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Metabolism of the Gram-Positive Bacterial Pathogen *Listeria monocytogenes*

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Summary

Bacterial metabolism represents the biochemical space that bacteria can manipulate to produce energy, reducing equivalents and building blocks for replication. Gram-positive pathogens, like *Listeria monocytogenes*, show remarkable flexibility which allows for exploitation of diverse biological niches from the soil to the intracytosolic space. Although the human host represents a potentially rich source for nutrient acquisition, competition for nutrients with the host, and hostile host defenses can constrain bacterial metabolism by various mechanisms, including nutrient sequestration. Here we review metabolism in the model Gram-positive bacterium, *L. monocytogenes*, and highlight pathways that enable the replication, survival and virulence of this bacterial pathogen.

Metabolic Potential

The bacterial pathogen *L. monocytogenes* is well adapted to life in the soil and on vegetation as a saprophyte (i.e., extracellular environments), and to life in the cytosol of mammalian cells as a pathogen (i.e., intracellular environment). These environments differ greatly in their metabolite repertoire (e.g., carbon, sulfur and nitrogen sources) and abundance (e.g., amino acid availability). Therefore *L. monocytogenes* requires niche-specific adaptations to support growth, and also uses metabolic cues to trigger virulence mechanisms, such as the master virulence regulator, PrfA (1). The complete metabolic potential of *L. monocytogenes* has been inferred bioinformatically from genomic (2–5) and transcriptomic studies (1, 6–8), as well as experimentally using both defined media as a test for auxotrophy (9–12) and more recently by isotopologue metabolomics (13, 14). While these studies have certainly taught us much about *L. monocytogenes* metabolic potential, it is important to note that, given the wide array of environments *L. monocytogenes* is capable of inhabiting, our understanding of their metabolic potential and how strain to strain variation might affect this potential, is yet to be fully understood.

The first comparative genomic study of *L. monocytogenes* (EGDe) and *L. innocua* (CLIP 11262) demonstrated that complete glycolysis, pentose phosphate, respiratory chain and

multiple fermentation pathways were present in both pathogenic and non-pathogen *Listeria* isolates (2). In contrast, an incomplete TCA cycle was documented due to the lack of α -ketoglutarate dehydrogenase in both organisms while biosynthetic pathways for vitamins including riboflavin, biotin, thiamin and lipoate are absent or incomplete, consistent with observed auxotrophies (9). Finally, biosynthetic pathways for all amino acids were present, despite a variety of reported amino acid auxotrophies in various growth conditions, suggesting, not surprisingly, that regulation of these metabolic pathways under different growth conditions leads to conditional auxotrophies. Since this original comparative genomics study, more than one hundred additional *L. monocytogenes* genomes have now been sequenced in addition to many other non-*monocytogenes* *Listeria* species (4, 15). The core genome of *L. monocytogenes* is comprised of 1,791 genes with estimates of up to 6,500 genes in the pan genome of all *Listeria*, of which 54% are predicted to be dedicated to metabolism in one way or another, highlighting the potential diversity of metabolic adaptations of different *Listeria* species and even potentially different *Listeria monocytogenes* strains to different environments (3, 4). Defining how all of these accessory metabolic pathways contribute to colonization of niches, either environmental or host, will be of critical importance over the coming years.

Multiple efforts at developing a minimal media to define *L. monocytogenes* metabolic requirements have been attempted over the years, dating all the way back to initial efforts by Welshimer in the early 1960s (9). Different strains and different media have yielded some consistent findings and some that are confounding. As noted above, and consistent with genetic analysis, *Listeria* is auxotrophic for a variety of essential vitamins including thiamine, lipoate, biotin, and riboflavin which *L. monocytogenes* cannot produce *de novo* (9–11). In addition, multiple growth experiments in minimal defined media have demonstrated that *L. monocytogenes* requires the supplement of methionine and cysteine for growth (i.e., auxotrophic to Met and Cys). Moreover, addition of branched chain amino acids (BCAA; Ile, Leu and Val) arginine, histidine, phenylalanine and tryptophan substantially enhance *L. monocytogenes* growth in minimal media, while glutamine can serve as a nitrogen source (which can be replaced with ammonium or ethanolamine) (10–12, 16, 17). The requirement for Met and Cys is explained by the fact that most *L. monocytogenes* genomes are missing the gene/s for reducing sulfate to sulfide that is further condensed with O-acetylserine to form cysteine (2, 12). Supplementing *L. monocytogenes* bacteria with thiosulfate, which can be alternatively condensed with O-acetylserine to form cysteine, do not support *L. monocytogenes* growth, nor addition of methionine, indicating the lack of the transsulfuration pathway (converting methionine to cysteine) (12). Notably, strain-specific requirements for Cys and Met are observed. For example, the EGD-e strain requires only cysteine without the addition of methionine, while the 10403s strain requires both, implying a disruption in one or more of the *met* genes, whose gene products convert cysteine to methionine (*metI*, *metC* and *metD*). The requirement for BCAA, arginine, histidine, phenylalanine and tryptophan in defined media is more difficult to explain, as *L. monocytogenes* genomes encode for all the biosynthesis genes of these amino acids (more details below). The addition of BCAA and arginine appears to be essential for efficient growth, whereas histidine, tryptophan and phenylalanine are not essential but can improve the growth of distinct *L. monocytogenes* strains in chemically defined media (10–12, 16).

With respect to carbon metabolism, as a facultative aerobe *L. monocytogenes* can both ferment glucose or respire when oxygen is present. *L. monocytogenes* catabolizes glucose through the classical glycolytic (Embden-Meyerhof-Parnas) pathway to pyruvate and generates ATP through substrate level phosphorylation. Pyruvate can be shunted to acetate, acetoin, and lactate (18) or converted to acetyl-CoA by pyruvate dehydrogenase complex (PDH) or pyruvate formate lyase (Pfl). Under anaerobic conditions, fermentation favors the formation of ethanol and formate, and acetyl-CoA can be broken down to produce acetate and ethanol (18). Acetyl-CoA can be funneled into the oxidative branch of the tricarboxylic acid (TCA) pathway to generate amino acid precursors (19). It is predicted that two putative type II NADH dehydrogenases (*lmo2389*, *lmo2638*), glycerol-3-phosphate (*glpD*) and formate dehydrogenases (*lmo2586*) may shuttle electrons to either cytochrome *bd* oxidase or cytochrome *aa₃* oxidase via menaquinone (20). Electron transfer through the electron transport chain generates a membrane potential and drives oxidative phosphorylation of ATP by F₀F₁-ATPase. In the absence of oxygen or disruption of the electron transport chain, *L. monocytogenes* displays decreased growth, likely due to less ATP generation (20, 21). Interestingly *L. monocytogenes* is missing several key TCA enzymes: α -ketoglutarate dehydrogenase, succinyl-CoA synthase, and malate dehydrogenase (2) and therefore exhibits a branched variant of the TCA cycle. *L. monocytogenes* is also unable to bypass these steps using the glyoxylate shunt, though it has been proposed that succinate could be generated by the GABA shunt (22). The only way to replenish oxaloacetate is through anapleurosis, using pyruvate carboxylase to convert pyruvate and CO₂ to oxaloacetate (23, 24).

Life as a Saprophyte

While *L. monocytogenes* has been extensively studied as a both a human and a livestock pathogen for decades, significantly less is known about its life in the environment as a saprophyte. Seminal early studies by Welshimer demonstrated that *L. monocytogenes* was capable of surviving on soil (25), while a series of studies in the intervening decades demonstrated the ubiquity of *L. monocytogenes*. *L. monocytogenes* has been isolated from environmental samples ranging from uncultivated fields and meadows, where incidence ranges from 5–50% (26, 27), to cultured fields and dairy farms, where incidence is consistently lower, ranging from 3–24% (27–29). Prevalence varies not only by geography and soil type but also temporally, seasonally and with meteorological conditions. Importantly, in all of these studies while the frequency of positive soil samples was relatively high, the abundance of *L. monocytogenes* in these samples is very low and requires specific enrichment techniques to facilitate identification (30). Not surprisingly, given the diversity of locations that *L. monocytogenes* can be cultured from, a variety of soil types have been demonstrated to support maintenance of *L. monocytogenes*. In one study, 100 soil samples were tested and all were capable of maintaining *L. monocytogenes* survival for at least 14 days, while 71% were able to support growth for longer than 80 days (31). A variety of physiochemical characteristics, ranging from water content and pH were identified as contributing to *L. monocytogenes* survival in soil. Finally, the abundance of competing microorganisms is a major determinant in the success of soil colonization by *L. monocytogenes* as evidenced by the increased growth of *L. monocytogenes* in a variety of

autoclaved soils relative to non-autoclaved controls (25, 27, 32). Given the diversity of soil types and geographic locales in which *L. monocytogenes* can be cultured from soil, it is not surprising that there is little information regarding metabolic adaptations required for life in the soil. Much of what we do know about metabolism under saprophytic conditions comes from genomic and transcriptomic studies. For example, the *L. monocytogenes* EGD-e genome encodes over 330 putative transporters which is 2 and 3 times more transport genes than either *Escherichia coli* or *Bacillus subtilis*, respectively (2). Of these transporters, 84 assemble into 29 complete phosphoenolpyruvate-dependent phosphotransferase systems (PTS) (33). Despite this abundance of PTSs, they are downregulated during infection and are dispensable for intracellular replication in macrophages, suggesting they likely have a role facilitating the use of varied carbon sources in the nutrient poor environment (33, 34). Both transport and metabolic genes are overrepresented in both the core genome and accessory genomes (3, 35, 36). In addition to genomic studies suggesting that extreme diversity in transport and metabolic systems likely facilitate survival in diverse environments, a limited number of transcriptomic studies have begun to elucidate gene expression changes that facilitate growth/survival in the soil. In one study, although many genes were differentially regulated at any given time point following introduction of *L. monocytogenes* into soil, only 94 genes were specifically upregulated at all time points assessed (15 min, 30 min and 18 hours). Of these 94, 37 genes were involved in various PTS transport systems while an additional 26 were involved in other metabolic processes including chitinases and β -glucosidases (37). Similarly, consistent with limited availability of amino acids, 15 genes of the regulon of CodY, a repressor of branched chain amino acid biosynthesis, were upregulated at all timepoints following instillation of *L. monocytogenes* into soil (37). In a follow up study with different soil, upregulation of PTS systems was also observed, including those for cellobiose, mannitol and maltose, among others (38). Finally, both of these studies observed repression of genes regulated by the master virulence regulator, PrfA, consistent with the longstanding observation that in the presence of PTS substrates PrfA expression is repressed (39).

PrfA also reciprocally regulates the PTS system as Hpr levels are lowered in vivo (40) and constitutive PrfA expression results in impaired growth on glucose (14) and impaired fitness in the soil (41). Thus, while *L. monocytogenes* is ubiquitous in the environment, and its core genome accommodates an expanded repertoire of metabolic and transport capabilities to facilitate growth in these diverse environments, our understanding of the metabolic adaptation to any specific non-host environment remains rather limited.

Life inside the Host

As an foodborne opportunistic pathogen, *L. monocytogenes* primarily enters host organisms by passage through the gastrointestinal tract (42). For most immunocompetent individuals, commensal microbes may provide a first line defense against *L. monocytogenes* colonization and infection, at least in part by competing for available nutrients (43). *L. monocytogenes* is competent for growth in anaerobic environments like the intestinal tract, and a forward genetic screen identified genes encoding components of the F_0F_1 -ATPase as essential for anaerobiosis (20). An additional aspect of energy metabolism relevant in the intestinal tract is the recently discovered flavin-based extracellular electron transfer

(FLEET) locus (44). FLEET supports growth on non-fermentable carbon sources by mediating electron transfer to extracellular acceptors; a FLEET mutant exhibited a competitive defect within the mouse gastrointestinal tract (44). From within the gut, *L. monocytogenes* is able to traverse the intestinal epithelium to establish an intracellular infection (45), where its intracellular metabolism has been extensively studied.

L. monocytogenes doubles in the host cytosol approximately every 40 minutes demonstrating its exquisite metabolic adaptation to the cytosolic niche. Given that cytosolic replication is essential for virulence, metabolism during intracellular growth has been extensively studied. Traditional genetic and biochemical studies of *L. monocytogenes* virulence, coupled with newer transcriptomic and metabolomics approaches have been used to understand both host and bacterial metabolism during infection. Surprisingly, a comprehensive analysis of the nutritional availability of the cytosol has not been performed and may differ in different cell types. However, based on the observation that only cytosol-adapted pathogens can fully exploit the cytosol as a replication niche, it has been hypothesized that the cytosol is a nutrient-limiting environment (46, 47). The cytosol is a highly reducing (48), neutral pH environment (49) that is low in free amino acids (50) and free metal ions such as magnesium, sodium, calcium and iron (51). Despite this nutrient limiting environment, *L. monocytogenes* must acquire nutrients from its host to facilitate replication, and must do so in a way that both promotes survival of the host cell and minimizes detection of the pathogen by the innate immune system (52, 53). In addition, *L. monocytogenes* has evolved a metabolic network that not only utilizes nutrients for replication but also integrates signals regarding nutrient availability to regulate virulence gene expression once inside the cell (39, 54–56).

Intracellular carbon metabolism

Carbon utilization is one of the most well described aspects of *L. monocytogenes* cytosolic metabolism where hexose-phosphates and glycerol are the primary carbon sources scavenged from the host. Hexose-phosphates are acquired through the use of a dedicated permease Hpt (gene *uhpT*), that is both required for virulence (55) and is directly regulated by the master virulence regulator, PrfA, upon entry into the host cytosol (57). Based on isotopologue metabolomic studies in infected cells, host derived glucose-6-phosphate is primarily shunted into the pentose phosphate pathway where it is used to build nucleotides and aromatic compounds, but likely contributes little from an energy perspective (24). In contrast, glycerol, the second major intracellular carbon source for *L. monocytogenes*, is the primary energy source during intracellular replication (13). Glycerol does not significantly contribute to gluconeogenesis, but instead feeds into lower glycolysis and the TCA cycle, and as such it is also used in *de novo* synthesis of some amino acids (13). As expected given its critical role in energy generation and anabolism, glycerol utilization mutants (*glp* and *dha* genes) are also attenuated and defective for intracellular replication (54, 58). Despite the key role of both glycerol and hexose phosphates in *L. monocytogenes* cytosolic metabolism, a combinatorial mutant that can neither use glucose-6-phosphate or glycerol ($\Delta C3 \Delta uhpT$) was still partially able to replicate in macrophages (13), albeit at significantly decreased levels, suggesting that alternative carbon sources are likely available during intracellular replication. Some evidence indicates that lactate, but not pyruvate, could be used as an

additional carbon source during cytosolic replication, while amino acids are unlikely to be a major source of carbon and energy generation (13). *L. monocytogenes* lacks genes required for beta-oxidation of fatty acids, likely ruling fatty acids out as another potential carbon source for *L. monocytogenes* in the cytosol (2). However, breakdown of phosphatidylethanolamine (PE) in mammalian membranes could liberate not only glycerol, but also ethanolamine (59, 60) which can be converted into acetaldehyde and ammonia by adenosylcobalamin-dependent ethanolamine lyase (encoded by *eut*) (17). Consistent with phospholipids contributing to carbon metabolism during cytosolic replication, *L. monocytogenes eut* mutants are defective for intracellular replication in epithelial cells (58). Thus, *L. monocytogenes* has diversified the carbon sources that it scavenges from the host cell, likely to promote host cell survival and prevent activation of innate immune defense mechanisms.

Despite the presence of *de novo* synthesis pathways for most amino acids, *L. monocytogenes* relies on both *de novo* synthesis and uptake of amino acids from the host for the promotion of full virulence. Specifically, during intracellular growth in macrophages *L. monocytogenes* synthesizes *de novo* BCAA, histidine and arginine, likely reflecting the shortage of these amino acids in the macrophage cytosol. Consistent with this observation, mutants deleted in genes central to these amino acid biosynthetic pathways exhibit intracellular growth defects (56, 58). Additionally, ¹³C-isotopologue profiling of intracellular *L. monocytogenes* using ¹³C-glucose demonstrated that *L. monocytogenes de novo* synthesizes many of the other amino acids during intracellular growth (e.g., Ala, Glu, Asp, Ser, Gly, Pro, Thr and Lys) (13, 14, 24). Nevertheless, despite the importance of *de novo* synthesis, additional ¹³C-isotopologue profiling with labeled amino acids clearly demonstrated that cytosolic *L. monocytogenes* also imports these amino acids from the host cell, along with most other essential and non-essential amino acids, and that these scavenged amino acids are directly incorporated into newly synthesized proteins with little to no catabolism (13). The one exception is aspartate, which is exclusively synthesized *de novo* (13), consistent with a previous observation that *L. monocytogenes* lacks the ability to take up exogenous aspartate (61). Further demonstrating the importance of amino acid scavenging for intracellular growth, mutants in cysteine, arginine and glutamine transport systems are each impaired for intracellular replication and attenuated for virulence (61–63), while many other amino acid transporters have yet to be identified and/or characterized. For some scavenged amino acids the amounts required to support growth of *L. monocytogenes* are consistent with their availability within the host cell cytosol (e.g., glutamine), while for others the requirement exceeds the actual availability (e.g., arginine, cysteine and methionine) (64). It was therefore speculated that perhaps peptides are acquired from the host cell where they would then be degraded by aminopeptidases for utilization in protein synthesis (65, 66). Indeed, the ability of *L. monocytogenes* to acquire peptides was demonstrated *in vitro* (64, 67, 68), and several peptide transporters contribute to *L. monocytogenes* intracellular growth in macrophage cells or to infection of mice. Among these are the oligopeptide transporter Lmo0135–0137 (CtaP), that is also associated with cysteine acquisition (3, 61, 69), the oligopeptide transporter OppABCD (58, 61, 70, 71) and the di-tripeptide transporter, DtpT (72). In addition, the putative peptide transporter Lmo0152 is transcriptionally induced during intracellular growth and therefore may

contribute to *L. monocytogenes* pathogenesis (6). Altogether these studies establish that *L. monocytogenes* relies on both *de novo* synthesis and uptake of amino acids to support intracellular growth (13, 14, 24, 56, 73).

Intracellular vitamin metabolism

As previously noted, *L. monocytogenes* is auxotrophic for a variety of essential cofactors including thiamine, lipoate, biotin, and riboflavin (10, 11), and must acquire them from the host. Biotin and riboflavin scavenging are likely mediated by the putative biotin (BioMNY) and riboflavin transporters (RibU and the ECF complex), respectively (74–76). Interestingly, although *L. monocytogenes* is unable to synthesize thiamine *de novo*, due to absence of the 4-amino-hydroxymethyl-2-methylpyrimidine phosphate (HMP-P) synthase ThiC, provision of HMP-P/HMP *in vitro* facilitates growth of *L. monocytogenes* in the minimal media lacking thiamine. Furthermore, although the HMP transporter has not been definitively identified, both the thiamine transporter (ThiT) and enzymes for latter *de novo* thiamine biosynthesis (ThiD) are required for intracellular replication (77) suggesting that, as is the case with many amino acids, *L. monocytogenes* must concurrently scavenge host derived thiamine and synthesize additional thiamine from HMP to support robust growth. Finally, *L. monocytogenes* is also auxotrophic for lipoic acid and must acquire it from the host. Two, non-redundant lipoate ligase enzymes, are required for lipoylation of dehydrogenase complexes including the pyruvate dehydrogenase complex (PDH), branched-chain α -keto acid dehydrogenase (BKD) and glycine cleavage pathway (GCV) (78). LplA1 is likely the major lipoate host scavenging enzyme active in the cytosol due to its higher affinity for lipoate (79) and its essentiality for virulence (78, 80). Ultimately, both PDH and BKD are required for virulence, likely explaining the essentiality of LplA1 *in vivo* (21, 81).

Intracellular fatty acid metabolism

Fatty acid synthesis (FAS) is engaged during infection by many Gram-positive bacterial pathogens, due to its role in the biogenesis of phospholipid membranes, lipoproteins, and lipotechoic acids (82, 83). The FASII pathway used by bacteria, plants and parasites, is structured such that each enzyme of the pathway is encoded by a separate gene that encodes for one protein, responsible for the catalysis of a single enzymatic step (84). The initial condensation reaction of the FASII pathway involves short chain fatty acid thioesters, typically acetyl-CoA and malonyl-ACP (85). This reaction is catalyzed by FabH, with subsequent rounds of condensation and acyl chain elongation catalyzed by FabF. The condensation reactions catalyzed by FabH and FabF result in the formation of β -ketoacyl-ACP products. These products are reduced by FabG to form β -hydroxyacyl-ACP, then dehydrated and reduced to acyl-ACP by FabZ and FabI to complete the cycle (85).

Several key aspects of fatty acid synthesis are relevant to the infectivity and pathogenesis of *L. monocytogenes*. The *L. monocytogenes* FabH enzyme exhibits selectivity towards ante-iso branched chain fatty acid (aiBCFA) precursors (86), resulting in a membrane highly enriched in aiBCFA. High aiBCFA content promotes increased membrane fluidity, conferring upon *L. monocytogenes* the unusual property of being able to replicate at refrigeration temperatures (87). Moreover, aiBCFA contribute to *L. monocytogenes* stress resistance against anti-microbial host mechanisms, including lysozyme and cationic peptides

(81). Methicillin-resistant *Staphylococcus aureus* (MRSA), another Gram-positive pathogen, also displays similar membrane composition, enriched in aiBCFA (88). The high demand for aiBCFA precursors to populate the *L. monocytogenes* membrane results in a reliance on exogenously supplied BCAA, most notably isoleucine which is the precursor for the predominant membrane BCFA species, despite the capacity for *de novo* synthesis of these amino acids. In defined medium and in the host cytosol, BCAA scavenging is critical for robust replication of *L. monocytogenes* (89). The lipoylated BKD complex is central to conversion of BCAA into the short branched chain thioesters that are the precursors for FASII, and is required for virulence (90), likely due at least in part to decreased membrane stress resistance. Thus, *L. monocytogenes* intracellular metabolism is highly dependent on BCAA, particularly isoleucine, not only to provide building blocks for protein synthesis, but also for fatty acid synthesis, which confers resistance to membrane stress.

The intersection between metabolism and virulence

In addition to fulfilling essential metabolic needs for optimal growth, the availability of metabolites such as amino acids and intracellular carbon sources also play a key role in regulating virulence. As discussed above in the context of environmental PrfA repression, regulation of this essential master virulence regulator is mediated in large part by the presence or absence of carbon sources found in the cytosol of host cells. Both hexose-phosphates, as well as glycerol, promote full activity of PrfA, consistent with their function as the primary cytosolic carbon sources (54, 57). In contrast, metabolism of glucose and other PTS substrates not utilized for cytosolic replication actively inhibits PrfA activity, consistent with these PTS systems being dispensable for cytosolic replication (33, 39, 91). Inhibition of PrfA is not mediated by native sugars themselves, instead, one model suggests that uptake and metabolism of PTS sugars induces serine phosphorylation of the phosphocarrier protein Hpr (92) and that Ser-P Hpr directly inhibits PrfA activity (93). Other studies have not observed this effect, and instead have proposed that PrfA is regulated by other components of the PTS system (54, 94). PrfA is also responsive to redox conditions in the host cytosol (95, 96), highlighting the importance of this virulence regulator as a key hub for integrating environmental cues with bacterial metabolism.

In addition to carbon source availability, *L. monocytogenes* regulates virulence in response to low BCAA availability and high availability of glutamine as occurs in the host cytosol (56, 62, 97). The response to BCAA is dependent on CodY, a global regulator and a sensor of isoleucine. Whereas under high BCAA conditions, isoleucine-bound CodY acts as a repressor of BCAA biosynthesis pathways, under BCAA limiting conditions in the cytosol CodY-mediated repression of BCAA biosynthesis is alleviated and isoleucine-unbound CodY instead acts as a transcriptional activator of *prfA*, intimately coupling BCAA sensing to regulation of virulence gene expression (97). Sensing of BCAA levels is not limited to host BCAA as a recent study suggests that BCAA *de novo* synthesis is restricted to sustain virulence gene expression (98). A riboregulator (Rli60), functioning as a ribosome-mediated attenuator, was identified upstream of the BCAA biosynthesis genes that persistently restricts BCAA biosynthesis, even under low BCAA conditions, essentially keeping *L. monocytogenes* starved for BCAA thus ensuring PrfA expression in the low BCAA environment of the cytosol. In fact, Rli60 was found to be the reason for *L. monocytogenes*

partial-auxotrophy for BCAA, a phenotype that likely evolved to enable isoleucine to serve as a host signal and effector of virulence (98). In contrast, the mechanism by which L-glutamine regulates virulence gene expression is less clear as its direct sensor in *L. monocytogenes* is not known, though GlnR is likely a good candidate (62). L-glutamine uptake by *L. monocytogenes* via GlnPQ, triggers virulence gene expression only when the internal concentration of L-glutamine in *L. monocytogenes* crosses a certain threshold. Inactivation of GlnPQ arrested L-glutamine uptake, virulence gene expression, and led to attenuation of virulence in mice (62). Finally, though not directly tied to PrfA regulation, arginine sensing mediated by the highly conserved ArgR arginine-responsive transcription regulator (99) represses the arginine biosynthesis genes (*argCJBDF* and *argGH*), as well as the arginine deaminase pathway (i.e., ADI genes-*arcA*, *arcB* and *arcC*) the latter of which is required for *L. monocytogenes* acid tolerance and intracellular survival (100, 101). As both arginine biosynthesis and catabolism genes are regulated during intracellular growth, arginine sensing by ArgR likely plays a key role during *L. monocytogenes* infection (56, 58, 100, 101).

In addition to the classical central metabolic processes required for virulence, many secondary metabolic pathways and their metabolites contribute to virulence both through direct regulation of PrfA as well as through other, often unknown mechanisms. For example, S-adenosylmethionine (SAM) is a methionine precursor that regulates gene expression by binding to SAM-responsive riboswitches (102). The *L. monocytogenes* genome contains 7 such riboswitches upstream to Cys-Met biosynthesis genes, which in all cases terminate gene transcription upon SAM binding. Consistent with the theme of tying metabolism to virulence regulation, the premature terminated transcripts (i.e., the SAM-bound RNAs) act *in-trans* on *prfA* 5'UTR (via base-pairing), down-regulating its transcription specifically under rich nutrient conditions (102). In contrast, glutathione, an abundant tri-peptide either synthesized by *L. monocytogenes* from cysteine, glutamate and glycine or acquired directly from the host cytosol where it is necessary for maintaining redox balance (103) is an allosteric regulator that can increase PrfA activity as much as 150-fold (104, 105). Additionally, glutathione post-translationally regulates LLO via S-glutathionylation of its conserved cysteine residue, thus rendering it non-active, minimizing LLO toxicity in the cytosol and promoting virulence (106). Shikimate biosynthesis, which generates precursors for the biosynthesis of both aromatic amino acids (i.e., tyrosine, tryptophan and phenylalanine), is essential for *L. monocytogenes* intracellular growth (56, 107). However, shikimate biosynthesis is also needed for the generation of both menaquinone (MK) and its biosynthetic precursor 1,4-Dihydroxy-2-Naphthoate (DHNA), which are also both independently necessary for virulence (21). *L. monocytogenes* genes for the electron transport chain, including genes for MK biosynthesis genes (*men*) and *L. monocytogenes* cytochrome *bd* and *aa₃* oxidases, are induced in cell culture and *in vivo* (6, 70), consistent with a role for the electron transport chain and generation of a membrane potential during infection (21, 66, 107). As discussed previously, utilization of glycerol as a primary energy source requires ATP synthesis through oxidative phosphorylation, at least partially explaining the requirement for MK for virulence. However, the electron transport chain has also been implicated in redox balance (108), oxidative/nitrosative stress responses (109), subcellular localization of cell division machinery (110), nucleotide biosynthesis (111), and

solute transport of betaine/carnitine (112) in other bacteria. As such, the requirement for shikimate biosynthesis for virulence is complicated and likely multifactorial as even the role of MK in infection is not straightforward. To add complexity to ascribing virulence functions to metabolic pathways, it was recently reported that the MK biosynthetic intermediate 1,4-dihydroxynaphthoate (DHNA) is required for virulence and survival in the cytosol independent of full length MK (21), further emphasizing that the complex portfolio of intermediate products generated through metabolism may be a rich source for signaling and virulence regulation during infection.

Bacterial metabolism and the host immune response

The metabolic potential of bacterial pathogens like *L. monocytogenes* is robustly adapted to promote survival and replication within the host, however, the highly conserved nature of many products of bacterial metabolism presents an Achilles heel that can be recognized by the host immune system. The capability of the innate immune system to sense microbial ligands has been extensively reviewed elsewhere (113), with the most well-studied paradigm being recognition of bacterial products like lipopeptides by the Toll-like receptors. However, studies over the last two decades have revealed a surprisingly diverse repertoire of sensing mechanisms within the mammalian host cytosol that are responsive to bacterial virulence factors and metabolites. A striking example is host recognition of the bacterial metabolite, cyclic diAMP (c-di-AMP), a cyclic dinucleotide that controls growth, cell wall homeostasis and virulence (114). *L. monocytogenes* secretion of c-di-AMP through multi-drug transporters into the host cytosol enables recognition of this bacterial metabolite by a host protein, STING, then triggering upregulation of Type I interferon and the interferon response (115–117). A recent study suggests that there is a richer diversity of cyclic nucleotides produced by bacteria than previously suspected (118). While only a few *L. monocytogenes* metabolites have yet been established as ligands for cytosolic pattern recognition, the evolutionary conservation of many metabolic functions implicate these molecules as likely targets for the innate immune system. Evidence points to host recognition of chemically distinct metabolites from other bacterial species, including citrate, heptose-1–7-bisphosphate and ADP-heptose (53, 119, 120). Thus, bacterial pathogens must evolve to navigate a fine balance between fully exploiting the host niche for nutrients and their visibility to the host immune system through the by-products of their own metabolism.

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