leave unchecked the expression of PtsG, the low PEP/pyruvate ratio prevents glucose import and energy generation (34).

As with many bacterial sRNAs, SgrS action is mediated by Hfq, an RNA-chaperone protein (35). SgrS has at least seven direct regulatory interactions, with the translation of six transcripts being decreased by SgrS (*ptsG*, *manXYZ*, *adiY*, *purR*, *folE*, and *asd*) and one transcript (*yigL*) being increased (Fig. 2) (21, 23, 35–38). SgrS directly controls the *ptsG* transcript degradation response as it base pairs with the *ptsG* mRNA to promote *ptsG* translational inhibition and *ptsG* mRNA degradation. However, decreasing the expression of the PtsG transporter leaves unaffected the functional transporters already present in the membrane. A solution to this problem is provided in the form of a small protein, SgrT, which inhibits the activity of PtsG (but not ManXYZ). Thus, both the expression and the activity of PtsG are inhibited (39, 40). Remarkably, SgrT is encoded in the 5′ end of the *sgrS* sRNA.

SgrS and SgrT alleviate toxicity by interfering with *ptsG* expression and activity, which prevents the cells from depleting PEP during transport of glucose into the cell (41). In addition, amino acid supplementation can overcome toxicity but resumption of growth is slow (41). Lastly, induction of the Pho regulon alleviates toxicity, but the mechanism is unknown (33, 42). Whether these supplements act by independent mechanisms or are related to PEP and pyruvate remains to be established.

SgrS binds the *yigL* mRNA and protects it from RNase E action, thus enhancing the translation of *yigL*. YigL is a member of the HaloAcid Dehalogenase (HAD)-like family of phosphatases

that detoxify by dephosphorylating the stress-causing sugar phosphates (43–45). Since the accumulation of sugar phosphates leads to depletion of carbon metabolites and sequestration of phosphorus in the form of nonmetabolizable phosphorylated intermediates (36, 43, 46), releasing the phosphate would seem to be part of the solution. The HAD-like family consists of both highly specific and promiscuous phosphatases, exhibiting catalytic activity against several substrates. Such substrate ambiguity in hydrolyzing different metabolic phosphomonoesters allows these enzymes to serve in a "house-cleaning" capacity in all domains of life (44, 45). Newer activities likely arise as a result of gene duplication, followed by substrate specialization (44). The catalytic diversity seems to reflect the complexity of carbohydrate metabolism in bacteria (45). For example, the *E. coli* genome encodes 5 membrane-bound and 23 soluble HAD-like hydrolases, which collectively represent 40% of the total proteins with known or predicted phosphatase activity toward small-molecule phosphorylated metabolites (44). The importance of YigL is evident from the observation that prestress induction of this phosphatase nearly eliminates the stress response (36).

The above-mentioned two-pronged strategy to combat αMG toxicity mirrors the dual necessity of plugging a leak in a boat even while bailing out already built-up water: in the case of αMG toxicity, the accumulated sugar phosphate is dephosphorylated and pumped out even while fresh uptake is slowed (32, 47). This strategy of initiating both immediate and lasting responses is in fact duplicated with different stressors. In *E. coli*, 2-deoxyglucose (2dGlc) is taken up by the cells and phosphorylated to yield 2-deoxyglucose-6-phosphate (2dGlc-6P), which is toxic. Moreover, the inability to generate PEP from 2dGlc metabolism causes a deficit of PEP, which is consumed during uptake of 2dGlc. YniC (HAD-like phosphatase; *yniC*) appears to be the principal phosphatase involved in dampening 2dGlc-6P toxicity as *in vitro* studies revealed it to have higher catalytic efficiency (k_{cat}/K_m) toward 2dGlc-6P than YigL (44). A *yniC* mutant was more sensitive to the presence of 2dGlc in the growth medium than wild-type (IC_{50} of 20 μM versus 590 μM), and a YniC-overproducing strain could tolerate even 20 mM 2dGlc.

In *E. coli*, the role of DksA (*dksA*; RNA-polymerase transcription factor) and (p)ppGpp [alarmones synthesized by RelA (*relA*) and SpoT (*spoT*); (p)ppGpp synthetases] in response to glucose-phosphate stress is well documented (48). DksA and (p)ppGpp are described as global regulators of the stringent response to nutrient-limiting conditions and are believed to assist in recovery from the glucose-phosphate stress. The function of this stringent response is to inhibit the ribosome, as well as pilus and flagellum biosynthesis, thus conserving energy during nutrient-limiting conditions. *sgrS dksA*, *sgrS relA*, and *spoT* mutants exhibit severe growth defects under glucose-phosphate stress conditions due to a diminished stress response (48). Furthermore, deleting either *dksA* or *relA* and *spoT* lowers the expression of *sgrS* and *sgrR*, key mediators of the sugar-phosphate stress response, again highlighting the cross talk between the stringent response and alleviation of toxicity.

In the specific instance of a *pgi zwf* double mutant grown on glycerol and glucose, the mechanism of growth inhibition may be that Glc-6P can inhibit the gluconeogenesis enzyme FBPase that is required for growth on glycerol (12, 13). Although inhibition of FBP was observed *in vitro*, it needs to be confirmed *in vivo* (13).

Salmonella enterica. *S. enterica* and other gammaproteobacteria encode *sgrR-sgrS* homologs (19, 49). *S. enterica* genes regulated by *sgrS* include the *ptsG*, *manXYZ*, and *yigL* genes, as in *E. coli*, but also the *Salmonella*-specific gene *sopD* (50). *S. enterica* encodes two type 3 secretion systems (T3SS), encoded by *Salmonella* pathogenicity islands 1 and 2 (SPI1 and SPI2), that are used to inject proteins into host cells. SopD is one of the effector proteins secreted by the T3SS encoded by SPI1, although its function is unknown (51). *S. enterica* differs from *E. coli* in that, regardless of sugar-phosphate stress, *sgrS* is upregulated by SgrR during growth under conditions that are known to induce SPI1 (high salt and low oxygen). Thus, SopD expression is decreased during SPI1-inducing conditions by SgrS. Interestingly, another T3SS-1 effector, SopD2, is 42% identical to SopD, but its expression is not regulated by SgrS due to a single-nucleotide difference that prevents interaction of SgrS and the *sopD2* transcript (50).

While several HAD-like phosphatases display a high affinity for phosphorylated sugar substrates in *E. coli* (OtsB, YniC, YidA, YfbT, and YbiV) (44), only *yigL* is essential for growth in the

presence of αMG (36). YigL appears to have the same role in *S. enterica*, since its overexpression helped offset αMG stress while the active-site mutant YigL^{K191A} did not. Furthermore, the growth of *sgrS/pgi* and *yigL/pgi* mutant strains was strongly inhibited in the presence of glucose or trehalose, once again indicating the centrality of YigL in response to sugar-phosphate stress that results from the build-up of glycolytic intermediates (36).

Bacillus subtilis. The mechanism by which glucose inhibits a *B. subtilis pgi zwf* double mutant appears to be mediated by Glc-1P, which inhibits one of two enzymes involved in muramic acid production, either phosphoglucosamine mutase (*glmM*) or glucosamine-1P acetyltransferase (*glmU*), and thereby causes a cell wall defect (26). This inference is further strengthened by the finding that Glc-1P stress is associated with a heightened susceptibility to antibiotics targeting the cell wall (26). Attempts to induce Glc-6P stress in *B. subtilis* have been futile since a *pgi zwf pgcA* triple mutant could not be constructed (52).

Topics for Further Investigation

Glucose is readily available in mammalian blood at concentrations of 2 to 12 mM, and some bacterial mutants that experience glucose-induced sugar-phosphate stress are attenuated during infection of model systems (53) (Table 1). However, in all these instances, it remains to be determined what proportion of the fitness defect is due to a lack of glycolysis (energy deficit) or sugar-phosphate toxicity. For example, the intracellular pathogens, *Shigella* and *S. enterica*, rely on glycolytic enzymes for virulence. *Shigella* requires *pfkA* for expression of virulence regulators important in invasion of epithelial cells, and *S. enterica* requires both Pfk isozymes for full virulence in a mouse typhoid model of infection (54, 55). The *pfkA* gene is required for virulence of *Serratia marcescens* in a mouse model (56), but a *pfkA* mutant of *Francisella tularensis*, the causative agent of tularemia, has no fitness defect (57). In the plant pathogen *Xanthomonas oryzae*, mutation of *pgi* did not entirely prevent growth in plants but pathogenicity was significantly reduced (58). In the uropathogens responsible for urinary tract infections (UTI), mouse models of UTI revealed that *Proteus mirabilis* and uropathogenic *E. coli* (UPEC), *pgi* and *pfkA* were required for *P. mirabilis* but dispensable for UPEC (59).

With regard to glucose-induced toxicity, there are various topics that warrant further investigation. For instance, what is the mechanism by which αMG intoxicates cells? Is it purely through PEP depletion, or are there other mechanisms? How does stress modulate SgrR levels? What is the function of the *Salmonella* effector SopD, and why is it regulated by *sgrS* (50)? Does the regulation of *sopD* by *sgrS* suggest that sugar-phosphate stress occurs during infection? In *B. subtilis*, a *pgm* mutation can alleviate Glc-6P stress in a *pgi zwf* double mutant by preventing the conversion of Glc-6P to Glc-1P. Is this applicable in *E. coli*?

GALACTOSE

Background

Bacteria. Galactose, the C-4 epimer of glucose, is converted to Glc-6P by a three-step pathway named after Luis Leloir, an Argentine biochemist. Although this pathway (Fig. 1A) is highly conserved in all three domains of life, galactose is also catabolized via the De Ley-Doudoroff pathway in many prokaryotes, although this pathway is not known to contain toxic intermediates and is therefore not discussed further. As is typical of the catabolism of numerous sugars, the first step in the Leloir pathway entails the galactokinase (*galK;* GalK)-mediated phosphorylation of galactose to galactose-1-phosphate (Gal-1P). The displacement of Glc-1P from the sugar nucleotide UDP-glucose (UDP-Glc) by galactose-1-phosphate-uridylyltransferase (*galT;* GalT) yields UDP-galactose (UDP-Gal) and Glc-1P. In the final step, Glc-1P is converted to Glc-6P by PGM. Alternatively, Glc-1P can condense with UTP to generate a new molecule of UDP-Glc and pyrophosphate in the reaction catalyzed by UDP-glucose pyrophosphorylase (*galU;* GalU). With this route, the result is that one molecule of galactose was used to form one molecule of UDP-Gal (and not Glc-6P).

Mutations in three of the genes (*galE*, *galT*, and *galU*) in the galactose utilization pathway of *E. coli* and *S. enterica* each result in a different type of toxicity when grown in the presence

of galactose (3, 7, 60). As expected, none of these three mutants can grow on galactose as the sole carbon source (i.e., they are galactose negative). However, the presence of galactose inhibits growth even when other carbon sources are available (i.e., they are galactose sensitive). Since a mutation in *galK* prevents toxicity observed with both *galE* and *galT* mutants (3, 7), it was deduced that toxicity must stem at least in part from build-up of Gal-1P.

Eukaryotes. Humans also encode the Leloir pathway, and individuals lacking GALK, GALT, or GALE suffer from galactosemia (61, 62) (Table 1). While all three enzyme deficiencies share the common feature of elevated blood galactose, the clinical phenotypes associated with each are quite different even when patients are placed on a galactose-free diet (GALK, cataracts; GALT, progressive neurological disease [developmental delays, delayed language acquisition, impaired motor function] and ovarian insufficiency in females; and GALE, benign to severe phenotypes similar to the GALT defect). While the pathological basis remains unclear, glycolipid synthesis and glycoprotein synthesis are clearly impaired in the GALT and GALE mutants.

Evidence for Sugar-Phosphate Accumulation

Escherichia coli. Metabolic profiles for *E. coli* mutants deficient in *galE* and *galT* were evaluated with mass spectrometry (MS). In the presence of galactose, UDP-Gal accumulated in the *galE* mutant, while Gal-1P accumulated in both *galE* and *galT* mutants (63). This finding is consistent with earlier work in which Gal-1P was measured using a coupled enzyme assay with purified GalT. In this study, the *galT* mutant (0.67 μmol per 100 mg of dry weight cells), but neither the *galK* mutant (0.02 μmol per 100 mg of dry weight cells) nor the WT strain (0.01 μmol per 100 mg of dry weight cells), had significant accumulation of Gal-1P in the presence of galactose (64). Another group utilized thin-layer chromatography (TLC) to measure Gal-1P accumulation in a *galE* mutant (1.2 μmol per 100 mg of dry weight cells), and in several suppressor mutants with decreased GalK activity (ranging from 0 to 0.87 μmol per 100 mg of dry weight cells). The Gal-1P concentrations in these strains correlated with their inability to grow in the presence of galactose (7).

Eukaryotes. Since the deficiency of GALK (the kinase required to convert Gal to Gal-1P) is relatively benign and does not lead to a sugar-phosphate accretion, we focus here only on GALT and GALE. When GALT is not functional, Gal-1P accumulation is expected. Indeed, Gal-1P builds up in GALT-deficient *S. cerevisiae* (65), *Drosophila melanogaster* (66), mouse (67), zebrafish (68), and human red blood cells (RBCs) (69). For example, a *galT*-null *S. cerevisiae* mutant grown for 1 h in the presence of 0.005% (wt/vol) galactose exhibited a 1,000-fold increase in Gal-1P compared to the wild type or a GALK-deficient strain (65). Likewise, a 40-fold increase in Gal-1P was noted in RBCs of children (8 to 20 months of age) with classical galactosemia and on a galactose-free diet compared to age-matched unaffected children on a galactose-containing diet (69). This finding also highlights how the endogenous synthesis of galactose (70) contributes to sugar-phosphate build-up in galactosemic individuals despite dietary restriction of galactose.

When GALE is deficient, UDP-Gal generated from Gal-1P by GALT is unable to be converted back to UDP-Glc. Consistent with this notion, the absence of GALE resulted in accumulation of UDP-Gal in *S. cerevisiae* (65) and *Caenorhabditis elegans* (71).

Mechanism of Toxicity

***Escherichia coli* and *Salmonella enterica*.** Galactose is toxic to a *galT* mutant of *E. coli* due to the accumulation of Gal-1P, the substrate of GalT. Because of feedback inhibition of GalK by Gal-1P and depletion of ATP, galactose build-up is also observed (1, 3, 63, 72). When GalT is dysfunctional, UDP-Glc is not consumed. Mounting levels of UTP and CTP (which is formed by deamination of UTP) would be expected to signal the cells to dampen expression of pyrimidine biosynthesis genes, an expectation confirmed by transcriptomic and metabolomic studies of a *galT* mutant (1, 63). The wasteful investment of ATP in the GalK reaction is also consistent with the observed lower levels of ATP and GTP and the modest growth enhancement afforded by addition of purine nucleosides to a *galT* mutant grown in the presence of galactose and fructose/glycerol.

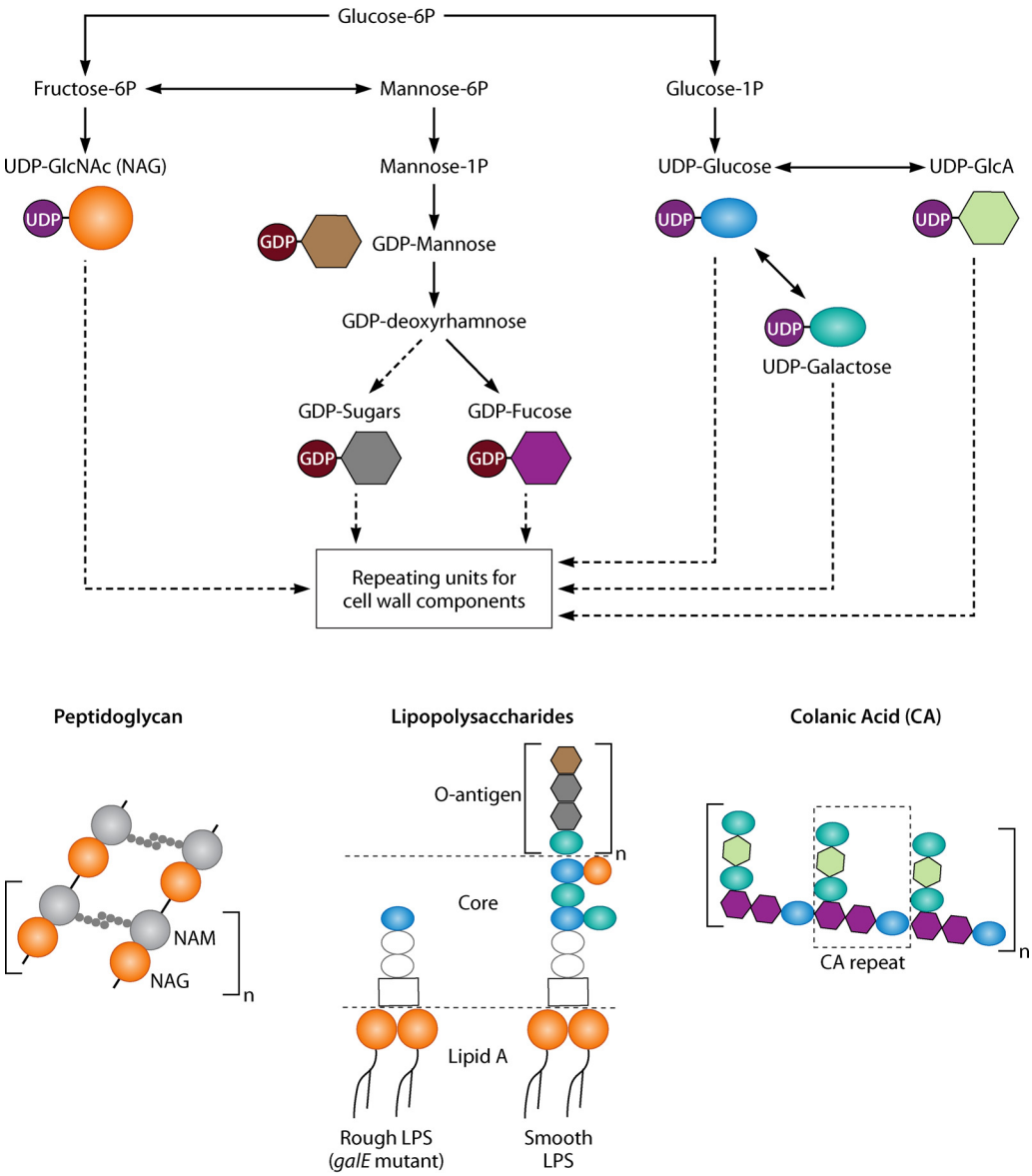

FIG 3 Contribution of sugar phosphates to synthesis of *S. enterica* cell wall components. The LPS of the *S. enterica galE* mutant is truncated ("rough") since UDP-galactose cannot be incorporated. In the peptidoglycan, NAM is *N*-acetylmuramic acid. In LPS, the rectangle symbol in represents 3-deoxy-D-manno-octulosonic acid (KDO), and the white ovals depict heptose. The orange circles in Lipid A are *N*-acetylglucosamine, which is derived from UDP-GlcNAc. The colors for fucose, galactose, glucose, glucuronic acid (GlcA), mannose, and *N*-acetylglucosamine are shown in the pathways (top) and used likewise in the peptidoglycan, LPS, and colanic acid (bottom).

The *galE* mutant is the most sensitive to galactose. The *E. coli galE* mutant cells fail to grow in minimal medium containing galactose plus one other carbon source, while they lyse in rich medium containing galactose (3–7). In a study using nutrient broth rather than minimal medium, the concentration of UDP-*N*-acetylglucosamine (UDP-GlcNAc), a major component of peptidoglycan synthesis, decreases in the *galE* mutant for the first 2 h after addition of galactose to a culture, while UDP-Gal, UTP, and UMP all accumulate (73), perhaps reflective of a general uptick in galactose catabolism until UTP shortages ensue. At 3 h after galactose addition, there is a striking decline in the concentrations of all of these metabolites and an increase in Gal-1P. Since GalE functions to interconvert UDP-Gal and UDP-Glc, the initial upregulation of UTP synthesis merely ties up the UDP in the form of UDP-Gal with no possibility of recycling it back to UDP-Glc. This lack of UDP-Glc leads to a shortage of UDP-GlcNAc, which causes peptidoglycan defects and lysis (Fig. 3 and 4) (73).

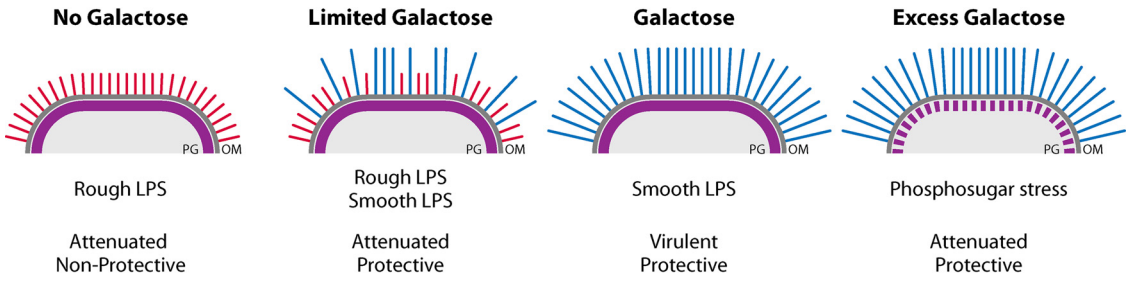

FIG 4 LPS and peptidoglycan of a *S. enterica galE* mutant with various concentrations of galactose. A *galE* mutant of *S. enterica* has truncated ("rough") LPS unless galactose is provided. Immune responses to rough LPS do not provide protection against wild-type *Salmonella* and are referred to as nonprotective in the figure. Trace amounts of galactose may allow some of the LPS to be full length and elicit a protective immune response, while the bacterium remains attenuated due to the regions of rough LPS still being accessible to complement and other innate immune defenses. The Goldilocks amount of galactose will restore a complete, full length, LPS, providing full virulence. Too much galactose can lead to galactose-induced toxicity, which includes accumulation of UDP-Gal and Gal-1P, and a decline in UDP-GlcNAc that ultimately leads to peptidoglycan defects and lysis.

While peptidoglycan synthesis is defective when a *galE* mutant is grown in the presence of galactose, LPS is defective when the same mutant is grown in the absence of galactose (Fig. 3 and 4). In the absence of galactose, the *galE* mutant cannot produce UDP-Gal and is therefore unable to condense galactose to the growing LPS O-antigen, thus preventing all subsequent ordered additions of sugar residues to the O-antigen. The LPS core of a *galE* mutant is "rough" (truncated, as opposed to full length, and "smooth") and contains only heptose and glucose residues (7, 74, 75). Providing low concentrations of exogenous galactose (together with glucose) rapidly restores the LPS to wild-type without causing toxicity. Phage P22, a transducing phage for *S. enterica* serovar Typhimurium, binds specifically to the O-antigen and cannot bind to *galE* mutant cells (76). On the other hand, Phage P1, a transducing phage for *E. coli* K-12, binds only rough LPS. These observations provide the basis for the genetic tool in which a *S. enterica* serovar Typhimurium *galE* mutant can be transduced with either P1 or P22, depending on the growth conditions (77, 78). Under typical growth conditions, the *S. enterica* serovar Typhimurium *galE* mutant is rough and susceptible to infection by P1 phage; likewise, for a *galE* mutant of enterohemorrhagic *E. coli* (EHEC) (79). With the addition of glucose and galactose, a wild-type LPS is produced and the *S. enterica* serovar Typhimurium *galE* mutant can be infected with P22 phage. However, the galactose should be provided at low concentrations and for a short duration to produce the full-length LPS. Prolonged incubation or high concentrations of galactose can lead to lysis and to spontaneous generation of *galK* mutants; these findings reinforce the notion that tying up UDP in the form of UDP-Gal (through the action of GalT) is unfavorable even if glucose is available (6, 7, 78).

Omics approaches. Although we lack a full understanding of the metabolic enzymes that are directly inhibited by accumulated Gal-1P, one mechanism of toxicity was discovered when it was noted that the growth inhibition was more severe in minimal medium supplemented with glycerol compared to fructose. Transcriptomic studies revealed surprisingly that the *glp* genes involved in glycerol metabolism are downregulated when the *galT* mutant was grown in the presence of 0.3% (wt/vol) galactose and 0.3% (vol/vol) glycerol (1). Typically, the GlpR (*glpR*) transcriptional repressor is induced by *sn*-glycerol-3-phosphate (Gly-3P), which is generated from glycerol as part of a feed-forward regulatory mechanism. However, in the presence of Gal-1P, it was hypothesized that GlpR fails to derepress the glycerol utilization genes, thus preventing growth on glycerol. Indeed, the observation that deletion of *glpR* largely restores growth and ameliorates toxicity supports the idea that Gal-1P acts via an anti-inducer mechanism. Addition of exogenous purine nucleosides provided a slight boost to growth of the *glpR* mutant, suggesting that purine depletion is a minor effect compared to the *glp* gene repression. Another yet-to-be-tested explanation is that Gal-1P inhibits glycerol kinase and dampens biosynthesis of Gly-3P (the GlpR inducer). This notion is tenable only if the *glpR* mutant permitted high constitutive expression of the glycerol kinase to override the putative Gal-1P inhibition. While this second hypothesis has not been ruled out, the suppression of glycerol utilization by Gal-1P exemplifies the idea

that exceeding the steady-state ceiling of a sugar phosphate could have unexpected effects on gene regulatory networks.

A subsequent metabolomics study performed at 30 min after galactose addition to a *galE* mutant of *E. coli* found very similar results in that galactose, Gal-1P, and UDP-Gal all accumulated in the *galE* mutant, with UDP-Gal being the most pronounced (63). Moreover, UDP-Glc was nearly absent in the *galE* mutant. Two other studies revealed that UTP and CTP decline as uridine is trapped as UDP-Gal (6, 60). As expected, DNA, RNA, and protein synthesis decline shortly thereafter. Numerous indirect effects have been characterized in *E. coli* using transcriptomics and metabolomics (60, 63). Upon galactose addition to the *galE* mutant, 135 genes are upregulated, and 106 are downregulated (60). The upregulated genes include those involved with pyrimidine biosynthesis; the *gad* and *hde* genes of the *E. coli* acid fitness island (mirroring the *galT* mutant); the small RNAs *csrC*, *isrB*, and *ssrA*; and ribosomal RNAs. The downregulated group includes genes encoding ribosomal proteins, glutathione biosynthesis enzymes, and several PTS transporters. The significance of the anti-correlated effects on the expression levels of the rRNA and ribosomal protein genes is unclear. Nucleotide analysis in the *galE* mutant (60) demonstrated that (p)ppGpp concentrations decline, which is similar to the *galT* mutant but opposite to the increase typically observed during nutrient deprivation.

There are thematic differences between *galE* and *galT*, however. Providing exogenous pyrimidines can at least partially restore growth of the *galE* mutant in minimal fructose medium, while exogenous purines enhance growth arrest (60). This finding is rationalized by the shortage of UTP and CTP in the *galE* mutant. In the *galT* mutant, UTP and CTP accumulate, and pyrimidine biosynthesis genes are downregulated, while ATP and GTP concentrations decline. Thus, providing purines to the *galT* mutant enhances growth (1).

Vaccine production. Mutants of *S. enterica* serovar Typhimurium with either an incomplete or rough LPS are dramatically attenuated in mouse models of infection but, with the exception of *galE* mutants, are poor vaccine strains given their low immunogenicity in the absence of the O-antigen of the LPS (80–83) (Fig. 4). The design of a successful vaccine strain requires establishing a fine balance between immunogenicity and attenuation, as illustrated by various *galE* mutants. When injected intraperitoneally, *galE* mutants (unlike other rough mutants) are highly immunogenic because they can elaborate at least some full-length lipopolysaccharide (LPS) using galactose scavenged *in vivo* (81). However, despite the full-length LPS, they are attenuated due to galactose sensitivity (81). In contrast, double mutants lacking *galE* and either one of two UDP-sugar LPS transferases (the names for the mutated transferases are unclear given differences between previous and current nomenclature [84]) cannot incorporate exogenous galactose into LPS and are not immunogenic (81). In a similar vein, a *galK galE* double mutant, albeit galactose insensitive, is avirulent and nonimmunogenic due to its truncated LPS (Fig. 4).

This property of being attenuated and immunogenic has rendered *galE* mutants a promising component of live-attenuated vaccines in mice, calves, and poultry (83, 85–87). In fact, the only live-attenuated vaccine strain of *S. enterica* serovar Typhi that is licensed for human use, Ty21a, was originally recovered on plates designed to isolate *galE* mutants of the parent strain Ty2 (88) and then mutagenized further (88–90). Caution is warranted, however, since later studies found that a newly constructed Ty2 double mutant lacking both *galE* and *via* (which encodes the V-antigen) was virulent in human volunteers who ingested the organism and was only modestly attenuated when administered to mice by the intraperitoneal route (18-fold) (91). The lack of attenuation of this *galE* mutant in humans demonstrates that mutations other than *galE* contribute to attenuation of the vaccine strain Ty21a. It is unknown why a *galE* mutant of serovar Typhi is not attenuated. *Salmonella enterica* serovar Choleraesuis is another example of a serovar in which the phenotype of a *galE* mutant was not as expected, since the mutants are virulent in mice (92). The LPS of this serovar includes galactose residues in the core but not in the O-antigen, so very little galactose may need to be scavenged from the host to elaborate a complete and full-length LPS (92). It would be instructive to examine the LPS composition of individual bacteria during infection to test these possibilities.

Eukaryotes. We consider below only a few aspects pertinent to the mechanism of toxicity of Gal-1P (93). First, although direct targets have not been established *in vivo*, Gal-1P was shown to interfere with the function of inositol monophosphatase (IMPase), which generates inosine from inosine-1-phosphate (94, 95). Gal-1P acts as a competing substrate for IMPase and triggers inositol depletion with associated downstream alterations to signaling and cellular homeostasis. This idea is supported by at least three observations: (i) GALT-deficient fibroblasts are able to grow in 2.5 mM inosine despite accumulation of Gal-1P (96), (ii) overexpression of human inositol monophosphatase rescues *galT-null S. cerevisiae* mutant grown in the presence of galactose (94), and (iii) genes involved in inositol biosynthesis are upregulated in a *galT*-null *S. cerevisiae* mutant grown on galactose (97). Second, because the severity of galactosemia does not correlate with the amount of accumulated Gal-1P, it is likely that other factors too contribute to the final clinical picture. For instance, the inability to convert Gal-1P to UDP-Gal is expected to cause defects in galactoprotein/galactolipid synthesis; indeed, the glycoproteins/lipids from galactosemic individuals show lower levels of galactose and *N*-acetylgalactosamine (98, 99). Since protein glycosylation in the endoplasmic reticulum (ER) is a key determinant for protein folding and trafficking, it is not surprising that aberrant protein glycosylation would aggravate ER stress and initiate a cascade of signaling changes associated with the unfolded protein response (UPR); in fact, results of studies in *S. cerevisiae* and *C. elegans* show that UPR is a shared outcome when either GALT or GALE is dysfunctional (71, 93, 100). Lastly, the inability of galactosemic individuals to fully metabolize galactose via the Leloir pathway results in use of alternative metabolic paths. Galactose is converted to galactitol by aldose reductase and to galactonate by galactose dehydrogenase (69). While it is unclear whether there are toxicities associated with galactitol and galactonate, the conversion of galactose to galactitol will consume NADPH and lead to oxidative stress. We showcase these complexities with Gal-1P to spotlight the difficulties in fully parsing direct and indirect effects associated with any sugar-phosphate toxicity.

Topics for Further Investigation

Galactose-negative mutants of bacteria, including *E. coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Streptococcus pneumoniae*, are attenuated in animal infection models (101–104). Inhibition of the enzymes would lead to growth inhibition or potentially cell lysis. Bacteria would naturally encounter galactose in the host since galactose is consumed in foods, including milk, and also many fruits and vegetables (105). As described above, *Salmonella galE* mutants obtain galactose *in vivo* (81). UPEC relies on galactose for survival during infection, since a *galK* mutant was attenuated in its ability to form biofilm-like intracellular bacterial communities (106). Similarly, deletion of the entire galactose operon (*galEKTM*) in EHEC resulted in a decreased ability to colonize the intestine of an infant rabbit (79). *Haemophilus influenzae galE* and *galE galK* mutants are significantly attenuated in infant rabbit bacteremia models. Attenuation was attributed to changes in LPS rather than phosphosugar accumulation because mutation of *galK* would abrogate any phosphosugars produced in a *galE* mutant alone (107). *galU* mutants are defective in LPS or capsule components and are also highly attenuated *in vivo* (108–112). Thus, targeting these enzymes may induce sugar-phosphate stress while also preventing the utilization of an essential nutrient, depending on the bacterial species and the type of infection. However, humans also encode enzymes of the Leloir pathway, and the bacterial and human homologs are highly conserved in sequence and structure. Therefore, it is highly unlikely that species-specific drugs could be designed. Curtailing paracellular and transcellular transport of such drugs across enterocytes might provide a possible solution to selectively target intestinal pathogens that have not reached systemic sites.

Does Gal-1P interact directly with GlpR to prevent GlpR release of DNA in the presence of the true inducer, Gly-3P (1)? Or could Gal-1P inhibit glycerol kinase? What is the state of the LPS in a *S. enterica galE* mutant during infection of mice? The mutant is immunogenic because of its ability to synthesize at least some full-length LPS but is attenuated,

presumably due to UDP-Gal accumulation. Is the attenuation due entirely to galactose sensitivity, or is the LPS defective enough to allow killing by complement?

FRUCTOSE-ASPARAGINE

Background

Bacteria. Fructose-asparagine (F-Asn) is an Amadori product that forms spontaneously between glucose and asparagine. It is found in human foods that have an abundance of these two compounds (asparagus, apricots, and other fruits and vegetables), and heating in the absence of water greatly increases its concentration (113–116). For example, raw apricots, lettuce, and canned peaches have between 400 and 800 pmol/mg, while commercially available dried apricots contain 8,000 pmol/mg (116). Mouse chow also contains F-Asn (400 pmol/mg) (116, 117).

The catabolic pathway for F-Asn in *S. enterica* serovar Typhimurium is encoded by the *fraBDAE* genes (118). These genes are not present in *E. coli*, but they are found in *S. enterica*, some members of the closely related *Klebsiella* and *Citrobacter* genera, as well as the distantly related *Clostridia* (119). The genes are also present within metagenomes of the murine intestinal tract (and some soil and rhizosphere metagenomes) (116, 119). The presence of the genes correlates with utilization of F-Asn as sole carbon source *in vitro* (119). The pathway is not found in eukaryotes.

There are four steps to the F-Asn catabolic pathway (Fig. 1B). FraE (*fraE*) is a periplasmic member of the asparaginase family that releases ammonia from F-Asn to create fructose-aspartate (F-Asp) (120). F-Asp is transported into the cell via the FraA (*fraA*) transporter and then phosphorylated by the kinase, FraD (*fraD*), to create 6-phospho-fructose-aspartate (6P-F-Asp) (120, 121). In the last step, the FraB (*fraB*) deglycase cleaves 6P-F-Asp into Glc-6P and aspartate (122). *S. enterica* serovar Typhimurium mutants lacking *fraD*, *fraA*, or *fraB* are F-Asn negative (fail to grow on F-Asn as sole carbon source). Mutants lacking *fraE* are only partially impaired for F-Asn utilization because *ansB* (encoding the asparaginase AnsB) is able to compensate as an alternative fructose-asparaginase (120). Only the *fraB* mutant is F-Asn sensitive (fails to grow in the presence of F-Asn even when alternative carbon sources, including glucose, are available) (11). F-Asn inhibits the growth of the *fraB* mutant with an IC_{50} of 19 μM. This inhibition is bacteriostatic since the cells recover upon removal of F-Asn. However, during infection of mice the inhibition is bactericidal, presumably due to the presence of additional stressors in the host environment (11). 6P-F-Asp appears to be the toxic metabolite since this compound accumulates in *fraB* mutant cells grown in the presence of F-Asn, and a *fraD* (kinase) mutation prevents the production of 6P-F-Asp and eliminates the toxicity experienced by a *fraB* mutant (11).

Mice with a healthy microbiota are highly resistant to *S. enterica* serovar Typhimurium-mediated inflammation (123, 124). The F-Asn in mouse chow (~400 pmol/mg) is rapidly consumed by specific members of the *Clostridia* class within the intestinal tract and is largely unavailable to *S. enterica* (116). However, if *S. enterica* is able to inflame the intestinal tract, these *Clostridia* are eliminated, allowing *S. enterica* access to F-Asn (117). It is under these conditions that a *Salmonella fraB* mutant is attenuated (117, 118). A *fraD* mutant is not significantly attenuated, suggesting that the lack of F-Asn as a nutrient source is not a detriment but rather that it is the accumulation of 6P-F-Asp in the *fraB* mutant that attenuates the organism (11).

Interestingly, the F-Asn utilization pathway has been lost in some serovars of *Salmonella*, including serovars Typhi and Paratyphi A, as part of the genome reduction that accompanied host adaptation. These serovars contain a deletion spanning *fraD*, *fraA*, and part of *fraE* (119). The loss of the *fra* locus is consistent with the loss of many other serovar Typhi genes involved with respiration of nutrients found in the inflamed intestine (125–127).

Evidence for Sugar-Phosphate Accumulation

Salmonella enterica. 6P-F-Asp has been shown by MS to accumulate in *fraB* mutant cells of *S. enterica* when grown on F-Asn, while it is below the limit of detection in wild-type cells or a mutant lacking the entire locus (*fraR* and *fraBDAE*) (11).

Mechanism of Toxicity

Salmonella enterica. The mechanism of intoxication by 6P-F-Asp is unknown.

Topics for Further Investigation

In the case of *S. enterica*, there are very few therapeutic targets due to diverse nutrient accessibility in the host and redundant metabolic pathways (128). FraB is a highly specific target for the nontyphoidal serovars of *S. enterica*. It is found in only a few bacteria and not in eukaryotes (Table 1). However, the mechanism of 6P-F-Asp toxicity to *S. enterica* is completely unknown, as are the cellular responses to this toxicity. A *fraB* mutant is dramatically attenuated in mouse models as it obtains F-Asn from mouse chow, and the resulting inflammation eliminates competitors for F-Asn among the normal microbiota (11, 116, 117, 119). Why is F-Asn bacteriostatic to *S. enterica in vitro* but bactericidal in mouse models of infection (11)? Since F-Asn is present at high concentrations in some human foods such as dried apricots, FraB inhibitors may be able to treat *S. enterica*-mediated gastroenteritis in humans. Given typical dietary variations, however, it would be useful to package F-Asn with the FraB inhibitor, as long as the safety, pharmacokinetics, and pharmacodynamics of both compounds are evaluated and there is little cross-interference between them. However, once *S. enterica* has reached systemic sites it is primarily intracellular, and it is unlikely that F-Asn would penetrate host cells. Efforts are under way to develop host-directed therapeutics that can either inhibit intracellular *S. enterica* or interfere with regulatory networks that *S. enterica* uses to survive within host cells (129–132). Such host-directed compounds will not affect extracellular *S. enterica* within the intestine, but combining them with FraB-specific inhibitors would provide a complementary cocktail.

FRUCTOSE

Background

Bacteria. In some bacteria, including *E. coli* and *Enterobacter aerogenes* (originally known as *Aerobacter aerogenes* and then proposed to be named *Klebsiella aeromobilis* [133]), fructose is transported into the cell by a fructose-specific PTS (*fruA*; FruA) and converted to fructose-1-phosphate (Fru-1P). A second phosphorylation is catalyzed by fructose-1-phosphate kinase (*fruK;* F1PK) to generate Fru-1,6BP, a glycolytic intermediate (Fig. 1F). Sugar-phosphate stress results from mutation of *fruK*, resulting in accumulation of Fru-1P (134).

Eukaryotes. In mammals, fructose is first converted to Fru-1P by ketohexokinase and then cleaved by aldolase B to DHAP and glyceraldehyde. The accumulation of Fru-1P in human cells has drawn attention due to hereditary fructose intolerance (HFI), an inborn disorder of carbohydrate metabolism caused by mutations in the *ALDOB* (aldolase B) gene (135); although there are three aldolase isoforms (A, B, and C), only isoform B is deficient. Aldolase B differs from the other two isoforms in its ability to cleave either Fru-1,6BP to DHAP and GAP or Fru-1P to DHAP and glyceraldehyde. Aldolase B is expressed primarily in the liver, kidney, and small intestine, with liver dysfunction in HFI patients being largely attributed to a build-up of Fru-1P and not Fru-1,6BP. This surprising finding has been rationalized by (i) the observation that some of the *ALDOB* mutations disproportionately affect the cleavage activity toward Fru-1P compared to Fru-1,6BP (136) and (ii) the postulate that aldolase A, which is predominantly expressed in muscle but also in other tissues, might provide backup for clearing the Fru-1,6BP (135). Just as bacterial sugar-phosphate toxicity is easily avoided by impeding the action of the upstream kinase, blocking the activity of ketohexokinases A and C in mice by either a genetic knockout or treatment with the inhibitor osthole prevents the production of Fru-1P and thereby ameliorates HFI-like symptoms (137).

Evidence for Sugar-Phosphate Accumulation

Escherichia coli. In *E. coli*, studies were performed in an *uhp*^{c} (constitutive expression) background to enable direct uptake of Fru-1P. Both fructose and Fru-1P individually inhibited growth of a *fruK* mutant (134). Approximately 97% of the radiolabeled [^{14}C]fructose in *fruK* mutant cells was converted to Fru-1P (134).

Enterobacter aerogenes. The presence of fructose inhibits the growth of *fruK* mutants of *Enterobacter aerogenes* (134, 138). These kinase-deficient mutants accumulated 18.2 μM Fru-1P per g of cells compared to only 0.92 μM Fru-1P per g of WT cells (138).

Eukaryotes. After consumption of 50 g of fructose, Fru-1P accumulation in the liver of patients with HFI has been measured using ^{31}P nuclear magnetic resonance (NMR) spectroscopy (2). The increase in liver sugar phosphate was accompanied by the expected decrease in P_i.

Mechanisms of Toxicity

Bacteria. No direct targets of Fru-1P in bacteria have been identified. However, Fru-1P is a strong glycating agent that can cause DNA damage of a plasmid *in vitro*, as assessed by transformation competence (139).

Eukaryotes. In humans affected with HFI, consumption of fructose (or sorbitol) results in a build-up of Fru-1P thus depleting P_i and ATP. Hypoglycemia arises from a twin blow to glycogenolysis: phosphate sequestration impairs phosphorolysis of glycogen, and Fru-1P inhibits glycogen phosphorylase (140). In addition, apoptosis in mouse lymphoma cells has been observed presumably due to glycoxidation of the genomic DNA (141).

Topics for Further Investigation

Does *E. coli* experience Fru-1P toxicity in the absence of the *uhp*^{c} allele? Do PTS mutations alleviate toxicity? Are there any pathogens in which *fruK* mutants are attenuated during infection? Animal experiments should be performed in the presence or absence of exogenous fructose, since accessibility of dietary fructose to invading pathogens may be limited. In mice, the small intestine clears most of the dietary fructose, while the liver and colonic microbiota also contribute (142). If *fruK* mutants are indeed attenuated, then bacterial FruK, which converts Fru-1P to Fru-1,6BP, merits exploration as a drug target since humans do not have a FruK homolog and use aldolase B to cleave Fru-1P, the entry metabolite generated from fructose.

TREHALOSE AND MALTOSE

Background

General. Trehalose is a disaccharide consisting of two glucose molecules, joined by an α1$\rightarrow$1 linkage, that is found widely in many organisms but not in vertebrates. It is used as a storage/signaling molecule, a transport sugar, an osmolyte, and a stress protectant (8, 143–145). Trehalose can be synthesized by at least five different pathways, but the most highly studied route, found in both prokaryotes and eukaryotes, entails the use of a trehalose-6-phosphate synthase (OtsA/TPS1) to condense an ADP/UDP-Glc to a molecule of Glc-6P, resulting in trehalose-6-phosphate (Tre-6P). A trehalose-6-phosphate phosphatase (OtsB/OtsB2/TPS2/OrlA) dephosphorylates Tre-6P to yield trehalose (Fig. 1C and Fig. 5). In some bacteria and fungi, disruption of the *otsB* homolog causes an accumulation of Tre-6P, which is highly toxic to the cells (8, 146–156). This example differs from the other sugar phosphates in this review in that it is not the provision of trehalose to an *otsB* mutant that leads to toxicity but rather the generation of Tre-6P from Glc-6P, which is generated from glycolytic or gluconeogenic pathways.

We include maltose in this section because its metabolic pathways intertwine with trehalose, and providing trehalose to a *glgE* or *glgB* mutant of *Mycobacterium* causes maltose-1-phosphate (Mal-1P) toxicity (157). In fact, it was recently discovered that all α-glucans in *Mycobacterium*, both intracellular and extracellular, are derived from Mal-1P, lending appeal to consideration of these converging pathways as therapeutic targets (158).

***Mycobacterium* spp.** There are two pathways by which *Mycobacterium tuberculosis* synthesizes trehalose (Fig. 5) (152). The dominant pathway utilizes OtsA-OtsB2 as described above, but a second route leads to trehalose formation by removal of two sugar units from α-1,4-glucans using the TreX-TreY-TreZ catalytic trio (Fig. 5). *otsB2* is an essential gene that cannot be mutated in the absence of a second copy. A conditional *otsB2* mutant was used to demonstrate accumulation of Tre-6P and that exogenous trehalose could not rescue the toxicity associated with Tre-6P. Thus, the

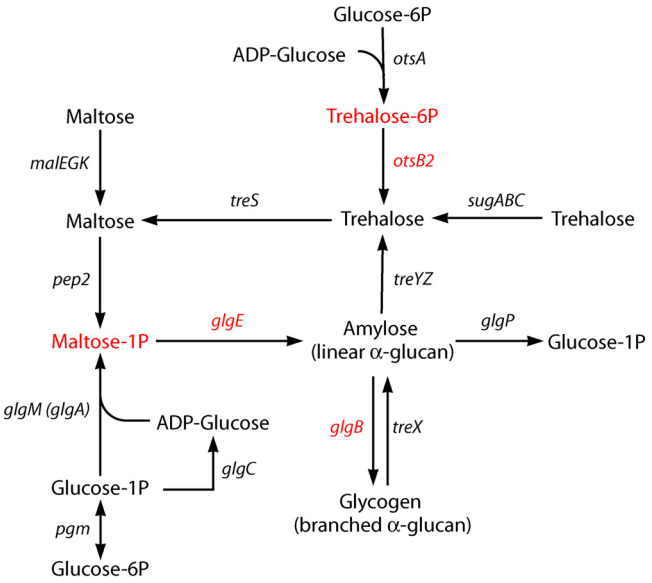

FIG 5 Trehalose/maltose pathways of *M. tuberculosis*. Genes whose mutation/dysfunction cause accumulations of toxic phosphosugars and the respective built-up phosphosugar are highlighted in red. The *E. coli* versions of these pathways are shown in Fig. 1C for comparison.

phenotype is not due to the inability to synthesize trehalose but likely attributable to adverse outcomes from Tre-6P accumulation.

The *otsB2* gene was required during the acute phase of mouse infection but not during the chronic phase, a surprising observation which might indicate a more significant role for the TreX-TreY-TreZ pathway during the chronic phase (152). Moreover, mutation of *otsA* relieves the toxicity experienced by an *otsB2* mutant and thus could provide a ready mechanism for bacteria to become resistant to therapeutics directed at OtsB2. Tn-seq was used to identify genes that are essential for viability in an *otsA* background (synthetic lethals) and numerous genes were identified, including *treX*, *treY*, and *glgC* (152). The products of these genes, along with OtsB2, could be targeted as part of a combinatorial therapeutic cocktail.

Mal-1P is another sugar phosphate that is toxic to *Mycobacterium* species; maltose is similar to trehalose in that it contains two glucose units, although the two units are joined by a $\alpha 1 \rightarrow 4$ linkage rather than the $\alpha 1 \rightarrow 1$ linkage in trehalose. A trehalose synthase (*treS*, TreS) transforms trehalose to maltose (159). Once generated, maltose is exploited for the biosynthesis of intracellular and capsular α-glucans using the Pep2-GlgE duo: Pep2 (*pep2*) phosphorylates maltose to Mal-1P, and GlgE (*glgE*), a maltosyltransferase, then uses Mal-1P as the precursor to generate α-1,4-glucan polymers for carbon and energy storage and capsule synthesis (158). GlgB (*glgB*) is a branching enzyme that is responsible for the formation of α-1,6 linkages. Mal-1P accumulates in *glgE* and *glgB* mutants (Fig. 5) (157). In *Mycobacterium smegmatis*, a *glgE* mutant is viable in minimal medium but intoxicated upon supplementation with trehalose. In contrast to *M. smegmatis*, it was not possible to mutate *glgE* in *M. tuberculosis* except in a *treS* background. This observation confirms that Mal-1P is also toxic in *M. tuberculosis*. As expected, *treS* or *pep2* mutations that prevent Mal-1P formation also prevent trehalose-mediated toxicity of *M. smegmatis* and *M. tuberculosis glgE* mutants (157).

During infection of mice, Mal-1P presumably accumulated in a *glgE* (GlgE) mutant and was bactericidal to *M. tuberculosis*, suggesting that trehalose is available to the bacteria *in vivo*. Because vertebrates do not synthesize trehalose, it was concluded that trehalose must be synthesized by the bacteria and released extracellularly during cell wall biosynthesis and then transported back into the cell using an ABC transporter (160). Consistent with this notion, *M. smegmatis glgE* mutants lacking the *sugABC* transporter became resistant to trehalose-mediated toxicity (160). If GlgE is targeted

therapeutically, mutations in *treS*, *pep2*, *glgC*, or *glgA* would eliminate Mal-1P toxicity (158). Fortunately, none of these mutations restores α-1,4-glucan polymer production, leaving the bacterium attenuated.

Eukaryotes. In fungi, including *S. cerevisiae*, *Candida albicans*, *Cryptococcus neoformans*, *Aspergillus nidulans*, and *Aspergillus fumigatus*, Tre-6P accumulates in mutants lacking the Tre-6P phosphatase (TPS2/OrlA) (8, 146–150, 153, 155, 156). Interestingly, the accumulation is temperature dependent, presumably due to increased trehalose synthesis in response to heat stress (8). The resulting phenotypes of TPS2/OrlA mutants are quite severe, including growth defects, lysis, and attenuation in models of animal infection (8, 147–150, 153, 154, 161).

Tre-6P-mediated toxicity has also been observed with RNAi knockdown of the Tre-6P phosphatase, *gob-1*, in *C. elegans* (162). This leads to a "gut-obstructed" phenotype and disruption of larval development.

Evidence for Sugar-Phosphate Accumulation

***Mycobacterium* spp.** Conditional *otsB* mutants of *M. tuberculosis* but not the wild type accumulated a high abundance of Tre-6P as measured by TLC, followed by [^{1}H]NMR spectroscopy. Cytosolic Tre-6P in mutant cells ranged from 5 to 10 mM (152). TLC analyses of hot water extracts of *M. smegmatis glgE* mutants also revealed Mal-1P accumulation (157).

Eukaryotes. In *C. albicans*, accumulation of intracellular Tre-6P was measured under conditions of growth at 30°C and 43°C. After 2 h of growth at 43°C, a *tps2*/*tps2* mutant accumulated 35 mM Tre-6P. In contrast, the WT and a heterozygous *tps2* mutant grown at 43°C only contained 1 and 2.5 mM Tre-6P, respectively. For all strains, the Tre-6P concentrations were much lower at 30°C, with a *tps2*/*tps2* mutant containing 5 mM after 4 h of growth and both the WT and a heterozygous *tps2* mutant containing 100 to 200 μM Tre-6P (150).

Similarly, in haploid *S. cerevisiae* bearing a *tps2* mutation, after a shift in temperature from 27 to 40°C and 1 h of growth, Tre-6P accumulated to 0.45 g of Tre-6P per gram of protein (146). This increased to 1.4 g of Tre-6P per gram of protein at the stationary phase. For comparison, the concentration of Tre-6P in wild-type cells was below the limit of detection.

Accumulation of Tre-6P in a *C. neoformans tps2* mutant occurred at both 30 and 37°C with the highest intracellular concentrations reported at 37°C. The maximum concentration was reached after 3 h of growth for both temperatures, reaching 170 μmol per 10^8 cells at 30°C compared to 280 μmol per 10^8 cells at 37°C (148).

In *A. nidulans*, TLC analyses revealed accumulation of Tre-6P in an *orlA* mutant but not in the WT (156). In *A. fumigatus*, liquid chromatography-MS analyses of *orlA* mutants revealed accumulation of Tre-6P in both conidia and mycelia grown at various temperatures (153).

In *C. elegans*, the accumulation of Tre-6P in a *gob-1* knockdown is presumed since the mutation of two trehalose-phosphate synthases (*tps-1* and *tps-2*) suppress the growth phenotype; however, no measurements of Tre-6P were performed (162).

Mechanism of Toxicity

***Mycobacterium* spp.** The direct mechanisms of Tre-6P toxicity are unknown, but the cellular response suggests the involvement of toxin-antitoxin systems and RNA degradation (152). RNA-seq was used to characterize the impact of Tre-6P-mediated toxicity in an *otsB2* conditional mutant of *M. tuberculosis*: over 800 genes were found to be upregulated, and 37 were downregulated (152). This bias toward upregulated genes was postulated to arise from differential RNA stability rather than transcriptional regulation. Consistent with this expectation, two antitoxins that inhibit toxins with RNase activity were upregulated. An efflux pump of unknown function and arginine biosynthesis genes were upregulated. DNA repair enzymes were upregulated, suggesting that Tre-6P toxicity might include DNA damage.

Similarly, the direct target(s) of Mal-1P are not known, although the cellular responses suggest that respiration is inhibited and that cells with a build-up of Mal-1P suffer DNA damage (157). The cellular response to Mal-1P toxicity was investigated using transcriptomics of a conditional *treS glgE* mutant of *M. tuberculosis* in which Mal-1P accumulation could be controlled

(157). This study revealed changes in gene expression similar to those observed using potassium cyanide, a cytochrome *c* oxidase-specific inhibitor: downregulation of cytochrome *c* oxidase and ATP synthase components and upregulation of NADH dehydrogenase I complex, cytochrome *bd* oxidase, and nitrate reductase. Accumulation of Mal-1P likely results in activating the glycogen reserve only to further accentuate the situation as any new maltose released results in a lethal amplification of Mal-1P toxicity. RelA, which generates (p)ppGpp, a stress indicator, was upregulated, and this may signal translational downregulation.

Although most of the gene expression differences observed during Mal-1P stress were different from that with Tre-6P, two aspects of the response were similar: upregulation of arginine biosynthesis and DNA repair enzymes (e.g., *recA dnaE2*). Since arginine production in *E. coli* is enhanced during osmotic stress (163), an osmotic imbalance appears to be a common thread during the stress imposed by these two phosphodisaccharides. Likewise, DNA damage can be inferred from the increased synthesis of *recA* and *dnaE2*.

Eukaryotes. Tre-6P is thought to sequester phosphate which could lead to reduced ATP concentrations and, in *S. cerevisiae* and *A. fumigatus*, Tre-6P also directly inhibits hexokinases and regulates glycolysis (143, 150, 153, 164, 165). In *C. elegans*, the mechanism of Tre-6P toxicity has not been determined, although the inactivation of *gob-1* leads to blockage and structural defects of the intestine that may result in a more general metabolic defect (162).

Topics for Further Investigation

GlgE seems to be an excellent drug target in *Mycobacterium*. Fortunately, suppressor mutations that eliminate Mal-1P toxicity do not restore α-glucan production, so the organism remains attenuated (158). *Mycobacterium* GlgE transition state inhibitors have been reported (166). OtsB is also an interesting drug target in *Mycobacterium*, although suppressing mutations in *otsA* provide resistance without attenuating the organism (152).

TPS2/OtsB also appears to be a viable broad-spectrum antifungal target, since TPS2 mutants of numerous fungal species are severely attenuated in animal models, and there is no TPS2 in humans (8, 147–150, 153, 154, 161, 167) (Table 1). In addition, TPS1 mutants that cannot form Tre-6P are still attenuated in mouse models, at least for *Cryptococcus gattii* and *C. neoformans*. This finding bodes well for avoiding resistance to TPS2 inhibitors. Unfortunately, TPS1 mutants of *C. albicans* or *A. fumigatus* are not attenuated (167).

Despite the promise of GlgE and OtsB as therapeutic targets, the mode of action of Mal-1P and Tre-6P in *Mycobacterium* are not known. Thus far, Mal-1P toxicity has only been induced in *Mycobacterium* using trehalose as the trigger. Can Mal-1P toxicity be induced in *Mycobacterium* using maltose? Tre-6P toxicity has not been reported in *E. coli*, presumably because the accumulated Tre-6P in an *otsB* mutant is converted to glucose by TreC (Fig. 1C). Would a *treC otsB* double mutant of *E. coli* experience Tre-6P toxicity? Is the Mal-1P and Tre-6P toxicity more widespread among other pathogenic bacteria?

GLYCEROL

Background

***Escherichia coli* and *Salmonella enterica*.** Glycerol can be utilized by *E. coli* as the sole carbon and energy source via two converging pathways (Fig. 1G). Glycerol can enter the cytoplasm via a facilitated diffusion transporter (*glpF*), and then the GlpK kinase (*glpK*) generates *sn*-glycerol-3-phosphate (Gly-3P). Alternatively, Gly-3P can be actively transported from the extracellular milieu into the cytoplasm using an ATP transporter (*ugpABCE*) (9). Some strains of *E. coli* have a periplasmic glycerol phosphodiesterase (*glpQ*) that can convert glycerol phosphodiesters (derived from deacylated phospholipids) into Gly-3P, which is then transported in through a *sn*-glycerol-3-phosphate:phosphate antiporter (*glpT*) (168). Regardless of the route, the cytoplasmic Gly-3P is oxidized by the GlpD dehydrogenase (*glpD*) to DHAP. *E. coli glpD* mutants suffer growth inhibition when grown in the presence of either glycerol or Gly-3P (9).

In *S. enterica*, mutants lacking the catabolic pathway for glycerol (*glpFK*) are attenuated 4- to 10-fold during systemic infection of mice, indicating that glycerol is a major

nutrient source (128, 169–171). A mutant lacking *glpD* has a similar phenotype, suggesting that its attenuation is at least partially due to a lack of glycerol as a nutrient source rather than to Gly-3P toxicity (128).

Mycobacterium tuberculosis. High-throughput screening was used to identify compounds that inhibit the growth of *M. tuberculosis in vitro* (29). One class of compounds identified, the pyrimidine-imidazoles, required glycerol in the growth medium for effect and led to an accumulation of Gly-3P and a depletion of ATP. However, the compounds had no effect on *M. tuberculosis* growth in a mouse infection model. The lack of effectiveness in mice suggests that glycerol is not available to *M. tuberculosis in vivo*, which is in contrast to the results described above for *S. enterica* (29).

Evidence for Sugar-Phosphate Accumulation

Escherichia coli. An enzyme assay was used to measure Gly-3P. After 1 h of exposure to 5 mM glycerol, an *E. coli glpD* mutant accumulates 20 mM Gly-3P, whereas the wild type does not (9, 172).

Mycobacterium tuberculosis. GlpD converts Gly-3P to DHAP, which is then converted to methylglyoxal (Fig. 1). Gly-3P accumulates up to 3.5-fold in the presence of chemical inhibitors that interfere with methylglyoxal detoxification in *M. tuberculosis* (29). However, this inhibition is downstream of GlpD, and it is not known whether the inhibition was complete. It is possible that a mutation of *glpD* may cause a more dramatic accumulation.

Mechanism of Toxicity

Escherichia coli. Gly-3P is bacteriostatic as the cells can recover when plated for viable counts (9). As expected, mutation of the kinase, *glpK*, prevents intoxication by glycerol but not by Gly-3P, while mutation of the transporter (presumably *glpT* [173]) prevents intoxication by Gly-3P but not by glycerol. Glucose can alleviate Gly-3P toxicity (9). It is conceivable that glycerol kinase is inhibited by either EIIAGlc or Fru-1,6BP (174, 175), thus preventing Gly-3P accumulation and allowing nonphosphorylated glycerol to diffuse out of the cell.

Mycobacterium tuberculosis. Compounds that could inhibit the growth of *M. tuberculosis* were found to require glycerol in the growth medium and led to accumulation of Gly-3P and depletion of ATP. Resistant mutants had mutations in *glpK*, consistent with growth inhibition being due to Gly-3P accumulation. Surprisingly, these compounds did not appear to target GlpD but rather one or more glyoxylase enzymes involved in methylglyoxal detoxification (29). Overexpression of one of these enzymes, Rv0577, provided resistance to the compounds. The relative contributions of Gly-3P and methylglyoxal accumulations, or ATP depletion, to the toxic effects of the compound remain to be determined. An important point, however, is that glycerol does not appear to be available to *M. tuberculosis* during infection, in contrast to its availability during *S. enterica* infection.

Topics for Further Investigation

What is the mechanism of Gly-3P intoxication? Why does *S. enterica* but not *M. tuberculosis* rely on glycerol as a nutrient during systemic infection? Could GlpD inhibitors have efficacy against systemic *S. enterica* infections? Drug design would need to take into consideration that mammalian cells have similar pathways (Table 1). GlpD of *S. enterica* is utilized during aerobic respiration, while a paralog, GlpA, is utilized under anaerobic conditions (176). Do mutations in each cause sugar-phosphate toxicity during growth in the appropriate conditions? Mutations in both are similarly attenuated during systemic mouse infection (128). Why are both required?

L-ARABINOSE

Background

Bacteria. The metabolism of L-arabinose, a pentose, in *E. coli* depends on three intracellular enzymes encoded by the *araBAD* operon (Fig. 1E and Fig. 6). The first step is the L-arabinose isomerase (*araA*; AraA)-catalyzed conversion of L-arabinose to L-ribulose, which is then phosphorylated to L-ribulose-5-phosphate (Ru-5P) by L-ribulokinase (*araB*; AraB). In the

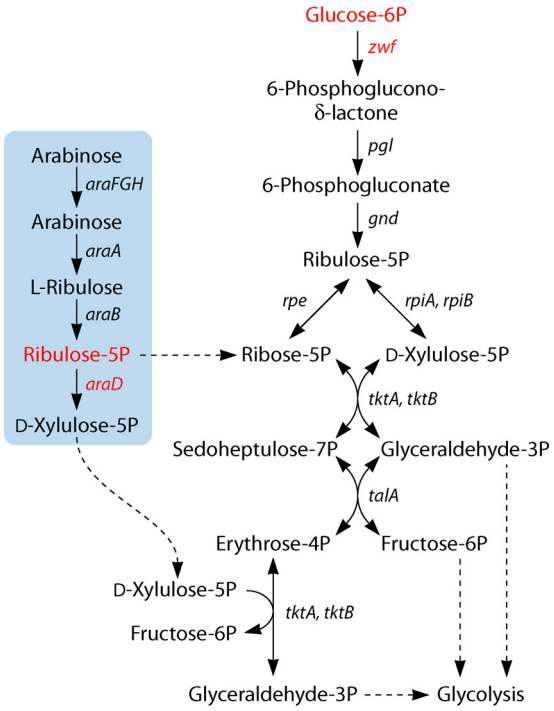

FIG 6 Arabinose utilization and pentose phosphate pathways in *E. coli*. Genes whose mutation/dysfunction cause accumulations of toxic phosphosugars and the respective built-up phosphosugar are highlighted in red.

final step, L-ribulose-5-phosphate-4-epimerase (*araD*; AraD, an inducible enzyme) converts Ru-5P to xylulose-5-phosphate, which enters the pentose phosphate pathway. The accumulation of Ru-5P inhibits the growth of *araD* mutants in the presence of various other nutrients, with the exception of glucose (10, 177). It is unknown how glucose helps overcome L-arabinose-mediated inhibition, although catabolite repression might play a role. Mutation of the kinase, *araB*, prevents L-arabinose-mediated inhibition (10). Interestingly, wild-type cells can suffer from L-arabinose-mediated toxicity in hypertonic media containing 5% (wt/vol) NaCl (177, 178). In this instance, 5% (wt/vol) NaCl adversely affects the expression of arabinose-metabolizing enzymes to provoke a sugar-phosphate stress similar to that observed with a genetic alteration such as *araD*.

Evidence for Sugar-Phosphate Accumulation

Escherichia coli. In an *araD* mutant of *E. coli*, Ru-5P was present at 13.4 μmol per g of dry weight of cells, while it was undetectable in the wild type (10).

Mechanism of Toxicity

Escherichia coli. The mechanism of toxicity from Ru-5P accumulation is unknown. However, UDP-Gal increases, reminiscent of galactose toxicity in *galE* mutants (178). *E. coli* is reported to possess 13 potential L-arabinose efflux transporters (179). Whether this large array of transporters acts as a safety net to avoid the build-up of the sugar precursor that could generate a toxic phosphosugar is unclear.

Topics for Further Investigation

AraD is rather rare in bacteria and not found in humans, suggesting that AraD could be a target with high specificity (Table 1). However, *araD* mutants of pathogens of interest need to be tested in animal infection models to determine whether these mutants are attenuated and whether exogenous L-arabinose, either ingested or injected, can enhance the clearance of the organism.

The ribulose-P-3-epimerase of *E. coli*, Rpe, performs the same reaction as AraD (Fig. 6). Why does this redundancy not prevent the toxicity of arabinose in an *araD* mutant? Are the

rpe and *araD* genes expressed under different conditions? Does the presence of glucose repress *araD* or cause activation of Rpe and the pentose phosphate pathway? Will an *rpe* mutation cause accumulation of Ru-5P (without secondary defects in *rpiA* and *rpiB*)? Would mutating *tktA* and *tktB* lead to accumulation of erythrose-4-phosphate and xylulose-5-phosphate (Fig. 6)?

MANNITOL

Background

Bacteria. Mannitol is a highly soluble polyol that is widely employed in nature, especially in plants and fungi, as a storage molecule, osmolyte, and redox protectant (180). In bacteria, mannitol is imported by a Mtl-specific PTS (*mtlA*) and converted to mannitol-1-phosphate (Mtl-1P) during transport. Mtl-1P is then converted to Fru-6P by the reversible NAD^+-dependent oxidase activity of mannitol-1-phosphate 5-dehydrogenase (*mtlD*; M1PDH) (Fig. 1D) (181–183). Fru-6P can then be catabolized by the glycolytic pathway. Mutation of *mtlD* leads to growth defects that coincide with the accumulation of the sugar phosphate, Mtl-1P, and intracellular mannitol. It remains unclear which of these two metabolites causes growth inhibition. In fungi and plants there is also a Mtl-1P phosphatase involved with mannitol metabolism, but it is unclear whether most bacteria have this capability. There are two phosphatases (*hxpA* or *hxpB*; hexitol phosphatase) that may play this role in *E. coli*, and although their activities have been characterized *in vitro*, their roles *in vivo* are uncertain (184). *Acinetobacter baylii* has an interesting M1PDH enzyme that has an extra HAD-like phosphatase domain. Thus, this one enzyme performs both steps: Fru-6P to Mtl-1P and Mtl-1P to mannitol (185).

Eukaryotes. Some fungi and lactic acid bacteria can produce mannitol to serve as an osmoprotectant under low-pH or high-salt stress (180, 186). They convert Fru-6P to Mtl-1P by reversing the M1PDH reaction, and then use a mannitol-1-phosphate phosphatase to produce mannitol (187). Plants synthesize mannitol by using a NADPH-dependent mannose-6-phosphate reductase to convert Man-6P to Mtl-1P (180). A phosphatase then converts Mtl-1P to mannitol. In contrast to bacteria, there is no accumulation of Mtl-1P when M1PDH is absent in plants or fungi. This is because mannitol is not phosphorylated when it enters the cell, but instead it is converted into fructose. Mtl-1P is only generated from Fru-6P, so a lack of M1PDH simply prevents Mtl-1P from being formed (188).

There is an "arms race" between fungi and plants in which mannitol is a key player. Fungal pathogens of plants secrete mannitol, presumably to shield the fungus from reactive oxygen species. In response, some plants secrete a mannitol dehydrogenase to convert mannitol to mannose when encountering such pathogens (180, 189).

Evidence for Sugar-Phosphate Accumulation

Escherichia coli. Providing mannitol to *E. coli mtlD* mutant cells results in growth arrest (181, 190). Interestingly, in the absence of mannitol in the medium, both wild-type and *mtlD* mutant *E. coli* strains synthesize mannitol and Mtl-1P (191). Surprisingly, Mtl-1P did not accumulate in the *mtlD* mutant compared to wild-type cells, although 50% of the carbon from mannitol ends up in nucleic acids within 2 h, suggesting an undiscovered pathway for dissimilation of Mtl-1P in the absence of *mtlD* (191, 192). The *mtlD* mutant used in these studies was unable to convert Mtl-1P to Fru-6P, as expected, but could perform the reverse reaction (Fru-6P to Mtl-1P), likely reflecting a second enzyme activity in the cell (191).

Salmonella enterica. Mutants deficient in M1PDH are mannitol sensitive, with 1 μM inhibiting growth and 20 μM inducing lysis (181, 193). [^{14}C]mannitol provided to an *mtlD* mutant at 0.5 mM accumulates as Mtl-1P at concentrations up to 20 mM (193).

Staphylococcus aureus. In *Staphylococcus aureus*, the relative concentrations of mannitol and Mtl-1P were determined in wild-type and *mtlD* mutant cells, in the absence of mannitol in the growth medium. Mannitol increases ~3-fold in a *mtlD* mutant compared to the wild type, whereas Mtl-1P goes from being undetectable in the wild type to being 6-fold more abundant than mannitol in the mutant (194). However, another study shows intracellular mannitol being decreased in a *mtlD* mutant compared to the wild type, unless mannitol is provided, at which point the intracellular

mannitol is 2- to 3-fold increased in the *mtlD* mutant relative to the wild type (187). Mtl-1P was not measured in this study.

Providing mannitol in the growth medium slows the growth rate of a *mtlD* mutant dramatically but does not slow the wild type or the *mtlABFD* mutant (194). While metabolite concentrations were not measured in this scenario, Mtl-1P is the likely growth inhibiting agent, especially if mannitol is transported by a PTS so that intracellular mannitol should be in the form of Mtl-1P. However, while it is expected that Mtl-1P concentrations would increase in a *mtlD* mutant, it is not clear how mannitol concentrations also increase. There could be a phosphatase that converts Mtl-1P to mannitol that remains to be discovered, or it is possible that the mannitol-specific PTS transporter itself is interconverting mannitol and Mtl-1P (195). It is also not clear how mannitol and Mtl-1P are synthesized in the absence of exogenous mannitol or *mtlD*. There may be aspects of these pathways that remain to be discovered.

Fungi and plants. There is no accumulation of Mtl-1P in fungi or plants when M1PDH is absent, as mannitol is not phosphorylated upon entry into the cell, but rather is formed by M1PDH from Fru-6P.

Mechanism of Toxicity

Salmonella enterica. In *S. enterica*, the mechanism for toxicity was studied by tracking the radioactive precursors for the cell wall, lipids, proteins, and nucleic acids (193). One of the first processes inhibited by the addition of mannitol in an *mtlD* mutant was lipid synthesis, as measured by the incorporation of [^{14}C]acetate. This inhibition of lipid metabolism was not observed in WT strains or in an *mltD* mutant grown in the absence of mannitol (193).

Escherichia coli* and *Staphylococcus aureus. While the presence of mannitol is bacteriostatic to an *E. coli mtlD* mutant (190), it can lyse an *S. aureus mtlD* mutant (187, 194). The mechanisms for these phenotypes are largely unknown. The *mtlD* mutants of *S. aureus* are susceptible to Triton X-100 and the antimicrobial fatty acids, such as sapienic acid, oleic acid, or linoleic acid, but oddly are not susceptible to SDS or the human antimicrobial peptide LL37 (187, 194). The mutant is also sensitive to high pH or high salt, even in the absence of exogenous mannitol (187). Since both mannitol and Mtl-1P accumulate in the mutant, it is not clear which is the toxic metabolite. Since mannitol is essential to sustain the cellular redox and osmotic potential, a homeostatic imbalance in *mtlD* mutants of *E. coli* and *S. aureus* is likely contributing to the swelling and lytic effects.

Topics for Further Investigation

Mannitol metabolism appears to play a major role in stress resistance and the maintenance of osmotic balance in bacteria, and yet surprisingly little is known about the pathways regulating mannitol and Mtl-1P concentrations. *E. coli* and *S. aureus* both appear to synthesize mannitol and Mtl-1P even when mannitol is not present in the growth medium and even when *mtlD* is mutated (191, 194). However, the *mtlD* mutant used in the *E. coli* study was obtained by chemical mutagenesis and was able to perform the reverse reaction of Fru-6P to Mtl-1P (191). Is there a second enzyme in *E. coli* that performs this reverse reaction? Does *E. coli* (or *S. aureus*) utilize a Mtl-1P phosphatase to then create mannitol from Mtl-1P? There are *E. coli* enzymes that have the appropriate activity *in vitro* and have homologs in related bacteria but not in *S. aureus* (184). Could differences in the catalytic repertoire explain the more severe effects of mannitol on *S. aureus* than *E. coli*? With regard to toxicity, are the effects caused by mannitol or Mtl-1P?

In *S. enterica*, Mtl-1P accumulates to 20 mM in a *mtlD* mutant that has been provided with mannitol (193). However, in a similar experiment with *E. coli*, Mtl-1P did not accumulate (191). These results may need to be reexamined. In *S. aureus*, both mannitol and Mtl-1P accumulate in a *mtlD* mutant, but this has only been measured in the absence of exogenous mannitol (194). What are the concentrations of mannitol and Mtl-1P upon inclusion of exogenous mannitol? Do mannitol or Mtl-1P have other effects in the cell beyond the dysregulation of osmotic balance? How do cells respond to mannitol/Mtl-1P toxicity? *S. aureus mtlD* mutants were attenuated in mice regardless of regular mannitol injections (187). Are the mutants attenuated in the absence of mannitol or are they gaining access to Mtl in the

host? A competitive inhibitor of *S. aureus* M1PDH, dihydrocelastrol, decreased the viability of this pathogen in murine macrophages (187). Thus, the development of inhibitors of M1PDH suitable for use in mammals may be warranted (Table 1).

MANNOSE

Background

***Escherichia coli* and *Salmonella enterica*.** Metabolism of mannose by *E. coli* and *S. enterica* begins by its import through a PTS (*manXYZ*), resulting in mannose-6-phosphate (Man-6P) (Fig. 1D) (196). Phosphomannose isomerase (encoded by *manA*; PMI) catalyzes the zinc-dependent conversion of Man-6P to Fru-6P, which then enters the glycolytic pathway (197). However, the reversible reaction catalyzed by ManA can also redirect glycolysis by converting Fru-6P to Man-6P. Phosphomannose mutase (two isozymes in *S. enterica*: *manB* [*cpsG*] and *rfbK*; PMM) catalyzes the conversion of Man-6P into mannose-1-phosphate (Man-1P), which is used to synthesize the GDP-mannose (GDP-Man) sugar nucleotide that is a precursor for colanic acid synthesis (Fig. 3) (198, 199). GDP-Man is also used to synthesize several building blocks of the repeating O-antigen units of LPS (200, 201). The addition of mannose to a *manA* mutant restores O-antigen synthesis, a striking parallel to restoration of O-antigen synthesis by providing galactose to a *galE* mutant. One difference is that *S. enterica* appears to obtain galactose, but not mannose, during mouse infection (81, 200). Providing mannose during the growth of a *S. enterica* inoculum does increase virulence modestly (200).

***Bacillus subtilis*.** In *B. subtilis*, *manA* is essential for balancing the sugar content in the cell wall, especially the teichoic acid contents (202), which are integral to the peptidoglycan-associated polymers and cell shape in many Gram-positive organisms (203). *B. subtilis manA* mutants display an enlarged size, and the nucleoid is disconnected from the cell wall components, resulting in loss of coordination between the cell growth, DNA replication, and segregation, which in turn results in polyploidy (202).

Eukaryotes. In eukaryotes, mannose is converted to Man-6P by a hexokinase (encoded by *Hk1*) rather than by a PTS transporter. Alternatively, Fru-6P is converted to Man-6P by PMI (encoded by *Mpi* in mice, MPI in humans, and *pmi* in yeast). Man-6P is the first step in the N-glycosylation pathway, and is utilized as a targeting signal for cellular trafficking of proteins to lysosomes (204). Humans with reduced PMI activity suffer CDGS-1b (carbohydrate-deficient glycoprotein syndrome type 1b), which results in hypoglycemia, liver fibrosis, and coagulopathy (205, 206). Interestingly, the symptoms are largely alleviated with dietary mannose supplementation. This finding suggests that, similar to bacteria, the lack of PMI is not causing Man-6P accumulation and toxicity but rather is preventing biosynthesis (in bacteria) or glycosylation (in humans). In contrast, however, toxicity due to Man-6P accumulation has been reported in human tumor cells (207), in a mouse model (208), in honeybees (209), in *A. fumigatus* (210), and in *S. cerevisiae* (211). It is unclear what factors result in the same metabolite (Man-6P) becoming toxic in tumor but not normal cells.

Evidence for Sugar-Phosphate Accumulation

***Escherichia coli* and *Salmonella enterica*.** To date, there have been no measurements of Man-6P accumulation (212–214).

***Plesiomonas shigelloides*.** Man-6P has been measured in one Gram-negative species, *Plesiomonas shigelloides* (215). This organism is unusual in that it imports mannose through a PTS, converting it to Man-6P, but it is metabolized no further as the organism naturally lacks *manA*. Providing mannose to this species inhibits growth. When provided 10 mM ^{13}C-labeled mannose, Man-6P accumulated to 12 mM (2.8 mmol per 100 mg dry weight of cells) (215).

Eukaryotes. Humans affected by CDGS-1b benefit from dietary mannose supplementation that restores glycosylation to their proteins (205, 206). At the same time, they appear to be protected from Man-6P intoxication by residual PMI activity or an intestinal uptake barrier. Homozygous *Mpi* mouse mutants die during embryonic development despite glycosylation levels being normal (208). Man-6P accumulates in these embryos to 55 nmol/g of protein, while it is only present in wild-type embryos at 2 nmol/g of protein. When primary murine embryonic fibroblasts derived from these embryos were incubated with increasing

concentrations of mannose, the concentration of intracellular Man-6P increased. In the presence of 100 μM mannose, the physiological concentration in plasma, the intracellular Man-6P accumulated to 18 mM (208, 216).

Mannose—but not galactose, glucose, fructose, or fucose—can impair the growth of some cancer cells that have low expression levels of PMI (207). Accumulation of Man-6P was observed after 6 h growth in the presence of ^{13}C-labeled mannose (207). The addition of 20% (wt/vol) mannose to drinking water inhibited tumor growth in CD-1 nude mice transplanted with an oncogenic cell line without causing any adverse effects on animal health.

In *A. fumigatus*, loss of PMI led to a 20-fold increase in intracellular Man-6P concentration when grown in the presence of 3 mM mannose (1151 versus 56 nmol per 50 mg of dry mycelium in the *pmi* mutant and the wild type, respectively) (210). Man-6P also accumulates in *S. cerevisiae* and honeybees (209, 211).

Mechanism of Toxicity

***Escherichia coli* and *Salmonella enterica*.** *S. enterica* utilizes similar metabolic pathways to colonize, survive, and replicate within both plant and animal host environments (171, 212). In particular, *S. enterica* requires *manA* during systemic mouse infection and during the early stages of attachment to and colonization of alfalfa seedlings (212). However, providing mannose to a *manA* mutant *in vitro* has no adverse effect. The attenuation of the *manA* mutant could be complemented by providing supplemental mannose, but not fructose, suggesting that ManA converts Fru-6P to Man-6P during *S. enterica* infection of plants (and animals). Since Man-6P is used to produce both colanic acid and the O-antigen of LPS, mutants lacking these functions (*gmd* for colanic acid and *rfbP* for O-antigen) were used to determine whether either of these end products are responsible for virulence. The *rfbP*, but not the *gmd* mutant, was less competent to colonize alfalfa seedlings. This finding suggests that O-antigen rather than colanic acid synthesis downstream of PMI contributes to seedling colonization by *Salmonella* (Fig. 3) (212). The question remains as to whether the *manA* mutants are attenuated solely due to defects in colanic acid and O-antigen synthesis or whether they can suffer from Man-6P accumulation and toxicity. To test the hypothesis that Man-6P accumulation induces stress, it may be necessary to mutate or inhibit the ManB-type enzymes that utilize Man-6P as a substrate (CpsG and RfbK) to produce colanic acid and O-antigen.

***Bacillus subtilis*.** To study the impact of phosphosugar stress in *B. subtilis*, a triple mutant lacking *manA*, *mtlD*, and *malA* was constructed. When this strain was grown in Luria-Bertani (LB) medium or LB glucose, the cells appeared normal by microscopy. However, when grown in LB mannose or LB mannitol, the cells were club-shaped, indicating cell wall defects, and growth was arrested (52). This result is somewhat surprising since the *manA* and *mtlD* mutations block the entry of the phosphosugar into glycolysis, but they do not block the synthesis of cell wall components from Man-6P and Mtl-1P. If Man-6P does indeed accumulate, the mechanism of toxicity is unknown. Transcriptomes of the triple mutant in the presence of each of the three sugars were compared to the wild type after 3.5 h of growth. The stress imposed by Man-6P resulted in upregulation of three gene clusters: a glucose-catabolite repression system (*glcR-phoC*), a β-glucoside utilization system (*bglPH*), and the ribose utilization system (*rbsRKDACB*). The product of the *glcR-phoC* (initially known as *ywb*) operon includes a regulator, *glcR*, and a putative HAD-like sugar-phosphate phosphatase, *phoC*. It was initially hypothesized that PhoC might act on Man-6P to alleviate the sugar-phosphate stress, but *in vitro* assays showed that PhoC prefers to dephosphorylate Gly-3P and ribose-5-phosphate and that Man-6P is a weak substrate (52). Despite the upregulation of *phoC*, the stress imposed by mannose was not alleviated. The *bglPH* and *rbsRKDACB* are catabolic operons, and it is unclear how upregulation of these genes is beneficial under phosphosugar stress.

Eukaryotes. (i) *Saccharomyces cerevisiae*. Addition of mannose to a *pmi* (*manA*) mutant of *Saccharomyces cerevisiae* caused the accumulation of Man-6P and significantly decreased the growth rate. These mutants were viable only when supplemented with glucose. The mechanism of toxicity associated with Man-6P accumulation is through the inhibition of PGI activity, which dampens glucose metabolism (211). Decreased energy generation, protein synthesis, and cell wall biogenesis imply system-wide aberrations, partly from

transcriptional rewiring. For instance, a decrease in the expression levels of a transcription factor, *RAP1*, which is involved in the regulation of several glycolytic enzymes and cell cycle regulators, might account for these wide-ranging effects (211).

(ii) *Aspergillus fumigatus*. In *Aspergillus fumigatus*, loss of PMI led to an intracellular accumulation of Man-6P and defects in cell wall integrity, unusual morphology, and conidiation abnormalities (210). The mechanism of toxicity due to the accumulation of Man-6P is postulated to be through a decrease in α-glucan content in the cell wall, a significant feature observed under both mannose-deficient and -excess conditions. Even the wild-type cells are affected by excess exogenous mannose and exhibited a diminished α-glucan content. Besides a reduction in Man-1P, GDP-Man and glycolytic intermediates (Glc-6P, Fru-6P, and Fru-1,6BP), a 2-fold increase in Man-6P content was noticed in the mutants supplemented with 0.5 mM mannose. The addition of glucose and mannose rescued the mycelial growth of the *pmi* mutants but conidiation defects were not remedied (210). While exogenous mannose is required for the survival of *pmi* mutant strains of *A. fumigatus*, the expression of PMI is critical for conidia formation. The defects in cell wall integrity and conidiation in the mutant likely arise from excessive mannosylation of cellular proteins and alterations in the content of α-glucan, β-glucan, and chitin. Clearly, PMI serves as a critical check-valve for shunting appropriate amounts of mannose toward energy generation and mannosylation.

(iii) Honeybees and *Gilliamella apicola*. Mannose elicits strong toxicity in honey bees (209). This toxicity has been attributed to the low levels of PMI, whose transcript abundance is only 6% compared to hexokinase, which leads to Man-6P accumulation (217). Toxicity from Man-6P accumulation is believed to arise from inhibition of hexokinase and the associated decrease in intracellular ATP levels (218). Therefore, neither glucose nor fructose is exploitable as a nutrient in the presence of a high concentration of mannose. Interestingly, *Gilliamella apicola*, a Gram-negative and dominant gut bacterium in honeybees, as well as bumble bees, can consume several sugars (mannose, arabinose, rhamnose, and xylose) that are toxic to the host bees. This symbiosis is interesting since this bacterium aids the host by digesting these carbohydrates (219).

(iv) Mice. Mice lacking PMI accumulate Man-6P and die as embryos (208). The mechanism of toxicity from Man-6P accumulation is postulated to be through the inhibition of hexokinase, PGI, and G6PD but not phosphofructokinase. As expected, accumulated Man-6P cannot enter glycolysis but rather is trapped in a futile cycle of dephosphorylation (by a putative phosphatase) and rephosphorylation with depletion of intracellular ATP levels; accentuating this downward spiral is the additional inhibition of glycolytic enzymes (208).

(v) Humans. Humans with mutations of the MPI gene suffer from a lack of protein glycosylation, rather than from Man-6P toxicity. Regular dietary mannose is usually insufficient to restore glycosylation, but supplemental mannose can largely alleviate severe disease outcomes (205, 206). This finding in humans is very different from mice, in which *Mpi* mutations are embryonic lethal. This difference may be because the naturally occurring human mutations leave some residual PMI activity to detoxify Man-6P, whereas the mice are complete knockouts.

Topics for Further Investigation

In all of these organisms, it is difficult to distinguish toxicity due to Man-6P accumulation from other cellular defects that occur when glycolytic intermediates cannot be diverted via PMI to meet anabolic needs. In *S. enterica*, it appears that the latter scenario explains the attenuation of *manA* mutants in plant and animal models of infection. In fact, with the exception of *P. shigelloides*, there are currently no reports that Man-6P accumulation is toxic to Gram-negative bacteria (212–215). Are these bacteria simply diverting all excess Man-6P to biosynthesis of molecules such as colanic acid and O-antigen? To test the hypothesis that Man-6P accumulation induces stress, it will be necessary to mutate or inhibit the ManB-type enzymes that utilize Man-6P as a substrate (CpsG and RfbK). Providing mannose to a *manA* mutant of *B. subtilis* leads to cell wall defects (52). Why does this defect manifest in a phenotype in *B. subtilis* but not *E. coli* or other Gram-negative bacteria? Further studies, including the measurement of Man-6P accumulation, are needed. In humans and mice, it appears that disruption of MPI can lead to loss of protein glycosylation and Man-6P toxicity, with the severity

depending on the level of residual PMI activity. Before mannose can be used as part of any cancer treatment, it is essential to build an inventory of PMI levels in different tumors. For instance, the low PMI levels observed in colorectal tumors suggest that the Man-6P intoxication strategy might be useful for this cancer.

L-RHAMNOSE

Background

***Escherichia coli* and *Salmonella enterica*.** L-Rhamnose (an aldose, 6-deoxy-L-mannose) is a naturally occurring sugar found in some plant cell wall pectins. In *E. coli*, L-rhamnose is imported via proton symport (*rhaSPQT*) (220). Once in the cell, L-rhamnose is isomerized to L-rhamnulose (a ketose) by L-rhamnose isomerase (*rhaA*, RhaA). Rhamnulokinase (*rhaB*, RhaB) then generates L-rhamnulose-1-phosphate (Rhu-1P), which is then cleaved by L-rhamnulose-1-phosphate aldolase (*rhaD*; RhaD) to generate two C3 molecules: DHAP and lactaldehyde (Fig. 1H). While DHAP is committed to glycolysis, lactaldehyde is converted to lactate and then pyruvate during aerobic growth or to 1,2-propanediol during anaerobic growth. L-Rhamnose inhibits the growth of *rhaD* mutants of *E. coli* and *S. enterica*, presumably due to the accumulation of Rhu-1P (220, 221). *S. enterica* serovar Typhi is a natural *rhaD* mutant that still encodes RhaA and RhaB and is also inhibited by the presence of L-rhamnose (222, 223). Interestingly, *S. enterica* serovar Typhi has been experiencing genome reduction as it evolved into a specialist that causes systemic disease in humans and a few other primates (125–127). The *rhaD* gene has acquired a frameshift mutation which has left the organism sensitive to L-rhamnose (222, 223).

***Arthrobacter pyridinolis*.** Growth inhibition by rhamnose has been identified in the Gram-positive organism *Arthrobacter pyridinolis* (224). This organism has two uptake routes for rhamnose that feed into the *rhaA-rhaB-rhaD* trio: the first is a PTS and the second is a respiration-coupled uptake that depends on the oxidation of either L-malate or succinate. When taken up by the PTS system, L-rhamnose-1-phosphate (Rha-1P) is formed, which is then converted back to rhamnose by a Rha-1P phosphatase. A mutant lacking this putative phosphatase accumulated what was likely Rha-1P (224). Since *rhaD* in *A. pyridinolis* has not been mutated, it is unknown whether Rhu-1P would accumulate and inhibit growth.

Evidence for Sugar-Phosphate Accumulation

***Escherichia coli* and *Salmonella enterica*.** Mutants of *E. coli* and *S. enterica* serovar Typhimurium that lack *rhaD* are inhibited by the presence of L-rhamnose (220, 221). However, the accumulation of the RhaD substrate, Rhu-1P, has not been measured quantitatively in any of these organisms. Cell extracts of an *E. coli rhaD* mutant and of *S. enterica* serovar Typhi both contain RhaB activity when the cells are grown in the presence of L-rhamnose but not glucose (220, 222). Since this activity is known to result in Rhu-1P (225), this result suggests that Rhu-1P is likely formed, and potentially accumulates, in these cells under these growth conditions.

***Arthrobacter pyridinolis*.** When a mutant of *A. pyridinolis* that lacks Rha-1P phosphatase activity is provided [^{3}H]-L-rhamnose, a single anionic radioactive compound (presumably Rha-1P) accumulates (224).

Mechanism of Toxicity

The mechanisms by which L-rhamnose intoxicates a *rhaD* mutant are unknown. It has not been reported whether growth inhibition of *rhaD* mutants by L-rhamnose is bacteriostatic or bactericidal (220–223).

Topics for Further Investigation

The presence of a *rhaD* mutation in *S. enterica* serovar Typhi is consistent with this organism undergoing genome reduction during specialization. It also suggests that L-rhamnose is available during gastrointestinal but not systemic infection, which is consistent with a plant-based source of the sugar. Are *rhaD* mutants of any enteric pathogens attenuated during gastrointestinal infection? If so, could inhibitors of RhaD be used as therapeutics? Would L-rhamnose need to be provided in excess along with the inhibitor? *S. enterica* serovar Typhi has not

mutated further to become L-rhamnose resistant, presumably by acquiring mutations in *rhaA* or *rhaB*. Does this finding indicate that *S. enterica* serovar Typhi no longer encounters L-rhamnose in nature? Was the inactivation of *rhaD* before any of the other members of the pathway a chance occurrence? Has nature provided us a simple way to treat *S. enterica* serovar Typhi with L-rhamnose? Could L-rhamnose be injected safely into humans? Would the L-rhamnose reach the pathogen? What is the concentration of Rhu-1P in *rhaD* mutant cells? What is the mechanism of intoxication? What are the cellular responses to intoxication?

CONCLUDING REMARKS

The ability to induce metabolic dysregulation using an enzyme inhibitor coupled with the availability or provision of a particular nutrient offers new possibilities for therapeutic design. For any sugar-phosphate toxicity to be exploited as a therapeutic modality, however, a common set of questions will need to be addressed. First, it is important to determine in animal infection models whether the appropriate sugar-utilization mutants in pathogens of interest are indeed attenuated and whether the corresponding sugar-phosphate build-up is bactericidal or bacteriostatic. Second, even if a particular sugar-phosphate toxicity is not lethal, it may potentiate sensitivity to other antibiotics, especially if the sugar-phosphate stress directly or indirectly weakens the cell wall (for example, Glc-1P stress in *B. subtilis* [26]). Alternatively, inducing two phosphosugar toxicities may cause lethality. Thus, it is useful to determine which combinations of sugar-phosphate toxicities together, or in combination with antibiotics, have synergistic effects. Third, it is critical to ascertain whether the sugar of choice is available at the site of infection (e.g., the pathogen-containing compartment) or, if the sugar is exogenously supplied, whether paracellular and transcellular transport need to be investigated to determine the likelihood of the sugar being carried to the appropriate location. Fourth, are there nutrients that alleviate the toxicity (e.g., glucose with regard to arabinose and glycerol toxicities), and are these nutrients going to be present at the site of infection? Such experiments are particularly valuable in verifying that the hierarchy of nutrient utilization for energy production and growth *in vitro* is mirrored *in vivo* where the metabolic landscape is influenced by the host. Here, one must also consider the intersection between inherited and infectious diseases; for example, metabolomic alterations in inborn disorders (e.g., elevated glucose in diabetes) might either attenuate or exacerbate infection by a pathogen. Fifth, are there redundancies in metabolic pathways that will serve as a safety net against the sugar phosphate in the *in vivo* milieu? Finally, what are the molecular targets of the sugar phosphates? Systematic biochemical studies are needed to catalog the targets for each sugar phosphate. Payoffs from such an undertaking will include understanding what factors mitigate catalytic deficiencies or auxotrophies caused by these disruptions, as well as a better understanding of the phenotypes observed in select human disorders.

Further studies are necessary to understand how cells become resistant to sugar-phosphate toxicities. The disruption of an upstream enzymatic step is a recurring theme. For example, an *araD* mutant of *E. coli* can gain resistance to arabinose by acquiring mutations in *araB* (10). However, if the upstream enzyme is essential during infection, the acquisition of resistance becomes much more difficult. The essentiality of these enzymes may be indicated by the requirement of a particular nutrient for a pathogen's colonization and survival (for example, glycerol for *S. enterica* systemic infections [171]). Even if the gene which confers resistance is not essential, there is promise. Consider a drug targeting GalE. A *galK* mutation will render the bacterium resistant to intoxication by galactose, and a *galK* mutation by itself does not attenuate fitness during infection. Thus, it would appear that resistance to galactose would be easily achieved with a mutation in *galK*. However, the *galK* mutation, combined with the inhibition of GalE, will prevent the bacterium from scavenging galactose and synthesizing LPS during infection, thus attenuating fitness (81). Thus, acquiring resistance to galactose intoxication will lead to fitness defects by an alternative route. In instances where the gene that confers resistance is not essential, it may be possible to identify epistatic interactions (synthetic lethals), as illustrated with Tre-6P toxicity in *M. tuberculosis* (152). Although this approach would add a second target to drug-discovery efforts, it nevertheless warrants a close look given the paucity of new antibiotics in development.

Our analysis has identified five sugar-phosphate toxicities to be specific to bacteria (those caused by arabinose [*araD*], rhamnose [*rhaD*], F-Asn [*fraB*], maltose [*glgE*], and fructose [*fruK*]) (Table 1), all deserving further attention. Some of these toxicities are more specific to particular genera of bacteria than others, which may be useful for the creation of narrow spectrum therapeutics that do not disrupt the microbiota (those caused by F-Asn, maltose, rhamnose, and arabinose). Tre-6P sugar-phosphate toxicity occurs in fungal pathogens but not in humans, thus supporting its consideration as an antifungal (or antibacterial) target. Since the glucose, glycerol, galactose, and mannose utilization pathways exist in humans and microbes, the challenge of designing species-specific inhibitors of these pathways makes them less attractive targets.

Because chance as much as necessity has shaped the myriad metabolic pathways that sustain life, it is not surprising that certain molecular assets become cellular liabilities under specific circumstances. Such vulnerabilities represent the critical loose first threads that ultimately unravel well-knit cellular tapestries. In this regard, customizing phosphosugar-mediated metabolic derangements offers exciting possibilities to fracture the striking resilience of bacterial pathogens.

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