4
Metabolism and Physiology of *Listeria monocytogenes*

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Abstract: Compared to the rich wealth of knowledge concerning the molecular basis of *Listeria monocytogenes* virulence, little is known on the physiological background necessary for allowing this facultative intracellular human pathogen to survive and replicate in its natural surroundings, particularly in the host cell's cytosol. This cellular compartment appears to be the preferred site of replication, during a systemic infection caused by *L. monocytogenes*. Complementing earlier physiological studies, especially the more recent results obtained by comparative genomics, transcriptome, and proteome analyses, and by ^{13}C-isotopolog perturbation studies, allow us today to draw a first (although still rather incomplete) picture of how the metabolism of these bacteria may function to facilitate efficient growth under extra- and intracellular conditions. In this chapter, we concentrate on the carbon- and nitrogen-metabolism of *L. monocytogenes* as deduced from these studies. Although many carbon- and nitrogen-metabolic pathways of *L. monocytogenes* appear to be similar to those of the extensively studied *Bacillus subtilis*, which like *L. monocytogenes* belongs to the group of low G + C gram-positive (Gp) bacteria, there seem to be some profound differences that are essential for understanding the interplay of the listerial metabolism with that of the host cells and hence may have an important impact on listerial virulence.

4.1. Introduction

The genus *Listeria* to which the human pathogen *L. monocytogenes* belongs comprises six characterized heterotrophic species, including, besides *L. monocytogenes,* L. ivanovii, *L. innocua, L. welshimeri, L. seeligeri*, and *L. grayi*. With the exception of *L. ivanovii*, which is pathogenic to animals, all other *Listeria* species are harmless saprophytes occurring in nature at various sites (Farber and Peterkin, 1991; Gray and Killinger, 1966; Seeliger, 1984;

Seeliger and Jones, 1986). Earlier genetic and biochemical studies had already pointed to a close physiological relationship of the genus *Listeria* to that of other bacteria belonging to the group of Gp bacteria with low G+C genomic DNA, in particular to the members of the genus *Bacillus* (Collins et al., 1991). Recent comparative genome analyses including the genome sequences of other *Listeria* species show indeed a rather close genomic relationship of *L. monocytogenes* and the other *Listeria* species to *B. subtilis* (Glaser et al., 2001; Karlin et al., 2004) (see Chap. 3 in this book). However, unlike *B. subtilis*, *L. monocytogenes* does not grow readily in a defined mineral salt medium with glucose as sole carbon source, consequently growth of *L. monocytogenes* is routinely carried out in a rich brain–heart infusion (BHI) medium. Most molecular studies on virulence genes and virulence gene expression were performed in the past on *L. monocytogenes* cultivated in this complex medium. Several defined minimal media have been designed in the past (Friedman and Roessler, 1961; Jones et al., 1995; Phan-Thanh and Gormon, 1997; Premaratne et al., 1991; Tsai and Hodgson, 2003), which support the (often rather slow) growth of *L. monocytogenes*. These media contain a more or less complex mixture of amino acids and lipoate as essential additional substrates (O'Riordan et al., 2003), and growth efficiency in these media seems to depend to some extent on the *L. monocytogenes* strain used (Tsai and Hodgson, 2003). Our own studies using the sequenced *L. monocytogenes* EGDe strain show that in liquid culture with glucose as carbon source and glutamine as nitrogen source, a strict requirement for the amino acids cysteine, methionine, isoleucine, and leucine (or valine) and the vitamins, biotin, riboflavin, thiamine, and lipoate; growth is stimulated by the addition of arginine. These data already hint to a more complex metabolism of *L. monocytogenes* even under extracellular conditions which cannot be readily explained by the genome sequence of EGDe, which, for example, shows complete sets of genes for all enzymes of the branched chain amino acids (BCAA; Ile, Leu, and Val) of arginine-, cysteine-, and methionine pathways.

Suitable oligopeptides can be utilized by *L. monocytogenes* as source for essential amino acids (e.g., Val) as shown in the *L. monocytogenes* Scott A strain. Two different oligopeptide transporters were identified, one being driven by proton-motive force (PMF) and the other by ATP (Verheul et al., 1998). Furthermore, specific glycine- and proline-containing peptides were shown to stimulate growth at high osmolarity (Amezaga et al., 1995). Protein-bound lipoate is an essential cofactor of several dehydrogenase complexes, e.g., the pyruvate- and the 2-oxoglutarate dehydrogenases. *L. monocytogenes*, like several other bacteria, lacks the genes for the enzymes involved in lipoate biosynthesis and hence listerial growth depends on its external supply. *L. monocytogenes* possesses genes for two lipoate ligases which link lipoate to the dehydrogenases; their differential expression is essential for intracellular growth (O'Riordan et al., 2003) as discussed later. Among the other vitamins, riboflavin, thiamine, and biotin were also found to be essential for growth in minimal media (Tsai and Hodgson, 2003). *L. monocytogenes* is able to grow under aerobic and anaerobic

conditions (Gottschalk, 1986; Pine et al., 1989; Romick et al., 1996). Respiration seems to occur only aerobically, and the respiration chain contains menaquinone but not ubiquinone. Since biosynthesis of menaquinone depends on the common branch of the aromatic amino acids pathway, *L. monocytogenes* strains with mutations in this pathway switch to a predominantly anaerobic metabolism even in the presence of oxygen (Stritzker et al., 2004). When grown aerobically in the presence of glucose, *L. monocytogenes* secretes large amounts of acetoin (as overflow product) into the growth medium (Romick et al., 1996; Romick and Fleming, 1998), while lactate (together with acetate and other products) is the major fermentation product under anaerobic conditions. Under these conditions, the gene for pyruvate–formate lyase is highly induced (Joseph et al., 2006; Karlin et al., 2004), suggesting that mixed acid fermentation is the major mode of fermentation in *L. monocytogenes*.

4.2. Carbon Metabolism

4.2.1. The Use of Phosphotransferase System Carbohydrates

Glucose like many other sugars and sugar alcohols is taken up by bacteria via the phosphotransferase system (PTS; for a review on PTS-mediated sugar transport in Gp bacteria, see Reizer et al., 1988; Titgemeyer and Hillen, 2002; Vadeboncoeur et al., 2000).

Glucose and other PTS sugars such as fructose, mannose, and cellobiose are preferred carbon sources for *L. monocytogenes*, when growing in defined liquid minimal media (Tsai and Hodgson, 2003; our own unpublished results). The *L. monocytogenes* genome contains an unusually large number of genes (>40) encoding PTS*s* (Glaser et al., 2001). Among those, four PTS (determined by *lmo0096–0098, lmo0781–0784*, and *lmo1997–2002*) are specific for mannose transport, nine PTS for fructose (determined by *fruAB, lmo0021–0023, lmo0358, lmo0399–0400, lmo0426–0428, lmo0503, lmo0631–0633, lmo2135–2137*, and *lmo2733*), and seven PTS for cellobiose (*lmo0034, lmo0901, lmo1095, lmo2683–2685, lmo2708, lmo2762, 2763, 2765, lmo2780, 2782*, and 2783). Surprisingly, however, *ptsG* which encodes the PTS-dependent glucose transport in many low G+C Gp bacteria, including *B. subtilis* (Gonzy-Treboul et al., 1991), is incomplete in *L. monocytogenes*, and only the gene (*lmo1017*) for the EIIA component of the PTS-G system is present in *L. monocytogenes* and is not organized in an operon with *ptsH* and *ptsI* as in *B. subtilis*. Deletion of this gene does not affect the growth rate of *L. monocytogenes* in minimal media with glucose as carbon source suggesting that this residual part of *ptsG* is not involved in glucose uptake (Mertins et al., 2007). Although non-PTS glucose uptake driven by PMF has been previously suggested for *L. monocytogenes* (Christensen and Hutkins, 1994), a *ptsH* mutant which cannot produce a functional HPr

protein (essential for all PTS-dependent systems) is unable to grow in glucose-containing minimal medium (Mertins et al., 2007). These data clearly indicate that glucose transport in *L. monocytogenes* is predominantly, if not exclusively, PTS-mediated.

4.2.2. Glucose Catabolism

Listeria monocytogenes growing in complex media (e.g., BHI) catabolizes first glucose (and the other PTS sugars, which are all ultimately converted to glucose-6-phosphate) mainly by the glycolytic pathway. The principal glycolysis genes (*gap, pgk, tpi, pgm*, and *eno*) of *L. monocytogenes* as in most low G+C Gp bacteria belong to the predicted highly expressed genes (Karlin et al., 2004). In glucose-containing minimal medium, these genes are, however, down-regulated and genes of the pentose phosphate pathway (PPP) are induced when compared to BHI (Joseph et al., 2006), indicating the need of the oxidative decarboxylation of glucose by glucose-6-phosphate dehydrogenase (possibly for the production of CO_2—see below) and/or the generation of increased amounts of erythrose-4-phophate (for the biosynthesis of aromatic amino acids which are not present in the minimal medium). Interestingly, a similar down-regulation of most glycolysis genes and up-regulation of PPP genes are also observed when *L. monocytogenes* grows in the cytosol of mammalian host cells (Joseph et al., 2006).

Entry of pyruvate into the citrate cycle affords the oxidative decarboxylation to acetyl-CoA by the lipoate-dependent pyruvate—dehydrogenase; this step seems to be critical for intracellular *L. monocytogenes*, since a mutant defective in the lipoate ligase 1 (LplA1) is strongly impaired in intracellular growth (O'Riordan et al., 2003).

The citrate cycle of *L. monocytogenes* is interrupted due to the lack of 2-oxoglutarate dehydrogenase (Eisenreich et al., 2006; Trivett and Meyer, 1971). Hence oxaloacetate, which is essential for the entry of acetyl-CoA into the "cycle" leading to citrate and an important intermediate for the synthesis of Asp and other amino acids belonging to the Asp family, cannot be regenerated from citrate, and its synthesis becomes a crucial step in *L. monocytogenes* metabolism. As recently shown by ^{13}C-isotopolog perturbation studies with uniformly labeled ^{13}[C]glucose (Eisenreich et al., 2006), oxaloacetate is mainly produced by carboxylation of pyruvate catalyzed by pyruvate carboxylase (determined by *pycA*, while a gene for PEP-carboxylase seems to be missing in *L. monocytogenes*). Oxaloacetate is probably converted into malonate and succinate by the reducing branch of the citrate cycle for the generation of these important intermediates. Thus, for the generation of oxaloacetate by pyruvate carboxylase, CO_2 is an essential substrate, and we suggest that the observed induced oxidative decarboxylation of glucose-6-phosphate (first step in the PPP) may be therefore required for growth of *L. monocytogenes* in glucose-containing minimal medium. The special role of CO_2 for growth of *L. monocytogenes* and *Yersinia pseudotuberculosis* was pointed out earlier (Buzolyova and

Somov, 1999). This CO_2 requirement may also explain the inability of *L. monocytogenes* to grow in minimal media with pentoses like ribose or rhamnose as carbon sources, although fermentation of rhamnose is observed in rich media (Groves and Welshimer, 1977).

4.2.3. Nonphosphotransferase System Carbon Sources

A *ptsH* mutant of *L. monocytogenes* (unable to metabolize PTS sugars) is able to grow in BHI medium, albeit at a reduced growth rate (Mertins et al., 2007), suggesting that other C-components present in BHI besides PTS sugars can serve as efficient carbon sources. BHI is a rich medium containing many poorly defined C-components which may even vary in composition from batch to batch. It contains probably many peptides, and efficient transporters for oligopeptides were identified in *L. monocytogenes* (Verheul et al., 1998). However, based on previous studies (Premaratne et al., 1991; Tsai and Hodgson, 2003), *L. monocytogenes* fails to grow with casamino acids suggesting that amino acid catabolic pathways may not occur in this microorganism, and hence, amino acids probably cannot serve as sole carbon sources.

Listeria monocytogenes is, however, able to grow on phosphorylated hexoses, like glucose-1(6)-phosphate and fructose-6-phosphate (but again not on the phosphorylated C5 sugars, like ribose-5-phosphate or xylose-5-phosphate). A gene (*hpt or uhpT*) for a special transporter for phosphorylated hexoses has been identified in *L. monocytogenes* (Chico-Calero et al., 2002). This listerial Hpt transporter is highly homologous to a similar sugar transporter in *E. coli* (UhpT) (Weston and Kadner, 1988) and is under the control of PrfA, the central virulence regulator of *L. monocytogenes* (see Chap. 7), and all PrfA-dependent genes including *hpt* are highly up-regulated when *L. monocytogenes* replicates in the cytosol of mammalian host cells (Joseph et al., 2006). Mutants lacking *hpt* are less efficient in replication in host cell's cytosol (Chico-Calero et al., 2002), suggesting that phosphorylated hexoses, presumably mainly glucose-6-phosphate but possibly also glucose-1-phosphate (deriving from cellular glycogen—M. Beck, 2005, personal communication) are major carbon sources in mammalian cells. This assumption is supported by the above mentioned highly induced expression of the *hpt* gene in the host cell's cytosol and the strong activation of the *hpt* promoter in this cellular compartment (S. Pilgrim, personal communication). It is also possible that the listerial Hpt can also transport ribose-5-phospate as shown for the highly related Hpt of *E. coli*; however, in contrast to *E. coli* which can grow on this carbon source, *L. monocytogenes* is unable to use ribose-5-phosphate as a sole carbon source. Glycerol can also replace glucose in defined minimal media (Tsai and Hodgson, 2003); our own unpublished observation). Glycerol, probably taken up by *L. monocytogenes* as by most bacteria via facilitated transport (Heller et al., 1980; Lin, 1976), is phosphorylated by glycerol kinases and oxidized by glycerol-3-phosphate dehydrogenase to glyceraldehyde-3-phosphate which can be further metabolized by the enzymes of the glycolytic pathway. Two glycerol kinase genes (*lmo1034*

and *lmo1538*) were identified in *L. monocytogenes*. These two genes as well the glycerol-3-phosphate dehydrogenase are significantly induced not only in *L. monocytogenes* growing in minimal media with glycerol as carbon source but also upon growth within the host cell's cytosol, suggesting that glycerol is a possible carbon source for *L. monocytogenes* metabolism inside the host cells. This assumption is supported by the observation that mutants blocked in both glycerol kinase genes and the glycerol dehydrogenase gene are impaired in cytosolic growth (Joseph et al., 2006). One possible source for the supply of glycerol by the host cell may be phospholipids which by degradation via the listerial phospholipase C (PlcB) together with cellular A-type lipases can yield glycerol, fatty acids, and ethanolamine (or choline). Expression of *plcB* is highly induced in the host cell's cytosol (Joseph et al., 2006; Klarsfeld et al., 1994), and phospholipids as substrates may arise by the disruption of the primary phagosome by which *L. monocytogenes* is taken up in the host cells. In this context, it is interesting to note that the genes for ethanolamine–ammonia lyase which convert ethanolamine into ammonia (which may serve as nitrogen source—see later) and acetyl-CoA are also highly induced in cytosolically growing *L. monocytogenes*. Vitamin B_{12} is required for this reaction (Roof and Roth, 1988, 1989) and interestingly, the genes for cobalamin biosynthesis are also induced in cytosolically growing *L. monocytogenes* (Joseph et al., 2006).

Acetyl-CoA alone cannot be used as a sole carbon source by *L. monocytogenes* since the genes of the glyoxlyate shunt are missing in *L. monocytogenes* (Glaser et al., 2001), a fact that also rules out the utilization of fatty acids, which were shown to be important intracellular carbon sources for *Mycobacterium tuberculosis* and *Salmonella typhimurium* (Fang et al., 2005; McKinney et al., 2000).

4.2.4. Catabolite Repression and Its Impact on PrfA-Dependent Virulence Gene Expression

Previous studies have repeatedly shown that sugars that can be used by *L. monocytogenes* as carbon source, like glucose, fructose, mannose, and cellobiose, have an inhibitory effect on PrfA activity and hence the PrfA-dependent gene expression (Behari and Youngman, 1998b; Milenbachs et al., 1997; Milenbachs et al., 2004). The strongest inhibition is exerted by cellobiose. These sugars are taken up by PTS-mediated transport and result ultimately in the conversion to glucose-6-phosphate and in catabolite repression of many genes and operons in *L. monocytogenes*. The inhibition of PrfA activity by these sugars thus suggests that PrfA may interact either with components involved in carbon catabolite repression (CCR) or with PTS-mediated sugar transport or with both.

The mechanism of CCR control in Gp bacteria of low G+C content (Figure 4.1) depends on the regulator protein CcpA (*c*atabolite *c*ontrol *p*rotein *A*), a member of the LacI/GalR family of bacterial regulatory proteins, which affects the expression of genes containing a *c*atabolite-*r*esponsive *e*lement (CRE-box)

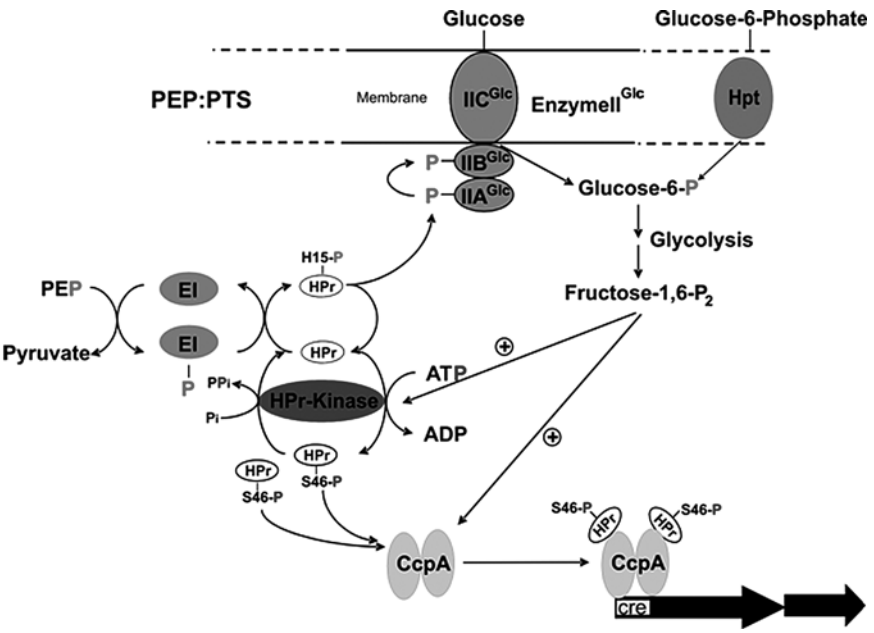

FIGURE 4.1. Schematic representation of CCR in low G+C gram-positive bacteria. *PEP* phosphoenolpyruvate; *PTS* phosphotransferase system; *Hpt* hexose phosphate transporter; *EI*: enzyme I; Enzyme II^{Glc} *A B C* glucose-specific enzyme II A, B, and C; *HPr* PTS phosphocarrier protein HPr; *CcpA* catabolite control protein A; *CRE c*atabolite-*r*esponsive *e*lement; *H*15-*P* HPr phosphorylated at His 15; *S*46-*P* HPr phosphorylated at Ser 46; *ATP* adenosine triphosphate; *ADP* adenosine diphosphate; *PP$_i$*: pyrophosphate; *fructose-1,6-P$_2$* fructose-1,6-bisphosphate.

near their regulatory region (about 200 genes in *B. subtilis*) (Hueck and Hillen, 1995; Weickert and Chambliss, 1990). The CcpA activity in itself is dependent on different cofactors, which leads to different modes of gene regulation (Blencke et al., 2003; Gosseringer et al., 1997; Moreno et al., 2001). The major cofactor of CcpA is HPr phosphorylated at position Ser-46. HPr, a component of the general PTS pathway, is phosphorylated at His-24 during PTS sugar transport. This phosphate is transferred to EIIA and further to EIIB of the sugar-specific permeases. During active glycolysis, HPr also becomes phosphorylated at Ser-46 by a specific ATP-dependent HPr-kinase/phosphorylase (HPrK/P) (Reizer et al., 1998). This HPr phosphorylation is stimulated by the intermediates of the glycolytic pathway, especially by fructose-1,6-bisphosphate (FBP), while free phosphate stimulates the phosphorylase activity of HPrK/P (Dossonnet et al., 2000; Galinier et al., 1998; Reizer et al., 1988). HPr-Ser46-P associated with CcpA binds to the CRE sites of CCR-controlled genes and leads to the repression of these genes (type I CcpA-controlled genes) (Deutscher et al., 1995; Jones et al., 1997) as depicted in Figure 4.1.

These genes are therefore up-regulated in *ccpA*- and *HPrK*-deficient mutants (Blencke et al., 2003; Moreno et al., 2001).

A *ccpA* mutant also shows impaired glucose transport and down-regulation of the transcription of several genes that are essential for the C- and the N-metabolism (Blencke et al., 2003). Typical genes of this group (class II CcpA-dependent genes) are involved in the biosynthesis of the BCAA (Ile, Leu, Val) pathway, in glycolysis (*gapA* operon) and glutamate synthesis (*gltAB*). PTS-mediated glucose uptake requires HPr phosphorylation at His15 which is catalyzed by enzyme I in the presence of PEP. EIIA phosphorylated by HPr-His15-P is also involved in other regulatory functions (Titgemeyer and Hillen, 2002). During glucose starvation and by increased inorganic phosphate concentration and low concentrations of glycolytic intermediates, HPr-Ser46-P is dephosphorylated (Fieulaine et al., 2002).

The genomes of *L. monocytogenes* and *L. innocua* contain genes for orthologs of all components involved in CCR of *B. subtilis* (with the exception of *crh* Glaser et al., 2001), suggesting a similar CCR control mechanism in *Listeria* as in *B. subtilis*. This assumption is supported by biochemical and genetic studies on CcpA, HPr, and HPrK/P from *L. monocytogenes* (Behari and Youngman, 1998a; Christensen and Hutkins, 1994; Mertins et al., 2007). CRE sequences highly similar to those of *B. subtilis* were identified in direct proximity to genes and operons which by analogy with the *B. subtilis* counterparts are expected to be under CRR control (Andersson et al., 2005; Joseph et al., 2006). A direct CCR control of PrfA and PrfA-regulated genes and the possible interaction of CcpA with PrfA protein can be ruled out, since a *ccpA* mutant has little effect on *prfA* expression and PrfA activity (Mertins et al., 2007). Recent data (Marr et al., 2006) show, however, that overexpression of PrfA leads to a highly significant growth inhibition of *L. monocytogenes* in glucose-containing media, which seems to be caused by inhibition of PTS-mediated glucose uptake, suggesting that PrfA may interact with components of the PTS-mediated sugar transport rather than with CCR.

4.3. Anabolic Pathways

The genome sequence of *L. monocytogenes* contains all genes for the amino acid-, purine-, pyrimidine-, and several vitamin biosynthetic-pathways (those for biotin, riboflavin, thiamine, and lipoate are absent). The gene for the last enzyme in serine biosynthesis (serine–phosphate phosphatase) has not been annotated in the genome sequence, but all growth studies clearly show that *L. monocytogenes* is not auxotrophic for serine. Surprisingly, previous studies (Phan-Thanh and Gormon, 1997; Premaratne et al., 1991) indicated the requirement of the BCAA (Ile, Val, and Leu) as well as cysteine, methionine, and arginine when *L. monocytogenes* was grown in defined minimal media with glucose as carbon and energy source.

Listeria monocytogenes lacks sulphate and nitrate reductases and hence is dependent on reduced N and S sources, which readily explains the growth requirement for cysteine and methionine. In the presence of cysteine, methionine can be biosynthesized de novo albeit at low rate (J. Slaghuis, personal communication). The ability of *L. monocytogenes* to biosynthesize Arg has been also demonstrated (Tsai and Hodgson, 2003; our own unpublished data), but again, addition of Arg to the minimal medium clearly enhances the growth rate. In the absence of Ile, the growth rate is very low in this minimal medium, and addition of Ile together with one of the other two BCAA (Leu or Val) is required to obtain efficient growth of *L. monocytogenes* in this culture medium. Our recently performed ^{13}[C]-isotopolog perturbation studies (Eisenreich et al., 2006) using uniformly labeled ^{13}[C]glucose show low-level biosynthesis of all three BCAA, even in the presence of externally added BCAA. This synthesis (especially that of Ile) is significantly enhanced in the presence of high PrfA concentration, which as discussed above reduces PTS-mediated glucose uptake and hence may inhibit PMF-dependent BCAA transport. These data clearly show that the biosynthesis of Ile, Leu, and Val is functional, but its efficacy is low in glucose-containing minimal medium.

The BCAA are indicators of the general nutritional status of the bacterial cell because their synthesis depends on several basic catabolic precursors (oxaloacetate, pyruvate, and acetyl-CoA), and hence the rate of BCAA synthesis is an important factor in the overall bacterial physiology. In *B. subtilis*, the central BCAA biosynthesis operon (*ilvB*) is under complex control of the global regulators CcpA, CodY, and TnrA (Shivers and Sonenshein, 2005; Tojo et al., 2005) which also regulate many genes that respond to nutrient availability and growth rate (Molle et al., 2003). All three regulators have binding sites in the *ilvB* regulatory region. CcpA binding to a CRE site within this region activates the transcription starting at the *ilvB* promoter. CodY interacts directly with Ile and GTP, and these two components (indicators of efficient growth and high energy level in the bacterial cell) act as independent corepressors for CodY (Shivers and Sonenshein, 2005).

Listeria monocytogenes contains orthologous genes for CcpA, CodY, and GlnR (highly similar to TnrA), and it is therefore likely that similar control mechanisms may act in *L. monocytogenes* as in *B. subtilis*. The low rate of BCAA biosynthesis in glucose-containing minimal medium may therefore reflect the shortage of necessary catabolic intermediates (especially oxaloacetate) due to the interrupted citrate cycle.

Previous studies indicated that *L. monocytogenes* mutants auxotrophic for some amino acids, like Phe, Gly, and Pro, replicated within host cells like the parental *L. monocytogenes* strain, while a mutant deficient in all three aromatic amino acids was impaired in intracellular replication and virulence (Marquis et al., 1993). Similar results were obtained in our recent investigation with *aro* mutants which are defective in the basic pathway of all aromatic components. These mutants—in addition to exhibiting impaired cytosolic replication, cell-to-cell-spreading, and virulence—showed a predominantly anaerobic

metabolism (Stritzker et al., 2004). The reason for this more unexpected result is apparently the lack of synthesis of menaquinone which is the only quinone produced by *L. monocytogenes* and hence its absence strongly impairs aerobic respiration.

Menaquinone biosynthesis involves the condensation of 1,4-dihydroxy-2-naphthoate with polyprenyl-PP which is produced by the isoprenoid biosynthesis pathway. The precursor of all isoprenoids, isopentenyl-PP, is biosynthesized either by the classical mevalonate or by the alternative 2-C-methyl-D-erythritol-4-phosphate (via gyceraldehyde-3-P and pyruvate) pathway. Interestingly, the *L. monocytogenes* genome carries the information for both pathways and both seem to be functional (Begley et al., 2004). It remains to be seen whether there is a preferential activation of one of the pathways when *L. monocytogenes* replicates inside host cells.

Comparative transcript profiling using RNA from extra- and intracellularly grown *L. monocytogenes* shows highly significant up-regulation of the genes for the biosynthesis of all essential amino acids; in particular, the aromatic amino acids and the three BCAA but not the nonessential ones (Joseph et al., 2006), suggesting that the latter ones are provided by the host cell. Strong up-regulation is also observed for the genes involved in purine and pyrimidine biosynthesis, in accordance with previous data (Klarsfeld et al., 1994; Marquis et al., 1993) indicating that nucleotides are not efficiently provided by the host cell to intracellularly growing *L. monocytogenes*.

4.4. Nitrogen Metabolism

For most bacteria including *L. monocytogenes*, glutamine (Gln) is the optimal nitrogen source (Merrick and Edwards, 1995), but in the absence of Gln, *L. monocytogenes* is capable of utilizing alternative nitrogen sources, such as ammonium (Tsai and Hodgson, 2003), arginine, and even ethanolamine (our own unpublished results). These latter N sources might become important, particularly when *L. monocytogenes* replicates in mammalian host cells where the supply of free Gln is limited and its consumption by the intracellular *L. monocytogenes* may strongly impair the "host function" of the invaded cell for the intracellular *L. monocytogenes*. Gln as primary nitrogen source is converted to Glu—the major donor of nitrogen for amino acids and nucleotides—by glutamate synthetase (GOGAT) with 2-oxoglutarate (OG) as additional substrate. The cellular level of Gln, Glu, and OG is stringently controlled in *E. coli* on the transcription—and the glutamine synthetase (GS) activity—level by uridinylation/deuridinylation of the PII protein and by the two-component system NtrB/NtrC (Arcondeguy et al., 2001). Unlike in *E. coli*, the activity of GS of low G + C Gp bacteria (studied extensively in *B. subtilis*) is not modulated by covalent modification, and the global NtrB/C regulatory system is absent here. Synthesis of GS is regulated by the repressor GlnR, by the global regulator TnrA, and possibly by other transcription regulators.

Ammonium as an alternative nitrogen source is transported in *B. subtilis* at low external concentration (at high concentration uptake occurs by diffusion or facilitated transport) by the transporter NrgA which is encoded by the *ngrAB* operon (Detsch and Stulke, 2003), and transcription of the *ngrAB* promoter is activated during nitrogen-limited growth by the global regulator TnrA (Wray et al., 1996; Yoshida et al., 2003). Ammonium is then channeled into glutamine and further to glutamate via glutamine- and glutamate-synthetases as described above. The product of the second gene of the *nrgAB* operon, NrgB belongs to the PII family of regulatory proteins, but there is no indication that NrgB is covalently modified in *B. subtilis*. It is rather believed that NrgB transforms the information of cellular ammonium concentrations by fine-tuning downstream regulatory factors essential for the expression of glutamine- and glutamate-synthetases (Fisher, 1999). Orthologous genes for these regulators of nitrogen metabolism were also identified in the *L. monocytogenes* genome suggesting a similar mode of nitrogen control in *L. monocytogenes* as in *B. subtilis*.

The *nrgAB* operon (*lmo1516–1517*) in *L. monocytogenes* is up-regulated under all conditions which lead to the up-regulation of *glnAR* (operon for GS and the repressor) and *gltAB* (encoding the GOGAT subunits). Up-regulation of these genes is also observed in cytosolically replicating *L. monocytogenes* (Joseph et al., 2006), suggesting that ammonium rather than glutamine might be the major nitrogen source within mammalian host cells.

Ammonium could be provided under the intracellular conditions by excess host cell arginine (normally removed via the urea cycle) or ethanolamine (deriving from phosphatidylethanolamine (PEA)). Indeed the induction of *arpJ*, a gene encoding a specific arginine ABC transporter, has been shown in cytosolically replicating *L. monocytogenes* (Joseph et al., 2006; Klarsfeld et al., 1994). The listerial arginine deiminase (*lmo0043—arcA*) could then degrade this arginine into ammonia and citrulline, two substrates which could serve as nitrogen sources as citrulline can be further converted into another ammonia molecule (together with CO_2 and ATP) and ornithine via the enzymes ornithine carbamoyl transferase and carbamoyl carboxy kinase encoded by the *L. monocytogenes*-specific *arcBCD* operon (*lmo0036-0039*). The possible involvement of the *arcA-D* genes in intracellular ammonium supply does not rule out the participation of these genes in acid resistance of *L. monocytogenes* as well, as recently suggested (Gahan and Hill, 2005). The excess arginine of the host cell would be otherwise removed from the host cell by arginase-catalyzed degradation to ornithine and urea. This urea could not be utilized as nitrogen source by *L. monocytogenes* due to the absence of a listerial urease.

Another possible intracellular nitrogen source provided by the host cell could be ethanolamine generated by degradation of PEA. PEA is an excellent substrate for PlcB, a listerial PlcB encoded by the PrfA-dependent gene *plcB* (Goldfine et al., 1993) that is highly up-regulated inside host cells. Hydrolysis of ethanolamine into ammonia and acetaldehyde occurs by the vitamin B_{12}-dependent ethanolamine–ammonia lyase (Bradbeer, 1965) encoded by the *eutBC* genes. These genes have been shown in *Salmonella enterica* serovar

Typhimurium (Kofoid et al., 1999) to be part of a 55-kb locus which in addition to *eutB* and *eutC* (the genes for the two subunits of the lyase) carry 15 genes that encode a positive regulator (necessary for the induction of the *eut* operon together with B12), enzymes for propandiol degradation, and shell proteins for the carboxysome. A similar gene cluster showing high homology to the *Salmonella eut* operon has been identified in *L. monocytogenes*, and may have been introduced by lateral gene transfer as pointed out previously (Buchrieser et al., 2003). Several genes of the *eut* gene cluster, particularly *eutBC*, but also those for cobalamine synthesis, are highly up-regulated in *L. monocytogenes* replicating inside mammalian host cells (Joseph et al., 2006). Our recent unpublished data indicate that ethanolamine can function as a sole nitrogen source in a minimal medium with glycerol as carbon source, whereas an *eutBC* deletion mutant cannot grow under these conditions.

4.5. Conclusions

Bioinformatic and functional genomic data indicate that *L. monocytogenes* is a heterotrophic and largely prototrophic bacterium belonging to the group of low G + C Gp bacteria. But its metabolism is also optimally adapted for highly efficient growth within the cytosol of many mammalian cells (Goetz et al., 2001). The metabolism of *L. monocytogenes* reveals some unusual features which seem to have profound consequences for extra- and intracellular replication of *L. monocytogenes* and hence for virulence:

1 the inability to use oxidized sulfur and nitrogen sources due to the lack of nitrate and sulfate reductases,
2 the interrupted citrate cycle due to the lack of 2-oxoglutarate dehydrogenase, and
3 the complex PTS-mediated glucose transport.

(1) This feature readily explains the observed auxotrophy of *L. monocytogenes* for Cys (and in the absence of Cys for Met) and indicates that intracellularly replicating *L. monocytogenes* will entirely depend on the host cell for Cys supply. Cystein is a nonessential amino acid for mammalian cells whereas methionine is an essential one. In *L. monocytogenes*, the situation is reverse and the two "partners" could therefore provide each other with the necessary sulphur-containing amino acids.

(2) The incomplete citrate cycle renders the synthesis of oxaloacetate by pyruvate carboxylase to be a critical metabolic step when *L. monocytogenes* grows in environments where glucose (or another carbohydrate) is the sole carbon source. Shortage of this catabolic intermediate (and as a consequence also pyruvate and acetyl-CoA) may be the reason for the unexpected dependency of *L. monocytogenes* on BCAA (Ile and Leu or Val), Met and to a lesser extent Arg for efficient growth in the minimal media containing glucose as a sole carbon

source. Although the biosynthetic pathways for these amino acids are functionally intact, their efficient synthesis depends directly or indirectly on the availability of oxaloacetate. In fact, a *B. subtilis* mutant deficient in 2-oxoglutarate dehydrogenase required Asp (which derives directly from oxaloacetate and represents an important intermediate for the biosynthesis of these amino acids) for growth at wild-type rates in minimal media due to the inability of the mutant to regenerate oxaloacetate from citrate (Fisher and Magasanik, 1984). The syntheses of these amino acids which are needed for protein synthesis and branched-chain fatty acids (especially Ile) (Nichols et al., 2002) in large amounts depend on oxaloacetate (via Asp), pyruvate (needed for synthesis of oxaloacetate), or actyl-CoA (deriving from pyruvate). As mentioned above (see discussion on the ilv-leu operon), nitrogen metabolism and carbon metabolism are coregulated by the global regulators CcpA, TnrA, and CodY (Shivers and Sonenshein, 2005; Tojo et al., 2005). This regulatory network (best studied in *B. subtilis*) guarantees well-balanced intracellular concentrations of the central carbon (especially glucose and its catabolites) and nitrogen (especially Gln, Glu) intermediates essential for the entire cellular metabolism.

The inability of *L. monocytogenes* to regenerate oxaloacetate from citrate may be overcome within the host cell by the supply of malonate. This intermediate, which is directly converted to oxaloacetate in the citrate cycle, could be provided by the host cell via the oxoglutarate/malate shuttle at the expense of the oxoglutarate produced in excess by *L. monocytogenes* due to the absence of oxoglutarate dehydrogenase. It has been shown that a *B. subtilis* mutant deficient in this enzyme secretes considerably larger amounts of OG into the medium than the wild-type strain (Fisher and Magasanik, 1984).

(3) The most important feature is, however, the use of the appropriate carbon source by *L. monocytogenes* within the host cell. The transport of glucose, a preferred carbon source for *L. monocytogenes* metabolism, is achieved in a yet unknown way. Although a large number of (in part *L. monocytogenes* specific) PTS has been identified in the *L. monocytogenes* genome, a functional PTS-G glucose uptake system (characteristic for many bacteria) is missing and glucose may be cotransported by several other PTS permeases (R. Ecke, personal communication).

Uptake of glucose-1-P generated by degradation of host cell's glycogen (a nonessential storage product of the host cell) by the specific transporter (Hpt) avoids the competition for glucose with the host cell, and at the same time, the inactivation of PrfA by PTS-mediated sugar uptake. The efficiency of intracellular replication and virulence may be therefore strongly influenced by the carbon source and its transport.

Phospholipids may act as alternative intracellular carbon source for *L. monocytogenes* (and may even become the primary carbon source in mammalian cells lacking glycogen). Phospholipids are probably generated in sufficient amounts by the disruption of the primary phagosome by which *L. monocytogenes* is internalized. In particular PEA may serve as an important carbon and nitrogen source since it can be converted by cellular lipases of the A-type and especially

the listerial PlcB to glycerol, fatty acids, and ethanolamine phosphate, which after dephosphorylation can serve as nitrogen source for *L. monocytogenes* in presence of glycerol as carbon source (Schaffer et al., unpublished results). Unlike *M. tuberculosis* and S. *enterica* which use the fatty acids as major intracellular carbon sources (Fang et al., 2005; McKinney et al., 2000), *L. monocytogenes* could utilize mainly glycerol and ethanolamine as intracellular nutrients.

We hypothesize that these three metabolic features, although unfavourable for growth of *L. monocytogenes* under certain extracellular conditions, are essential for the efficient intracellular replication of *L. monocytogenes* since they lead to an intimate interference between the metabolism of the bacterium and that of the host cell. This metabolic interference will allow an extended survival of the infected cell, which can then serve as a "host cell" for *L. monocytogenes* for a longer period of time.

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