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Listeria monocytogenes cytosolic metabolism promotes replication, survival and evasion of innate immunity

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Summary

Listeria monocytogenes, the causative agent of listeriosis, is an intracellular pathogen that is exquisitely evolved to survive and replicate in the cytosol of eukaryotic cells. Eukaryotic cells typically restrict bacteria from colonizing the cytosol, likely through a combination of cell autonomous defenses, nutritional immunity, and innate immune responses including induction of programmed cell death. This suggests that *L. monocytogenes* and other professional cytosolic pathogens possess unique metabolic adaptations, not only to support replication, but also to facilitate resistance to host-derived stresses/defenses and avoidance of innate immune activation. In this review, we outline our current understanding of *L. monocytogenes* metabolism in the host cytosol and highlight major metabolic processes which promote intracellular replication and survival.

Access to the cytosol is essential for virulence

L. monocytogenes is a Gram-positive facultative intracytosolic pathogen and the causative agent of listeriosis in humans and livestock (Freitag *et al.*, 2009). Ingestion of *L. monocytogenes*-laden food products and entry into the host via the gastrointestinal tract is responsible for periodic outbreaks of listeriosis (Lecuit, 2007). Infections with *L. monocytogenes* normally results in mild gastroenteritis, however severe *L. monocytogenes* infection, especially in at-risk populations (immunocompromised, elderly or pregnant individuals) may result in complications such as septicemia, meningitis, endocarditis, or spontaneous abortion. Disseminated infections with *L. monocytogenes* are deadly, with a lethality rate of up to ~30% even with antibiotic treatment (Swaminathan and Gerner-Smidt, 2007).

L. monocytogenes enters host cells either via phagocytosis or through receptor-mediated endocytosis facilitated by the bacterial surface proteins internalin A (InlA) and internalin B (InlB) (Mengaud *et al.*, 1996; Shen *et al.*, 2000). *L. monocytogenes* is initially encapsulated in a host vacuole and subsequently secretes the cholesterol-dependent pore-forming toxin listeriolysin O (LLO, encoded by *hly*) and the phospholipases PlcA and PlcB to promote

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escape into the cytosol (Portnoy *et al.*, 1988; Camilli *et al.*, 1989; Mengaud *et al.*, 1991; Vazquez-Boland *et al.*, 1992; Marquis *et al.*, 1995). Once in the cytosol, ActA promotes *L. monocytogenes* actin-based motility and cell-to-cell spread (Kocks *et al.*, 1992). Genetic deletion of any of these virulence factors or their master regulator, PrfA (Freitag *et al.*, 2009), attenuates *L. monocytogenes* virulence, signifying necessity for *L. monocytogenes* to access the cytosol to cause disease. Importantly, not only access to, but maintenance of the cytosolic niche is essential for *L. monocytogenes* virulence as mutants that trigger host cell death, either due to LLO toxicity or activation of innate immune cell death pathways, are highly attenuated in vivo (Glomski *et al.*, 2003; Sauer *et al.*, 2010).

Metabolic adaptions to the cytosolic environment are crucial for *L. monocytogenes* intracellular replication and survival. Although our understanding of *L. monocytogenes* physiology and metabolism in the cytosol is currently incomplete, robust genetic tools coupled with exciting new approaches to transcriptomics, metabolomics and proteomics are leading to a renaissance in our molecular understanding of *L. monocytogenes* intracellular life. A complete and comprehensive discussion of *L. monocytogenes*' metabolic potential is beyond the scope of this review, instead, we will focus on major metabolic processes critical for *L. monocytogenes* replication and survival in the eukaryotic cytosol.

The inhospitable cytosolic environment

Listeria monocytogenes inhabits the host cytosol, the dense (400 mg/ml of macromolecules) (Guigas *et al.*, 2007) gel-like fluid surrounding the organelles in the cytoplasm that is the major site for glycolysis, gluconeogenesis and the pentose phosphate pathway. Indirect measurements determine that the cytosol of various eukaryotic cells is approximately 1- to 10-fold greater viscosity than bulk water (Luby-Phelps, 2000). Additionally, the cytosol is typically a pH neutral environment (~7.4) (Llopis *et al.*, 1998) that is highly reducing (oxidized:reduced glutathione is approximately 30:1) (Hwang *et al.*, 1992) and contains low concentrations of free amino acids (Piez and Eagle, 1957) and free metal ions such as magnesium, sodium, calcium, potassium, and iron (Ray *et al.*, 2009).

Despite limited availability of amino acids and metals, it was originally thought that the cytosol was a nutrient replete environment, where bacteria should thrive if they had access. This idea was supported by the observation that *Bacillus subtilis* engineered to express LLO were capable of replicating in the cytosol of host cells following phagosomal escape (Bielecki *et al.*, 1990). However, subsequent studies by Goebel and colleagues challenged this idea and demonstrate that multiple different non-cytosol-adapted bacteria, microinjected or engineered to enter the host cytosol, will not replicate in the cytosol and appear to be cleared (Goetz *et al.*, 2001; Slaghuis *et al.*, 2004). They further demonstrated that rare replication of cytosolic *B. subtilis* occurred only in dead or dying cells, potentially explaining the original observations by Bielecki *et al.* Even more recently it has become clear that some vacuolar pathogens that accidently escape into the cytosol of macrophages are also unable to replicate and appear to be cleared (Beuzon *et al.*, 2000; Beuzón *et al.*, 2002; Creasey and Isberg, 2012; Ge *et al.*, 2012). In the case of *Salmonella typhimurium* this appears to be cell-type specific, as *S. typhimurium* are restricted in the cytosol of macrophages but replicate in the cytosol of epithelial cells, suggesting that certain host cells

are better able to prevent bacterial cytosolic replication and survival (Beuzón *et al.*, 2002; Knodler *et al.*, 2010).

Finally, several metabolic pathways in *L. monocytogenes* and *Francisella* spp. have been reported to promote not just cytosolic replication but survival, as disruptions in these pathways lead to cytosolic bacteriolysis, in some cases in a cell-type specific manner (Sauer *et al.*, 2010; Peng *et al.*, 2011; Pensinger *et al.*, 2016; Chen *et al.*, 2017). Importantly, bacteriolysis in the cytosol can potently activate innate immunity through a series of cytosol specific DNA sensing pathways including cGAS/STING and AIM2, leading to type I interferon induction and inflammasome activation, respectively (Sauer *et al.*, 2010; Woodward *et al.*, 2010; Sauer, Sotelo-Troha, *et al.*, 2011; Rae *et al.*, 2011; Hansen *et al.*, 2014). As discussed above, maintenance of the cytosolic niche is essential for *L. monocytogenes* and other cytosolic pathogens to causes disease and as such, activation of inflammasome attenuates virulence (Warren *et al.*, 2011; Sauer, Pereyre, *et al.*, 2011). Together these data suggest through unknown mechanisms the cytosol of various cells vary in their ability to restrict bacterial replication and survival. To promote its virulence, *L. monocytogenes* must modulate its metabolism not only to acquire host nutrients but also to tolerate cytosolic stresses and evade host immune defenses.

Host-derived metabolites required for virulence

Like all intracellular pathogens, *L. monocytogenes* steals nutrients from its host cell. To facilitate this, the *L. monocytogenes* genome encodes over 330 putative transporters which is 2 and 3 times more transport genes than either *Escherichia coli* or *Bacillus subtilis*, respectively (Glaser *et al.*, 2001). Of these transporters, 84 assemble into 29 complete phosphoenolpyruvate-dependent phosphotransferase systems (PTS) (Stoll and Goebel, 2010). Interestingly despite this abundance of PTSs, they are downregulated during infection and are dispensable for intracellular replication in macrophages (Stoll and Goebel, 2010; Aké *et al.*, 2011). During intracellular replication, *L. monocytogenes* transports host-derived hexose-phosphates, such as glucose-1-phosphate and glucose-6-phosphate, through Hpt (gene *uhpT*), a permease required for virulence (Chico-Calero *et al.*, 2002) whose expression is tightly controlled by the master virulence regulator, PrfA (Ripio *et al.*, 1997). A second major intracellular carbon source for *L. monocytogenes* is glycerol. Glycerol utilization mutants (*glp* and *dha* genes) are defective for intracellular replication in both epithelial cells and macrophages (Joseph *et al.*, 2006; Joseph *et al.*, 2008). ^{13}C isotopologue studies suggest that host derived glucose-6-phosphate and glycerol are diverted to separate metabolic pathways in *L. monocytogenes* such that glucose-6-phosphate is oxidized by the pentose phosphate pathway and likely used as precursors for nucleotide biosynthesis and aromatic compounds, whereas glycerol feeds lower glycolysis for energy and amino acids biosynthesis (Grubmüller *et al.*, 2014). Surprisingly a combinatorial mutant that can neither use glucose-6-phosphate or glycerol (ΔC3Δ*uhpT*) was still partially able to replicate in macrophages (Grubmüller *et al.*, 2014), suggesting that alternative carbon sources may still be available during intracellular replication. Although it is unlikely that lipids act as the direct alternative carbon source due to a lack of genes required for beta-oxidation of fatty acids (Glaser *et al.*, 2001), breakdown of phosphatidylethanolamine (PE) in mammalian membranes (Tsoy *et al.*, 2009), possibly by the *L. monocytogenes* phosphatidylcholine

phospholipase C (PlcB), may liberate both ethanolamine and glycerol (Geoffroy *et al.*, 1991). Ethanolamine can be further degraded into acetaldehyde and ammonia by *L. monocytogenes*' adenosylcobalamin-dependent ethanolamine lyase (encoded by *eut* genes) (Joseph and Goebel, 2007). In support of this hypothesis *eut* mutants are defective for intracellular replication in epithelial cells (Joseph *et al.*, 2006). Whether ethanolamine and/or other host metabolites are used by *L. monocytogenes* as carbon sources during intracellular replication remains an important unanswered question. Furthermore, it is likely that diversification of carbon sources would help *L. monocytogenes* fulfill its nutritional requirements in the cytosol while minimizing metabolic perturbations that can be detected by the host cell (Grubmüller *et al.*, 2014).

During intracellular replication *L. monocytogenes* likely assimilates nitrogen from various host sources, such as glutamine. Indeed genes for glutamate synthase, used to assimilate nitrogen from glutamine (Schreier, 1993), are up-regulated during infection (Joseph *et al.*, 2006). Furthermore, the glutamine ABC transporter (GlnPQ) is required for optimal intracellular replication and virulence suggesting that *L. monocytogenes* needs to scavenge glutamine from the host (Haber *et al.*, 2017). In the absence of glutamine, other nitrogen sources such as ammonia, ethanolamine and arginine may sustain growth of *L. monocytogenes* in the cytosol (Tsai and Hodgson, 2003; Kutzner *et al.*, 2016). Inorganic ammonia transporters (encoded by *nrgAB*) are upregulated in epithelial cells (Joseph *et al.*, 2006) but downregulated in macrophages (Chatterjee *et al.*, 2006), possibly signifying differences in nitrogen source availability between these two cell types. Ammonia may also be acquired through ethanolamine degradation (Joseph *et al.*, 2006). Finally, genes for the arginine ABC transporter appear to be induced intracellularly (Klarsfeld *et al.*, 1994; Joseph *et al.*, 2006). As with carbon acquisition, nitrogen assimilation from different host sources may benefit *L. monocytogenes* replication but also facilitate avoidance of host defense pathways as arginine metabolism plays a critical role in nitric oxide synthase and polyamine synthesis. Indeed, several pathogens are known to deplete arginine levels to avoid killing by host cells (Gobert *et al.*, 2001; Abu-Lubad *et al.*, 2014; Goldman-Pinkovich *et al.*, 2016).

Although *L. monocytogenes* possesses all the metabolic pathways required for *de novo* synthesis of both essential and non-essential amino acids (Glaser *et al.*, 2001) during infection, host-derived amino acids serve as building blocks for listerial proteins and other macromolecules, as well as for assimilation of nitrogen as discussed above. Mutants in cysteine, arginine and glutamine transport systems are each partially impaired for intracellular replication and attenuated for virulence (Klarsfeld *et al.*, 1994; Schauer *et al.*, 2010; Haber *et al.*, 2017), while other amino acid transporters have yet to be characterized. ^{13}C isotopologue profile analysis demonstrates that host-derived amino acids are efficiently taken up by cytosolic *L. monocytogenes* and quickly incorporated into protein with little to no detectable catabolism of amino acids (Eylert *et al.*, 2008; Grubmüller *et al.*, 2014). Amino acids may also be acquired in the form of host-derived oligopeptides (Marquis *et al.*, 1993), possibly through an ATP-dependent oligopeptide transporter, OppABCDF (Verheul *et al.*, 1998) or the di/tripeptide transporter, DtpT (Wouters *et al.*, 2005) where they are then degraded by aminopeptidases (Perry and Higgins, 2013; Cheng *et al.*, 2015). Assimilation of host amino acids and *de novo* synthesis of certain amino acids (discussed later) are crucial for *L. monocytogenes* replication in the cytosol, however the relative

contribution of amino acid scavenging versus synthesis is not clear. This is important since nutritional immunity through amino acid depletion is a well-defined host defense mechanism which *L. monocytogenes* likely must overcome (Appelberg, 2006).

L. monocytogenes does not possess complete pathways required to *de novo* synthesize a variety of cofactors including thiamine, lipoate, biotin, and riboflavin (Premaratne *et al*, 1991; Phan-Thanh and Gormon, 1997), suggesting *L. monocytogenes* must scavenge these cofactors from the host cytosol. Transport of biotin and riboflavin from the host cytosol likely occurs through the putative biotin (BioMNY) and riboflavin transporters (Lmo1945, Ecf and RibU), respectively (Dowd *et al*, 2011; Karpowich *et al*, 2015; Matern *et al*, 2016). *L. monocytogenes* is unable to synthesize thiamine *de novo*, due to absence of ThiC, a HMP-P synthase, however, supplementation of HMP-P/HMP *in vitro* facilitates growth of *L. monocytogenes* in the minimal media lacking thiamine, suggesting that latter steps in the thiamine biosynthesis pathway are intact (Schauer *et al*, 2009). Interestingly growth of *L monocytogenes* in epithelial cells requires both the thiamine transporter (ThiT) and enzymes for latter *de novo* thiamine biosynthesis (ThiD) (Schauer *et al*, 2009), suggesting that thiamine concentrations in the host are scarce and *L. monocytogenes* must concurrently scavenge host derived thiamine and synthesize additional thiamine from HMP to support robust growth of *L. monocytogenes* in the cytosol. Finally, *L. monocytogenes* possesses two lipoate ligase enzymes, LplA1 and LplA2 which are required for lipoylation of dehydrogenase complexes (Keeney *et al*, 2007) including the pyruvate dehydrogenase complex (PDH), branched-chain α-keto acid dehydrogenase (BKD) and glycine cleavage pathway (GCV). Although *L. monocytogenes* possess two of these enzymes their functions are not fully overlapping or redundant; LplA1 is the major lipoate host scavenging enzyme active in the cytosol as evidenced by its higher affinity for lipoate (Christensen *et al*, 2011) and its essentiality for virulence (O'Riordan *et al*., 2003; Keeney *et al*., 2007). Interestingly, both PDH and BKD mutants are significantly attenuated for virulence and may explain the necessity for lipoate scavenging (Sun and O'Riordan, 2010; Chen *et al*., 2017). These host-derived cofactors are responsible for enzymatic activity in numerous metabolic pathway, some of which may be required for intracellular replication and/or survival, however, in many cases the specific metabolic pathways impaired when *L. monocytogenes* is unable to scavenge these cofactors from the host have not been characterized.

Undoubtedly nutrient acquisition from the host is essential for replication of *L. monocytogenes*, though, why *L. monocytogenes* has evolved redundant mechanisms to acquire diverse and sometimes overlapping host nutrients remains unclear. One possibility is that overlap of carbon and nitrogen utilization pathways may be important to overcome host nutritional immunity (Appelberg, 2006). Additionally, diversification of nutrient sources may reduce metabolic burdens on the host and prevent detection by the host immune system. Multiple recent studies have highlighted a variety of mechanisms by which host cell sense disruptions of metabolic flux and/or bacterial metabolites directly as indicators of infection to trigger innate immune responses (Wynosky-Dolfi *et al*., 2014; Sanman *et al*., 2015; Gaudet *et al*., 2015; Wolf *et al*., 2016). Interestingly, although recent host metabolomics studies clearly indicate that theft of nutrients from the host cytosol by *L. monocytogenes* and *Shigella flexneri* influences host metabolism (Gillmaier *et al*., 2012; Kentner *et al*., 2014; Grubmüller *et al*., 2014), these pathogens effectively evade known metabolically triggered

host innate immune responses. Together these findings suggest that *L. monocytogenes* metabolism is not only important for replication but also countering host immunity, though the mechanisms by which this occurs remain to be elucidated. Importantly, the intersection of metabolism and innate immunity in the context of *L. monocytogenes* infection in an intact animal has also not been addressed.

Bacterial metabolic programs required for virulence

Although *L. monocytogenes* acquires many essential nutrients directly from the host, there are also many intrinsic metabolic pathways essential for cytosolic replication, survival and ultimately virulence. Genetic screens have uncovered an exhaustive list of metabolic pathways vital to *L. monocytogenes* intracellular replication (Camilli *et al.*, 1989; Joseph *et al.*, 2006; Schauer *et al.*, 2010), though in many cases these pathways' contributions to intracellular survival, innate immune evasion and virulence of *L. monocytogenes* have not been thoroughly examined.

Although most amino acids utilized in *L. monocytogenes* protein synthesis appear to be host-derived, pathways for biosynthesis of Thr, His, Arg, Ser, Met, the branched chain amino acids (BCAA), and the aromatic amino acids are induced during intracellular replication and are necessary for efficient replication during infection (Marquis *et al.*, 1993; Joseph *et al.*, 2006; Chatterjee *et al.*, 2006; Camejo *et al.*, 2009; Schauer *et al.*, 2010; Lobel *et al.*, 2012; Chen *et al.*, 2017). These data suggest that although host derived amino acids are transported and used directly in protein synthesis, certain amino acids are inadequately supplied in the host cell cytosol. In some cases, inefficient replication and/or defective stress responses are not due to defects in protein synthesis, but instead are due to the roles that amino acids or their precursors play in non-protein synthesis processes. For example, BCAAs are necessary not only for protein synthesis, but are also a major precursor of branched chain fatty acid biosynthesis and are necessary for proper *L. monocytogenes* lipid/membrane homeostasis and resistance to intracellular stresses (Sun and O'Riordan, 2010). BCAAs levels also signal the global metabolic regulator CodY, which represses and activates genes for amino acid biosynthesis, nutrient transport, stress response and virulence (Bennett *et al.*, 2007; Lobel *et al.*, 2012; Lobel and Herskovits, 2016). Moreover, *L. monocytogenes codY* mutants are partially attenuated for virulence both *ex vivo* and *in vivo* (Bennett *et al.*, 2007; Lobel *et al.*, 2012; Whiteley *et al.*, 2015). Alternatively, the *aro* genes, required for chorismate biosynthesis are upregulated during infection and are essential for virulence. However, chorismate is a precursor not only for aromatic amino acids (Tyr, Trp, Phe) but also menaquinone (MK) and folate, and the attenuation of the *aro* mutants is primarily due to disruption of MK and/or 1,4-dihydroxy-2-naphthoate (DHNA) biosynthesis (Stritzker *et al.*, 2004; Chen *et al.*, 2017). Taken together, these observations demonstrate that virulence defects associated with amino acid biosynthesis mutants may be mistakenly attributed to protein synthesis and that the functions of amino acids/precursors in other metabolic pathways should be carefully evaluated to fully understand *L. monocytogenes*' intracellular metabolic requirements.

Lipids play a critical role in *L. monocytogenes* replication and survival during infection. *L. monocytogenes* and other Gram-positive bacterial membranes are highly enriched for

branched chain fatty acids (Whittaker *et al.*, 2005). Lipoate-dependent BKD synthesizes constituents for branched chain fatty acids from BCAAs (Massey *et al.*, 1976) and BKD mutants are severely attenuated *ex vivo* and *in vivo* (Sun and O'Riordan, 2010) due to killing by lysozyme and cathelicidin-related antimicrobial peptides (CRAMP) (Sun *et al.*, 2012). Finally, modulation of lipid composition between anteiso-, iso-, and straight-chain fatty acids drastically alters virulence phenotypes of *L. monocytogenes* within macrophages (Sun and O'Riordan, 2010; Sun *et al.*, 2012). These data together highlight the importance for proper membrane homeostasis and biogenesis during intracellular replication of *L. monocytogenes*.

Likewise, connections between central metabolism and cell wall homeostasis are likely important for *L. monocytogenes* survival and replication within host cells. For example, L-rhamnose utilization genes are active during intracellular replication of serogroup 1/2 *L. monocytogenes* strains that decorate their wall teichoic acids (WTA) with rhamnose (Uchikawa *et al.*, 1986; Lobel *et al.*, 2012). As WTA modifications have been implicated in resistance to cell wall stress, this could indicate a critical role for WTA modification or other L-rhamnose dependent processes during infection. Indeed, mutants defective for L-rhamnosylation of their WTA are attenuated during infection in a cationic peptide-dependent manner (Carvalho *et al.*, 2015). Similarly, the bacterial second messenger cyclic di-adenosine monophosphate (c-di-AMP) is emerging as a critical signaling molecule in *L. monocytogenes* that integrates regulation of central metabolism with cell wall homeostasis and osmoregulation (Kaplan Zeevi *et al.*, 2013; Huynh *et al.*, 2014; Tadmor *et al.*, 2014; Sureka *et al.*, 2014; Whiteley *et al.*, 2015; Whiteley *et al.*, 2017). Mutants lacking c-di-AMP (Δ*dacA*) are prone to bacteriolysis in the cytosol of macrophages leading to hyperactivation of DNA-sensing innate immune signaling pathways. Not surprisingly given the central role of c-di-AMP in regulating metabolism and cell-envelope stress Δ*dacA* mutants are highly attenuated for virulence (Witte *et al.*, 2013). Finally, the PASTA (penicillin-binding-protein and serine/threonine associated) kinase PrkA and its associated protein of unknown function YvcK have been shown to be important for glycerol metabolism in *L monocytogenes* (Görke *et al.*, 2005; Mir *et al.*, 2014; Pensinger *et al.*, 2016). Through some unknown mechanism these proteins also direct cell wall homeostasis, cytosolic survival, and virulence of *L. monocytogenes* (Sauer *et al.*, 2010; Pensinger *et al.*, 2016). The molecular mechanisms by which *L. monocytogenes* intracellular metabolism facilitates proper cell wall maintenance and resistance against cytosolic stresses remains to be elucidated.

L. monocytogenes is a facultative aerobe that can respire on oxygen but not on nitrate, nitrite or other terminal electron acceptors since it is missing genes for these reductases (Glaser *et al.*, 2001). While it is clear that *L. monocytogenes* possesses the capacity for both aerobic and fermentative growth (Romick *et al.*, 1996), which mode of growth is dominant during intracellular replication has not been resolved, though several lines of evidence point towards aerobic respiration. *L. monocytogenes* genes for the electron transport chain are induced in cell culture and *in vivo*, including genes for MK biosynthesis genes (*men*) and *L. monocytogenes*' cytochrome *bd* and aa_3 oxidases *in vivo* (Chatterjee *et al.*, 2006; Camejo *et al.*, 2009). These data suggest that the electron transport chain and generation of a membrane potential is necessary for infection, and indeed MK deficient mutants are attenuated *in vivo* (Stritzker *et al.*, 2004; Perry and Higgins, 2013; Chen *et al.*, 2017). Additionally, the fact that

the non-fermentable substrate, glycerol, is a primary carbon and energy source during intracellular replication (Grubmüller *et al.*, 2014) suggests that respiration is required for intracellular replication. Likewise, computational modeling of *L. monocytogenes* metabolic flux also predict that oxygen consumption and CO2 efflux occur in the cytosol (Lobel *et al.*, 2012). In contrast anaerobic growth likely occurs during transit through the gastrointestinal tracts and plays a role in priming *L. monocytogenes* for intracellular invasion (Wallace *et al.*, 2017). Understanding the relative contribution of aerobic and anaerobic metabolism and its temporal and spatial regulation during infection is a key next step in understanding the progression of listeriosis.

While a key role for electron transport is to facilitate ATP synthesis through oxidative phosphorylation, the electron transport chain also governs reduction-oxidation (redox) balance (Bueno *et al.*, 2012), detoxification of oxidative/nitrosative stress (Giuffrè *et al.*, 2014), protein localization during cell division (Strahl and Hamoen, 2010), nucleotide biosynthesis (Kilstrup *et al.*, 2005), and solute transport of betaine/carnitine (Wargo and Meadows, 2015) in bacteria. As such, it is difficult to know which of the key functions of the electron transport chain are essential for virulence. Even more confounding is a recent study demonstrating that MK biosynthetic mutants are killed in the macrophage cytosol independent of respiration defects or even the synthesis of full length MK. Instead, the cytosolic survival defects and at least some of the virulence defect of the MK biosynthetic mutants is due to lack of the MK biosynthetic intermediate, 1,4-dihydroxynaphthoate (DHNA) (Chen *et al.*, 2017). It is likely that DHNA/MK, a functioning electron chain and oxidative phosphorylation all contribute to the virulence of *L. monocytogenes in vivo*, however, since these processes are intimately linked, studies to tease apart their relative contributions will be necessary in the future.

Integration of metabolism and virulence gene expression

PrfA is a Crp/Fnr family transcription factor that is responsible for regulating the entire virulence regulon in *L. monocytogenes*, and not surprisingly it is both regulated by and regulates metabolism (de las Heras *et al.*, 2011) (Milohanic *et al.*, 2003). Most notably, while PrfA activity is necessary to fully activate expression of *uhpT* as discussed above, PrfA function is inhibited by glucose and other phospho-transfer-system (PTS) substrates not present or utilized by *L. monocytogenes* in the host cytoplasm (Milenbachs *et al.*, 1997; Mertins *et al.*, 2007). The inhibition of PrfA is not mediated directly by the sugars, instead, uptake and metabolism of these sugars affects the serine phosphorylation (Ser-P) of the phosphocarrier protein Hpr (Deutscher *et al.*, 1995) and it is hypothesized that PrfA inhibition is directly mediated by Ser-P Hpr (Herro *et al.*, 2006) or other components of the PTS (Joseph *et al.*, 2008; Vu-Khac and Miller, 2009). PrfA in turn likely regulates the PTS system since Hpr levels are lowered *in vivo* (Fuchs *et al.*, 2012) and a constitutively active PrfA mutant (PrfA*) has impaired growth on glucose (Eylert *et al.*, 2008), although the mechanism by which this is regulated is not defined. Consistent with the regulation of PrfA activity by carbon source availability, glycerol metabolism also increases PrfA activity, again through an unknown mechanism (Mertins *et al.*, 2007). Through this regulation, *L. monocytogenes* ensures that virulence factors required for colonizing a host are only turned on once the bacteria gain access to a host. Indeed, loss of this regulation through genetic

manipulation leads to loss of fitness during *L. monocytogenes*' environmental lifestyle (Bruno and Freitag, 2010), demonstrating the importance of tying metabolism to virulence factor regulation.

In addition to regulation of PrfA function through sensing of carbon metabolism, several studies have investigated other metabolic regulators of PrfA function. For example, in response to low levels of BCAAs *L. monocytogenes* upregulates BCAA synthesis and the transcriptional regulator CodY which promotes transcription of *prfA* (Bennett *et al.*, 2007; Lobel *et al.*, 2012; Lobel *et al.*, 2014). Additionally, translation of PrfA is repressed *in trans* by S-adenosyl methionine dependent riboswitches which bind the *prfA* UTR (Loh *et al.*, 2009). Finally, PrfA is post-translationally regulated allosterically by glutathione (Reniere *et al.*, 2015), an abundant peptide which maintains redox balance in the host cytosol (Meister and Anderson, 1983) such that high exogenous and endogenous glutathione increase PrfA activity as much as 150 fold (Reniere *et al.*, 2015). Ultimately, defining additional molecular mechanisms by which metabolism regulates PrfA activity transcriptionally, translationally or post-translationally will be key to further understanding how *L. monocytogenes* rapidly adapts to its intracellular niche.

Perspective

Despite previous assumptions about the bountiful and protected nature of the cytosol, it is now clear that specific adaptations are required to colonize this niche. Increasing evidence suggests that different cell types vary in their ability to restrict bacteria from colonization, highlighting the need for *L. monocytogenes* to have metabolic flexibility (Beuzón *et al.*, 2002; Chen *et al.*, 2017). Much work is still required to characterize the unique host cytosolic environment and understand what metabolic adaptations are necessary to live in this restrictive environment (Fig. 1). While historically *L. monocytogenes* metabolic adaptations have been studied in the context of nutrients required for replication, it is likely that metabolic adaptations are also necessary to facilitate stress responses as *L. monocytogenes* encounters cytosolic antimicrobial effectors such as ubiquicidin (Hiemstra *et al.*, 1999), interferon-inducible guanylate binding proteins (GBP) (Man *et al.*, 2016), lysozyme (Rae *et al.*, 2011) autophagy (Knodler and Celli, 2011) and other yet to be defined cell autonomous defenses. In the future, it will be important to clearly delineate functions of metabolism related to stress responses versus replication, what host cell processes impose these selective pressures and how the physiochemical, nutritional, and immunological characteristics of the cytosol differ between cell types.

Finally, infection by intracellular pathogens could impose a tremendous metabolic burden on the host cell, and it is likely that some pathogens, including *L. monocytogenes*, modulate their metabolism to minimize this impact and promote survival of the host cell (Gillmaier *et al.*, 2012; Kentner *et al.*, 2014). Furthermore, it is becoming increasing clear that host cells monitor metabolic activity such that both host and pathogen metabolites can be potent activators of the host immune system (Wynosky-Dolfi *et al.*, 2014; Sanman *et al.*, 2015; Gaudet *et al.*, 2015; Wolf *et al.*, 2016). This further highlights the need for pathogens to tightly regulate their metabolism to promote survival and replication without alerting the innate immune system to their presence, and points to our need to further understand

pathogen metabolism during infection as a potential option for therapeutic interventions. Using a combination of newly developed metabolomics, transcriptomics and genetic approaches we can start probing both the host and *L. monocytogenes* metabolism simultaneously during infection to get a systematic picture of host-pathogen interactions in the cytosol.

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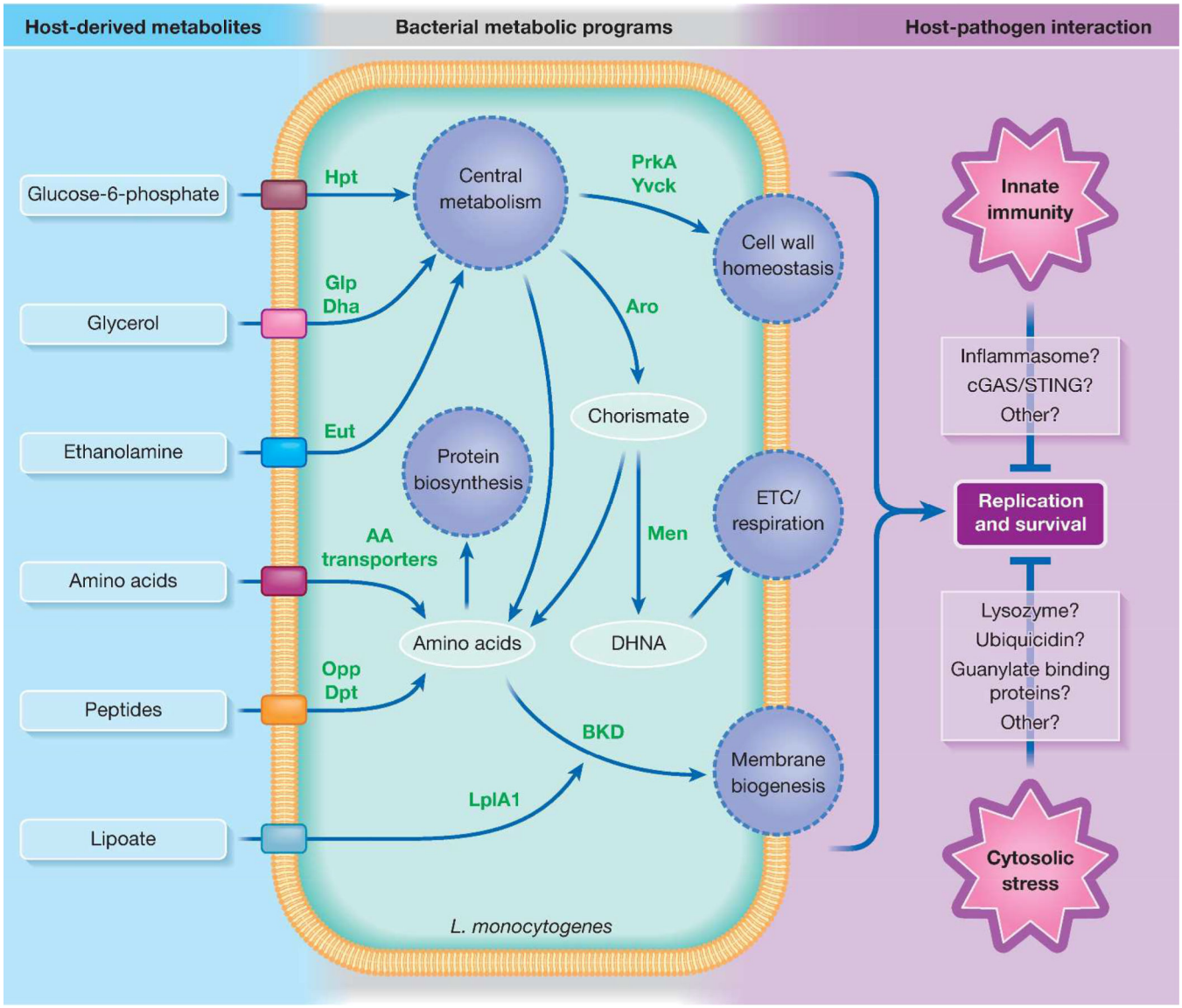

Fig 1.

Overview of select *L. monocytogenes* metabolic pathways that determine intracellular replication and survival. During colonization of host cells, *L. monocytogenes* transports host-derived metabolites through various nutrient transport systems. These metabolites are used by *L. monocytogenes* both for catabolic and anabolic pathways to replicate in the host cytosol and resist cytosolic stresses. Green text denotes *L. monocytogenes* enzymes crucial for intracellular replication and/or survival. Abbreviations: ETC - electron transport chain, DHNA - 1,4-dihydroxy-2-naphthoate, BKD - branched-chain α-keto acid dehydrogenase complex.