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REVIEW

How Bacterial Pathogens Coordinate Appetite with Virulence

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SUMMARY Cells adjust growth and metabolism to nutrient availability. Having access to a variety of carbon sources during infection of their animal hosts, facultative intracellular pathogens must efficiently prioritize carbon utilization. Here, we discuss how carbon source controls bacterial virulence, with an emphasis on *Salmonella enterica* serovar Typhimurium, which causes gastroenteritis in immunocompetent humans and a typhoid-like disease in

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mice, and propose that virulence factors can regulate carbon source prioritization by modifying cellular physiology. On the one hand, bacterial regulators of carbon metabolism control virulence programs, indicating that pathogenic traits appear in response to carbon source availability. On the other hand, signals controlling virulence regulators may impact carbon source utilization, suggesting that stimuli that bacterial pathogens experience within the host can directly impinge on carbon source prioritization. In addition, pathogen-triggered intestinal inflammation can disrupt the gut microbiota and thus the availability of carbon sources. By coordinating virulence factors with carbon utilization determinants, pathogens adopt metabolic pathways that may not be the most energy efficient because such pathways promote resistance to antimicrobial agents and also because host-imposed deprivation of specific nutrients may hinder the operation of certain pathways. We propose that metabolic prioritization by bacteria underlies the pathogenic outcome of an infection.

KEYWORDS cAMP, carbon, central metabolism, CRP, magnesium, PhoP, PTS, *Salmonella*, virulence

INTRODUCTION

Here, we introduce carbon source utilization as a virulence determinant by discussing how the utilization of different carbon sources can result in different physiological consequences and how bacterial virulence comes at a metabolic cost. We propose that the regulation of virulence and of carbon metabolism are interconnected, highlighting the importance of considering these two processes as one in the context of bacterial infection of animal or plant hosts.

Bacterial survival requires continuous adaptations to environmental changes (1–3). Even within the confines of the mammalian gut, individual bacteria of the microbiome experience various conditions because they encounter different microbes in different parts of the gastrointestinal tract and because most hosts do not ingest nutrients continuously. Moreover, a given niche typically contains multiple metabolizable carbon sources that bacteria can utilize as energy or building blocks to sustain growth, making metabolic prioritization imperative.

The study of how bacteria sense and acquire preferred carbon sources from their surroundings has provided a wealth of fundamental knowledge about microbial physiology. The classical example of glucose-lactose diauxie (4, 5) revealed that the gut commensal bacterium *Escherichia coli* preferentially utilizes D-glucose (here, glucose) over lactose when both sugars are present, thereby accelerating bacterial growth. This observation established the phenomenon of carbon catabolite repression (CCR) (4, 6), whereby cells prioritize which carbon source to utilize first when faced with several at the same time. That bacteria prefer one carbon source over another is often ascribed to differences in the energetic output that result from the utilization of individual carbon sources (7).

A predominant inequality in the utilization of carbohydrates as carbon sources concerns their biochemical transformation into glucose derivatives (7). To be metabolized via glycolysis, glucose must first be phosphorylated in two consecutive reactions, with the second phosphoryl donor in bacteria being adenosine triphosphate (ATP) (Fig. 1). This energy-intensive "preparatory" phase of glycolysis activates glucose to be further metabolized in the "payoff" phase of glycolysis, which yields ATP and reducing equivalents in the form of reduced nicotinamide adenine dinucleotide (NADH). For alternative carbon sources such as disaccharides and oligosaccharides, the biochemical processes that convert these carbohydrates into glucose or other derivatives require specific proteins that transport and metabolize the carbohydrates and often require additional energy (i.e., ATP) or reducing equivalents. In the case of noncarbohydrate carbon sources, such as intermediates in the tricarboxylic acid (TCA) cycle (e.g., succinate, malate, etc.), the "payoff" phase of glycolysis is skipped, depriving the bacterium of critical ATP pools. This explains, at least in part, why growth on the latter substrates is suboptimal.

How do bacteria coordinate physiological status with the availability of specific carbon sources? Bacteria sense the presence of specific carbon sources and appropriately

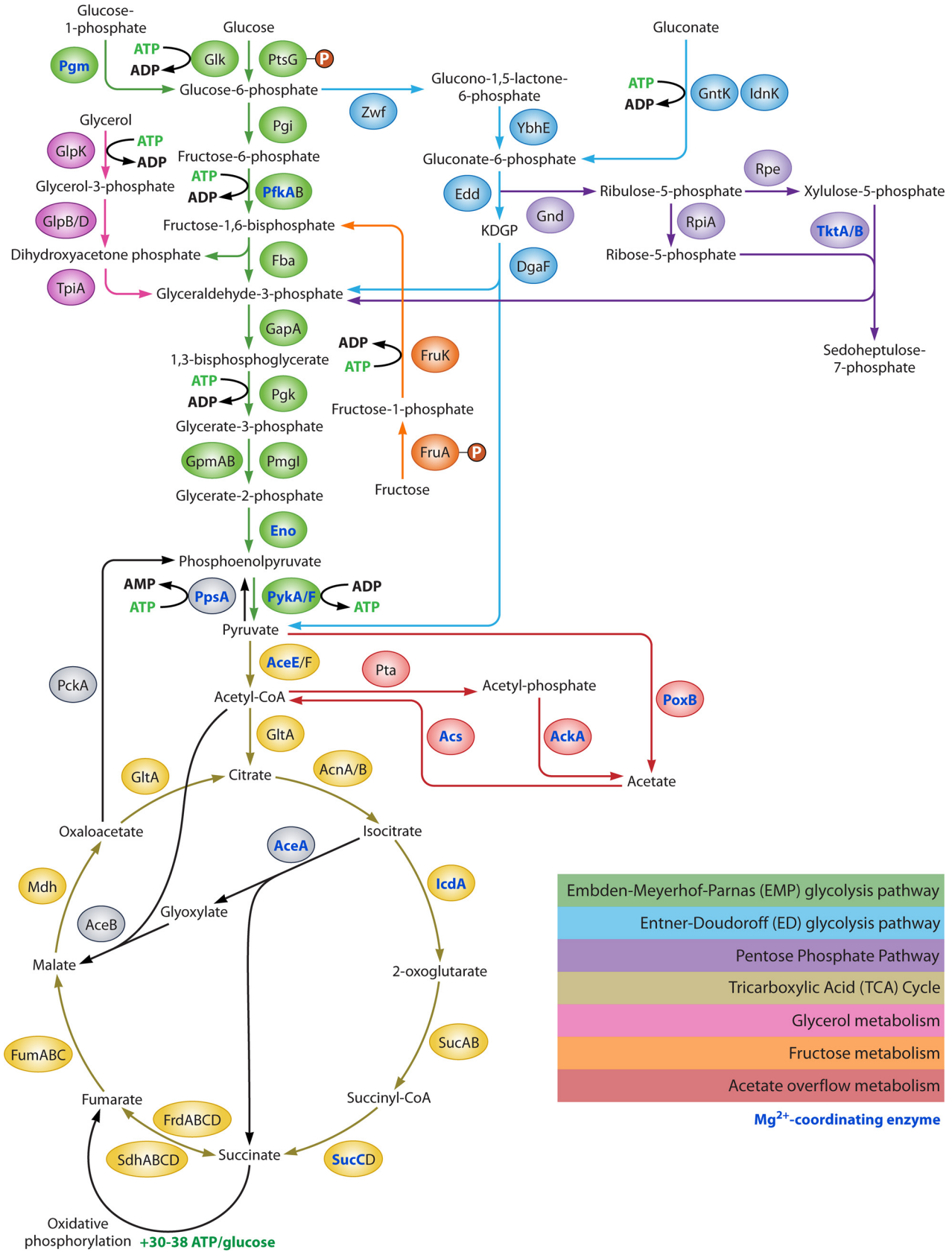

FIG 1 Core carbon utilization pathways operating in *S.* Typhimurium. Depiction of central metabolic pathways operating in *S.* Typhimurium (based on strain 14028s, information derived from KEGG Pathway database). Depicted pathways include glycolysis (Embden-Meyerhof-Parnas versus

(Continued on next page)

tune transport and metabolic activities, which dictate distinct growth properties. For example, in *E. coli*, carbon source availability determines the relative metabolic cost of amino acid biosynthesis, which corresponds to the point at which a given carbon source enters the glycolytic pathway (8). Therefore, increasing or decreasing the metabolic cost of synthesizing various amino acids according to carbon source availability can augment protein synthesis by determining the relative availability of amino acids required to translate mRNAs into functional proteins. Because proteins carry out the vast majority of cellular work and are key structural components in all cells, the fundamental cellular process of protein synthesis directly correlates with bacterial growth rate (9).

In Gram-positive bacteria, carbon source availability has been linked to cell division and production of the peptidoglycan cell wall (10, 11), highlighting regulatory mechanisms that coordinate catabolic and anabolic processes. In Gram-negative bacteria, the regulation of carbon source utilization has also been associated with cell division (12), and such association may dramatically impact pathogen behavior during infection.

The link between carbon source utilization and virulence has been established in a few cases (13–15). These studies suggest that carbon source, like other nutritional cues, may operate as an extracellular signal controlling virulence traits. In the Gram-negative pathogen *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium), the master virulence regulatory system PhoP/PhoQ is activated by multiple intra- and extracytoplasmic signals, including low Mg^{2+} and certain cationic antimicrobial peptides (CAMPs) in the periplasm and a mildly acidic pH in the bacterial cytosol resulting from acidification of the *Salmonella*-containing vacuole (SCV) when the pathogen is inside mammalian cells (16, 17). These PhoQ-activating conditions depress *S.* Typhimurium growth and proliferation in both laboratory media and host tissues (16–18), implying that bacterial replication and induction of virulence programs can be in conflict. In agreement with this notion, the induction of virulence determinants independently of specific environmental signals hinders growth (19, 20) and virulence (21) in *S.* Typhimurium.

A limited number of metabolic enzymes are essential for *S.* Typhimurium virulence (22), suggesting that this pathogen accesses various sufficient nutrients from the host to sustain growth and display virulence properties. These findings raise several questions, such as to what extent access to preferred carbon sources alleviates a conflict between growth and virulence and how bacteria have adapted their virulence programs to the availability of specific carbon sources from the eukaryotic host and from other microbes that coexist with the pathogen in a given locale.

In this article, we explore the relationship between carbon source utilization and virulence using *S.* Typhimurium as a case study. We propose that the regulation of carbon source utilization and virulence is reciprocal because regulators of virulence dictate carbon source utilization, and regulators of carbon metabolism govern virulence. Importantly, these two processes are pivotal and interdependent (rather than discrete) mediators of bacterial physiology.

We first discuss the regulatory mechanisms that enable preferential utilization of carbon sources in *S.* Typhimurium. We then review how these systems regulate *S.* Typhimurium virulence in the context of bacterial metabolism and host metabolic reprogramming during infection. Finally, we elaborate how virulence programs sustain bacterial viability during infection by regulating carbon source utilization. This perspective synthesizes basic features of bacterial physiology (e.g., central carbon metabolism) with a sophisticated understanding of bacterial virulence. This synthesis aims to resolve apparent contradictions in the understanding of bacterial pathogenesis by interrogating the underlying molecular, metabolic, and physiological bases for behaviors displayed by pathogens during infection.

FIG 1 Legend (Continued)
Entner-Doudoroff), TCA cycle, pentose phosphate, acetate overflow, and the intersection between glycolysis and the utilization of alternative carbon sources, using glycerol and fructose as examples. Protein annotations specify either single metabolic enzyme, enzyme complex or protein isoforms (separated by forward slash). Blue letters are used to designate Mg^{2+}-coordinating enzymes.

REGULATION OF CARBOHYDRATE UTILIZATION CONFERS FLEXIBILITY IN GROWTH AND METABOLISM

Here, we discuss the major mechanisms dictating carbon source utilization that operate at the transcriptional and posttranscriptional levels. We propose that the regulation of carbon metabolism enables broad flexibility in carbon source utilization under nutrient-replete conditions. That is, if other requisite (micro)nutrients are available to a pathogen, the regulation of carbon metabolism ensures efficient utilization of a wide array of carbon sources, guaranteeing that the viability and basic growth requirements of the cell are satisfied.

The Phosphoenolpyruvate-Carbohydrate Phosphotransferase System Mediates Carbohydrate Uptake

The central systems regulating preferential carbon source utilization are of the phosphoenolpyruvate (PEP)-carbohydrate phosphotransferase system (PTS) class. These systems are widespread in bacteria, including the human gastroenteritis- and murine typhoid-causing *S.* Typhimurium and the human-adapted typhoid agent *S. enterica* serovar Typhi (*S.* Typhi). We provide an overview of the ability of PTS systems to discriminate among carbon sources, with an emphasis on the regulatory mechanisms that coordinate these processes, and refer the reader to extensive reviews on the function and control of PTS systems (23–25). Where direct evidence is lacking for *Salmonella*, we consider findings obtained in *E. coli*. However, mechanisms present in *E. coli* but absent from *Salmonella* (such as regulation of beta-glucoside utilization by BglG-dependent antitermination [26]) are not discussed in detail.

In general, PEP-carbohydrate PTSs are phosphorelay networks that phosphorylate specific carbohydrates as they enter the cell, preventing carbohydrate escape from the cytoplasm and favoring carbohydrate entry into the glycolytic pathway. For example, the glucose PTS system in the family *Enterobacterales* includes the following components (with the corresponding genes italicized and in parentheses): PEP, enzyme I (EI/*ptsI*), histidine protein (HPr/*ptsH*), enzyme IIA (EIIA/EIIA^{Glc}/*crr*), and enzyme IIB/C (EIIBC/PtsG/*ptsG*) (Fig. 2A) (24). EI and HPr are often involved in the uptake of different PTS sugars (25). The EIIB and EIIC proteins of some PTS systems are encoded by distinct genes, and some bacteria contain additional permease subunits, designated EIID (25).

The phosphorelay begins with PEP donating a phosphate to a conserved histidyl residue in EI, which then donates this phosphoryl group to His-15 in HPr (27, 28). In *Firmicutes* and some proteobacterial species (but not *S. enterica*), HPr is also phosphorylated by the protein HprK/P at Ser-46 (29, 30). In these species, HPr plays a broader regulatory role and can exist in four phospho-states. In all cases, the phosphoryl group in HPr's His-15 is donated to EIIA, again at a conserved histidyl residue (31). Phospho-EIIA then donates the phosphoryl group to EIIB (or, in the case of the glucose PTS, the EIIB/C protein, PtsG), typically at a conserved cysteyl residue (32). For EIIBs of the mannose PTS, however, phospho-EIIA donates the phosphoryl group to a conserved histidine in EIIB (32). Finally, the phosphoryl group is transferred from EIIB to the incoming sugar as it is being imported from the periplasm into the cytoplasm by the integral membrane protein EIIC.

The subsequent metabolism of glucose-6-phosphate (G6P) in the glycolytic pathway leads to the generation of PEP (Fig. 1). Therefore, the PEP/pyruvate ratio determines the relative activity of the PTS phosphorylation cascade (Fig. 2A) (33). That is, when the PEP concentration is low relative to that of pyruvate, EIIA^{Glc} is predominantly dephosphorylated, having donated its phosphoryl group to PtsG (EIIB/C), which, in turn, donates it to the incoming glucose molecule. By contrast, when the PEP concentration is high relative to that of pyruvate, EIIA^{Glc} exists mostly in the phosphorylated state because there is a surplus of PEP, the initial phospho-donor in the PTS phosphorelay. Therefore, EIIA^{Glc} has emerged as a principal regulator of the process of CCR due to its close association with the terminal steps of glucose uptake (34).

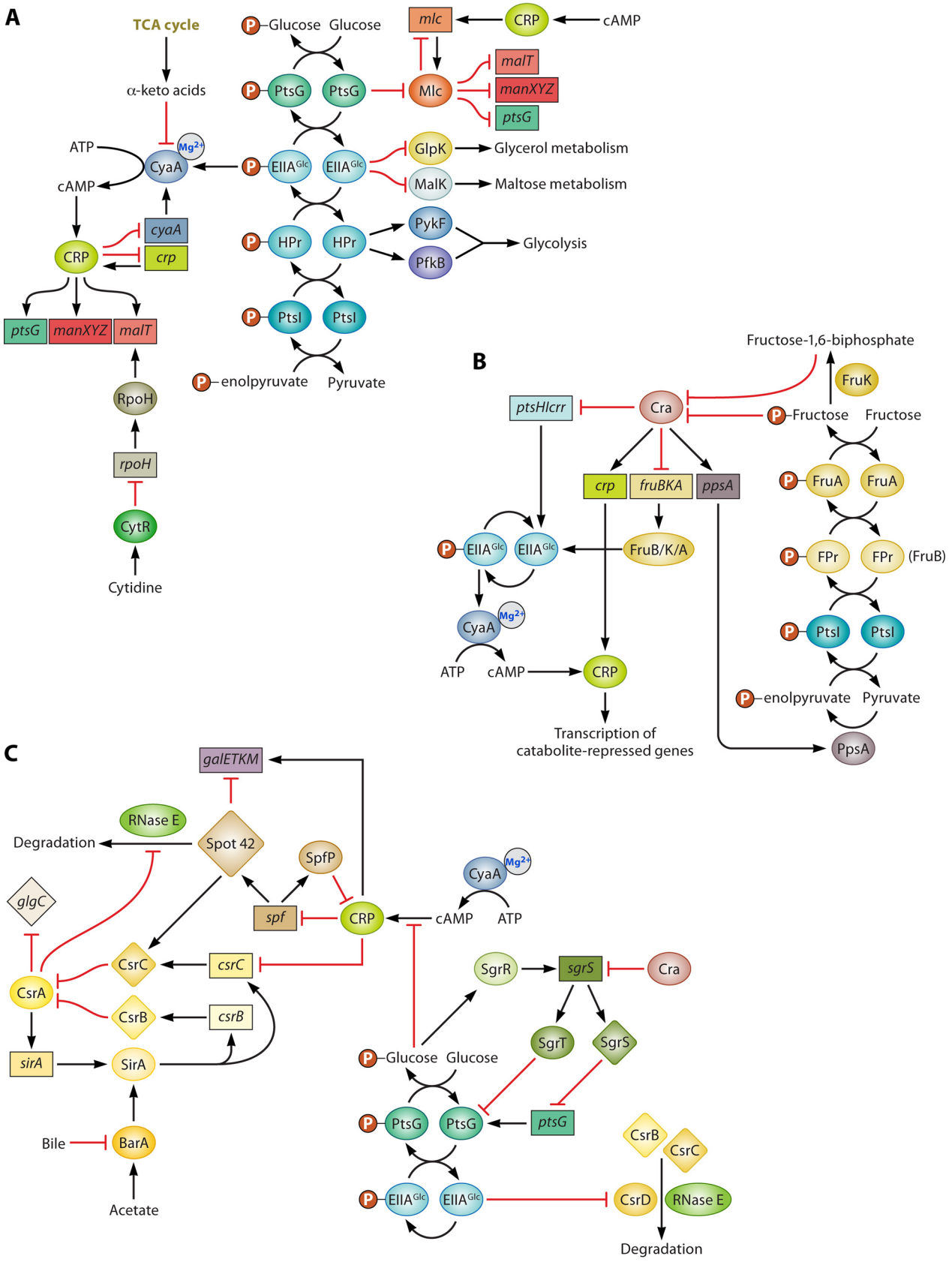

FIG 2 Regulatory circuits governing carbon source utilization in *S.* Typhimurium. (Some of the depicted regulatory interactions are presumed to be operating in *S.* Typhimurium based on findings in *E. coli*.) (A) Control of carbon source utilization by cAMP. The glucose-phosphoenolpyruvate

(Continued on next page)

Enzyme IIA-Glucose Dictates Alternative Carbon Source Utilization

Inducer exclusion is a phenomenon by which bacteria (*E. coli* being the prototypical example) grown on a preferred carbon source actively reduce the uptake of alternative (nonpreferred) carbon sources (35, 36). This process is proposed to explain, in part, the phenomenon of glucose-lactose diauxie. The primary molecular mechanism underlying inducer exclusion involves inhibition of the permeases responsible for import of carbohydrates other than glucose by dephosphorylated EIIA^{Glc} (Fig. 2A) (37). For example, inhibition of lactose uptake in the presence of both glucose and lactose results from dephosphorylated EIIA^{Glc} binding to the cytoplasmic face of the lactose/H$^+$ symporter LacY complexed with lactose at the periplasmic face of the membrane, thereby preventing lactose import from the periplasm to the cytoplasm (38–41). *Salmonella* cannot utilize lactose due to the absence of the *lac* operon; however, this regulatory scheme also applies to the uptake of galactose, melibiose, and raffinose (42, 43), which *Salmonella* can utilize.

EIIA^{Glc} blocks maltose translocation from the periplasm into the cytoplasm through a slightly different mechanism from that inhibiting lactose uptake. EIIA^{Glc} targets the membrane-associated ATPase MalK rather than binding to the integral membrane permease subunits MalF and MalG (44). EIIA^{Glc} prevents ATP hydrolysis by MalK, a member of the ATP-binding cassette protein family, which utilizes the energy derived from ATP hydrolysis to move substrates across the cytoplasmic membrane with the participation of the integral membrane components (44–46).

EIIA^{Glc} excludes glycerol from the cytoplasm by inhibiting glycerol kinase GlpK (47, 48), the enzyme that catalyzes the first step of glycerol metabolism using ATP to phosphorylate glycerol into glycerol 3-phosphate (49). EIIA^{Glc} binds GlpK distal to the catalytic site and appears to regulate its activity allosterically (50, 51). This distinct regulatory strategy reflects that the glycerol permease GlpF mediates the ATP-independent facilitated diffusion of glycerol from the periplasm to the cytoplasm (52, 53) and that GlpK is activated by glycerol diffusion through GlpF (53). Thus, by inhibiting GlpK-dependent phosphorylation of glycerol, EIIA^{Glc} hinders glycerol retention within the cytoplasm.

Critically, the phosphorylated and unphosphorylated states of EIIA^{Glc} exert completely different effects on the utilization of alternative carbon sources. Whereas dephosphorylated EIIA^{Glc} promotes inducer exclusion, phosphorylated EIIA^{Glc} (P-EIIA^{Glc}) stimulates the activity of adenylate cyclase (CyaA, encoded by the *cyaA* gene), the enzyme that converts ATP into the second messenger cyclic AMP (cAMP) (Fig. 2A) (54–56). When bound to cAMP, the cAMP receptor protein (CRP; also known as catabolite activator protein [CAP], encoded by the *crp* gene) (57) binds to target DNA sequences, altering transcription of a large number

FIG 2 Legend (Continued)
phosphotransferase system (glucose-PEP PTS) is a central node in the regulation of carbon source utilization. When glucose is present, dephosphorylated enzyme IIA-glucose (EIIA^{Glc}) excludes inducers for other carbohydrate utilization genes, thereby suppressing uptake of carbon sources other than glucose. When glucose is absent, phosphorylated EIIA^{Glc} promotes cAMP synthesis by adenylate cyclase (CyaA), thereby increasing transcription of catabolite-repressed genes by the cAMP-bound cAMP receptor protein (CRP). CyaA activity is inhibited by α-keto acids, which are part of the TCA cycle. The unphosphorylated form of the histidine phosphocarrier protein (HPr) activates the glycolytic enzymes PykF and PfkB. Activation of the transcriptional regulator CytR by the nucleoside cytidine increases transcription of the *rpoH* gene, specifying the heat shock sigma factor RpoH. RpoH promotes transcription of some CRP-activated genes, such as *malT*. (B) cAMP-independent control of carbon source utilization. The catabolite repressor/activator (Cra) protein represses fructose uptake and promotes glycolytic/gluconeogenic flux. Transcriptional repression of the *fruBKA* operon by Cra leads to an apparently indirect decrease in cAMP amounts by promoting the dephosphorylated form of EIIA^{Glc}. Paradoxically, Cra also promotes transcription of the *crp* gene, which increases transcription of catabolite-repressed genes. Cra promotes transcription of the *ppsA* gene, which specifies a protein that initiates the fructose PTS cascade that results in the formation of fructose-6-phosphate, which is converted into fructose-1,6-bisphosphate and enters glycolysis. Both fructose-6-phosphate and fructose-1,6-bisphosphate negatively regulate Cra. (C) Posttranscriptional control of carbon source utilization. CRP-cAMP represses transcription of the *spf* gene, which encodes both the small regulatory RNA Spot 42 and the small peptide SpfP. Spot 42 reduces the mRNA abundance of CRP-activated mRNAs but increases the abundance of the sRNA CsrC. SpfP antagonizes CRP-cAMP recognition of the promoter of the *galETKM* operon. The RNA-binding protein CsrA reduces the abundance of the *glgC* mRNA, which specifies an enzyme that converts ATP and glucose-1-phosphate into ADP-glucose and diphosphate. CsrA increases the abundance of the response regulator SirA, which is activated by the bile- and acetate-responding sensor BarA. SirA promotes transcription of the *csrB* and *csrC* genes, specifying sRNAs that bind to CsrA, preventing CsrA from exerting its regulatory effects. CsrA also protects Spot 42 from degradation by RNase E. Glucose uptake through the Glucose-PEP PTS decreases CsrA activity because dephosphorylated EIIA^{Glc} sequesters the CsrD protein away from the sRNAs CsrB and CsrC, thereby preventing degradation of these sRNAs by RNase E and thus increasing CsrA sequestration away from its targets. In addition, accumulation of phospho-sugars, such as glucose-6-phosphate, increases the activity of the SgrR protein, a transcriptional activator of the sRNA-encoding *sgrS* gene. The *sgrS* gene specifies the sRNA SgrS, which reduces the stability of the *ptsG* transcript, and the small peptide SgrT, which reduces the activity of the PtsG protein. The Cra protein represses transcription of the *sgrS* gene. In all panels, ovals represent proteins, rectangles represent genes, where necessary, and diamonds represent regulatory sRNAs or the mRNAs transcribed from respective genes.

of genes (58, 59). The concentrations of both cAMP and P-EIIA^{Glc} are thought to reflect the degree of catabolite repression experienced by bacteria, being low in catabolite-repressed bacteria (i.e., grown in the presence of glucose) and high in bacteria not experiencing catabolite repression (i.e., grown in the absence of glucose) (60).

Curiously, P-EIIA^{Glc} alone is not sufficient to stimulate cAMP synthesis by purified CyaA *in vitro*, and cAMP synthesis increases in the presence of a crude *E. coli* extract (61). An additional component(s) of the PTS system appears to activate CyaA because the cAMP concentration is lower in mutant *E. coli* lacking the entire *pts* operon than in a strain lacking only *crr*, the gene specifying EIIA^{Glc} (62). In addition, both *sn*-glycerol-3-phosphate (G3P) and G6P antagonize CyaA activation by P-EIIA^{Glc} (63), which explains, in part, catabolite repression in the presence of non-PTS carbon sources (Fig. 2B).

Regulation of CyaA activity by P-EIIA^{Glc} is purported to explain the increase in cAMP amounts when bacteria grow in the absence of glucose. However, this may not be the case because the cAMP concentration begins to increase even at saturating glucose concentrations for the glucose importer PtsG (64). Given that dephosphorylated EIIA^{Glc}, rather than P-EIIA^{Glc}, should predominate under saturating glucose concentrations, the relevance of P-EIIA^{Glc} in stimulating CyaA activity is presently unclear. Perhaps cAMP abundance responds to the energy status of the cell rather than carbon source *per se* (65). Alternatively or in addition, P-EIIA^{Glc} amounts available to stimulate CyaA may not be strictly linked to glucose saturation of PtsG.

The regulatory schematic deployed by EIIA^{Glc}, whereby its phosphorylation status dictates regulatory activity, is also utilized by the PTS component HPr. The dephosphorylated form of HPr allosterically activates one of the two pyruvate kinase isozymes—PykF—and one of the two phosphofructokinase isozymes—PfkB (Fig. 2A) (66). In addition, dephosphorylated HPr binds to the anti-sigma factor protein Rsd, antagonizing its association with the σ^{70} sigma factor during logarithmic growth (67). Phosphorylated HPr, on the other hand, allosterically activates the adenylate kinase Adk (66), an essential enzyme that catalyzes the reversible conversion of two molecules of ADP into one molecule of AMP and one of ATP. The phosphorylation state-dependent regulatory activity of HPr thereby enables tuning of metabolism and physiology with carbon source availability.

CRP Controls Transcription of Genes Involved in Catabolism

The global transcriptional regulator cAMP receptor protein (CRP) and its orthologues in various species are the only proteins known to bind cAMP in bacteria. The CRP-encoding *crp* gene in *E. coli* was identified as one of two genes that, upon inactivation, prevented induction of the *lac* operon in the presence of lactose (68), the other being *cyaA*. Cyclic AMP binds each monomer of the apo-CRP dimer with negative cooperativity at two distinct sites, the first of which exhibits high affinity and promotes sequence-specific DNA-binding by CRP-cAMP (69–71). Thus, upon binding cAMP, CRP experiences an allosteric conformational transition in its C-terminal DNA-binding domain (72). Occupation of the second cAMP-binding site at high cAMP concentrations reduces the affinity of CRP-cAMP for DNA (73), implying that transcriptional activation by CRP is optimal only within a certain cAMP concentration range. By contrast, the apo-CRP dimer lacks sequence-specific DNA-binding activity, leading to the suggestion that apo-CRP functions as a nucleoid-associated protein that silences gene transcription in a *cyaA* mutant, which mimics low-cAMP conditions (74, 75).

CRP-cAMP helps prioritize carbon source utilization, as in the case of glucose-lactose diauxie, wherein glucose is preferentially utilized before lactose when *E. coli* is grown in the presence of both carbon sources (76). This is accomplished by the implementation of coherent feed-forward loops composed of CRP-cAMP, the catabolic gene(s) of interest that is transcriptionally activated by CRP-cAMP, and a carbohydrate-dependent transcriptional regulator that is regulated by CRP-cAMP and then regulates transcription of the catabolic gene(s) of interest (77). For example, CRP-cAMP activates transcription of both *malT*, the gene encoding the ATP-dependent, maltotriose-responsive transcriptional activator MalT (78), and the *malK-lamB* and *malEFG* operons encoding the various proteins required to

take up and catabolize maltose in the cell (79). The architecture of this regulatory circuit imposes a requirement for the relevant carbohydrate on the cell such that various carbohydrate-specific catabolic genes are not needlessly expressed in the absence of the cognate carbohydrate, e.g., maltose or maltotriose, in the case of the *malK-lamB* and *malEFG* operons (80).

Critically, CRP activity is modulated by molecules other than cAMP. For instance, Jacques Monod proposed in his final publication that a "catabolite modulator factor" present in the water-soluble fraction of an *E. coli* cell lysate hinders transcriptional control by CRP-cAMP (81, 82). It has been proposed that this factor is the pyrimidine nucleoside cytidine, which binds to the transcriptional regulator CytR, resulting in transcriptional repression of the heat shock sigma factor gene *rpoH* (83), which is required, in turn, for transcription of CRP-regulated genes such as *malT* (Fig. 2A) (84). However, it is unclear whether this indirect effect (i.e., via CytR and RpoH) is sufficient to explain the original observations made by Monod using *E. coli* cell lysates. In agreement with this notion, cytidine does not influence promoter recognition of the wild-type CRP *in vitro* (84).

CRP is also subjected to posttranslational modification that impacts its activity as a transcriptional regulator and alters its subcellular localization. CRP can be acetylated on at least nine lysine residues in *E. coli* (85, 86), but few modified residues have been assigned a molecular function. Acetylation at residue K100 promotes the steady-state abundance of the CRP protein while reducing its affinity for class II promoters (87), which depends on multiple positively charged residues making contact with various negative residues of the RNA polymerase α subunit (88).

CRP self-acetylates at residue K100 using the metabolic intermediate acetyl phosphate as acetyl donor. Therefore, conditions that favor acetate fermentation, such as growth on glucose where overflow metabolism leads to the accumulation of acetate (Fig. 1), may favor CRP acetylation and thus transcriptional activation of class I promoters (87, 89). Acetylation of *E. coli* CRP requires both the *ackA-pta* operon, encoding proteins responsible for the synthesis and degradation of acetyl phosphate, and the Nε-lysine acetyltransferase-encoding *yfiQ* gene (90). Moreover, synthesis of the stringent response alarmone guanosine (penta)tetraphosphate [(p)ppGpp] promotes CRP acetylation in *E. coli* because a *relA* mutant lacking one of the (p)ppGpp synthetases (the other being encoded by the *spoT* gene) exhibits reduced amounts of acetylated CRP and reduced expression of CRP-activated genes (90).

In *Vibrio cholerae* and *E. coli*, CRP is reported to associate with the bacterial inner membrane (91). In *V. cholerae*, the membrane association of CRP depends on acetylation of residues K26 and K35 and succinylation of residue K52 (91). Membrane association of CRP promotes sequestration of the DNA-binding protein PepA, altering transcription of PepA-regulated genes. Thus, CRP activity is responsive to multiple intermediate metabolites in the cell, including acetate and succinate.

Signaling by cAMP is proposed to link the composition of the bacterial proteome to metabolic demands (92). Carbon-limited growth promotes expression of genes involved in carbon catabolism (e.g., *lacZ*), whereas limitation of nutrients other than carbon (e.g., nitrogen limitation) suppresses expression of such genes. Critically, both carbon limitation and nitrogen limitation decrease ribosomal protein content, which decreases protein synthesis. Metabolic intermediates such as α-keto acids induce a transient decrease in the expression of catabolic operons concomitantly with decreased cAMP synthesis. Therefore, it is possible that such metabolic intermediates balance carbon intake with nitrogen intake by reducing CRP-cAMP activity. This would coordinate catabolic and anabolic proteome capacity to ensure that carbon intake matches nitrogen intake in the production of amino acids for protein synthesis (92). CyaA regulation by such precursor metabolites presumably impacts CRP-cAMP activity by reducing the cAMP pool (Fig. 2A) (93). Beyond catabolite repression, transcriptional regulation by CRP-cAMP has been implicated in a wide variety of biological processes, including nitrogen metabolism (94), the stringent response (95), flagellum biosynthesis (96, 97), and, as discussed below, bacterial virulence.

CRP is often considered a prototypical gene regulator in bacteria (98). However, some CRP-mediated behaviors are paradoxical. For example, CRP-cAMP activates *ptsG* transcription during the glucose growth phase in a glucose-lactose diauxic growth condition, resulting in PtsG-mediated glucose uptake (99), which is puzzling because the imported glucose is expected to *lower* the cAMP concentration, thereby reducing the amount of cAMP-bound CRP (i.e., the active form of the CRP protein). The higher rate of glucose uptake results in larger amounts of dephosphorylated $EIIA^{Glc}$, which inhibit the uptake of alternative carbon sources via inducer exclusion, as described above.

How, then, does CRP-cAMP retain enough activity to induce *ptsG* transcription in cells growing on glucose? There are at least three contributing factors. (i) The presence of two CRP binding sites within the *ptsG* promoter may alleviate the necessity for high CRP-cAMP amounts to activate *ptsG* transcription (100). (ii) Transcription of the *crp* gene is both positively and negatively autoregulated (101), with negative autoregulation predominating at low cAMP concentrations (102). This feedback mechanism balances apo-CRP amounts with cAMP concentration so that CRP is predominantly in the regulation-competent cAMP-bound state. (iii) Finally, *ptsG* transcription is repressed by additional factors (103–105), notably the global repressor of carbohydrate uptake known as Mlc.

Dephosphorylated PtsG sequesters Mlc at the bacterial inner membrane and away from target promoters (106, 107). Importantly, it is the association of Mlc with PtsG *at the membrane* that results in inactivation of Mlc, as the cytoplasmic domain of PtsG ($EIIB^{Glc}$) alone is insufficient to hinder Mlc activity (108). This is perhaps surprising given that there exist many notable examples of active membrane-bound DNA-binding proteins, such as the transcriptional activator of cholera toxin gene expression ToxR in *V. cholerae* (109) and the highly expanded family of hybrid two-component systems from the commensal gut bacterium *Bacteroides thetaiotaomicron*, which encode all components of classical two-component systems in a single polypeptide located in the inner membrane (110, 111). Nevertheless, as glucose is imported into the cell, Mlc repression is relieved, and CRP-cAMP promotes *ptsG* transcription. The resulting increase in PtsG protein abundance results in Mlc sequestration, producing a positive feedback loop that increases *ptsG* transcription by hindering Mlc access to the *ptsG* promoter (Fig. 2A).

The Global Repressor of Carbohydrate Uptake Mlc Counteracts CRP-cAMP-Dependent Activation of Transcription

Mlc is a transcriptional repressor of the catabolite-repressed *malT* and *manXYZ* genes (112, 113). This is paradoxical because, as noted above, Mlc's activity is negatively correlated with glucose uptake. That is, transcriptional repression by Mlc is high when alternative carbon sources are being utilized even though the genes responsible for utilization of these carbon sources are activated by CRP-cAMP, ultimately determining the transcriptional output (114). One possible explanation for this behavior is that negative autoregulation of *mlc* gene transcription by the Mlc protein (112) results in sufficiently low levels of Mlc that are superseded by the available CRP-cAMP molecules. However, CRP-cAMP also activates transcription from the *mlc* promoter (Fig. 2A) (115), suggesting that posttranscriptional regulatory mechanisms play more determinative roles in the amounts of active Mlc protein, potentially by regulating the stability or turnover rate of the *mlc* mRNA (115). Regardless of the mechanism, Mlc sequestration by PtsG ties Mlc to glucose uptake and positions Mlc as a key regulator of carbon metabolism.

The Catabolite Repressor/Activator Cra Determines cAMP-Independent Catabolite Repression and Gluconeogenic Flux

Early observations that catabolite repression was displayed by mutant *E. coli* lacking *cyaA* or *crp* led to the hypothesis that cAMP-independent mechanisms of CCR exist in enteric bacteria (116, 117). The catabolite repressor/activator Cra (formerly referred to as FruR) emerged as the principal regulator of cAMP-independent catabolite repression (118, 119). *S.* Typhimurium and *E. coli* strains deficient in *cra* are unable

to grow in media containing gluconeogenic substrates as the sole carbon source (120, 121), implicating *cra* in the regulation of metabolic flux (122). In these strains, the *fru* operon, encoding the proteins that constitute the fructose PTS system, is constitutively expressed, and growth on gluconeogenic substrates cannot be restored by addition of cAMP. Cra can either repress or activate gene transcription, and its DNA-binding activity is antagonized by micromolar amounts of fructose-1-phosphate or millimolar amounts of fructose-1,6-bisphosphate (123–125), key intermediates in glycolysis (Fig. 1). Curiously, Cra activates *crp* transcription (126) but indirectly decreases CyaA activity. The latter regulation results from Cra promoting transcription of the *fru* operon, which results in fructose uptake and increases the abundance of dephosphorylated $EIIA^{Glc}$ (127), the $EIIA^{Glc}$ form that does not stimulate CyaA activity (Fig. 2B) (54–56). Therefore, Cra is closely intertwined with the cAMP-dependent CCR network even though Cra activity is cAMP-independent.

Bacteria Rely on Various Forms of Posttranscriptional Regulation of Carbohydrate Utilization Determinants

Several posttranscriptional mechanisms direct carbon source utilization downstream of the $EIIA^{Glc}$, CRP-cAMP, Mlc, and Cra proteins, with small RNAs (sRNAs) being one of the best characterized (we direct the reader to a recent, comprehensive review on the subject [128]). For instance, the *spf*-encoded sRNA Spot 42 was first discovered for its role in hindering *E. coli* adaptation to nutritional shifts (129) and has since been implicated in CCR by virtue of its role in repression of the galactokinase-encoding *galK* gene (Fig. 2C) (130–132). Importantly, *spf* is one of the few genes repressed by CRP-cAMP (133), tying Spot 42 activity to cAMP concentration in the cell. In most cases, Spot 42 represses translation of mRNAs for genes transcriptionally activated by CRP-cAMP, leading to coherent feed-forward loops that regulate CCR (131, 134). Spot 42 is predicted to regulate at least 29 genes, including the glycerol permease-encoding *glpF* (134, 135), linking Spot 42 activity to the inducer exclusion mechanism described above for glycerol uptake and retention. Surprisingly, the Spot 42 sRNA from *E. coli* also functions as an mRNA: it encodes a 15-amino-acid peptide—termed SpfP—that binds CRP-cAMP to inhibit transcriptional activation of catabolite repressed genes, such as those in the *galETKM* operon (Fig. 2C) (136). The carbon-storage regulator CsrA regulates Spot 42 abundance by protecting the *spf* mRNA from degradation by RNase E (137).

CsrA is an RNA-binding protein that plays a key role in carbon source utilization (138). Originally identified as a repressor of glycogen biosynthesis in *E. coli* (139), CsrA has emerged as a widespread regulator of *Salmonella* physiology (140). CsrA controls protein abundance using a variety of mechanisms but primarily inhibits translation by binding to mRNAs and occluding their Shine-Dalgarno sequences (141–143). CsrA is antagonistically regulated by the sRNAs CsrB and CsrC (Fig. 2C), which harbor Shine-Dalgarno-like sequences, thereby competing with targeted mRNAs for binding to CsrA (141, 142).

CsrA can repress transcription through Rho-dependent transcription termination (144) as well as activate transcription of certain genes (145). In *E. coli*, CsrA indirectly activates transcription and translation of the *uvrY* gene (146) (designated *sirA* in *Salmonella*), which encodes the response regulator of the BarA/UvrY two-component system. UvrY promotes *csrB* transcription in *E. coli* (147), thereby generating a negative feedback loop that controls CsrA abundance (Fig. 2C). Unphosphorylated $EIIA^{Glc}$ also regulates turnover of CsrB and CsrC by sequestering CsrD, a protein that targets these sRNAs for RNase-E-mediated degradation (Fig. 2C) (148). In addition, transcription of *csrC*, but not *csrB*, is activated by Spot 42 in a CRP-cAMP-dependent manner (Fig. 2C) (149).

The sRNA SgrS mediates a response to phospho-sugar stress (150), the growth inhibition resulting from accumulation of phosphorylated carbohydrates such as G6P (151). Synthesized in response to excess glucose-phosphate, SgrS specifically promotes decay of the *ptsG* mRNA, reducing PtsG amounts, and subsequently decreasing glucose uptake (Fig. 2C) (152). In *E. coli*, the *sgrS* gene is transcriptionally activated by the

regulatory protein SgrR (153), which likely binds phospho-sugars that stimulate its activity, and is repressed by Cra (Fig. 2C) (154). Like Spot 42 the *sgrS* sRNA is unusual in that it also functions as an mRNA that specifies a 40-residue peptide designated SgrT (Fig. 2C) (155). The SgrS sRNA reduces *ptsG* mRNA amounts (155), whereas the SgrT peptide inhibits PtsG activity, thereby reducing glucose uptake (Fig. 2C) (156).

In sum, multiple regulators dictate various aspects of carbon source utilization to tune bacterial metabolism to growth requirements. These regulators include DNA-binding proteins, RNA-binding proteins, sRNAs, and metabolites that control the activity of these regulators. The targets of these regulators often overlap, highlighting the complex network that enables bacteria to respond to changes in carbon source while moving between environments with various nutrient compositions.

HOST CELL METABOLISM AND *SALMONELLA* VIRULENCE

Here, we discuss how *S. enterica* metabolism and virulence are interdependent and reflect the environment the pathogen experiences inside host cells. Virulence-inducing conditions alter *Salmonella* metabolism, and host cell metabolism dictates the environment with which the bacterium interacts. Therefore, a delicate balance exists between host metabolic conditions that hinder versus promote the success of intracellular bacteria.

Infectious Life Cycle of *Salmonella enterica*

To establish a replicative niche within mammalian cells, *Salmonella* coordinates the expression of a large cohort of gene products that elicit bacterial uptake by host cells, manipulate host cellular processes, aid survival of host-originated insults, and maintain bacterial viability when key nutrients are sequestered by the host (157). After colonization of the gastrointestinal lumen, *S. enterica* relies on effector proteins injected into host cells by the *Salmonella* pathogenicity island 1 (SPI-1)-encoded type III secretion system 1 (T3SS-1) to invade the epithelial mucosa (158, 159). Upon invasion, *S. enterica* enters the mildly acidic, lysosome-like SCV (160). The intravacuolar environment is modified by effector proteins translocated into the host cell cytoplasm via the SPI-2-encoded T3SS-2 to sustain bacterial replication and viability (161). A small fraction of bacteria escapes the SCV into the cytosol of the host epithelial cell, where the pathogen hyper-replicates (162, 163).

Salmonella recognition by the host immune system triggers a proinflammatory immune response that results in lymphocyte recruitment from the proximal lamina propria (164, 165). *Salmonella* can invade various cells of the monocytic lineage, through which it disseminates into distal tissues to cause systemic infection (166). Ultimately, the host either succumbs to infection or prevents further proliferation and clears the pathogen. This multistage infectious life cycle is tightly regulated and involves multiple regulatory circuits (167–169). Here, we focus on the central role that the regulation of central metabolic processes plays in *S. enterica* virulence.

Metabolic Requirements of *Salmonella* during Infection

As discussed above, *S. enterica* can take up and metabolize a wide array of carbohydrates and gluconeogenic substrates (100, 170, 171). Thus, the availability and utility of particular carbon sources inside host tissues contribute to the outcome of *S. enterica* infection. For example, *S.* Typhimurium requires the *pfkAB*-encoded glycolytic enzyme phosphofructokinase (Fig. 1) for full virulence in cultured murine macrophages and a mouse model of infection (172). By contrast, inactivation of the PTS responsible for importing glucose causes a relatively moderate defect, implying that central metabolic functions such as glycolysis play a more pivotal role in virulence than carbon source prioritization through the PTS (172–174).

S. Typhimurium retains full virulence upon inactivation of genes encoding enzymes that participate in gluconeogenesis, with the exception of a mutant defective in both the PEP carboxykinase-encoding *pckA* gene and PEP synthase-encoding *ppsA* gene (175). The *pckA ppsA* double mutant fails to feed the tricarboxylic acid (TCA) cycle because it cannot convert substrates such as pyruvate and oxaloacetate into PEP (Fig. 1). Although the full TCA cycle is required to maintain wild-type virulence, neither the

reductive branch of the TCA cycle nor the glyoxylate bypass is necessary for *S.* Typhimurium virulence (Fig. 1) (175–177). However, an *sdhCDA* mutant, lacking succinate dehydrogenase, is mildly attenuated for virulence, and an *frdABCD* mutant, lacking fumarate reductase and incapable of running the reductive branch of the TCA cycle, is fully virulent, whereas the *sdhCDA frdABCD* double mutant is avirulent (177). The latter behavior has been ascribed to the ability of the *frdABCD*-encoded fumarate reductase to compensate for the absence of the *sdhCDAB*-encoded succinate dehydrogenase, thereby running the full TCA cycle (Fig. 1). Such compensatory action likely reflects the plasticity of *S.* Typhimurium metabolism during murine infection, presumably a requirement for the pathogen to successfully navigate diverse host environments (22, 170, 173).

During infection of mammalian cells, *S.* Typhimurium predominantly utilizes the Entner-Doudoroff (ED) glycolytic pathway for carbon catabolism (178, 179) (Fig. 1). This pathway converts glucose to pyruvate through a distinct set of enzymes from the classical Embden-Meyerhof-Parnas (EMP) glycolytic pathway (Fig. 1) (180). The preference for the ED pathway likely reflects that this pathway links glycolysis to the pentose phosphate pathway (PPP) (Fig. 1) (180). *S.* Typhimurium residing within colorectal adenocarcinoma epithelial cells (Caco-2) relies heavily on the PPP for biosynthesis of the amino acids phenylalanine, histidine, and tyrosine (181). In fact, metabolic flux through the PPP can outweigh flux through the EMP glycolytic pathway in *S.* Typhimurium, as glucose is shunted toward ED rather than EMP glycolysis (182). It appears that glucose is a crucial carbon source because *Salmonella* does not appear to readily access gluconate (the immediate glucose derivative feeding the ED pathway) as a carbon source to fuel the PPP during infection (181).

Intriguingly, competition studies in which mice were coinfected with wild-type *S.* Typhimurium and a mutant strain in which the genes *glpFK*, *gldA*, *glpT*, and *ugpB* were inactivated implicated glycerol as a pivotal carbon source for *S.* Typhimurium within a mammalian host (173). That is, *S.* Typhimurium mutants defective in the uptake of glycerol and glycerol-3-phosphate (Fig. 1) were more readily outcompeted by wild-type *S.* Typhimurium in a mouse model of intravenous infection than mutants defective in the uptake of glucose (173). In fact, glucose uptake-deficient *Salmonella* replicates to similar levels as wild-type *Salmonella* in Caco-2 cells (174), reflecting their ability to readily access 3-carbon substrates, such as glycerol and lactate, to generate biomass (181).

It is important to keep in mind that competition studies require careful interpretation because they assume that phenotypes in mutant strains are due solely to the metabolic function of the protein encoded by the inactivated gene(s) rather than potential nonmetabolic (i.e., moonlighting) roles, which have been reported for other key regulators of carbohydrate metabolism, including $EIIA^{Glc}$ (183). In addition, competition studies can hide mutant phenotypes when a wild-type bacterium supports growth of a mutant strain, as in the case of wild-type *Salmonella* supporting passive entry of noninvasive bacteria into host cells (184). Moreover, such studies typically entail infections with bacterial doses orders of magnitude higher than those necessary for lethality. Nonetheless, *S.* Typhimurium can utilize diverse carbon sources throughout infection and may depend on access to alternative, 3-carbon substrates when glucose is limited. It is now critical to define when and where these carbon sources are essential for pathogen growth and survival.

The Gastrointestinal Microbiome Provides Carbon Sources to *Salmonella* during Gut Colonization

The intestinal microbiome can promote or hinder pathogen infection depending, in part, on the availability of microbiome-derived or -eliminated carbon sources (185). *S.* Typhimurium induces inflammation in the gut, promoting pathogen proliferation in the intestinal lumen (186). Intriguingly, the ability to utilize carbon sources scavenged from the inflamed gut differs among *S. enterica* serovars according to their host specificities and disease conditions these serovars promote. For example, the genes associated with the utilization of inflammation-derived metabolites, such as ethanolamine, are no longer functional in *Salmonella* serovars that are primarily extraintestinal (e.g.,

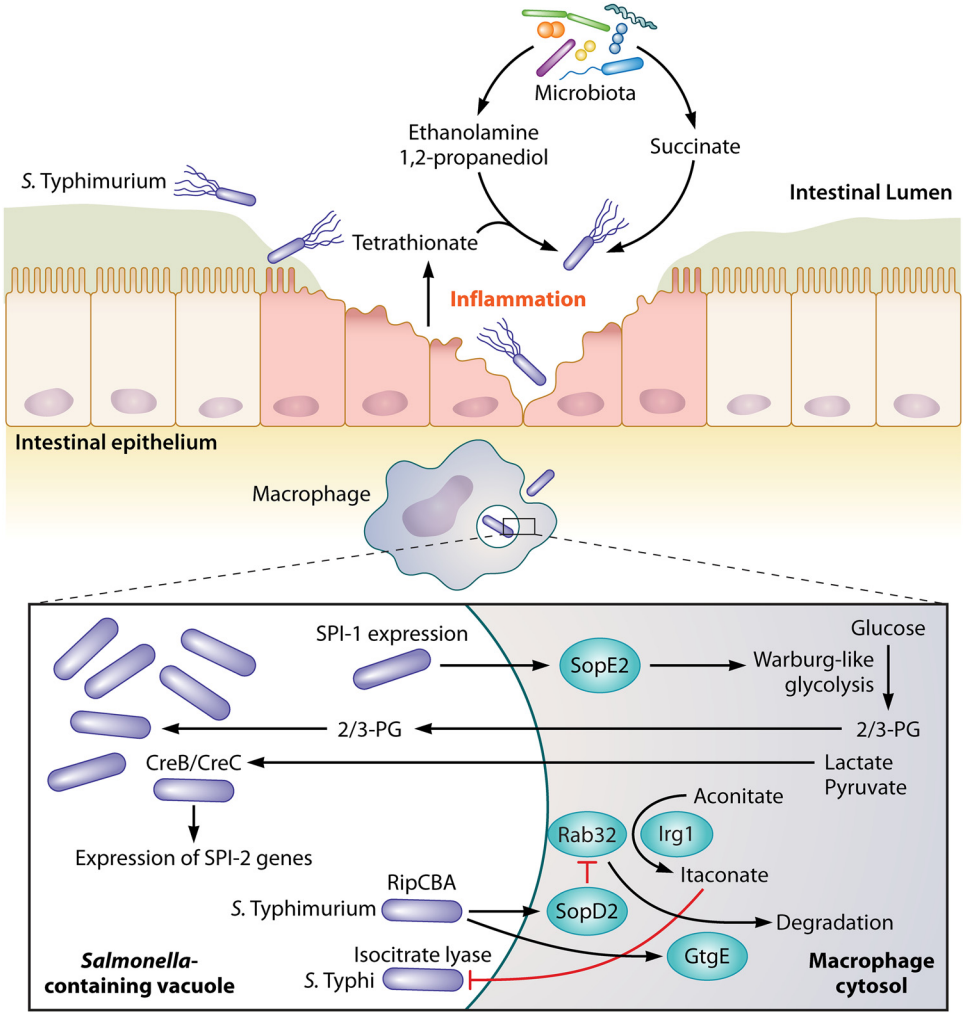

FIG 3 Metabolic state of *S.* Typhimurium during infection of a mammalian host. (top) During colonization of the mammalian gastrointestinal tract, *S.* Typhimurium provokes inflammation, resulting in the release of the electron acceptor tetrathionate by host cells. Tetrathionate enables *S.* Typhimurium to utilize certain microbiota-derived carbon sources, such as ethanolamine and 1,2-propanediol. *S.* Typhimurium performs the complete, oxidative TCA cycle in the gut lumen, enabling the utilization of microbiota-derived succinate as a carbon source. (bottom) When inside host cells, the T3SS encoded in SPI-1 secretes effector proteins, such as SopE2, that trigger host cell metabolic reprogramming, eliciting "Warburg-like" glycolysis (aerobic glycolytic fermentation) that results in accumulation of glycolytic intermediates (e.g., 2/3-phosphoglycerate). *S.* Typhimurium can utilize such intermediates as carbon sources. In addition, accumulated pyruvate and lactate activate the regulatory system CreB/CreC of *S.* Typhimurium, resulting in increased transcription of SPI-2 genes that further intracellular replication. Activated upon infection with *S.* Typhimurium, the host immune responsive gene 1 (Irg1) converts the TCA cycle intermediate aconitate into itaconate. The GTPase Rab32 traffics Irg1 to the SCV, where itaconate inhibits the bacterial isocitrate lyase of *S.* Typhi, thereby reducing intracellular replication. By contrast, *S.* Typhimurium harbors an itaconate degradation gene cluster (*ripCBA*) that enables itaconate metabolism, the secreted cysteine protease GtgE that degrades Rab32, and the secreted effector SopD2 that exhibits GAP activity toward Rab32, inactivating the GTPase.

S. enterica serovar Choleraesuis) (187). By contrast, these genes are functional in gastrointestinal *S.* Typhimurium, which can readily utilize ethanolamine when intestinal inflammation promotes the production of the respiratory electron receptor tetrathionate by the mammalian host (Fig. 3, top) (188). A similar schema applies to the utilization of the microbial fermentation by-products 1,2-propanediol (189) and fructose-asparagine (190), whereby *S.* Typhimurium must promote intestinal inflammation to utilize these carbon sources in a manner dependent on host-derived tetrathionate as an electron acceptor in anaerobic conditions (Fig. 3, top).

Specific microbiota-derived carbon sources, such as butyrate, also display serovar-specific utilization. For example, the extraintestinal serovar *S.* Typhi lacks the *ydiQRSTD* operon and thus cannot utilize the microbial short-chain fatty acid butyrate (191). By

contrast, the *ydiD* gene confers upon the intestinal serovar *S.* Typhimurium protection from butyrate-induced suppression of SPI-1 gene transcription and host cell invasion (191). In addition, *S.* Typhimurium actively inhibits the production of butyrate in the gut by promoting inflammation, which antagonizes the growth of butyrate-producing members of the resident microbiota such as *Clostridia* spp. (192).

Infection-Driven Metabolic Reprogramming of Mammalian Host Cells Governs *Salmonella* Virulence

Mammalian cells undergo metabolic reprogramming upon pathogen infection, shifting their metabolism in ways that influence pathogen biology (193–197). In general, bacterial infection or stimulation of mammalian cells with specific bacterial compounds, such as lipopolysaccharide (LPS), shifts cellular metabolism away from oxidative phosphorylation (OXPHOS) and toward aerobic glycolytic fermentation (i.e., the Warburg effect often exhibited by cancer cells) (194, 198), usually in a pathogen-specific manner (193). This process has been observed in cells infected by the intracellular bacterial pathogen and tuberculosis agent *Mycobacterium tuberculosis*, in which a predominant result of metabolic reprogramming is the accumulation of molecules, such as lactate and fatty acids, that support mycobacterial growth (199, 200). This is apparently also the case for two other intracellular pathogens—*Legionella pneumophila* and *Chlamydia trachomatis*—that rely on aerobic glycolysis for access to amino acids (201) and nucleotides (202), respectively.

S. enterica also reprograms mammalian cell metabolism to access carbon sources that promote bacterial growth and pathogenesis. For instance, *S.* Typhimurium requires the host cell nuclear fatty acid receptor PPARδ to access host glucose pools during infection, supporting bacterial replication (203). Curiously, *S.* Typhimurium reduces macrophage glucose amounts by activating Warburg-like glycolysis, resulting in suppression of serine biosynthesis and accumulation of 2- and 3-phosphoglycerate (2/3-PG), PEP, and succinate, among other metabolites, furthering bacterial growth (Fig. 3, bottom) (204). The SPI-1 T3SS-secreted effector protein SopE2 is required for the accumulation of 2/3-PG and PEP, and subsequent accumulation of lactate and pyruvate activates the bacterial CreB/CreC two-component regulatory system (Fig. 3, bottom). The DNA-binding protein CreB promotes transcription of the *vprB* gene, which encodes a LysR-type regulator that directly regulates transcription of genes in SPI-2, such as *spiR* (also referred to as *ssrA*) and *ssrB* (204). These two genes specify a *S. enterica*-specific two-component system that governs transcription of genes within as well as outside SPI-2 (205), including ancestral genes that control *S.* Typhimurium virulence (206).

It has been proposed that *S.* Typhimurium elicits succinate accumulation in host macrophages; that the accumulated succinate is sensed by *S.* Typhimurium independently of its ability to metabolize it; and that *S.* Typhimurium responds to the accumulated succinate by promoting transcription both of genes regulated by the two-component system PmrA/PmrB and of genes located within SPI-2, resulting in enhanced bacterial virulence (207). The proposed connection between succinate accumulation and *S.* Typhimurium virulence is hard to reconcile with reports from multiple groups over the past 25 years (208–211). Below, we discuss evidence that contradicts the premise that succinate controls *Salmonella* virulence.

Succinate is reported to increase *S.* Typhimurium resistance to the antimicrobial peptide (AMP) polymyxin B (PMB) (207), which is taken as an indication of succinate promoting *S.* Typhimurium virulence. However, the relevance of these findings is highly debatable given that the antibiotic polymyxin B is not used to treat *Salmonella* infections and that there is no correlation between PMB resistance and *S.* Typhimurium virulence in mice. For instance, a *pmrA*-null mutant is 10,000 times more sensitive to PMB than a *phoP*-null mutant when grown in the presence of the PmrB-activating signal Fe^{3+} (212, 213), but a *phoP* mutant has a median lethal dose (LD_{50}) following intraperitoneal inoculation of mice that is ~100,000 times higher than that of wild-type *S.* Typhimurium (214), whereas a *pmrA*-null mutant is actually hypervirulent (208).

If succinate were important for *S.* Typhimurium virulence, preventing succinate uptake would attenuate *S.* Typhimurium virulence. However, inactivation of the

succinate uptake gene *dcuB* had no effect on wild-type *S.* Typhimurium virulence (207). Inactivation of the *dcuB* gene showed mild attenuation in a *phoP* mutant background in a competition experiment in which mice where inoculated with both a *dcuB phoP* double mutant and a *phoP* single mutant (see above for caveats of competition assays) (207). As discussed above, *phoP* specifies the master regulator of *S.* Typhimurium virulence (215), and a *phoP* single mutant is already attenuated 100,000-fold in LD_{50} experiments, raising questions about the significance of the mild attenuation in a competition assay resulting from inactivation of the *dcuB* gene, which does not alter virulence in a wild-type strain (207).

Because the PmrA protein binds to the *ssrB* promoter and hinders *ssrB* transcription (208), the proposal that succinate increases transcription of both SPI-2 genes and genes activated by PmrA is paradoxical, unless the proposed increases take place at different times during infection and/or in different tissues or cell types. Nevertheless, *S.* Typhimurium also utilizes succinate as a carbon source, and microbiota-derived succinate does support *S.* Typhimurium growth in the intestinal lumen (216), suggesting that succinate availability benefits *S.* Typhimurium as it competes for carbon sources in the gut.

Host cell metabolic reprogramming results in itaconate accumulation (207), as aconitate is diverted away from the TCA cycle by the enzymatic activity of the host protein immune-responsive gene 1 (Irg1) (217). Itaconate has been ascribed a largely anti-inflammatory role in the modulation of activated macrophages and plays crucial immunomodulatory roles during inflammation, tumorigenesis, and infection (195, 218). Itaconate inhibits the bacterial isocitrate lyase, the key enzyme in the glyoxylate shunt of the TCA cycle (217).

Bacterial pathogens such as *Yersinia pestis* and *Pseudomonas aeruginosa* detoxify itaconate by degrading it to acetyl-coenzyme A (acetyl-CoA) and pyruvate, thereby supporting bacterial growth and pathogenicity (219). *S.* Typhimurium, but not the human-adapted *S.* Typhi, possesses an itaconate degradation gene cluster encoded by the *ripCBA* operon that confers a similar advantage during infection (220). However, the host GTPase Rab32 functionally interacts with Irg1 during *S.* Typhimurium infection to deliver itaconate to the SCV, a process regulated by the host transcription factor EB (221), suppressing growth of *S.* Typhi (Fig. 3, bottom) (222). By contrast, *S.* Typhimurium encodes the secreted cysteine protease GtgE (223), and the GTPase-activating protein SopD2, which respectively target Rab32 for degradation (224) and suppress Rab32 GTPase activity (Fig. 3, bottom) (225). These findings support the previously reported dispensability of the glyoxylate shunt during murine infection by *S.* Typhimurium (175), which can degrade itaconate and degrade or inactivate the Rab32 GTPase. Furthermore, these results suggest that the glyoxylate shunt may play a more fundamental role in the metabolism of *S.* Typhi during infection, which remains sensitive to itaconate because it does not encode GtgE or the itaconate-degradation cluster, and because *sopD2* has been pseudogenized (226). The accumulation of itaconate during infection illustrates how host metabolic reprogramming can influence the outcome of bacterial infection.

Salmonella achieves metabolic reprogramming of the host cell via secreted effectors such as SopE2 (227). SopE2-dependent reprogramming enables bacterial access to distinct nutritional niches that promote pathogen growth (e.g., 2/3-PG and PEP) (204). In turn, host metabolic reprogramming produces metabolites such as itaconate that suppress *S.* Typhimurium growth (222) but, counterintuitively, may also be degraded into derivatives that promote replication (219, 220). Considered together, these findings may reflect the coevolution of pathogen and host resulting in metabolic reprogramming of infected cells. Host cell metabolic reprogramming likely influences the activity of key regulators of carbon metabolism in *S. enterica*, and these regulators in turn impact *S. enterica* virulence.

REGULATORS OF CARBON METABOLISM CONTROL *SALMONELLA* VIRULENCE

There is considerable overlap in the regulatory outputs of factors thought to act primarily on only one of two processes—metabolism or virulence—which are often seen as operating in discrete fashions. This overlap underscores that, together with physical

and other chemical cues, the availability of specific carbon sources is crucial to *S. enterica* virulence. In this section, we discuss the regulators of carbon source utilization and metabolism that play roles in *S. enterica* virulence.

An array of extracellular and intracellular signals control virulence programs. Such signals often alert a pathogen to activate or suppress processes that dictate survival and proliferation and can impact preference for one carbon source over another. The mildly acidic environment of the SCV (226, 228, 229) and starvation of the essential cation magnesium (Mg^{2+}) (18, 230) regulate virulence in a wide variety of pathogenic *Salmonella* biovars with different host specificities. If a particular carbon source is readily available in the SCV, *S. enterica* may link the sensation of mildly acidic pH to uptake of that carbon source. In addition, the effect of carbon source on the activity of key regulators, such as CRP-cAMP, may have prompted the recruitment of specific virulence determinants into a previously unrelated (i.e., metabolic) regulatory circuit. Such relationships would enable a highly granular distinction between specific biological processes that depend on a given set of nutritional signals and stress conditions that denote the multifarious host environments experienced by a pathogen during infection.

Adenylate Cyclase and CRP are Required for *Salmonella* Virulence

cAMP and CRP orthologues regulate virulence attributes, such as T3SSs, toxin production, and intracellular survival determinants, in several pathogens, including *M. tuberculosis*, cholera agent *V. cholerae*, and opportunistic pathogen *P. aeruginosa*, that have different lifestyles and inhabit different locales (231). CRP-cAMP also regulates *S.* Typhimurium virulence because *cyaA* and *crp* mutants fail to disseminate from the murine intestine into lymph nodes and peripheral organs such as the spleen (232, 233). This defect renders *S.* Typhimurium avirulent, suggesting that the ability to regulate carbon source utilization is crucial to bacterial virulence. Given that *cyaA* and *crp* null mutants still manage to infect the intestinal epithelium (i.e., the bacteria are not broadly attenuated in gaining access to host cells), this virulence defect appears to result from the inability to utilize alternative carbon sources in deep tissues. However, cAMP and CRP may contribute to virulence in additional ways.

For example, *cyaA* and *crp* null mutants fail to form flagella, leading to a strong motility defect (96), and derepress the *spv* operon located in the virulence plasmid of *S.* Typhimurium (234). Transcriptional activation of the *sdiA* gene by CRP-cAMP (235) may also impact *S.* Typhimurium growth in the gut by enabling the LuxR-type transcription factor SdiA to detect a specific *N*-acyl homoserine lactone(s) produced by other species (236, 237). Most organisms harboring LuxR-type regulators also produce the specific *N*-acyl homoserine lactones that bind to such regulators, resulting in quorum sensing abilities (238). *S. enterica* is unusual in that it lacks the genes responsible for synthesis of *N*-acyl homoserine lactones but harbors the gene specifying the regulatory protein that detects such molecules (236). In *S.* Typhi, CRP-cAMP directly promotes transcription of the *yihU-yshA* operon, which supports biofilm development and gallbladder colonization (239).

CRP-cAMP and CsrA Control *Salmonella* Pathogenicity Island-1 Gene Expression

The BarA/SirA two-component system and the SPI-1-encoded regulators HilA, HilC, HilD, and InvF control transcription of the T3SS encoded in SPI-1 (159). The complete details of this regulatory network are beyond the scope of this review, and it will suffice to note that BarA/SirA promotes transcription of the key SPI-1 regulatory gene *hilA* in response to an unknown stimulus (240, 241). Notably, the *barA* and *sirA* genes are encoded in separate regions of the genome (242), allowing the sensor BarA to also activate under physiological conditions the noncognate response regulator RcsB, which governs transcription of a large number of genes, including several impacting *S.* Typhimurium virulence (243). Thus, conditions increasing BarA activity may reflect effects on RcsB, and potentially other regulators, rather than SirA's transcriptional output (244).

By promoting *hilA* transcription, BarA/SirA triggers several positive feedback loops within the HilA-HilC-HilD-InvF regulatory network and responsible for expression of SPI-1 genes necessary for *S.* Typhimurium invasion of epithelial cells (158, 245, 246). Key negative regulators, such as HilE (247) and the carbon storage regulator CsrA (248,

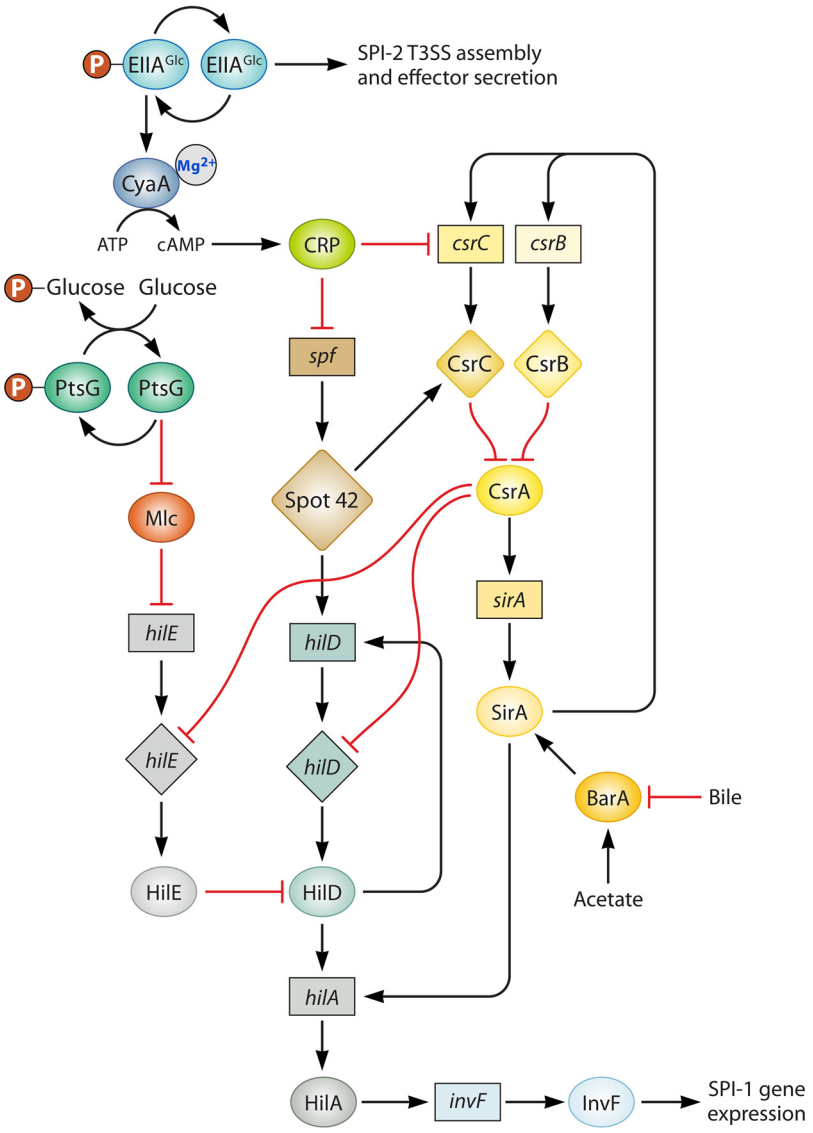

FIG 4 Intersection of the regulatory circuits governing virulence and carbon source utilization in *S.* Typhimurium. Unphosphorylated EIIA^{Glc} promotes the assembly of the T3SS encoded in SPI-2 and secretion of effector proteins. Phosphorylated EIIA^{Glc} stimulates CyaA activity, thereby increasing cAMP amounts. CRP-cAMP represses transcription of the *spf* and *csrC* genes, specifying the sRNAs Spot 42 and CsrC, respectively. Spot 42 increases the abundance of both CsrC sRNA and the HilD protein. HilD is a transcriptional activator of the *hilA* gene, specifying an activator of SPI-1 genes, including the *invF* gene. InvF is a transcriptional regulator of SPI-1 genes, including those specifying the components of the T3SS machinery and secreted effectors. The RNA-binding protein CsrA increases the amounts of SirA, which is activated by the bile- and acetate-responding sensor BarA. Activated SirA promotes *hilA* transcription and negatively regulates *hilD* and *hilE* translation. A negative regulator of SPI-1 genes, HilE antagonizes HilD. The global regulator of carbohydrate uptake Mlc represses transcription of the *hilE* gene. Because unphosphorylated PtsG sequesters Mlc, *hilE* transcription should be derepressed in the absence of glucose. Simultaneously, CRP should be activated by cAMP because phosphorylated EIIA^{Glc} promotes CyaA activity. The sRNAs CsrB and CsrC bind CsrA, preventing it from exerting its regulatory functions, with only *csrC* transcription being repressed by CRP-cAMP. Ovals represent proteins, rectangles represent genes, where necessary, and diamonds represent regulatory sRNAs or the mRNAs transcribed from respective genes.

249), are counterbalanced by other participants in the regulatory circuit. For instance, BarA/SirA promotes transcription of the sRNA genes *csrB* and *csrC*, thereby antagonizing *hilD* silencing by the RNA-binding protein CsrA, thus favoring *hilD* expression (249–251) (Fig. 4). As discussed above, the regulation of *csrB* and *csrC* turnover by EIIA^{Glc}'s sequestration of CsrD (148) and the specific regulation of *csrC* by CRP-cAMP (149) tie CsrA regulation of SPI-1 genes to carbon source signals during infection.

How does CRP-cAMP impact *Salmonella*'s behavior inside infected cells? On the one hand, the RAW 264.7 macrophage cell line infected with a *crp*-null mutant experiences lower levels of apoptosis and altered glycolytic metabolism than when infected with wild-type *S.* Typhimurium (252). Reduced levels of apoptosis and glycolysis may result from reduced fitness of the *crp* mutant and/or altered abundance of specific virulence factors. On the other hand, a *S.* Choleraesuis *crp* null mutant exhibited reduced secretion of the SPI-1-encoded effector proteins SipB and SopB, leading to decreased cytotoxicity toward infected macrophages (253). The reported decreased secretion of SPI-1 effectors exhibited by the *S.* Choleraesuis *crp* mutant is paradoxical given CRP-cAMP's role in silencing SPI-1 genes. That is, CRP-cAMP promotes expression of SPI-1 genes by repressing transcription of the Spot 42 sRNA, stabilizing the *hilD* mRNA by interacting with its 3′ untranslated region (Fig. 4) (254).

The sRNA-encoding *csrC* gene is transcriptionally activated by BarA/SirA (250) and positively regulated by Spot 42 (149). If the CsrA-dependent protection of Spot 42 RNA from RNase E-mediated degradation described in *E. coli* (137) also takes place in *S. enterica* (Fig. 2C), CsrA sequestration by CsrC may render Spot 42 susceptible to RNase E-mediated cleavage and limit Spot 42's role in activation of SPI-1 via HilD (Fig. 4) (254). However, CsrC also antagonizes CsrA-mediated repression of *hilD* translation and stability (249), suggesting that CRP-cAMP sits atop an incoherent feed-forward loop that both inhibits and promotes *hilD* expression via CsrC (Fig. 4). Curiously, the promoter activity of the *prgH* gene (a proxy for transcription of SPI-1 genes) is efficiently suppressed in glucose-supplemented minimal media (a condition in which CRP should be inactive due to low cAMP amounts) compared to lysogeny broth (LB) (255). These regulatory interactions enable *S. enterica* to control SPI-1 activation in response to carbon source, which may reflect the general nutritional environment in the gut.

Transcription of the *sirA* gene is repressed during growth on glucose in a *cyaA*- and *crp*-dependent manner (256). This repression appears to be indirect because CRP-cAMP exhibits no appreciable affinity for the *sirA* promoter. Given that BarA/SirA activity is modulated by a wide array of stimuli, including short-chain fatty acids (257), bile (258), and changes in osmolarity (259), it seems plausible that indirect catabolite repression of the *sirA* gene may be overridden by other signals. In addition, the transcriptional outcome of a BarA/SirA two-component system may differ depending on the specific signal activating the system (215). Importantly, the reported studies of the regulatory effect of CRP-cAMP-repressed Spot 42 on SPI-1 expression were performed by monitoring changes between exponential and stationary-phase growth in LB, without supplementation of a specific carbohydrate (149, 254). Thus, the signal controlling cAMP production, and by extension CRP-cAMP activity, in the latter studies may be unrelated to carbohydrate metabolism as carbohydrates were not present in the media.

Glucose availability coincides with suppressed SPI-1 expression (255), suggesting that glucose-responsive regulators besides CRP-cAMP similarly repress SPI-1. In agreement with this notion, the sRNA SgrS represses transcription and translation of the *sopD* gene, which specifies an effector secreted by both T3SS-1- and T3SS-2 (260). SgrS amounts increase in the presence of phospho-sugar stress (in particular excess G6P) (152), a condition triggered by excess glucose in a pathogen's surroundings. Thus, *Salmonella* utilizes carbon source-responsive regulators to avoid expression of virulence traits when glucose is in excess. It is therefore plausible that *Salmonella* distinguishes between environments requiring and not requiring the expression of virulence traits based on the relative amount of available glucose. In support of this notion, an *S.* Typhimurium mutant deficient in glucose uptake suffers only a mild competitive defect against wild-type bacteria in mice, whereas mutants unable to utilize glycerol or mannose are more readily outcompeted (173).

The Global Repressor of Carbohydrate Uptake Mlc Represses SPI-1 Gene Expression

The global regulator Mlc controls transcription of genes in SPI-1 by binding to the *hilE* promoter and repressing *hilE* transcription (Fig. 4) (261). HilE is a key negative regulator of SPI-1 genes (247). Because Mlc is sequestered by dephosphorylated PtsG after PtsG-P has donated its phosphoryl group to an incoming glucose molecule, glucose

import through PtsG is expected to derepress *hilE* transcription, resulting in repression of SPI-1 genes. However, the increased expression from a *hilE-lacZ* transcriptional fusion exhibited by *S.* Typhimurium grown on glucose, mannose, or arabinose (but not glycerol) (261) was only partially ameliorated upon inactivation of the *mlc* gene, implying that additional factors coordinate expression of SPI-1 genes with nutritional conditions. These data suggest that inactivation of the *hilE* gene should prevent the increased transcription of SPI-1 genes mediated by Mlc taking place in the presence of glucose.

The Regulator of Gluconeogenic Flux Cra is Required for *Salmonella* Virulence

The regulatory protein Cra monitors metabolic flux through the glycolytic and gluconeogenic pathways and is required for *Salmonella* virulence (262, 263). In *Shigella flexneri* and enterohemorrhagic *E. coli* (264–266), Cra controls distinct virulence determinants, raising the possibility that a similar control operates in *Salmonella*; however, this possibility has not been explored in detail. The virulence role of Cra is attributed to its regulation of the genes encoding proteins that are part of the TCA cycle (175–177, 262).

Control of the SPI-2-Encoded Type III Secretion System by the PEP-Carbohydrate PTS and EIIA^{Glc}

Several regulators of carbohydrate metabolism that operate at the posttranscriptional level contribute to *S.* Typhimurium virulence. For example, the mRNA abundance of several genes, including those specifying the T3SS-2 and members of the PhoP regulon, is reduced in a mutant harboring a transposon insertion in the *ptsI-crr* locus compared to the isogenic wild-type parent (267). In addition, EIIA^{Glc} supports the assembly of T3SS-2 by interacting with inner membrane-bound components of this secretion apparatus and with secreted effectors such as PipB (Fig. 4) (183). Consequently, EIIA^{Glc} is required for secretion by the T3SS-2 apparatus, which may be responsible for a *crr*-null mutant being rapidly outcompeted by wild-type *S.* Typhimurium following intravenous infection in mice (183), a model that examines the pathogen's proliferation in deep tissues.

Unexpectedly, substitution of EIIA^{Glc}'s critical phospho-accepting histidine (H90) for alanine did not alter *S.* Typhimurium virulence or effector secretion (183), suggesting that the mechanism by which EIIA^{Glc} promotes secretion by the T3SS-2 is distinct from that controlling carbohydrate metabolism (the latter requires phosphorylated EIIA^{Glc} [41, 56]). It is possible, however, that inducer exclusion mediated by dephosphorylated EIIA^{Glc} contributes to virulence as well as metabolism (see discussion on EIIA^{Glc} activity above). What role, then, does EIIA^{Glc} play in *S.* Typhimurium virulence experiencing different carbohydrates?

On the one hand, poor growth of the EIIA^{Glc}-H90A strain on glycerol-supplemented minimal media prevented examination of the efficiency with which effectors are translocated by the T3SS-2 system (183). (The H90A substitution prevents phosphorylation of EIIA^{Glc}.) On the other hand, secretion of effector SteC, and to a lesser extent SseJ, was higher during growth on glycerol (when the phosphorylated form of EIIA^{Glc} predominates) in the wild-type strain and in a strain expressing the phosphomimetic EIIA^{Glc}-H90D grown on glucose than in the wild-type strain grown under the same conditions (183). That the phenotypes of these mutants are modest may reflect that regulation by EIIA^{Glc} (and potentially other proteins) is not binary and that various functions are supported by the same regulatory states (and the carbon sources that promote them) to greater or lesser extents. In other words, dephosphorylated EIIA^{Glc} (i.e., H90A) may satisfy basal T3SS-2 secretion needs but lacks the dynamic range of phosphorylatable EIIA^{Glc}. Nevertheless, EIIA^{Glc}'s involvement in promoting T3SS-2 effector translocation demonstrates the broadly intersecting nature of regulators of carbohydrate metabolism and bacterial virulence.

In sum, bacterial pathogens experience a variety of metabolizable carbon sources during infection. Although CRP-cAMP negatively regulates some virulence functions (254), the association between heightened virulence states and growth on non-PTS carbohydrates (e.g., saccharate [255, 268] and glycerol [173, 183]) and repression of the *hilE* gene by Mlc (261) implies that *S.* Typhimurium pathogenicity benefits from access to alternative carbon sources. This benefit is further realized by transcriptional repression of the sRNA-encoding

gene *csrC* by CRP-cAMP, which antagonizes inhibition of the SPI-1 activator HilD by the RNA-binding protein CsrA (149).

Critically, no single carbon source, member of the resident microbiota (with variable and redundant capacity to metabolize complex carbohydrates), or host diet regulates the full range of pathogen virulence functions in a binary way (i.e., as an "on/off" switch). Rather, the composition of metabolizable substrates in the environment likely tunes virulence along a spectrum, ensuring pathogen survival and maintaining a high level of plasticity and responsivity in specific virulence outputs. The expression of different metabolic and virulence determinants reflects the highly variable nature of the environments *S.* Typhimurium occupies. In addition, regulators of *Salmonella* virulence, and the stimuli that induce them, likely impact carbohydrate uptake and the general metabolic or energetic state of the bacterial cell. In this way, virulence cues may help pathogens "decide" when, where, and how to utilize the most advantageous carbon source in the environments they experience.

PHYSIOLOGICAL CONNECTION BETWEEN VIRULENCE AND CARBON SOURCE PRIORITIZATION

In this section, we propose an intimate link between carbon source utilization and expression of virulence traits, whereby each process influences the other in a seemingly continuous loop. We discuss the environmental conditions that induce virulence behaviors, regulators that mediate such induction, and how the response can impact the suitability of a given carbon source in supporting a successful bacterial infection. We focus on the PhoP/PhoQ system, the master regulator of virulence and Mg^{2+} homeostasis in *S.* Typhimurium (215).

Premise: Host Environments that Require Expression of Virulence Traits do not Always Favor Rapid Bacterial Growth

Salmonella grows slowly inside mammalian cells (269). This slow growth is presumably due to the multiple stresses *Salmonella* experiences within mammalian cells, including mildly acidic pH (229), CAMPs (270), and nutrient limitation (271), as *Salmonella* competes with host cells for key carbon sources such as glucose and fatty acids (171). *Salmonella* furthers its survival and proliferation in host tissues by coordinating nutrient utilization with expression of virulence determinants, many of which mediate resistance to antimicrobial agents produced by the host. *Salmonella* can benefit from reducing its growth rate to accommodate virulence strategies and/or evade host antimicrobial defenses. That specific *Salmonella* auxotrophs display slower growth and increased resistance to antibiotics (272–275) than the wild-type strain during infection of macrophages suggests that conditions that slow the growth of wild-type bacteria render them resistant to host-mediated defenses which often target actively replicating bacteria.

The slow growth rate displayed by wild-type *Salmonella* inside mammalian cells reflects both the hostile host environment and the expression of particular virulence factors, such as those encoded in SPI-1. Curiously, *S.* Typhimurium expresses SPI-1 genes heterogeneously, resulting in an altruistic behavior toward genetically identical bacteria that do not express SPI-1 genes (19). Thus, bystander (non-SPI-1-expressing) bacteria avoid the metabolic burden of expressing SPI-1 genes (19) and are internalized by host cells due to the biochemical changes triggered by the effectors injected by SPI-1-expressing bacteria into host cells (21, 184). In addition to the metabolic costs resulting from expression of SPI-1 genes, the T3SS apparatus encoded in SPI-1 decreases the permeability barrier of the bacterial inner membrane, rendering *S.* Typhimurium hypersensitive to membrane-damaging agents (276). We propose that, by reprograming their physiology, *Salmonella* species counteract the negative effects resulting from expression of virulence determinants and that this reprogramming entails a metabolic reprioritization that slows down the growth of intracellular bacteria.

The *Salmonella*-Containing Vacuole is Deprived of Mg^{2+} by Host Divalent Cation Transporters

The murine endosomal divalent cation transporter encoded by the solute carrier family 11 member 1 (*SLC11A1*) gene (formerly designated Nramp1, for "natural resistance

associated macrophage protein 1") is crucial to limit *Salmonella* growth (277). The SLC11A1 protein localizes to the membrane of pathogen-containing phagosomes (278) and transports divalent cations across the membrane in a pH-dependent manner (279). The anti-*Salmonella* effect of SLC11A1 had originally been attributed to the depletion of Fe^{2+} and Mn^{2+} from the phagosome (280–282) and promotion of a rapid inflammatory response (283). Recent studies suggest that, rather than transporting Fe^{2+} or Mn^{2+}, SLC11A1 controls intraphagosomal replication of *S.* Typhimurium by depriving the pathogen of Mg^{2+} (18). Similarly, the human cation channel encoded by the *MCOLN2* gene restricts *S.* Typhi replication by causing Mg^{2+} starvation in the pathogen (284). Competition for Mg^{2+} during intracellular infection raises the question of how *Salmonella* overcomes Mg^{2+} limitation (230).

The PhoP/PhoQ Two-Component System Maintains Cytoplasmic Mg^{2+} Homeostasis and Promotes *Salmonella* Virulence

PhoP/PhoQ is a two-component regulatory system that enables *Salmonella* to withstand Mg^{2+} starvation in laboratory media (16) and to survive inside mammalian macrophages. The response regulator PhoP is activated by multiple signals detected by the PhoQ sensor kinase in the cytoplasm or periplasm (Fig. 5) (215). Activating signals sensed by PhoQ include low periplasmic Mg^{2+} (16), CAMPs in the periplasm (285), mildly acidic cytosolic pH (17), hyperosmotic stress (286), and long-chain unsaturated fatty acids (287). Activating signals promote the phosphorylated state of PhoP (PhoP-P), which can result from increased PhoQ autophosphorylation and/or phosphotransfer to PhoP and from decreased dephosphorylation of PhoP-P by PhoQ (288). Phosphorylation promotes PhoP dimerization, resulting in PhoP-P binding to target sequences and transcriptional regulation of the corresponding genes (289).

In addition to controlling genes directly by binding to their promoter sequences, PhoP regulates genes indirectly, by controlling the abundance and/or activity or other regulatory proteins or RNAs (215). That is, PhoP is a direct transcriptional activator of the *rstA* (290, 291), *slyA* (292), and *ssrB* (293) genes, which specify DNA-binding regulatory proteins. PhoP also promotes expression of horizontally acquired genes by displacing gene silencer H-NS from AT-rich DNA sequences, rendering H-NS a substrate of the Lon protease and decreasing H-NS amounts 95% when *S.* Typhimurium is inside macrophages (294, 295). Moreover, PhoP posttranslationally activates PmrA (213, 296) and alters the stability of numerous proteins, including several transcriptional regulators (297, 298), by decreasing the amounts (297) and activity (299) of the protease adaptor ClpS.

The control of Mg^{2+} homeostasis and the control of bacterial pathogenicity by PhoP (215) appear to be intimately connected. All cells strive to maintain cytoplasmic Mg^{2+} homeostasis because Mg^{2+} is the most abundant divalent cation (300) and cannot be replaced by other cations as a cofactor for the $>$300 biochemical reactions that require Mg^{2+}. Therefore, cells respond to Mg^{2+} starvation by reprogramming cell physiology, with a central goal of maintaining cytoplasmic Mg^{2+} concentration above the levels required for essential processes. Moreover, PhoP promotes the expression of proteins required for both cytoplasmic Mg^{2+} homeostasis and virulence.

S. enterica harbors three distinct Mg^{2+} transporters: CorA (301), MgtA (302), and MgtB (303). CorA is a relatively ubiquitous cation channel that mediates Mg^{2+} uptake and efflux (304). By contrast, MgtA and MgtB are P-type ATPases (i.e., they require ATP hydrolysis to import Mg^{2+} against an electrochemical gradient) expressed under conditions resulting in cytoplasmic Mg^{2+} starvation (304, 305), including hyperosmotic stress (306, 307), high ATP (308), and/or Mg^{2+}-limited extracellular conditions (16, 304, 309). Though both MgtA and MgtB further survival during Mg^{2+} starvation (306), only MgtB is required for virulence in *S.* Typhimurium (18, 310, 311) and *Y. pestis* (312).

PhoP is a direct transcriptional activator of the *mgtA* and *mgtB* genes (16, 309) and is responsible for inhibition of CorA activity (313) by reversing membrane potential (314). The *mgtB* gene is required to counteract the antibacterial effects of SLC11A1 in murine hosts, but *mgtA* is not (18). Similarly, the *mgtB* gene, but not the *mgtA* gene, is

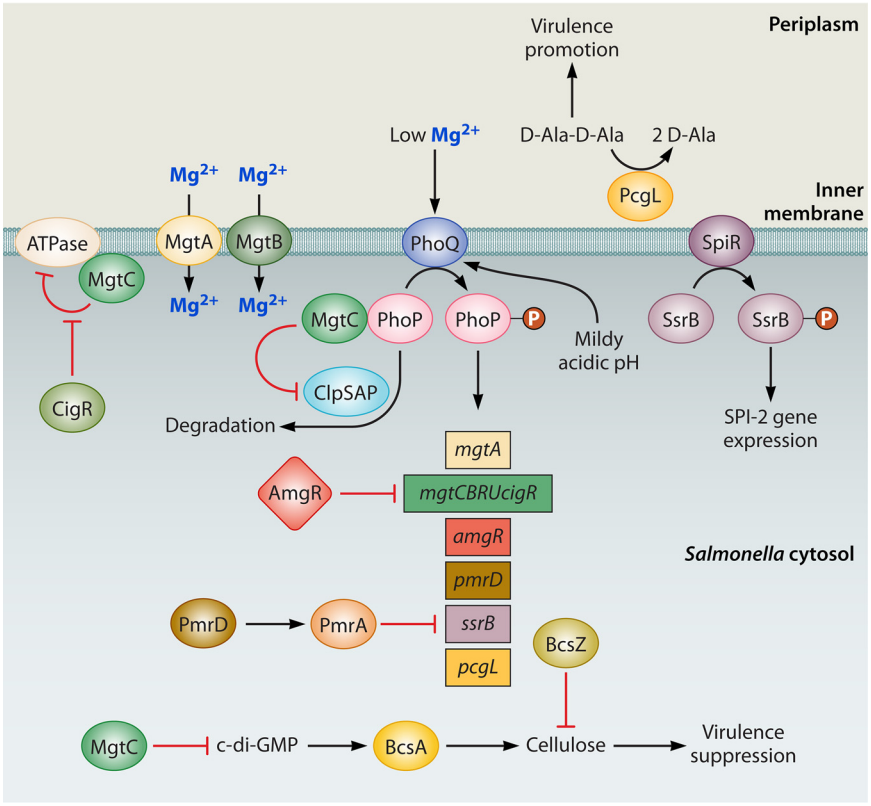

FIG 5 The master virulence regulatory system of *S.* Typhimurium—PhoP/PhoQ—promotes expression of both virulence-advancing and virulence-suppressing determinants. Multiple signals activate the sensor PhoQ, including low Mg^{2+} in the periplasm and mildly acidic pH in the cytosol. Activated PhoQ promotes the phosphorylated state of the regulator PhoP, which binds to specific DNA sequences modifying transcription of dozens of genes. The PhoP/PhoQ system governs both Mg^{2+} homeostasis and virulence. PhoP promotes transcription of the *mgtA* and *mgtB* genes, which encode distinct Mg^{2+} transporters that import Mg^{2+} from the periplasm to the cytoplasm, furthering Mg^{2+} homeostasis. The PhoP-activated *mgtC* gene encodes a protein required for virulence and Mg^{2+} homeostasis. The MgtC protein decreases the ATP concentration by inhibiting the F_1F_0 ATP synthase (ATPase) and a phosphate importer, liberating Mg^{2+} that is otherwise complexed with ATP. MgtC also binds to PhoP, protecting it from proteolytic degradation by ClpSAP. Paradoxically, many PhoP activated genes decrease *S.* Typhimurium virulence. Encoded by the last gene in the *mgtCBRU-cigR* operon, the CigR protein binds to MgtC, preventing MgtC both from binding the F_1F_0 ATP synthase and inhibiting ATP synthesis and from protecting PhoP from proteolysis by ClpSAP. The PhoP-activated *amgR* gene specifies a sRNA—AmgR—that promotes degradation of the *mgtC* portion of the *mgtCBRU-cigR* transcript. PhoP promotes transcription of the *pmrD* gene, which encodes a posttranslational activator of the regulatory protein PmrA, a transcriptional repressor of the virulence regulatory gene *ssrB*, which is directly transcriptionally activated by PhoP. Together with the sensor SpiR, the regulatory protein SsrB forms a two-component system necessary for transcription of genes located within SPI-2 and for derepression of other horizontally acquired genes. The PhoP-activated *pcgL* gene encodes a periplasmic D-alanyl-D-alanine (DAA) dipeptidase that converts DAA into two molecules of D-Ala, thereby decreasing the amounts of the virulence-promoting DAA. In addition, MgtC suppresses the synthesis of cyclic di-GMP (c-di-GMP), an allosteric activator for the cellulose synthase BcsA. Cellulose hampers *Salmonella* virulence in mice because a *bcsA* mutant is hypervirulent and also because inactivation of the cellulase-encoding *bcsZ* gene attenuates *Salmonella* virulence. Ovals represent proteins, rectangles represent genes, where necessary, and diamonds represent regulatory sRNAs or the mRNAs transcribed from respective genes.

required for bacterial survival for 24 h against Mg^{2+} starvation in laboratory media (311). Furthermore, the *mgtB* gene is more induced than the *mgtA* gene when the Mg^{2+} concentration in laboratory media decreases from 10 to 1 μM Mg^{2+} (304). In addition, *mgtB* is a horizontally acquired gene exhibiting a sporadic phylogenetic distribution, whereas *mgtA* is widespread within enteric bacteria (315). These data highlight the critical role that MgtB-mediated Mg^{2+} homeostasis plays during infection.

ATP exists as a Mg^{2+} salt in living cells (316). *S.* Typhimurium responds to a decrease in cytoplasmic Mg^{2+} concentration by reducing the amount of the Mg^{2+}-chelating ATP molecules (317, 318). This reduction in ATP decreases both the proteolytic turnover of

otherwise functional proteins (319) and the rate of protein synthesis (320, 321), liberating Mg^{2+} to participate in other biochemical processes (316). By inhibiting ATP synthesis, *S.* Typhimurium initiates a global reprogramming of cellular metabolism, as essentially all biological processes require ATP to proceed uninhibited. Growth in a virulence-priming, PhoQ-activating medium (e.g., low Mg^{2+} and mildly acidic pH) modifies *S.* Typhimurium's metabolism to accommodate the virulence program (322), further supporting the notion that virulence and metabolism interact. In addition, Mg^{2+} limitation promotes antibiotic tolerance in *S.* Typhimurium (280). Consistent with this behavior, a reduction in ATP concentration (and resulting dampening of metabolic activity) in *E. coli*, *Staphylococcus aureus*, and *Acinetobacter baumannii* furthered survival against bactericidal antibiotics (323).

Encoded by a *Salmonella*-specific gene (324) transcriptionally activated by PhoP (291), the MgtC protein reduces the bacterium's ATP amounts by targeting the Fo *a* subunit subunit of the F_1F_0 ATP synthase subunit termed AtpB (317) and a phosphate importer (318) whose identity is still unknown. MgtC is one of the most highly expressed *S.* Typhimurium proteins when within the mildly acidic phagosome of mammalian macrophages (310, 324, 325). MgtC is necessary to buffer the cytosolic pH of the bacterial cell (317). Because ATP is the primary energy currency of all living cells, the MgtC-dependent decrease in ATP concentration has profound effects in *Salmonella* by reducing protein synthesis (318), the cellular activity demanding the most energy (326, 327), and ATP-dependent proteolysis (319). Since bacterial growth is largely correlated with the rate of protein synthesis (9), the MgtC-dependent reduction in ATP results in a concomitant reduction in *S.* Typhimurium's growth rate (319). MgtC also decreases the amount of cyclic-di-GMP (328), the allosteric activator of the bacterial cellulose synthase (329), thereby decreasing cellulose biosynthesis when *S.* Typhimurium is within phagocytic cells (328). In addition, MgtC protects the master virulence regulator PhoP from degradation by the protease ClpSAP (298), impacting the stability of numerous regulatory proteins.

Thus, activation of PhoP provokes a dramatic physiological reprogramming designed to maintain Mg^{2+} homeostasis. This reprogramming involves increased Mg^{2+} uptake into the cytoplasm, as well as a dramatic reduction in ATP amounts, thereby reducing translational activity. Moreover, it challenges the assumption that conditions that favor rapid growth are always best for a cell. Instead, a cell may prioritize survival in the various environments it experiences, such as the SCV or the mammalian gut for *Salmonella*. The adaptation designed to maintain Mg^{2+} homeostasis is accompanied by increased expression of virulence factors necessary for *Salmonella* survival inside host tissues. Because the expression of virulence factors imposes its own physiological costs, how does a bacterium balance these various constraints?

Case Study: The Physiological Cost of *Salmonella* Virulence is Balanced by the Expression of Antivirulence Factors

S. Typhimurium and many other bacterial pathogens harbor antivirulence factors, which are proteins and RNAs that reduce virulence despite being synthesized by a pathogen during infection. Antivirulence factors act directly or alter the abundance of metabolites that determine the outcome of a bacterium-host interaction. That pathogens would produce antivirulence factors seems paradoxical given that pathogens require virulence factors to survive within host environments. However, if virulence is understood as balancing multiple biological constraints (330, 331), such as the metabolic and/or physiological costs of expressing virulence-associated proteins or the induction of a slow growth state, against the need to survive in the host environment, then antivirulence factors may serve to curtail an otherwise detrimental virulence program.

For example, the antivirulence gene *pcgL* specifies a periplasmic D-alanyl-D-alanine (DAA) dipeptidase that hydrolyzes the dipeptide DAA into two molecules of D-alanine (Fig. 5) (332). DAA accumulation and release into host tissues appear to be responsible for the hypervirulence phenotype of a *pcgL*-null mutant, rather than PcgL slowing bacterial growth, because injection of mice with synthetic DAA increases the number of wild-type *S.* Typhimurium in the liver and spleen 100× to 1,000× and also because the *pcgL* mutant

grows like the wild-type strain in laboratory media (333). Unexpectedly, the antivirulence gene *pcgL* is transcriptionally activated by the virulence regulator PhoP (333).

Cellulose is a major component of *S.* Typhimurium's biofilms (334, 335). Surprisingly, inactivation of the *bcsA* gene, which specifies the catalytic subunit of the cellulose synthase, increases *S.* Typhimurium virulence (328), whereas the cellulose-degrading BcsZ protein promotes virulence (336). The cellulose produced during infection may prevent host cells from responding to *S.* Typhimurium surface molecules, such as the LPS, occluded by cellulose. The *bcsA*-null mutant exhibits wild-type growth in laboratory media (328), supporting the notion that BcsA, like PcgL, exerts its antivirulence effects by controlling the abundance or accessibility of molecules that act on host cells.

Certain antivirulence factors operate within the pathogen to control the expression or availability of virulence determinants. For example, the anti-sense RNA AmgR promotes degradation of the *mgtC* portion of the *mgtCBRUcigR* polycistronic mRNA by RNase E (337). Paradoxically, PhoP is a direct transcriptional activator of both the *mgtC* (291) and *amgR* promoters (337), providing a singular example of a regulatory protein essential for bacterial virulence that promotes expression of both a virulence gene *and* its antagonizing antivirulence gene. Because larger amounts of active PhoP protein are required to activate the *amgR* promoter than the *mgtC* promoter (337), AmgR's role appears to be in limiting MgtC's effects over a defined time window.

CigR is an antivirulence protein that binds to the MgtC protein, thereby antagonizing MgtC's ability to reduce ATP amounts and protect PhoP from proteolysis by ClpSAP (338). A *cigR*-null mutant replicates to higher numbers in murine macrophages than does wild-type *S.* Typhimurium (338). As the *cigR* gene is also transcribed from a PhoP-independent promoter, CigR sets a threshold that MgtC must surpass to exert virulence-promoting activities (338).

Activated by PhoP at the posttranslational level, the DNA-binding protein PmrA represses transcription of the *Salmonella*-specific *ssrB* gene (208), which encodes the response regulator of the SPI-2-activating SsrB/SpiR two-component system (339). PhoP is a direct transcriptional activator of the *ssrB* (293) and *pmrD* (213) genes. This is curious because PmrD is responsible for the posttranslational activation of PmrA (340). In other words, PhoP increases expression of both a critical virulence regulator (SsrB) and of a protein (PmrD) that (indirectly) reduces expression of that very same regulator.

Cumulatively, the results discussed above argue that virulence traits must be carefully regulated to balance bacterial proliferation against survival inside a mammalian host. We propose that a similar logic applies to the regulation of bacterial metabolism: when navigating host environments, bacteria may dampen their metabolism to enhance survival against antimicrobial products and/or to balance biological constraints, such as virulence factor production, access to nutrients, and replication.

Hypothesis: PhoP Alters Carbon Prioritization to Maintain Cytoplasmic Mg^{2+} Homeostasis

The adenylate cyclase CyaA plays a critical role in carbon metabolism by synthesizing cAMP, which is necessary for transcription factor CRP to bind its specific target DNA sequences and regulate gene transcription. The adenylate cyclase from *E. coli* exhibits an absolute requirement for Mg^{2+} in permeabilized cells, cell extracts, or purified preparations (341–345). CyaA coordinates Mg^{2+} ions in its catalytic site to perform the cyclization of ATP to cAMP (341, 344, 346). The preferred substrate of CyaA is likely ATP:Mg^{2+}, since ATP:Mn^{2+} exhibits much lower specific activity for the class III adenylate cyclase CyaB from *P. aeruginosa* (346). In addition, unprotonated or uncomplexed ATP (i.e., ATP lacking Mg^{2+}) inhibits mammalian adenylate cyclases (347, 348). The Mg^{2+} dependence of CyaA activity leads us to propose that cytoplasmic Mg^{2+} starvation decreases CyaA activity in living bacteria because it entails a reduction in the concentration of both the substrate (i.e., ATP) and cofactor (i.e., Mg^{2+}) of the reaction, which is anticipated to lower cAMP amounts and result in decreased abundance of active CRP.

By extension, the restoration of cytoplasmic Mg^{2+} homeostasis may support the synthesis of cAMP, promoting CRP-cAMP activity. It makes intuitive sense that bacteria would dampen their metabolic activity when experiencing cytoplasmic Mg^{2+} starvation until

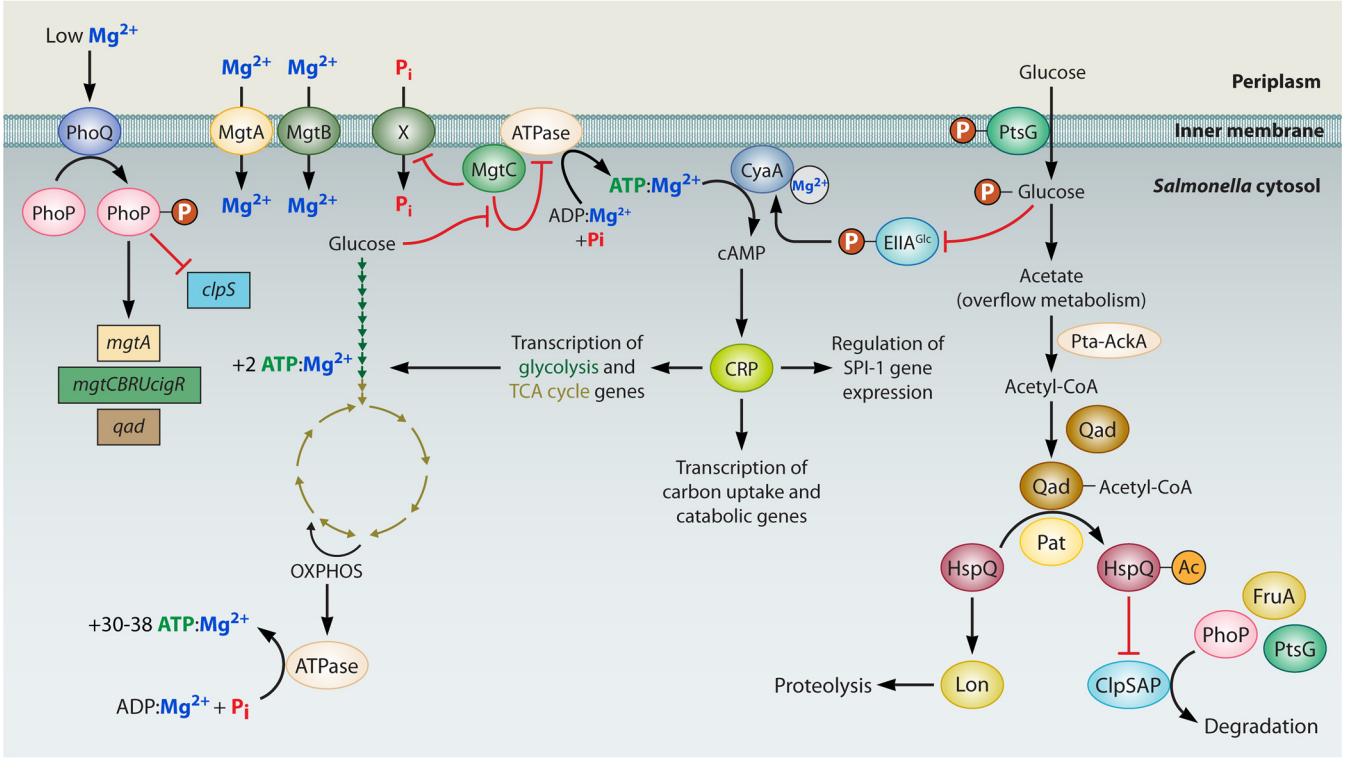

FIG 6 How the PhoP/PhoQ system may alter *Salmonella* metabolism by furthering cytoplasmic Mg^{2+} homeostasis. PhoP promotes expression of the Mg^{2+} importers MgtA and MgtB while decreasing the activity of the bidirectional Mg^{2+} channel CorA (not shown). PhoP also promotes expression of the MgtC protein, which decreases the ATP concentration by inhibiting both phosphate (P_i) uptake and ATP synthesis by the F_1F_0 ATP synthase. Because ATP exists predominantly as a chelate salt with Mg^{2+} in living cells, ATP abundance is closely tied to Mg^{2+} availability. ATP:Mg^{2+} is the substrate of the Mg^{2+}-dependent adenylate cyclase CyaA. Thus, a reduction in the cytoplasmic Mg^{2+} concentration below a certain threshold likely reduces CyaA activity and, by extension, the activity of CRP, which requires cAMP as coactivator. Thus, when the cytoplasmic Mg^{2+} concentration is above a certain threshold, CRP-cAMP promotes the transcription of many genes involved in glycolysis and the TCA cycle, along with genes responsible for the uptake and breakdown of certain carbon sources, and genes that encode factors that regulate SPI-1 gene expression. By contrast, when the cytoplasmic Mg^{2+} concentration is below a certain threshold, the resulting reduction in CRP activity should negatively regulate these processes, impacting the metabolic and energetic state of the cell, as well as virulence. *S.* Typhimurium access to glucose may also influence this process because glucose uptake reduces CyaA activity via P-EIIA^{Glc} and because acetate generated from overflow metabolism of glucose promotes the acetylation of the HspQ protein by the protein acetyltransferase Pat using as acetyl donor acetyl-CoA bound to Qad, a protein encoded by the PhoP-activated *qad* gene. Acetylated HspQ is no longer a substrate of the Lon protease. Both acetylated HspQ and nonacetylated HspQ bind the protease adaptor ClpS, inhibiting the proteolysis of ClpS-dependent substrate of the ClpAP protease. Genetic evidence suggests that one such substrate is the glucose transporter PtsG, favoring a state of increased glucose uptake and reduced cAMP synthesis. When glucose is abundant, it overrides the inhibition of ATP synthesis by MgtC likely because larger amounts of ATP can be synthesized from substrate-level phosphorylation rather than TCA-coupled oxidative phosphorylation. Ovals represent proteins and rectangles represent genes.

conditions improve and prioritize processes essential for stress survival. Nevertheless, *Salmonella* requires some level of active catabolism to remain viable during cytoplasmic Mg^{2+} starvation, and one way it may accomplish this is by importing Mg^{2+} into the cytoplasm via the Mg^{2+} transporters MgtA and MgtB (Fig. 6) (309).

Transcriptionally activated by PhoP, the *mgtA* gene and *mgtCBRUcigR* operon are also regulated at the transcription elon2gation level by several signals (311, 349–355) that enable temporal and Mg^{2+} concentration-dependent distinction between the expression of MgtA and MgtB (310, 356). An intriguing possibility is that, while this regulation promotes distinct phases of Mg^{2+} uptake (dependent on the degree of cytoplasmic Mg^{2+} starvation) (356), it may also result in different cAMP amounts because CyaA activity is Mg^{2+} dependent (341–345). In other words, MgtA- and MgtB-dependent Mg^{2+} uptake may determine the utilization and catabolism of specific alternative carbon sources by controlling the amount of CRP-cAMP. An expected consequence of such regulation would be the utilization of readily available, host-derived alternative carbon sources, such as glycerol (173), if access to primary carbon sources like glucose is limited. In conjunction with the inhibition of ATP synthesis by MgtC (i.e., OXPHOS), growth on poorly fermented carbon substrates may help ensure a smaller amount of ATP generation from substrate-level phosphorylation (357, 358).

To date, most studies on the regulation of Mg^{2+} homeostasis in *Salmonella* have utilized minimal media supplemented with either glucose or glycerol, and few comparisons have been made between these growth conditions. Particularly with regard to the level of ATP, it is conceivable that growth on an energy-dense carbon source like glucose will have profound impacts on the overall physiology of a Mg^{2+}-starved cell. In support of this notion, the dependence of MgtC on the *atpB*-encoded *a*-subunit of the F_1F_0 ATP synthase to reduce ATP levels is conditioned by the carbon source available to *S.* Typhimurium (Fig. 6) (318). That is, when *S.* Typhimurium is grown on an inefficiently fermented carbon source such as glycerol, an *atpB* mutant exerts a dominant effect over *mgtC* because the bulk of ATP synthesis results from low substrate-level phosphorylation, resulting in low ATP. By contrast, growth on readily fermentable glucose decouples the effect of *mgtC* from that of *atpB* as higher levels of ATP can accumulate by substrate-level phosphorylation independently of OXPHOS. Thus, *S.* Typhimurium may preferentially utilize suboptimal carbon sources during infection due to factors other than carbon source availability, resulting in a low ATP concentration. This would reduce the rate of protein synthesis, which is the cellular activity that demands the most energy, thereby decreasing bacterial growth rate and resulting in increased resistance to antimicrobial agents that preferentially kill growing bacteria (274, 359).

By contrast, some PhoP-dependent phenotypes benefit from bacterial access to glucose. For example, along with ATP, the amounts of acetyl-CoA, the immediate end product of glycolysis and initial substrate of the TCA cycle, increase during growth on glucose (299). When *S.* Typhimurium experiences low cytoplasmic Mg^{2+}, PhoP promotes transcription of the *qad* gene, which specifies the acetyl-CoA-binding protein Qad (360), resulting in acetylation of HspQ, a substrate of the Lon protease that stimulates proteolysis of other Lon substrates (299). Acetylated HspQ is no longer a Lon substrate or able to promote proteolysis of Lon substrates. Instead, acetylated HspQ binds to ClpS, inhibiting proteolysis by ClpSAP (299). Thus, access to glucose during Mg^{2+} starvation would be expected to alter the specificity of the Lon and ClpAP proteases, which together control the abundance of hundreds of proteins, including several participating in central metabolism. For example, the abundance of the EIIBC proteins PtsG and FruA, which import glucose and fructose, respectively, is lower in a *phoP* mutant but higher in a *clpS* mutant than in the wild-type strain (361). These results strongly suggest that PtsG and FruA are subject to ClpS-dependent proteolysis by ClpAP because PhoP increases the abundance of the ClpS inhibitor HspQ (360), as well as transcriptionally represses the *clpS* gene (297). Thus, growth on glucose would be expected to preserve PtsG and FruA amounts during Mg^{2+} starvation in a PhoP-dependent manner (Fig. 6).

Transcription of the *bcsA* gene is positively regulated by the stress-responsive RNA polymerase sigma factor RpoS (362), the amounts of which are stabilized in low Mg^{2+} by the PhoP-activated *iraP* gene, which encodes a protein that binds protease adaptor RssB and prevents RpoS delivery to the ClpXP protease (363). Because transcription of the *rpoS* gene is negatively regulated by CRP-cAMP (364), high cellulose biosynthesis should correspond to PhoP-activating and CRP-inactivating conditions, a plausible scenario for *Salmonella* residing intracellularly. However, as noted above, the PhoP-activated *mgtC* gene reduces cellulose biosynthesis by decreasing cyclic di-GMP amounts (328).

Apart from inhibiting ATP synthesis, MgtC also inhibits phosphate (P_i) uptake (Fig. 6) (318, 365). ATP synthesis requires P_i to phosphorylate ADP. When *S.* Typhimurium experiences cytoplasmic Mg^{2+} starvation, a P_i-starvation response is triggered due to reduced ATP consumption resulting from a decrease in translation, which lowers that concentration of free P_i (320). MgtC actively inhibits P_i uptake to maintain low levels of ATP (315) rather than accumulating P_i via induction of the PhoB/PhoR regulon.

Importantly, P_i is required for the catabolism of most carbohydrates because ATP donates a phosphoryl group to incoming carbohydrates to prevent their diffusion back across the cytoplasmic membranes (366, 367). Therefore, inhibiting P_i uptake may also prevent efficient carbohydrate catabolism. In addition, the CyaA-dependent conversion

of ATP into cAMP creates a pyrophosphate by-product that could conceivably contribute to P_i homeostasis in the cytoplasm (368).

The utilization of many alternative carbon sources entails less phosphorylation than does the utilization of glucose. For example, G3P enters the glycolytic pathway as dihydroxyacetone phosphate (DHAP), which represents a midway point of glycolysis and only requires the initial phosphorylation of glycerol to G3P by GlpK (Fig. 1) (369). Furthermore, uptake of glycerol by facilitated diffusion through GlpF does not require energy to be expended via ATP hydrolysis (52). It is plausible that access to carbon sources such as glycerol benefits *S.* Typhimurium as it attempts to balance central carbon metabolism against the demands of maintaining Mg^{2+} homeostasis inside macrophages. Because P_i is required for the generation of ATP, reducing P_i uptake and thereby ATP levels should also decrease cAMP generation. Thus, the activity of regulators of P_i homeostasis such as PhoB (370) may intersect with the regulation of carbon metabolism. In support of this notion, activation of the PhoB/PhoR two-component system alleviates sugar phosphate stress in an *sgrS*-null strain of *E. coli* (371).

Minor differences in the abundance of ~70 metabolites were recently reported among a set of *S.* Typhimurium strains with a wild-type, constitutively active, or genetically inactivated *phoP* gene when grown in low Mg^{2+} laboratory media (372). Unfortunately, the results are hard to interpret because the authors reported a decrease in ATP amounts in a *phoP* mutant strain (372), despite prior reports that *phoP* mutant *S.* Typhimurium exhibits greatly increased amounts of ATP in low Mg^{2+} (321), and also because some of the results were obtained by expressing PhoP at nonphysiological amounts.

The PhoP regulon likely has far-reaching, as-yet-underappreciated effects, on the regulation of carbohydrate metabolism in *Salmonella*, impacting both the general physiological and the metabolic state of the pathogen and influencing its behavior while in the SCV. By reducing the ATP concentration in response to cytoplasmic Mg^{2+} starvation, the PhoP-activated MgtC protein limits the amount of the precursor for cAMP synthesis, likely reducing the regulatory activity of CRP-cAMP. Moreover, and to balance this effect, the PhoP-activated Mg^{2+} transporters MgtA and MgtB may support the Mg^{2+}-dependent activity of CyaA to maintain a cAMP concentration sufficiently high for the catabolism of alternative carbon sources (Fig. 6).

The hypothesis proposed here underscores central roles for both ATP and Mg^{2+} in the coordination of bacterial virulence and metabolism. However, other intracellular bacterial pathogens such as *Listeria monocytogenes* lack adenylate cyclase (373). Interestingly, the major virulence regulator of *L. monocytogenes*, PrfA (374, 375), a CRP-like protein that also regulates facets of carbon metabolism (376–378), is allosterically activated by the tripeptide glutathione (379, 380). It is likely no coincidence that glutathione is synthesized by the multidomain γ-glutamylcysteine ligase/glutathione synthetase GshF in a reaction dependent on ATP and Mg^{2+} (381). That is, even in cases where cAMP is not the secondary messenger for a regulator controlling virulence and carbon metabolism, ATP and Mg^{2+} still impose crucial regulatory checkpoints on the integration of these two activities.

In sum, both virulence factors and metabolic prioritization contribute to a successful pathogen infection, which may explain the high level of overlap in their regulation and physiological effects. That virulence and carbon metabolism are interconnected is reflected in the dramatic physiological consequences on expression of PhoP-regulated virulence proteins such as MgtC simply resulting from a change in the carbon source available to *Salmonella* (318). Thus, virulence pathways can modulate carbon source utilization, and carbon source utilization in turn governs virulence traits (Fig. 7).

PhoP-Regulated Metabolic Changes Resemble Those Exhibited by Certain Human Tumors

Cancer cells reprogram cellular metabolism. This reprogramming is often manifested by the conversion of glucose into lactate under aerobic conditions. Termed the Warburg effect, this metabolic behavior is characterized by increased glycolytic flux despite the presence of oxygen enabling oxidative phosphorylation (382). This behavior is paradoxical because the ATP yield per glucose molecule is up to 14.5 times lower in glycolysis than in the TCA cycle and takes place in the presence of oxygen. However, the increased glycolytic

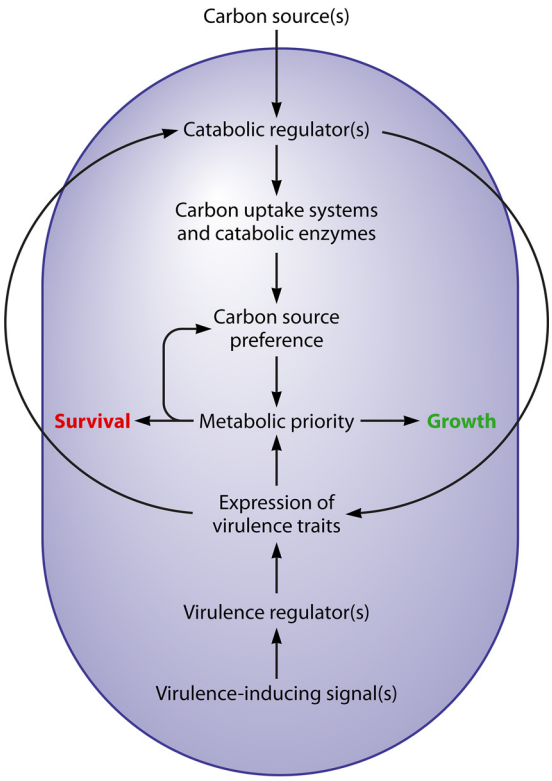

FIG 7 Virulence and carbon source utilization dictate a pathogen's metabolic priorities. The metabolic priority of a cell is dictated both by the availability of carbon sources in a given environment and the presence of virulence-stimulating signals such as Mg^{2+} starvation. Because regulators of carbon source utilization also regulate virulence traits (such as CRP-cAMP, Mlc, and CsrA) and because virulence-inducing signals such as low Mg^{2+} and the physiological reprogramming required to withstand these stresses impinge on the activity of catabolic regulators (such as CyaA and CRP), these two processes feed into one another in a continuous loop. The outcome for the cell, either rapid growth or survival, is codetermined by these various attributes.

flux in solid tumors is insufficient to compensate for reduced flux through the TCA cycle, leading to decreased ATP amounts in solid tumors compared to metastases or healthy tissue (383). What, then, is responsible for tumors employing a metabolic pathway that is less energy efficient than one normally operating in healthy cells?

Cytoplasmic Mg^{2+} availability may be responsible for the unexpected metabolic behavior of tumors. For example, low cytoplasmic Mg^{2+} can trigger cancer because this signal decreases antioxidant defenses and increases the mediators of inflammation, favoring angiogenesis (384). In addition, aberrant Mg^{2+} uptake via the transient receptor potential melastatin-subfamily member 7 (TRPM7) protein is associated with proliferation of metastatic cancers (385, 386). TRPM7 is both a channel permeant to divalent cations and a serine/threonine kinase. TRPM7 activity is sensitive to the metabolic state of the cell, being strongly activated when the Mg^{2+}-ATP concentration falls below 1 mM (387). Somatic mutations of TRPM7 have been implicated in cancers of the pancreas, lung, stomach, skin, prostate, and breast (387, 388). Moreover, TRPM7-mediated Mg^{2+} influx is necessary for growth signaling mediated by the PI3K/Akt/mammalian target of rapamycin (mTOR) pathway, leading to rapid quiescent/proliferative metabolic transitions (387, 389).

The kinase mTOR is a critical sensor and regulator of cellular energy (390, 391). Like many enzymes dependent on nucleotide triphosphates, mTOR requires two Mg^{2+} ions in its nucleotide triphosphate-binding site for catalysis to occur (392, 393). This requirement confers second order kinetics upon enzyme activity with respect to Mg^{2+} concentration: small changes in the concentration of free Mg^{2+} ions can have large effects on enzyme reaction rates.

Phosphatases of regenerating liver (PRL-1, -2, and -3)—biomarkers for poor cancer survival outcomes (394)—inhibit Mg^{2+} export through the cyclin M Mg^{2+} exporter

(CNNM) family of cation permeases (395). Inhibition of these permeases buffers against reduced ATP:Mg^{2+} amounts resulting from low extracytoplasmic Mg^{2+}, which involves reprogramming metabolism through an AMPK/mTORC2-dependent pathway that activates PRL-2 translation by a Mg^{2+}-sensing untranslated open reading frame (396).

Both quiescent and dividing cells require glucose but differ in their ability to conserve Mg^{2+} ions during glucose utilization. Quiescent cells recycle existing ADP:Mg^{2+} to ATP:Mg^{2+}, so Mg^{2+} is not lost to regenerate ATP, and new Mg^{2+} ions are not needed to support this conversion. By contrast, dividing cells require the import of a new Mg^{2+} ion for newly synthesized ATP molecules, thereby linking Mg^{2+} uptake with biosynthetic metabolism.

Finally, it is remarkable that cAMP functions as a second messenger in eukaryotes as it does in prokaryotes. cAMP activates protein kinase A (PKA) which, in turn, activates the transcription factors cAMP-response element-binding proteins (CREB) and inducible cAMP early repressor (ICER/CREM) via phosphorylation to regulate cell proliferation (397). Accordingly, cAMP-PKA signaling has been linked to cancer progression in lung (398), breast (399), and prostate cancer models (400, 401). Thus, reduced ATP amounts in solid tumors may decrease signaling through cAMP-PKA-dependent pathways to limit metastasis, thereby acting as a metabolic signature of proliferative and nonproliferative cancer states. As discussed above, cAMP is the essential allosteric activator of the CRP protein, a master regulator of carbohydrate utilization in bacteria.

A possible reason for cells utilizing cAMP as a second messenger, rather than relying simply on ATP concentration itself, may be related to the close association of ATP and cellular energetics with Mg^{2+}. The cAMP conjugate base (net charge -1) likely favors protonation by a hydrogen atom rather than the formation of a chelate salt. Thus, by converting ATP:Mg^{2+} into cAMP, the latter molecule can perform signaling functions in the cell *and* preserve Mg^{2+} for other cellular processes exhibiting a strict dependence on Mg^{2+}. That the cAMP-synthesizing adenylate cyclase is a Mg^{2+}-dependent enzyme may reflect that cAMP signaling promotes catabolic functions that require Mg^{2+} and ATP, and thus should be suppressed in the absence of either precursor. Intriguingly, bacteria such as *E. coli* secrete the overwhelming majority of cAMP synthesized in the cytoplasm (402), and there is no clear function for extracytoplasmic cAMP. Intracellular *M. tuberculosis* excretes cAMP to intoxicate the host cell through hyperactivation of PKA/CREB (403), but this does not explain why commensal bacteria like *E. coli* would excrete cAMP. Perhaps a closer interrogation of the intersection of cellular metabolism, Mg^{2+}, and the signaling pathways that connect them will reveal new insights into these processes.

CONCLUDING REMARKS

Virulence is a regulated, rather than constitutive, phenotype. The regulation of virulence factors changes the physiological state of the bacterial cell, often resulting in reduced growth, modification of the bacterial cell envelope, and other changes in the behavior of a pathogen. These changes include maintaining access to preferable carbon sources, which often results in regulators of carbon metabolism also regulating virulence traits and *vice versa* (Fig. 7). One key example is provided by the PhoP/PhoQ regulatory system and the targets of PhoP control, which are expected to impinge on the control of carbon metabolism by altering the amounts of active CRP-cAMP, a regulator responsible for bacterial access to less preferred carbon sources. The cumulative effects of PhoP-regulated targets reduce ATP amounts while supporting the uptake of alternative carbon sources.

The careful regulation of metabolism is essential for pathogens to survive both host-derived and synthetic antibacterial agents. For example, the *ptsI*-encoded EI of the PTS system (along with *cyaA* and *crp*) increases susceptibility to antibiotics (404) because it initiates a CRP-cAMP-regulated bacterial cell death pathway that involves shifting metabolism toward the TCA cycle, ATP surges, and the generation of reactive oxygen species (405). Thus, preventing cell death in response to environmental stress,

such as Mg^{2+} limitation, may very well rely on inhibiting this bacterial cell death pathway and by extension the PTS-CyaA-CRP axis.

In the absence of cAMP and PTS proteins, a bacterial species can still connect carbon source availability and metabolic and/or energetic prioritization. For example, the human gut commensal *Bacteroides thetaiotaomicron* harbors a CRP-like transcription factor—termed BT4338—that is necessary for both utilization of multiple carbohydrates (406) and fitness in the murine gut (407). Remarkably, *B. thetaiotaomicron* devotes ~18% of its genome to the uptake and utilization of carbohydrates (408), which enables it to utilize a much broader spectrum of carbohydrates than enteric bacteria such as *E. coli* or *S. enterica*. However, *B. thetaiotaomicron* lacks genes coding for the cAMP-synthesizing CyaA and PTS components (408). The most highly upregulated gene in a BT4338-dependent manner specifies an alternative form of the translation elongation factor EF-G (407), enabling *B. thetaiotaomicron* to carry out protein synthesis in an energy-efficient manner (409). Thus, carbon utilization and energy generation are tightly connected to protein synthesis, which, in turn, governs bacterial growth.

In dismantling the strict categorical framework that assigns each given gene, protein, or pathway to a discrete biological process, we can begin to understand holistically how central biological processes interdependently interact with phenomena as disparate as bacterial virulence and cancer.

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We declare there are no conflicts of interest.

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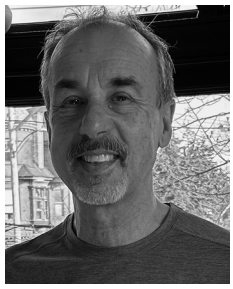

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