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# Adaptation of *Listeria weihenstephanensis* and *Listeria monocytogenes* to anaerobiosis

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*in memory of my beloved dad*

*&*

*for my mom*

*“Life is not easy for any of us. But what of that? We must have perseverance and above all confidence in ourselves. We must believe that we are gifted for something and that this thing, at whatever cost, must be attained.” -Marie Curie-*

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## I. SUMMARY

In the current study, the species specific adaptation of the not pathogenic *L. weihenstephanensis* and the pathogenic *L. monocytogenes* EGDe to oxygen availability was investigated with respect to a temperature-dependent influence.

As key prerequisite for the transcriptional profiling, the genome sequencing of *L. weihenstephanensis* was performed and the complete genome sequence was obtained. Global transcriptional analyses, via next generation RNA sequencing, were performed for cells grown aerobically and anaerobically at an environmental temperature (18°C) and a temperature close to the mammalian gut condition (34°). The oxygen dependent gene expression of selected genes was further validated via qPCR. For *L. weihenstephanensis*, 44 genes were found to be stronger transcribed aerobically while 35 genes were found to be stronger transcribed anaerobically at 18 °C. At 34 °C, 40 genes exhibited aerobically a stronger transcription while transcription of 23 genes was induced anaerobically. Overall, the most interesting outcome was the observation that *L. weihenstephanensis* is able to perform anaerobic respiration to support its survival under hypoxia, in contrast to members of *Listeria sensu stricto* clade, which do not possess genes encoding nitrate and nitrite reductases.

*L. monocytogenes* EGDe showed up-regulation of 136 genes aerobically and up-regulated of 81 genes anaerobically at 18 °C. At 34 °C, 48 genes were aerobically stronger transcribed, while transcription of 9 genes was promoted anaerobically. Among these, genes encoding for key enzymes of the central catabolic pathway of *L. monocytogenes* were identified and further analyzed by construction and characterization of their correspondent deletion mutants. Acetate kinase and pyruvate dehydrogenase were identified as key enzymes for *L. monocytogenes* catabolism since the deletion of the genes encoding for these proteins were deleterious and could not be completely compensated by other pathways.

*L. monocytogenes* activates a temperature-dependent adaptation to anaerobiosis, which might be important for the optimal adaptation to the host envoronment at 34°C and for the utilization of alternative carbon and nitrogen source at 18°C. Temperature is not supposed to be such an important environmental trigger in the not pathogenic *L. weihenstephanensis,* which, as environmental species, does not colonize the host

GI. Therefore, is not surprising that in this organism temperature did not significantly influence the oxygen-dependent regulation.

Furthermore, to investigate a possible contribution of strain-specific adaptations to anaerobiosis of *L. monocytogenes* to difference in the virulence, different *L. monocytogenes* strains with different virulence potential (EGD, EGDe, ScottA and 1670) were selected and their transcriptional profiling was determined via RNA-Next Generation Sequencing (NGS) under aerobic and anaerobic conditions at 37°C. Genes encoding for proteins already described to be important for anaerobic adaptation were regulated in at least three strains. Interestingly, ten genes encoding for proteins involved in flagellar biosynthesis were significantly down-regulated anaerobically in *L. monocytogenes* 1670 and slightly in ScottA, while in EGD and EGDe flagella genes were not expressed at all. Interestingly, further analyses via motility assays showed anaerobic swarming only for the outbreak strain ScottA incubated at 37°C after cultivation at 24°C. This motility under anaerobic conditions at 37°C, even if less pronounced than under aerobic conditions or at 24°C, suggests a role of flagella during the initial stage of infection in attaching to the host cells and a possible link to the virulence of this outbreak strain.

# II. INTRODUCTION

## 1. The genus *Listeria*

*Listeria* are Gram-positive, facultative anaerobic, non-spore-forming, rod-shaped bacteria, 0.5 µm in width and 1.0-1.5 µm in length, with a low G+C (36-42%) content (McLauchlin and Rees, 2009), which can be isolated from a variety of environments, including soil, water, wastewater, sludge, feces, silage, food and food processing environments (Linke *et al.*, 2014; O´Connor *et al.*, 2010; Paillard *et al.*, 2005; Vilar *et al.*, 2007).

The genus *Listeria* currently contains 17 species, namely *L. monocytogenes* (Pirie, 1940), *L. grayi* (Errebo Larsen and Seeliger, 1966). *L. innocua* (Seeliger, 1981), *L. seeligeri* (Rocourt and Grimont, 1983), *L. welshimeri* (Rocourt and Grimont, 1983), *L. ivanovii* (Seeliger *et al.* 1984), comprising the subspecies *L. ivanovii* subsp. *ivanovii* and *L. ivanovii* subsp. *londoniensis (*Boerlin *et al.,* 1992), *L. marthii* (Graves *et al.* 2010), *L. rocourtiae* (Leclercq *et al.,* 2010), *L. fleischmanii* (Bertsch *et al.,* 2013), comprising the subspecies *L. fleischmannii* subsp. *coloradonensis* and *L. fleischmannii* subsp. *fleischmannii* (den Bakker *et al.* 2013), *L. weihenstephanensis* (Lang-Halter *et al*., 2013)*,* and seven recently described *Listeria* species, namely *L. floridensis*, *L. aquatica*, *L. cornellensis*, *L. riparia, L. grandensis* (den Bakker *et al.,* 2014), *L. newyorkensis* and *L. booriae* (Weller *et al*., 2015). Besides *L. grayi*, all the *Listeria* spp. described till 2010 belong to the *Listeria sensu stricto* phylogenetic group. This phylogenetic clade includes the two pathogenic species, *L. monocytogenes* and *L. ivanovii.* While *L. ivanovii* is an animal pathogen (Gill *et al*., 1997; Chand and Sadana, 1999) and rarely occurs in humans (Cummins *et al.*, 1994; Guillet *et al.*, 2010; Snapir *et al.*, 2006), *L. monocytogenes* infects both humans and animals and is the causative agent of the relatively rare bacterial disease, listeriosis. Both pathogens must withstand microaerophilic or anaerobic conditions during the passage through the gastrointestinal tract during the infection cycle. However, like the nonpathogenic species, they might also encounter microaerophilic or anaerobic conditions in environmental niches.

## 2. *L. weihenstephanensis*

*L. weihenstephanensis* is an environmental species of *Listeria*, isolated from the water plant *Lemna trisulca* from a fresh water pond in Bavaria, Germany. The isolate

is non-hemolytic and has not been associated with animal or human diseases. *L. weihenstephanensis* has an optimal growth at pH 7-8 and temperature at 28-34°C, and is unable to proliferate at mammalian body temperature of 37°C (Lang-Halter *et al.*, 2013). The complete genome sequence is not available, only an assembly of a shotgun sequence (71 contigs) was published in January 2014 (Cornell University, GenBank: AODJ00000000.1), which was annotated in 2015 (NCBI Reference Sequence: NZ_AODJ00000000.1).

## 3. *L. monocytogenes*

*L. monocytogenes* is an osmotolerant and pathogenic Gram-positive bacterium. It is a facultative anaerobe, non-sporulating germ with a low G+C (~ 40%) content. It can be isolated from natural environment such as fresh water, soil, faeces, vegetables, food (especially read-to-eat food), fruit and mud (Gravani, 1999, McLauchlin and Rees, 2009). Its ability to grow at temperatures between -1.5°C and 45.0°C and pH between 4.0 and 9.6 (Lado and Yousef, 2007) is a constant challenge for food preservation (reviewed in Farber and Peterkin, 1991). It is a saprophyte in soil and decaying vegetation but it can also exist as an intracellular bacterial pathogen which is capable of causing a serious infection in humans and in many animal species (Gray *et al*., 2006). *L. monocytogenes* outbreaks have been associated with the consumption of a variety of foods including milk and dairy products, ready-to-eat (RTE) beef, pork, sausages and fish, as well as cabbage, radishes, pâté and soft cheeses (reviewed in Farber and Peterkin, 1991).

### 3.1 Infection with *L. monocytogenes*

#### 3.1.1 Human listeriosis

*L. monocytogenes* infects both humans and animals and is known as an important opportunistic human foodborne pathogen, which causes, after ingestion of contaminated food, human listeriosis (reviewed in Allerberger and Wagner, 2010). There are two forms of illness associated with a *L. monocytogenes* infection, invasive (severe form) and non-invasive (mild form, also called listerial gastroenteritis or febrile listeriosis) listeriosis. Depending on host susceptibility, number of organisms ingested and virulence of the infecting strain, one of the two forms develops. Non-invasive listeriosis has an incubation time of six hours (h)-10 days (Ooi and Lorber, 2005) and the main symptoms are fever, diarrhea, muscle

aches, nausea, vomiting, sleepiness and tiredness (Salamina *et al.*, 1996; Sim *et al.*, 2002). Invasive listeriosis is characterized by an infection of blood, fluid of the central nervous system (leading to bacterial meningitis) or the uterus of pregnant women Invasive listeriosis affects primarily pregnant women, newborns, persons with immunocompromising conditions and the elderly (Schuppler and Loessner, 2010). In case of pregnancy, it can cause miscarriage (20% of cases) or neonatal infection (63% of cases) and the typical symptoms are fever and gastrointestinal problems (Painter and Slutsker, 2007). The other cases of invasive listeriosis cause mainly meningoencephalitis and septicemia (rewieved by Vàzquez-Boland *et al.*, 2001). Meningitis is often complicated by encephalitis, a pathology that is unusual for bacterial infections (Lee *et al.*, 2010). Symptoms include fever, malaise, ataxia, seizures and altered mental status (Painter and Slutsker, 2007). The incubation time is between one and 67 days (Goulet *et al.*, 2013).

### 3.1.2 Colonization of the gastro intestinal tract

*L. monocytogenes*, in order to transiently colonize the host Gastro Intestinal (GI) tract, must survive several stress conditions like acidity, elevated osmolarity, the presence of bile salts and low oxygen level.

*Adaptation to acidic stress*

*L. monocytogenes* has the capacity to undergo an adaptive response to moderately acidic pH (pH 5.0–5.5) that enhances survival under conditions of lethal pH (pH 3.5) via two systems. The GAD system for ATR (acid tolerance response) is composed by three glutamate decarboxylases (encoded by *gadA, gadB* and *gadB'*) and a glutamate:GABA antiporter (*gadC*) (Cotter *et al.*, 2001; Conte *et al.*, 2002). A second system is the growth-phase-dependent (induced in stationary phase) ATR which is pH-independent but dependent on the stress-responsive alternative sigma factor, Sigma B (SigB) (Davis *et al.*, 1996; Ferreira *et al.*, 2003). These processes increase the invasive capacity of *L. monocytogenes in vitro* and its virulence potential *in vivo* (Conte *et al.*, 2000, Neuhaus *et al.*, 2013). This stress response is essential for infection since the bacterial gene expression during passage through the stomach influences the successive survival in the GI tract (reviewed in Gahan and Hill, 2014).

*Adaptation to salt stress*

The lumen of the GI tract is a region of relatively high salinity (0.3 M NaCl). Bacteria moving from the stomach into the small intestine, in order to survive and colonize, activate specific uptake systems to transport compatible solutes (osmolytes) to adapt to this osmotic upshift (reviewed in Foster and Spector, 1995 and in Sleator and Hill, 2002). The first most effective system is the glycine betaine uptake system BetL (encoded by *betL)*. Its expression is SigB-dependent (Sleator *et al.*, 1999; Hoffmann *et al.*, 2013). Gbu (encoded by the *gbuABC* operon), which is a second glycine betaine transport system belongs to the binding-protein-dependent ABC (ATP-binding cassette) transporter superfamily and is important in high salinity and at low temperature environments (Ko and Smith, 1999). L-carnitine is the second most effective osmolyte in *L. monocytogenes.* OpuC (encoded by the *opuCABCD* operon), is dedicated to carnitine uptake. It is also member of the ABC transporter superfamily, and its deletion results in a reduced virulence (Sleator *et al.*, 2001, Wemekamp-Kamphuis *et al*., 2002).

*Adaptation to bile stress*

As for other pathogens that target the small intestine, *L. monocytogenes* must possess mechanisms with which to resist the bactericidal activities of bile. The majority of the genes implicated in this resistance are predicted to encode proteins that play a role in stress resistance, maintenance of cell envelope integrity and transcriptional regulators (Begley *et al.*, 2002). A definitive link between survival of bile salts and oral infection was demonstrated by Dussurget *et al.* (2002) who investigated the role of bile salt hydrolase activity in *L. monocytogenes* infection of mice. The study showed that the gene *bsh* encoding bile salt hydrolase activity, to reduce bile toxicity for the bacterial cells, is required for a *L. monocytogenes* infection of guinea-pigs by the oral route and for systemic infection in mice. Furthermore, as *bsh* is absent from not pathogenic *Listeria* species and is regulated by PrfA (Positive regulatory factor A) and SigB, the gene product represents a key virulence factor indispensable for colonizing the GI tract by pathogenic *Listeria* species (Dussurget *et al.*, 2002). Another operon, designated *bilE* (bile Exclusion), is also necessary for colonization of the GI tract in mice and it is regulated by PrfA and SigB. This ABC transporter system, encoded by *lmo1421* and *lmo1422*, is a bile

exclusion system, required for full virulence potential in *L. monocytogenes* (Sleator *et al.*, 2005).

*Adaptation to anaerobiosis*

*L. monocytogenes* is able to survive under aerobic and anaerobic conditions, and this is a key prerequisite for the host infection. This bacterium can adapt its metabolism depending on the oxygen availability (reviewed in Lungu *et al.*, 2009) and its adaptation to anaerobiosis implicates oxygen-dependent regulation of enzymes involved in the central metabolic pathway of *L. monocytogens* (see 3.4, Muller-Herbst *et al.* 2014, Romick *et al.*, 1996). The mechanisms behind this adaptation are still not clear.

### 3.1.3 Intracellular infection cycle

*The key virulence genes in L. monocytogenes*

Genetic analyses of listeriosis in the mouse model and in tissue culture models have identified two clusters of genes necessary for invasion and intracellular replication. *inlA* and *inlB*, encoding for internalins, represent a first cluster of virulence genes which plays a role in the initial stages of attachment to host cells (reviewed in Bonazzi *et al.*, 2009). InlB binds three ligands, gC1qR (a cell receptor for complement protein, C1q), hepatocyte growth factor receptor (Met) and glycosaminoglycans present in the cell surface of mammalian cells (Khelef *et al.*, 2006). E-cadherin is the receptor for InlA, and it is principally expressed on epithelial cells and it forms part of the adherens junctions between polarized epithelial cells (Mengaud *et al.*, 1996).

A second cluster of virulence genes is located between the *prs* and *ldh* genes on the chromosome (Chakraborty *et al.*, 2000). The cluster encodes lysteriolysin O (LLO, encoded by *hlyA*), two phospholipases, PI-PLC and PC-PLC (encoded by *plcA* and *plcB*), that participate in the lysis of the phagosome of host cells, an actin polymerizing protein, ActA (*actA*), that promotes motility within the host cell cytoplasm, a zinc metalloprotease, Mpl (*mpl*), that is required for activation of PlcB, and PrfA, which is the transcriptional regulator of the virulence genes.

Once inside the epithelial cell, *L. monocytogenes* lyses the phagosomal membrane with LLO and Pl-PLC, so that the bacteria can escape from the phagosome and start to replicate in the cytoplasm (Portnoy *et al.*, 1992). At the same time, ActA expression is induced, leading to the polymerization of G-actin in F-actin filaments at

one cell pole (Kocks *et al*., 1993, Rafelski and Theriot, 2006). *L. monocytogenes* uses these filaments to push the bacteria through the cytoplasm and into the cytoplasm of neighboring cells (Lacayo *et al.*, 2012). The consequent endocytosis leads to the fact that the bacteria which enter the new cell are surrounded by a double membrane. To escape the secondary vacuole in the newly invaded cell, *L. monocytogenes* expresses the PC-PLC, Mpl and LLO (Gründling *et al.*, 2003; Poyart *et al*., 1993; Gedde *et al.*, 2000). This strategy is called cell-to-cell spread and it is an important advantage for the infection because the bacteria can spread through the host tissues without being released into the extracellular environment (Ryser and Marth, 2007). Due to this strategy, *L. monocytogenes* is able to cross three host barriers (intestinal, blood-brain and placental barrier) and to enter, replicate, and survive in several human cell types, such as macrophages, epithelial cells, and endothelial cells (reviewed in Lecuit, 2005).

*Regulation of virulence gene expression*

The main regulatory factor identified for regulation of the expression of most of these virulence genes and stress response is PrfA. This transcriptional regulator is active via a RNA thermosensor above 30°C and activates a switch-on of virulence genes once the bacteria enter the GI environment after ingestion (Leimeister-Wächter *et al.*, 1992; Johansson *et al.*, 2002; de las Heras *et al.*, 2011).

The PrfA protein belongs to the CRP/FNR (cyclic AMP [cAMP] receptor protein/fumarate and nitrate reductase regulator protein) family of transcriptional activators which binds to a palindromic PrfA recognition sequence of 14-bp (PfrA boxes) within target promoters localized at −40 (Sheehan *et al.*, 1996).

Another regulatory protein involved in regulation of gene expression during the infection is SigB. It controls the adaptive response of *L. monocytogenes* to osmotic, low pH and oxidative stress and growth at low temperature (Becker *et al.*, 2000; Ferreira *et al.*, 2001). Microarray analysis for genes regulated by PrfA revealed a subset of genes that are co-regulated by both PrfA and SigB (Milohanic *et al.*, 2003). Direct crosstalk between SigB and PrfA (Nadon *et al.*, 2002) and an overlap between these regulons may provide flexibility in adaptation to different environments encountered during infection of the GI tract.

### 3.2 Temperature-dependent adaptation of *L. monocytogenes*

Beside its role in activating the expression of the key regulator of the virulence genes (Leimeister-Wächter *et al.*, 1992), temperature is known as a trigger for expression of flagella, response to salt and acid stress (Williams *et al.*, 2005b*;* Bergholz *et al.*, 2012; Shen *et al.*, 2014) and for changes in central metabolic pathways, e.g. carbon and nitrogen metabolism, in *L. monocytogenes* (Grubmüller *et al.*, 2014; Kaspar *et al.*, 2014). Therefore temperature-dependent adaptations might have an important influence on the adaptation to the host environment and on the infection cycle.

Flagella are complex bacterial organelles well conserved among diverse bacterial species, which allow bacteria to migrate towards favorable and away from unfavorable environments and contribute to virulence in many pathogenic bacteria like e.g. *Salmonella typhimurium* and *Pseudomonas aeruginosa* (Carsiotis *et al.*, 1984; Feldman *et al.*, 1998).

*L. monocytogenes* has peritrichous flagella (Paterson, 1939) and the number of flagella is temperature-dependent (Griffin and Robbins, 1944). Best motility and flagellation is observed at 20°C while at 37°C bacteria are either atrichous or possess a single flagellum and are poorly motile (Leifson and Palen, 1955). The expression of the key subunit of flagella, flagellin FlaA (encoded by *flaA, lmo0690*), in *L. monocytogenes* is temperature-dependent with maximal transcription at 22° C or below, but very low transcription at 37°C (Peel *et al.*, 1988; Dons *et al.*, 1992).

Gründling *et al*. (2004) found out that the temperature-dependent expression of motility genes in *L. monocytogenes* is dependent on a regulator protein, MogR (encoded by *lmo0674*), that directly binds to the *flaA* promoter region. This regulator functions as a repressor of motility gene expression and is required for full virulence of *L. monocytogenes*. A second regulator, DegU (encoded by *lmo2515*), which is an activator of the transcription of motility and chemotaxis genes was identified (Williams *et al.*, 2005b). It was hypothesized that a RNA thermosensor could be responsible for the activation of both the regulators, inhibiting efficient mRNA translation at the nonpermissive temperature (Williams *et al.*, 2005b) as shown for PrfA (Johansson *et al.*, 2002).

Furthermore, bacterial flagellins serve as pattern recognition molecules for Toll-like receptor 5-mediated signaling (Hayashi *et al.*, 2001), leading to activation of innate immune responses to infection (McSorley *et al.*, 2002). In *in vivo* studies the presence of flagella is deleterious for systemic infection of *L. monocytogenes*, in fact

*flaA* is down-regulated at physiological temperature (37°C) (Dons *et al.*, 2004; Way *et al.*, 2004). It has been proposed that this down regulation during *in vivo* infection may serve as an adaptive mechanism to avoid host recognition and mobilization of host innate immune responses (Way *et al.*, 2004). Besides FlaA, the chemotaxis proteins CheY (encoded by *lmo0691*) and CheA (encoded by *lmo0692*), were shown to be involved in the contact of *L. monocytogenes* to the host cells. The expression of these proteins is optimal at 24°C and minor at 37°C (Flanary *et al.*, 1999; Dons *et al.*, 2004). As shown previously in *Pseudomonas aeruginosa*, the flagellar cap protein FliD can participate in this adhesion process (Arora *et al.*, 1998) and it was suggested that also in *L. monocytogenes* further components of the bacterial flagella can act as adhesins for host cells binding (Dons *et al.*, 2004).

Temperature is a driver not only for flagella regulation, also a temperature-dependent regulation of the salt stress response was described in *L. monocytogenes*. At 7°C, the genes *lmo2754* and *lmo2812,* encoding for proteins involved in bacterial cell wall biosynthesis (DD-carboxypeptidase activity) and genes regulated by the three-component system LiaFSR (Fritsch *et al.*, 2011) which is induced by cell envelope stress, are up-regulated compared to 37°C. On the other side, the virulence genes involved in infection, like *prfA*, *mpl*, *actA*, *plcB*, *plcA*, and *inlA,* are stronger transcribed at 37°C than at 7°C (Bergholz *et al.*, 2012).

Acid-stress adaptation is important for the infection process because of the acidic conditions that *L. monocytogenes* must survive within the stomach, in order to transiently colonize the host GI tract. It was shown that also this acid stress response is temperature-dependent since *L. monocytogenes* can adapt to sublethal acid stress at 37°C (mammalian gut temperature) but not at low temperatures (Ivy *et al.*, 2012; Shen *et al.*, 2014).

Furthermore, it has been shown recently that *L. monocytogenes* also regulates the expression of genes involved in central metabolic pathways like nitrogen metabolism (reviewed in Amon *et al.*, 2010) in a temperature-dependent manner (Kaspar *et al.*, 2014), suggesting also a niche dependent metabolic adaptation.

Moreover, also the carbon source usage is supposed to be regulated by the temperature. Glycerol was suggested as predominant alternative carbon source for *L. monocytogenes* metabolism inside the host cells. Its metabolism is based on degradation of the host-derivated phospholipids via PC-PLC (to generate glycerol) and it is highly induced in the host cell's cytosol (Joseph *et al*., 2006). Transcription

of *plcB* is temperature-depended controlled via PrfA (Gründling *et al.*, 2003) which is active, as described before, above 30°C (Leimeister-Wächter *et al.*, 1992; Johansson *et al.*, 2002). The activity of PrfA is strongly inhibited when glucose or other glycolytic carbohydrates are used as the major carbon source for *L. monocytogenes* grown intracellularly (Grubmüller *et al.*, 2014), explaining the importance of glycerol as alternative carbon source during infection.

### 3.3 Metabolism

The capacity of *L. monocytogenes* to adapt to different levels of oxygen is a prerequisite for the successful infection of its vertebrate host via the GI tract. As facultative anaerobe, this bacterium is able to switch between an aerobic and anaerobic metabolism, depending on the oxygen availability (reviewed in Lungu *et al.*, 2009). The first step in the metabolism of many organisms is glycolysis, which can take place in the presence or absence of molecular oxygen. Glycolysis itself yields two ATP molecules, by transforming one molecule of glucose in two molecules of pyruvate. This resulting product can be further used in different metabolic pathways, fermentation or TCA (tricarboxylic acid) cycle, depending on the oxygen level.

*ATP generation by respiration and fermentation*

The TCA cycle, or the Krebs cycle, is the best way to produce NADH and $FADH_2$, the key molecules for the respiration. The cycle starts with acetyl-CoA, the activated form of acetate, derived from glycolysis and pyruvate oxidation for carbohydrates and from beta oxidation of fatty acids. The two-carbon acetyl group in acetyl-CoA is transferred to the four-carbon compound oxaloacetate to form the six-carbon compound citrate. In a series of reactions two carbons in citrate are oxidized to $CO_2$ and the reaction pathway supplies NADH. The pathway also supplies important precursor metabolites for anabolic pathways, including 2-oxoglutarate. At the end of the cycle the remaining four-carbon part is transformed back to oxaloacetate. For each turn of the TCA cycle, one molecule of ATP, three molecules of NADH, and one molecule of $FADH_2$ are formed. Therefore, for one glucose molecule, the TCA cycle results in the formation of two molecules of ATP, six of NADH, and two of $FADH_2$ (Cooper and Sunderland, 2000; Berg *et al.*, 2002).

NADH and $FADH_2$ are the electron donors in the electron transport chain (ETC). This is the respiration stage and it couples electron transfer between an electron donor

and an electron acceptor (such as $O_2$, nitrate or fumarate) with the transfer of $H^+$ ions (protons) across a membrane. The produced proton gradient is exploited for ATP generation via $F_0F_1$-ATPase. For each molecule of NADH which enters the ETC, three molecules of ATP are formed and for each $FADH_2$ molecule, two molecules of ATP are formed (reviewed in Haddock and Jones, 1977; Berg *et al.*, 2002).

When there is no oxygen or other alternative electron acceptors, some cells are still able to produce energy by the process called fermentation. This process generates energy by reduction of pyruvate to various compounds and allow reoxidation of NADH to $NAD^+$ (Nelson and Cox, 2008), which permits the glycolysis to continue. Lactic acid fermentation is the production of lactic acid while alcoholic fermentation generates ethanol and carbon dioxide. Fermentation does not occur only in an anaerobic environment (Nelson and Cox, 2008; Madigan *et al.*, 1996).

*Interrupted TCA cycle and consequences for the metabolism in L. monocytogenes*

*L. monocytogenes* is able to catabolize glucose via the glycolytic pathway, but it possesses a split, non-cyclic TCA cycle due to the lack of 2-oxoglutarate dehydrogenase (Trivett and Meyer, 1971; Glaser *et al.*, 2001). Thus, the TCA cycle is separated in an oxidative and a reductive branch terminating at 2-oxoglutarate and succinate, respectively (Eisenreich *et al.*, 2006) (FIG. 1).

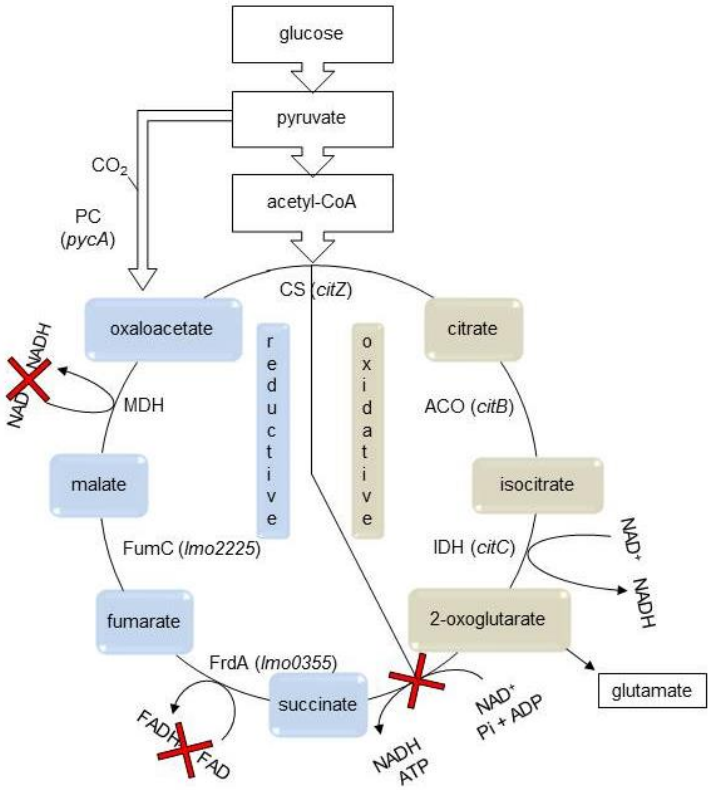


**FIG. 1: The interrupted TCA cycle in *L. monocytogenes*.** The cycle is separated in an oxidative (in blue) branch (CS, citrate synthase, ACO, aconitate hydratase, and IDH isocitrate dehydrogenase) and a reductive (in orange) portion (PC, pyruvate carboxylase, MDH, malate dehydrogenase, FumC, fumarate hydratase and FrdA, fumarate reductase) due to the absence of a 2-oxoglutarate dehydrogenase. The first is ending with oxoglutarate and the second with succinate. NADH is produced in lower amount and no $FADH_2$ is produced at all (red crosses). To allow the glycolysis to continue, 2-oxoglutarate is removed by *de novo* biosynthesis of glutamate. Figure adapted from Trivett and Meyer (1971).

This could explain why *L. monocytogenes* grows better with ammonium then glutamine. To allow the glycolysis to continue, 2-oxoglutarate must be removed by *de novo* biosynthesis of glutamate, which is used in the amino acid biosynthesis. When *L. monocytogenes* is grown on glutamine, as nitrogen source, it can generate two moles glutamate from one mole 2-oxoglutarate and one mole glutamine by glutamine-2-oxoglutarate aminotransferase (GOGAT). When grown on ammonium, the bacterium can incorporate one mole ammonium in one mole 2-oxoglutarate to generate one mole glutamate via glutamate dehydrogenease (GDH). As alternative, it can generate one mole glutamine by incorporation of one mole ammonium into one mole glutamate via the glutamine synthetase (GS) and then use one mole 2-oxoglutarate to generate two moles glutamate as described before via GOGAT. Therefore, when grown in ammonium, double amount of 2-oxoglutarate is consumed compared to growth on glutamine (Kaspar *et al.*, 2014).

The absence of a complete TCA cycle also explains why, during aerobic growth, glucose is not completely oxidized to carbon dioxide and the cells produce a lower amount of NADH, no $FADH_2$ and consequently less ATP compared to cells with a complete TCA cycle. Moreover there are evidences that *L. monocytogenes* produces, combined with aerobic respiration, fermentative end products under aerobic conditions to supply the lack of a complete TCA cycle and obtain a higher amount of ATP by constantly remaining glycolysis active (Pine *et al.*, 1989; Romick *et al.*, 1996).

*Respiration in L. monocytogenes*

The respiratory system of *L. monocytogenes* is quite simple and comprises many dehydrogenases like the NADH dehydrogenases (*lmo2389*, *lmo2638*), formate dehydrogenase (*lmo2586*) and glycerol–phosphate dehydrogenase (*glpD*, *lmo1293*)

that transfer electrons to membrane-soluble quinones (Patchett *et al.*, 1991; Glaser *et al.*, 2001). As electron carriers inside the membrane, *L. monocytogenes* contains only menaquinone, and not ubiquinone, which is at the end reoxidized by a terminal oxidase that transfers the electron to $O_2$ that is reduced to $H_2O$.

The two terminal membrane bound quinol oxidases are cytochrome aa3 or cytochrome bd (*lmo2718–lmo2715*, *cydABCD*) (Glaser *et al.*, 2001). Four structural genes organized in an operon (*lmo0013–lmo0016*, *qoxABCD*) are required for expression of cytochrome aa3 oxidase. This oxidase belongs to the heme-copper oxidase super family that pumps protons in response to electron transfer (Windstedt and von Wachenfeldt, 2000). Genes *cydA* and *cydB* (*lmo2718* and *lmo2717*), which code for the two subunits of the enzyme and two additional genes, *cydC* and *cydD* (*lmo2716* and *lmo2715*), which encode a putative ABC transporter, are required for cytochrome bd expression. The bd quinol oxidase is able to use even traces of $O_2$ with high affinity and is therefore expressed under microaerophilic condition in many bacteria (Windstedt and von Wachenfeldt, 2000), but is less effective in ATP generation compared to the heme-copper oxidase (reviewed in Borisov *et al*., 2015).

Moreover, *L. monocytogenes* lacks a nitrate reductase (Glaser *et al.*, 2001) so it is not able to use nitrate as alternative electron acceptors in the ETC. Also fumarate respiration is not described for this bacterium even if preliminary experiments from our laboratory indicate a role of fumarate in supporting anaerobic growth by working as a sink for reducing equivalents. Thus, *L. monocytogenes* is not able to perform anaerobic respiration and it is supposed to generate ATP under anaerobic condition only via substrate-level phosphorylation.

### 3.4 Adaptation of *L. monocytogenes* to anaerobiosis

The anaerobic adaptation of *L. monocytogenes* is not well known yet, however there are some published studies elucidating its anaerobic adaptation (Romick *et al.*, 1996; Jydegaard-Axelsen *et al.*, 2004; Sewell *et al.*, 2015) and, in particular, the oxygen-dependent regulation of many *L. monocytogenes* genes involved in the central catabolic pathway (Müller-Herbst *et al.,* 2014).

In particular, dehydrogenase genes *lmo2389*, *glpD* and *lmo2586*, and the *qox* genes, described before as involved in oxidative phosphorylation have been seen to be down-regulated anaerobically.

Moreover, Romick *et al.*, in 1996, analyzed the growth and metabolic end products of different strains of *L. monocytogenes* under aerobic and anaerobic conditions in different media. They found out that while under aerobic condition only lactate, acetate, acetoin and carbon dioxide were produced, under anaerobic condition also formate and ethanol were produced. Therefore they suggested that *L. monocytogenes* can perform mixed acid fermentation.

Müller-Herbst *et al.,* 2014, (FIG. 2) presented oxygen-dependent transcription of genes encoding for enzymes involved in the generation of these end products. Anaerobic down-regulation was shown for the gene encoding for acetolactate synthase, *alsS* (*lmo2006*), involved in acetoin generation. Acetate can be generated fro pyruvate via acetyl-phosphate or acetyl-CoA. Pyruvate oxidase (*lmo0722*) is involved in the first pathway and, even if no oxygen-dependent regulation was detected for this *lmo0722*, this enzyme was supposed to be active only under aerobic condition due to the dependency of the reaction to oxygen. The pyruvate dehydrogenase complex (*pdhABCD*) and pyruvate-formate lyases (*pflA*, *lmo1917,* and *pflB, lmo1406*), which generate formate at the same time, are involved in the second pathway. *pdhABCD* was shown to be anaerobically down-regulated while no oxygen-dependent regulation was detected for *pflA* and *pflB*.

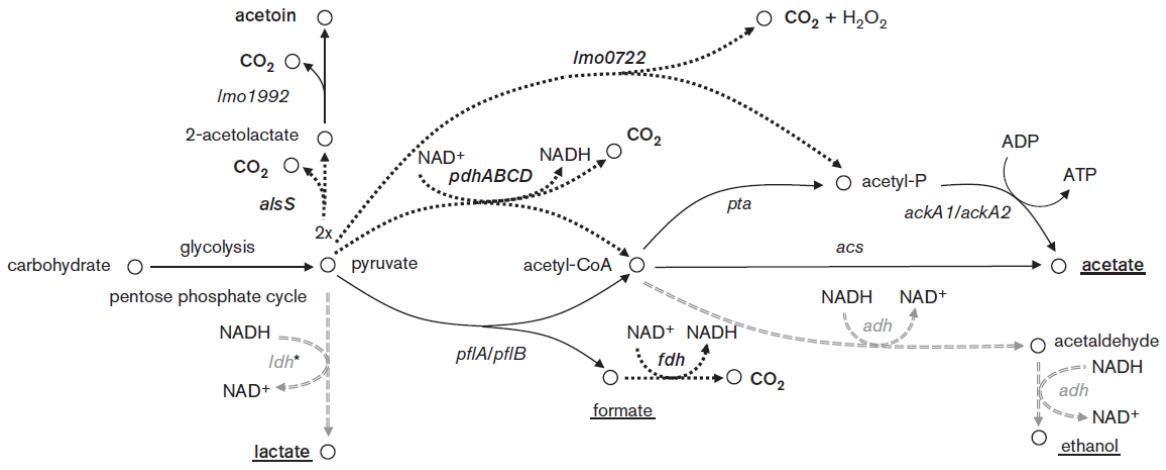


**FIG. 2: Model of aerobic and anaerobic glucose catabolism in *L. monocytogenes* from Müller-Herbst *et al.*, 2014.** The principal aerobic pathways are highlighted by black dotted lines and the anaerobic pathways by grey dashed lines. Genes with oxygen-dependent transcription are marked in bold. The other genes are involved in the catabolic pathways but not regulated in respect of oxygen availability. *lmo0722* is highlighted in bold even if its transcription is not differently regulated, because it requires oxygen for its activity. The genes whose products are involved in the metabolic pathways to generate, aerobically or anaerobically, the major end product of carbon metabolism of *L. monocytogenes* (lactate, acetate, formate and ethanol) are: *ackA1*/*ackA2* (acetate kinase), *acs* (acetyl-CoA synthetase), *adh* (bifunctional acetaldehyde-CoA/alcohol dehydrogenase), *alsS* (acetolactate synthase), *fdh* (formate dehydrogenase; α subunit), *ldh* (lactate dehydrogenase), *pdhABCD* (pyruvate dehydrogenase), *pflA*/*pflB* (pyruvate formate lyase), *pta* (phosphotransacetylase), *lmo0722* (pyruvate oxidase) and *lmo1992* (acetolactate decarboxylase).

Lactate dehydrogenase, LDH, (*ldh*, *lmo0210*), is required for lactate production during aerobic and anaerobic growth, but no regulation was identified for the gene encoding for this enzyme maybe due to down-regulation anaerobically of other pathways described before which might channel pyruvate to the lactate pathway. Acetyl-CoA is a precursor not only for acetate, but also for ethanol generation via reduction by the acetaldehyde-CoA/alcohol dehydrogenase (*adh*, *lmo1634*). *adh* transcription was up-regulated anaerobically. Interestingly, this protein was previously described as being involved in adhesion to host epithelial cells and as being induced anaerobically (Burkholder *et al.*, 2009; Burkholder and Bhunia, 2010). Formate and acetyl-CoA are produced by pyruvate-formate lyases (*pflA* and *pflB*). The oxidation of formate to carbon dioxide by formate dehydrogenase (*fdh*, *lmo2586*) is important to get rid of formate. This gene is active aerobically but not anaerobically when *lmo0912*, a gene encoding for a formate/nitrite transporter, is alternatively up-regulated and might allow formate export and so avoid toxicity (Müller-Herbst *et al.*, 2014). Moreover, it was shown that the gene encoding for fumarate reductase α chain (*lmo0355*) was anaerobically up-regulated, suggesting a role of fumarate in supporting anaerobic growth.

The anaerobic adaptation was shown to correlate with virulence. In particular it was shown that oxygen restriction increased the infectivity *in vitro* and *in vivo* of *L. monocytogenes* (Bo Andersen *et al.*, 2007; reviewed in Lungu *et al.*, 2009) and recently it was shown that anaerobiosis induced acid tolerance to surivive the GI tract environment (Sewell *et al.*, 2015). Moreover it was shown that deletions of genes encoding for enzymes involved in the aromatic amino acid biosynthesis (*aroA*,

*aroB*, or *aroE*) led to an improved synthesis of internalins (InlA and InlB) and consequently an increment in infectivity of *L. monocytogenes* (Stritzker *et al*., 2005). Quinones are essential components of the respiratory chain and their precursors derive from the common branch of the aromatic amino acid pathway. Therefore, all *L. monocytogenes aro* mutants perform anaerobic metabolism (Stritzker *et al*., 2004). This suggested that anaerobiosis induced a stronger expression of internalins and so led to an enhanced virulence. Anyway, up to now, only little is known about the metabolic and physiological responses of *L. monocytogenes* under anaerobiosis.

### 3.5 *L. monocytogenes* serotypes

Phenotypic and genetic subtyping methods let to a differentiation of *L. monocytogenes* isolates into four genetic evolutionary lineages (I, II, III, and IV). Isolates of different lineages displayed distinct genetic, phenotypic and ecologic characteristics. Lineages I and II harbors the serotypes more commonly associated with human clinical cases, with most of the human listeriosis outbreaks associated with lineage I. Lineage III and IV strains are sporadic and are mainly isolated from animal sources (Rasmussen *et al*., 1995; Wiedmann *et al*., 1997; reviewed in Gasanov *et al.*, 2005 and in Liu, 2006; Roberts *et al.*, 2006; Ducey *et al*., 2007; Ward *et al.*, 2008; Orsi *et al.*, 2008 and 2011; Di *et al.*, 2014). 13 serotypes have been recognized, but only serotypes 1/2a (lineage II), 1/2c (lineage II), 1/2b (lineage I) and 4b (lineage I) are responsible for over 98% of the human listeriosis cases reported (reviewed in Schuchat *et al.*, 1991; Jacquet *et al.,* 2002, reviewed in Kathariou 2002; Jacquet *et al.* 2004; Chen and Knabel*,* 2007; Hain *et al.*, 2012).

Huang *et al*. (2014) showed that a clinical strain and an environmental strain of *L. monocytogenes* exhibit differences in the expression of stress response proteins (e.g. superoxide dismutase and the general stress protein [Ctc]) and catabolic metabolism of glucose (e.g. glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate mutase). Therefore the enhanced virulence and pathogenicity identified for the clinical strains could be due to a better growth in the GI tract and a better adaptation to this stress environment.

## 4. Aim of the study

The focus of this study was the investigation of the ability of *Listeria* spp. to adapt to anaerobic conditions. Recently, the adaptation of *L. monocytogenes* to anaerobiosis was described (Müller-Herbst *et al.*, 2014), but the mechanisms behind this adaptation are still not completely clear. The aim of this study is to further elucidate the anaerobic adaptation and its role for the survival in the intestine of the host.

Adaptation to restricted oxygen concentrations positively influences the virulence of the pathogenic *L. monocytogenes*. Therefore, to identify genes which could be important for the colonization of the host, the oxygen-dependent adaptation of a non pathogenic species, *L. weihenstephanensis*, should be compared to that of the pathogenic *L. monocytogenes*. This investigation should be done via aerobic and anaerobic transcriptional profiling with respect to the influence of the temperature, which already has been seen to be a trigger for the activation of virulence genes and for the survival under host-like conditions in *L. monocytogenes.*

In the second part, the focus should be on the pathogenic *L. monocytogenes* and the analysis of the oxygen-dependent adaptation of strains with different virulence potential. Strain-specific mechanisms to improve the survival during the infection process are already known in this species. Thus, the hypothesis that strain-specific differences in anaerobic adaptation could be related to differences in virulence potential of different strains should be investigated.

# III. MATERIAL and METHODS

## 1. Material

### 1.1 Chemicals

All chemicals used for this thesis were obtained from Sigma-Aldrich (Taufkirchen, Germany), Roth (Karlsruhe, Germany), Fluka (Darmstadt, Germany), Merck Millipore (Darmstadt, Germany), Oxoid (Wesel, Germany), Qiagen (Hilden, Germany), Fermentas (Thermo Fisher Scientific, St. Leon-Rot, Germany), New England BioLabs (Frankfurt, Germany), Promega (Mannheim, Germany) or Illumina (München, Germany or San Diego, USA), if not stated otherwise.

### 1.2 Strains

Bacterial strains used in this thesis are summarized in Table 1 and 2.

**Table 1: Wild-type (WT) strains used in this thesis.**

| Strain name | Description |
|---|---|
| *L. weihenstephanensis* 4560 (=DSM 24698) | Environmental *Listeria* species (Lang-Halter *et al.*, 2012) |
| *L. monocytogenes* EGD | Lineage II, serotype 1/2a, kindly provided by Prof. Dr. Prof. Dagmar Beier, Department of Microbiology, Universität Würzburg (Williams *et al.*, 2005a) |
| *L. monocytogenes* EGDe | Lineage II, serotype 1/2a, derivative of EGD (Glaser *et al.*, 2001) |
| *L. monocytogenes* ScottA | Lineage I, serotype 4b, clinical isolate from outbreak in Massachusetts (USA) (Fleming *et al.*, 1985) |
| *L. monocytogenes* WSLC 1670 | Lineage II, serotype 1/2a, isolated from milk (Freising), strain collection ZIEL, Department of Microbiology |
| *Escherichia coli* (*E. coli*) DH5α | *E. coli* used for cloning strategies (Taylor *et al*., 1993) |

**Table 2:** ***L. monocytogenes*** **EGDe in frame deletion mutants used in this study.**

| Strain name | Gene knocked out encoding |
|---|---|
| *L. monocytogenes* EGDe Δ*lmo0210* | L-lactate dehydrogenase (strain collection ZIEL, Department of Microbiology, AG Müller-Herbst) |
| *L. monocytogenes* EGDe Δ*lmo0722* | Pyruvate oxidase (strain collection ZIEL, Department of Microbiology, AG Müller-Herbst) |
| *L. monocytogenes* EGDe Δ*lmo1055* | E3 subunit of pyruvate dehydrogenase complex (produced in this study in cooperation with Arista Samaras) |
| *L. monocytogenes* EGDe Δ*lmo1168* | Acetate kinase (produced in this study in cooperation with Matthias Steiger) |
| *L. monocytogenes* EGDe Δ*lmo1406* | Pyruvate formate-lyase (strain collection ZIEL, Department of Microbiology, AG Müller-Herbst) |
| *L. monocytogenes* EGDe Δ*lmo1581* | Acetate kinase (produced in this study in cooperation with Matthias Steiger) |
| *L. monocytogenes* EGDe Δ*lmo1634* | Bifunctional acetaldehyde-CoA/alcohol dehydrogenase and adhesion to host cells (produced in this study in cooperation with Arista Samaras and Matthias Steiger) |
| *L. monocytogenes* EGDe Δ*lmo1917* | Pyruvate formate-lyase (strain collection ZIEL, Department of Microbiology, AG Müller-Herbst) |
| *L. monocytogenes* EGDe Δ*lmo2006* | Alpha-acetolactate synthase (produced in this study in cooperation with Arista Samaras and Matthias Steiger) |
| *L. monocytogenes* EGDe Δ*lmo2720* | Acetate-CoA ligase (produced in this study in cooperation with Matthias Steiger) |
| *L. monocytogenes* EGDe Δ*lmo1917*Δ*lmo1406* | Double deletion mutant for Pyruvate-formate lyase (produced in this study in cooperation with Romy Wecko) |

## 1.3 Oligonucleotides

Oligonucleotides used in this thesis are listed in Table 5 and 6. All primers were salt-free, purchased from Eurofins Genomics (Ebersberg, Germany), dissolved to a concentration of 100 pmol/µl and diluted to a working concentration of 10 pmol/µl. Sequences were designed using the software Primer 3 (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) and for qPCR both forward and reverse primers were drafted to amplify a short (120-150 bp), centrally located fragment of the target gene obtained from the complete genome sequence (*L. weihenstephanensis*, genome sequence in this study) or ListiList (http://genolist.pasteur.fr/ListiList/, *L. monocytogenes* EGDe),

with an annealing temperature of 54 -56°C and containing 2 to 3 GC at the 3' end. Specific binding of the primers to the target regions in the genomes of all four tested *L. monocytogenes* strains (EGDe, ScottA, 1670 and EGD) was checked via Primer-Blast (http://www.ncbi.nlm.nih.gov/tools/primer-blast) if necessary.

**Table 3: PCR oligonucleotides used for verification of the *L. weihenstephanensis* genome.**

| Name | Sequence 5'→3' |
|---|---|
| 4_forward1 | gttgtgactgttcgaaatgaa |
| 4_reverse1 | aagagtcgttcgtgtggacag |
| 4_forward2 | atcatcgtctgcattctgcta |
| 4_reverse2 | ttcaacagtaccaatcgcgat |
| 5_forward | tgaaacggttttgatgtctat |
| 5_reverse | ccacgtcacctgcacttaatc |
| 6_forward1 | agagtacttcatgatgacaat |
| 6_reverse1 | tccatgcactgcggcatactt |
| 6_forward2 | gtcctatcatccgagcgaag |
| 6_reverse2 | gattagctccttggcaggtg |
| 10_forward1 | gtgatcgcgtaacttgctgtt |
| 10_reverse1 | accgccaggaataggtgtaga |
| 10_forward2 | tacgacgacgactagctaaaa |
| 10_reverse2 | tgtccacaggatatgagcttg |
| 11_forward1 | cggatgctcgaacatatggtg |
| 11_reverse1 | gccttactcagcgcatctccaag |
| 11_forward2 | cagagactctttatctcggac |
| 11_reverse2 | gggtaacttatctgcctatct |
| 14_forward | accagtggagtaatacgtatc |
| 14_reverse | cttctccactcgtgatttctc |
| Expand-F | atactcagggaatggtcgacgcagtggacg |
| Expand-R | gacgcgaatggtaagacaattgcg |

**Table 4: Oligonucleotides for construction of *L. monocytogenes* EGDe deletion mutants**.

| Name | Sequence 5'→3' |
|---|---|
| lmo1055_A: | tgctaacaacgatgcaacttc |
| lmo1055_nestAB: | actttaggatccgaaatcgaagtaactgcactt |
| lmo1055_B: | gtctctagatcttggaaaatcgcctactaccat |

| | |
|---|---|
| lmo1055_C: | acaatgagatctgccgaacttgctttaggtcgc |
| lmo1055_nestCD: | tcttatgaattctttagcatgcttccgaggcaa |
| lmo1055_D: | taacgcggcaacgacattact |
| lmo1168_A | cttacttcgcgaaagaatcgt |
| lmo1168_nestAB | gtaactggatcccttgcagttggaggatcattc |
| lmo1168_B | actcccagatcttatcgccataattttgtgcat |
| lmo1168_C | atgattagatctgatgtagaaaagtatgcaaaa |
| lmo1168_nestCD | cttcgtgaattcgcgaataattgctacatgcgg |
| lmo1168_D | ttagcgcaacttcgaataaag |
| lmo1581_A | tcaagtgcttgataatacagc |
| lmo1581_nestAB | agtggtggatccgtagatgatttgcttatttcg |
| lmo1581_B | acttccagatctaattgcaatcgttttttccat |
| lmo1581_C | gaattaagatctgctcgtgatgtagaaacaatt |
| lmo1581_nestCD | aagaaggaattcgagctttccaaaaagcaattc |
| lmo1581_D | atactctgatgcaatcggttc |
| lmo1634_A: | ttctcagaggacgagttatcg |
| lmo1634_nestAB: | cagatcggatcccagaagtccattcacatcatg |
| lmo1634_B: | tacttcagatctcgcattttctttaattgccat |
| lmo1634_C: | gtaagtagatctaaagaaatctaccttgaagct |
| lmo1634_nestCD: | tttaaagaattctcagtcatgaactcactcctt |
| lmo1634_D: | aagctttacaagttcaacgat |
| lmo2006_A: | atcgataatgcagtacctctt |
| lmo2006_nestAB: | gatgacgtcgacattcaagatctaccagcacgt |
| lmo2006_B: | tactttggtaccgtctttttctagtttcgccat |
| lmo2006_C: | gataacggtacccttggcgaaactttactacct |
| lmo2006_nestCD: | ttccggcccgggcgacatcatgtaatgcttcca |
| lmo2006_D: | caccgatgattggtgaagtaa |
| lmo2720_A | aaatcatgtgaaaaatcgccc |
| lmo2720_nestAB | cgtatggtcgactcgatcatcttgcagcaaatt |
| lmo2720_B | attataagatcttggagcgattaaattttctga |
| lmo2720_C | cgtcgcagatctttgcgtgatgcggagtttgct |
| lmo2720_nestCD | ttggagcccgggaatacggcgtaactatcgcta |
| lmo2720_D | gttgagttgttcttcttgagc |
| LSV4380rev* | agggttttcccagtcacg |
| LSV3* | agtaccattacttatgag |

*oligonucleotides previously published (Joseph *et al*., 2006).

**Table 5: qPCR oligonucleotides for *L. weihenstephanensis*.**

| Function of the gene | Name | Sequence 5´→3´ |
|---|---|---|
| Nitrate reductase NarH | UE46_11975_qRT_F | caactggaaaacgaataagg |
| | UE46_11975_qRT_R | cataaagcatcacaccaaga |
| Nitrite reductase | UE46_01305_qRT_F | cataaaaacggtccactagc |
| | UE46_01305_qRT_R | cagtttcatacgttttgacg |
| Ribonucleoside-triphosphate reductase | UE46_00920_qRT_F | cgaaactggtattttcgatt |
| | UE46_00920_qRT_R | taattcagcacatctgggta |
| Molybdenum cofactor biosynthesis protein MoaC | UE46_11930_qRT_F | tattaaagaagggcaaatcg |
| | UE46_11930_qRT_R | actttcgtcgtcataattgg |
| Nitric oxide dioxygenase | UE46_14170_qRT_F | attgcaccgaatatcttcac |
| | UE46_14170_qRT_R | aaatgctttcgtcaaactgt |
| Formate acetyltransferase | UE46_08310_qRT_F | atgggtaacagaatcaatcg |
| | UE46_08310_qRT_R | agtcgaccaaagtacggtta |
| 2-oxoisovalerate dehydrogenase | UE46_11895_qRT_F | aaggtcttttgatctcagca |
| | UE46_11895_qRT_R | ctttaccgatttcgattgtg |
| L-cystine-binding protein TcyJ | UE46_14320_qRT_F | cgaataaaatcgacttcgtg |
| | UE46_14320_qRT_R | cgaccgacttaatatccgta |
| Glutamate synthase | UE46_10735_qRT_F | cgattgacgtctttacgaat |
| | UE46_10735_qRT_R | caattttcgacatcaccttc |
| Iron ABC transporter ATP-binding protein | UE46_03380_qRT_F | gactgcaaaaggaagatgag |
| | UE46_03380_qRT_R | caatataagctcgctgctg |
| Glycine dehydrogenase | UE46_08610_qRT_F | acaagaaattgagccgttag |
| | UE46_08610_qRT_R | cttgggaatctcctacaaca |
| Dihydroxyacetone kinase family protein | UE46_05890_qRT_F | agttattccgctacacatcg |
| | UE46_05890_qRT_R | cacattaggctgagaagagg |
| Catalase | UE46_00145_qRT_F | gaggctacgacgatgattac |
| | UE46_00145_qRT_R | aacgcattttgatgtaaacc |
| ATP-dependent Clp protease ATP-binding subunit ClpX | UE46_01505_qRT_F | ggctgattttgatgtagagc |
| | UE46_01505_qRT_R | tgagtaaggcttgttgcac |

**Table 6: qPCR oligonucleotides for *L. monocytogenes*.**

| Function of the gene | Name | Seqence 5'→3' |
|---|---|---|
| aa3-600 quinol oxidase subunit I* | lmo0014_qRT_F | agaatggaatgacttggatg |
| | lmo0014_qRT_R | cctgcaatgaagaagaagac |

| | | |
|---|---|---|
| PTS system mannose-specific-factor IIAB*** | lmo0096_qRT_F<br>lmo0096_qRT_R | aaatattcttgcaccagctc<br>tcaacacgagttagaacgaa |
| Anaerobic ribonucleoside-triphosphate reductase* | lmo0279_qRT_F<br>lmo0279_qRT_R | attaaacaattggcgctaga<br>ccaagctggtaagaatgaac |
| Flavocytochrome C fumarate reductase chain A* | lmo0355_qRT_F<br>lmo0355_qRT_R | gattgaaaaagaacgtccag<br>gttgaacagttgggtgaact |
| Heavy metal-transporting ATPase** | lmo0641_qRT_F<br>lmo0641_qRT_R | ggaactttgacaaatggaac<br>tccggttctaagtcttgtgt |
| Flagellar hook-basal body protein FlgG | lmo0682_qRT_F<br>lmo0682_qRT_R | accgagatttctttatgca<br>cagaaaaccaaactgatcg |
| Pyruvate oxidase | lmo0722_qRT_F<br>lmo0722_qRT_R | agttggtttagtaggcgatg<br>tctaaccatttccaccattc |
| Pyruvate dehydrogenase E2 subunit*** | lmo1054_qRT_F<br>lmo1054_qRT_R | gaagcaagccatgaatctac<br>caattacaaggccattagga |
| Ethanolamine ammonia-lyase | lmo1176_qRT_F<br>lmo1176_qRT_R | gttggtgatggacttagctc<br>cacgacaatgtttaacgaaa |
| Precorrin isomerase | lmo1193_qRT_F<br>lmo1193_qRT_R | atgggattacacgctctatg<br>gaccactttctaccatctcg |
| Glutamine synthetase repressor (glnR) | lmo1298_qRT_F<br>lmo1298_qRT_R | cgctactatgaagaccaagg<br>ttttcttaattcccgcaata |
| Pyruvate formate-lyase | lmo1406_qRT_F<br>lmo1406_qRT_R | cttggttacctagctgcaat<br>cgatttcttgtgcttcttct |
| Superoxide dismutase | lmo1439_qRT_F<br>lmo1439_qRT_R | aattcggtacttttgacgaa<br>tggagaatcttggttagctg |
| Bifunctional acetaldehyde-CoA/alcohol dehydrogenase and adhesion to host cells*** | lmo1634_qRT_F<br>lmo1634_qRT_R | aaaagcatttggtatccgta<br>atccacaaccaagtgttagg |
| Methyl-accepting chemotaxis protein | lmo1699_qRT_F<br>lmo1699_qRT_R | atcccgattatacgtgaaga<br>ccaacactggaatgttcttt |
| Pyruvate formate-lyase | lmo1917_qRT_F<br>lmo1917_qRT_R | aacatctatggctggtatgg<br>ctttaaagctttcaggcaaa |
| Alpha-acetolactate synthase* | lmo2006_qRT_F<br>lmo2006_qRT_R | gtagttcacgccgaaagtat<br>tttctggtttagcaagtggg |
| Redox-sensing transcriptional repressor Rex | lmo2072_qRT_F<br>lmo2072_qRT_R | caaacagacaaatgttgcac<br>cactacctactttcgctgga |

| | | |
|---|---|---|
| Catalase (kat) | lmo2785_qRT_F<br>lmo2785_qRT_R | caagcttctcaaattcaagc<br>gtctttcggatccaacact |
| 16S rRNA** | 16S_LmonF1<br>16S_LmonR1 | agacacggcccagactcct<br>gatccgaaaaccttcttcataca |

*published by Müller-Herbst *et al*., 2014.

** published by Kaspar et al., 2014.

*** derived from the primer collection ZIEL Department of Microbiology, AG Müller-Herbst.

## 1.4 Plasmids

All plasmid used in this thesis are listed in Table 7.

**Table 7: Plasmid used in this study.**

| Plasmid | Description |
|---|---|
| pLSV101 | temperature sensitive shuttle vector |
| pLSV101-*Δlmo1055*** | pLSV101 containing ~1200 bp fused flanking regions of *lmo1055* amplified with nested primers |
| pLSV101-*Δlmo1168** | pLSV101 containing ~1200 bp fused flanking regions of *lmo1168* amplified with nested primers |
| pLSV101-*Δlmo1581** | pLSV101 containing ~1200 bp fused flanking regions of *lmo1581* amplified with nested primers |
| pLSV101-*Δlmo1634**** | pLSV101 containing ~1200 bp fused flanking regions of *lmo1634* amplified with nested primers |
| pLSV101-*Δlmo2006*** | pLSV101 containing ~1200 bp fused flanking regions of *lmo2006* amplified with nested primers |
| pLSV101-Δ*lmo2720** | pLSV101 containing ~1200 bp fused flanking regions of *lmo2720* amplified with nested primers |
| pLSV101-*Δlmo1917-Δlmo1406**** | pLSV101 containing ~1200 bp fused flanking regions of *lmo1917* and *lmo1406* amplified with nested primers |

All plasmids are derivated from the temperature-sensitive plasmid pLSV101 (Joseph *et al*., 2006).

* produced in this study in collaboration with Matthias Steiger

** produced in this study in collaboration with Arista Samaras

*** derived from the plasmid collection ZIEL Department of Microbiology, AG Müller-Herbst.

## 1.5 Media

All media were prepared in MilliQ water (MQ $H_2O$), if not stated otherwise.

**Luria-Bertani broth (LB)**

10 g/l Tryptone (Oxoid)

5 g/l Yeast extract (Oxoid)

5 g/l NaCl (Roth)

autoclaved for 15 min at 121°C; stored at room temperature (RT)

**Brain Heart Infusion broth (BHI)**

37 g/l BHI (Merck Millipore)

autoclaved for 15 min at 121°C; stored at RT

**Minimal medium**

Minimal medium was prepared modifying the protocol already published by Premaratne *et al*., 1991 and Kaspar *et al*., 2014 (Table 8).

**Table 8: Minimal Media composition.**

| Component | amount/l |
|---|---|
| $KH_2PO_4$ (Roth) | 6.56 g |
| $Na_2HPO_4 * 2H_20$ (Fluka) | 16.4 g |
| $MgSO_4 * 7H_20$ (Merck Millipore) | 0.41 g |
| Ferric citrate monohydrate (Fluka) | 0.1 g |
| L-Leucine (Serva Elecrophoresis, Germany) | 0.1 g |
| L-Isoleucine (Merck Millipore) | 0.1 g |
| L-Valine (Merck Millipore) | 0.1 g |
| L-Methionine (Sigma-Aldrich) | 0.1 g |
| L-Arginine (Sigma-Aldrich) | 0.1 g |
| L-Cysteine (Merck Millipore) | 0.1 g |
| L-Histidine (Roth) | 0.1 g |
| L-Glutamine (Roth) | 0.6 g |
| Riboflavin (Sigma-Aldrich) | 0.5 mg |
| Thiamine (Merck Millipore) | 1.0 mg |
| Biotin (Roth) | 0.5 mg |
| Thioctic acid (Sigma-Aldrich) | 0.0015 mg |

**Agar plates**

For LB or BHI plates, 1.5% (w/v) agar was added to the medium. After sterilization, cooling to below 60°C and optional supplementation with additives, ~25 ml per plate was poured. Agar plates were stored at 5°C. For motility plates, 0.25% agar, instead of 1.5%, was added to BHI.

**Antibiotics**

Erythromycin (Sigma-Aldrich) was dissolved in 100% EtOH (J.T. Baker, Center Valley, USA) and filter sterilized (0.22 µm) to generate a stock of 100 mg/ml (stored in aliquots at -20°C). 300 µg/ml dilutions were used as final concentration to cultivate *E. coli* and 10 µg/ml for *L. monocytogenes.*

**Resazurin solution**

A stock solution (0.01% w/v) of resazurin (7-Hydroxy-3H-phenoxazin-3-one-10-oxide; Sigma Aldrich) was prepared, filter sterilized and stored in the dark at 4°C.

## 2. Methods

### 2.1 Microbiological methods

#### 2.1.1 Culture conditions

For the cultivation on solid media, bacteria from the glycerol stock were streaked out on BHI agar plates (*Listeria*) or LB (*E. coli*) and incubated for one to two days at 24°C (*L. weihenstephanensis*) or 37°C (*L. monocytogenes* and *E. coli*) and then stored at 4°C. For overnight liquid cultures, a single bacterial colony was inoculated in BHI medium (Brain Heart Infusion, Merck, Darmstadt, Germany) overnight (17 h) at 24°C (*L. weihenstephanensis* and *L. monocytogenes* EGDe comparison, first and second part of the thesis) or 37°C (for *L. monocytogenes* EGD, EGDe, ScottA and 1670 comparison, third part of the thesis) under continuous shaking (150 rpm). Overnight cultures were diluted 1:100 in fresh BHI medium in a total volume of 51 ml for growth analysis. For aerobic growth the cultures were grown in 200 ml flasks, for anaerobic growth cultures were grown in 50 ml sealed CELLSTAR® tubes (Greiner Bio-One, Frickenhausen, Germany). The oxygen availability was analyzed in parallel cultures to which the redox indicator resazurin (0.0001% w/v) was added. When oxygen in the medium becomes limited, resazurin changes color from blue to pink to colorless when the culture is anaerobic. All cultures were incubated at the specific experimental temperature under continuous shaking (150 rpm). The optical density

at 600 nm ($OD_{600}$) was measured using the spectrophotometer Lambda Bio+ (PerkinElmer, Rodgau, Germany). For the growth analyses, the $OD_{600}$ was measured every hour until the stationary phase was reached. One additional measurement was taken after 24 h. Three biologically independent experiments were performed for each growth condition.

### 2.1.2 Bacterial glycerol stocks

Bacterial strains were stored frozen at -80°C in medium containing 20% glycerol (400 µl sterile 60% glycerol added to 800 µl of an overnight culture).

### 2.1.3 Small scale *in vitro* growth analyses (Bioscreen C)

For *in vitro* growth analysis in a microvolume of 200 µl, a Bioscreen C growth curve reader was used (Oy Growth Curves Ab Ltd., Helsinki, Finland). Overnight cultures grown at 24°C of *L. monocytogenes* EGDe and *L. weihenstephanensis* were diluted 1:200 in BHI or in Minimal Media (*L. weihenstephanensis*) supplied with 0 or 10 mM sodium nitrate ($NaNO_3$). In minimal media, as carbon source, 1 g/l D (+)-glucose monohydrate (Fluka) was used and 5 g/l yeast extract (Oxoid) were added. Cultures were incubated at 34°C or 18°C with continuous medium shaking (shaking step 60). For anaerobic growth analyses, cultures were overlaid with 200 µl sterile paraffin oil (Roth). The $OD_{600}$ of each well was automatically recorded every hour over a period of 24 h. Mean values and standard deviation were calculated from at least three independent biological experiments each including technical duplicates. Statistical student t-student was performed to compared cultures grown in presence or absence of sodium nitrate different conditions, aerobically and anaerobically, in different media for *L. weihenstephanensis* and for comparison between the mutants and the wild-type for *L. monocytogenes* EGDe.

### 2.1.4 Motility assays

To perform motility assays, a 3 ml overnight culture was prepared for each tested strain (*L. monocytogenes* EGDe, ScottA, 1670 and EGD) in test tubes (Reagenzgläser 100x15 mm, Wagner und Munz, GmbH, München) at 24°C and 37°C under constant shaking (150 rpm). The density of the overnight cultures were adapted to the lowest $OD_{600}$ of the four overnight cultures by making dilution in an eppendorf tube to ensure the inoculation of equal number of cells. Motility BHI plates (0.25% w/v agar) were divided in four sections and each section was inoculated with a specific strain by dipping a wire till the bottom of the respective eppendorf tube

containing the adapted overnight culture and pricking the agar once in the middle of it. The motility behavior was tested at 24°C and 37°C aerobically and anaerobically. Anaerobic milieu was established in an anaerobic jar with the Anaerocult® A Kit (Merck Millipore); an Anaerotest® test stripe served as control. For each condition, an individual plate was prepared. The plates were incubated for 24 and 48 h at the indicated temperature and oxygen condition and pictures were taken.

## 2.2 DNA methods

### 2.2.1 DNA isolation

#### 2.2.1.1 Extraction of chromosomal DNA from *L. monocytogenes*

*L. monocytogenes* cells from a 3 ml overnight culture were separated in two Eppendorf tubes, harvested (2 min, 15800 rcf) and resuspended in parallel in 290 µl of AKI solution (6.7% (w/v) Sucrose (Roth), 50 mM Tris-HCl (pH 8, Roth), 1 mM $Na_2EDTA$, Roth) supplemented with RNase A (100 µg/ml, Sigma-Aldrich) and lysozyme (10 mg/ml, Sigma-Aldrich) and vortexed. After incubation for 60-90 min at 37°C, 12.5 µl Proteinase K (20 mg/ml, AppliChem, Darmstadt, Germany) were added and, after invertion, the Eppendorf tubes were further incubated for 20 min at 50°C. 100 µl 5% (v/v) SDS (Roth) were added. After incubation for 10 min at 70°C and cooling down to RT, 100 µl neutral phenol-chloroform (Roth) were added. Phenol and aqueous phases were separated by centrifugation (20 min, 16100 rcf, RT). The upper phase was transferred into fresh Eppendorf tubes and phenol-chloroform extraction was repeated. DNA was precipitated by addition of $1/10^{th}$ volume 3 M sodium acetate (pH 8, Roth) and one volume isopropanol (Roth) and invertion. The precipitated DNA was transferred using a pipette tip to a new Eppendorf tube and washed twice with 500 µl 70% EtOH. Finally, the DNA of both the samples was transferred to a final Eppendorf tube and let be dissolved for 30 min at 37°C in 100 µl MQ $H_2O$ and then stored at -20°C.

#### 2.2.1.2 Isolation of plasmid DNA from *E. coli*

Plasmids were isolated from *E. coli* using Pure Link™ Hi Pure Plasmid Midiprep Kit (medium-scale, Invitrogen) or GenElute™ Plasmid Miniprep Kit (small-scale, Sigma-Aldrich) according to the manufacturer's instructions. 50 ml (high-copy plasmid) or 100 ml (low-copy plasmid) overnight cultures in selective LB media, inoculated with a single colony of *E. coli* containing the plasmid, were pelleted (10 min, 4186 rcf, RT)

for medium-scale extraction. For the small-scale isolation, 8 ml (instead of the suggested 1-5 ml) overnight cultures were used as starting material.

#### 2.2.1.3 DNA sequence analysis

DNA sequences were obtained from GATC Biotech AG (Konstanz, Germany). Premixed templates were prepared by adding 400-500 ng of purified plasmid DNA or 100-400 ng of purified PCR product and the specific primer (10 µM) in a total volume of 10 µl. BLAST analyses against the specific genome were done to check the obtained sequences.

### 2.2.2 Cloning techniques

#### 2.2.2.1 Polymerase chain reaction (PCR)

The polymerase chain reaction (PCR) was used to amplify specific DNA fragments. First, the reaction solution was heated to let the two complementary DNA strands separate (denaturation). The temperature was then lowered to allow the binding of the specific primers to the target DNA segments (annealing) and raised again to let the thermo-stable DNA polymerase extend the primers by adding dNTPs to the nascent DNA strand complementary to the DNA template strand. At each repetition of these three steps, the number of copied DNA molecules doubles. *Taq* polymerase displays relatively low replication fidelity while *Pfu* polymerase (Fermentas) possesses 3'-5' exonuclease proofreading activity which generates PCR product with lower mutagenesis rate. For this reason, *Pfu* polymerase was used in PCR reactions requiring high fidelity (e.g. cloning) and *Taq* polymerase was used for screening purposes or amplification of DNA probes. The reaction mixes for both the polymerases are shown (Table 9).

**Table 9: PCR mix with Taq and Pfu polymerases.**

| Taq polymerase mix | | Pfu polymerase mix | |
|---|---|---|---|
| forward primer (10 pmol/µl) | 1 µl | forward primer (10 pmol/µl) | 2 µl |
| reverse primer (10 pmol/µl) | 1 µl | reverse primer (10 pmol/µl) | 2 µl |
| dNTPs (20 mM) | 0.5 µl | dNTPs (20 mM) | 0.5 µl |
| 10x Taq buffer | 2.5 µl | Pfu buffer with $MgSO_4$ | 5 µl |
| $MgCl_2$ (25 mM) | 2.5 µl | $MgCl_2$ (25 mM) | 2.5 µl |
| Taq polymerase | 0.1 µl | Pfu polymerase | 0.5 µl |
| DNA template | 1 µl | DNA template | 1 µl |
| MQ $H_2O$ up to | 25 µl | MQ $H_2O$ up to | 50 µl |

For genomic DNA template, 1 µl of a 1:10 dilution, was added to the mix. For colony PCR, via Taq polymerase, the DNA template was preprared by streaking single colonies in 100 µl MQ $H_2O$ and vortexing the solution before adding 1 µl of the suspension to the mix.

After a short down spinning of the PCR mix, PCR tubes were placed into a thermal cycler (Primus 96 advanced, Primus 25 advanced [Peqlab, Erlangen, Germany]; MJ MiniTM Personal Thermal Cycler [Bio-Rad Laboratories GmbH, München, Germany]) and PCR were performed according to designed programs (Table 10).

**Table 10: Programs for Taq and Pfu PCR.**

| PCR program | Taq | Pfu |
|---|---|---|
| initial denaturation, 94°C | 3 min | 3 min |
| *PCR cycling (30x)* | | |
| denaturation, 94°C | 30 sec | 30 sec |
| annealing, 53°C | 30 sec | 30 sec |
| elongation, 72°C | 1 min/kb | 2 min/kb |
| final elongation, 72°C | 5 min | 5 min |
| cooling, 15°C | ∞ | ∞ |

For colony PCR, the initial denaturation was extended to 10 min in order to lyse bacteria and release DNA.

#### 2.2.2.2 Agarose gel electrophoresis

After amplification, the PCR products were analyzed by agarose gel electrophoresis. The negatively charged DNA fragments move differently through the agarose gel matrix in an electric field according to their size (smaller DNA molecules move faster to the plus pole than large molecules). A DNA ladder with molecules of defined lengths allows estimation of fragment size. Depending on the amplified fragments, a 1% or 2% agarose gel was used for separation (2% agarose gel for fragment sizes < 1 kb). Agarose (Biozym, Hessisch Oldendorf, Germany) was dissolved in 1x TAE buffer using a microwave to melt it completely. Agarose gel was added to a gel electrophoresis chamber (PeqLab) cointaining a comb and let solidify. 1-3 µl of the sample were mixed with 2 µl 6x DNA Loading Dye (Fermentas) and loaded into the slots. Electrophoresis was performed in 1x TAE buffer (50x TAE buffer**:** 2 M Tris base (Roth), 5.71% (v/v) Glacial acetic acid (Sigma-Aldrich) and 50 mM $Na_2$ EDTA (pH 8, Roth); pH 8.3) at 100 V for 30-45 min. As a reference, 1 kb DNA Ladder

(Fermentas) was used on a 1% and 100 bp DNA Ladder (Fermentas) on a 2% agarose gel, respectively. DNA was then stained for 20 min with Gel Red Nucleic Acid Stain (Biotium, Koln, Germany) and visualized with the UVsolo TS Imaging System (Biometra, Göttingen, Germany).

#### 2.2.2.3 Purification of DNA fragments

DNA fragments from PCR or enzymatic reactions were purified from primers, nucleotides, enzymes and salts using E.Z.N.A.® Cycle Pure Kit (Omega Bio-Tek, Norcross, USA) according to the manufacturer's instructions. DNA extraction from agarose gels was performed via the E.Z.N.A.® Gel Extraction Kit (Omega Bio-Tek, Norcross, USA).

#### 2.2.2.4 Restriction enzyme digestion of DNA

In a reaction mix (Table 11), site-specific restriction enzymes (Fermentas) were used to digest DNA fragments and plasmids at 37°C for two h. If necessary, restriction enzymes were heat-inactivated at the recommended inactivation temperature.

**Table 11: Restriction mix to digest plasmids and DNA fragments.**

| | |
|---|---|
| DNA dissolved in MQ $H_2O$ (30-60 ng/µl) | 86 µl |
| 10x restriction buffer | 10 µl |
| restriction enzyme | 4 µl |

#### 2.2.2.5 Dephosphorilation of plasmid DNA

After a purification step using the E.Z.N.A.® Gel Extraction Kit (Omega Bio-Tek), the linearized plasmid DNA was dephosphorylated by Shrimp Alkaline Phosphatase (SAP, Fermentas) to prevent re-circularization. The reaction mix (Table 12) was incubated for one h at 37°C. After incubation, the DNA was again purified by the E.Z.N.A.® Cycle Pure Kit (Omega Bio-Tek).

**Table 12: Dephosphorylation mix.**

| | |
|---|---|
| plasmid DNA dissolved in MQ $H_2O$ (10-20 ng/µl) | 88 µl |
| 10x SAP buffer | 10 µl |
| SAP (1 U/µl) | 2 µl |

#### 2.2.2.6 Ligation

Ligation of double-stranded DNA molecules with compatible ends was catalyzed by ATP-dependent T4 DNA ligase (Fermentas). Two DNA fragments were ligated in

equimolar amounts (2 µl) whereas linearized vector and insert were joined in ratios 1:3 or 1:7. The ligation mix (Table 13) was incubated at 15°C overnight.

**Table 13: Ligation mix to combine DNA fragments, plasmids and PCR products.**

| | |
|---|---|
| DNA fragment 1/insert | 2/1-7 µl |
| DNA fragment 2/vector | 2/1 µl |
| 10x Ligase buffer | 1 µl |
| T4 DNA Ligase (5 U/µl) | 1 µl |
| MQ $H_2O$ | up to 10 µl |

### 2.2.3 Transformation

#### 2.2.3.1 Preparation of $CaCl_2$ competent *E. coli*

*E. coli* was cultivated overnight in 50 ml LB shaking at 37°C and 1 ml of this culture was used for inoculation of 100 ml LB medium. Cells were grown (37°C, shaking 150 rpm) till $OD_{600}$=0.3-0.6 and centrifuged (10 min, 1860 rcf, 4°C) in 50 ml Falcon tubes. Pellet was resuspended in 10 ml cold 0.1 M $CaCl_2$ and incubated on ice for 30 min. Centrifugation was repeated as previously described and pellet was resuspended in 10 ml chilled 0.1 M $CaCl_2$ containing 20% (v/v) glycerol. Aliquots of 100-300 µl were dispensed, shock frozen in liquid nitrogen and stored at -80°C.

#### 2.2.3.2 Transformation of $CaCl_2$ competent *E. coli*

10 µl ligation product were added to 100 µl $CaCl_2$ competent *E. coli* and incubated 30 min on ice. Cells were heat shocked for 90 sec at 42°C and immediately placed on ice for 2 min. 1 ml LB medium was added and cells were shaken at 37°C for two to three h (to allow expression of antibiotic resistance genes encoded on the plasmid) before being plated (100 µl) on LB an agar plate containing 300 µg/ml erythromycin (LB Em300). The remaining cells were pelleted (3 min, 16100 rcf, RT), the supernatant was discarded and the cells were resuspended in the remaining medium and plated on another LB Em300 plate. The plates were incubated for one to two days at 37°C and the grown colonies were screened by colony PCR to verify the presence of insert-containing vectors.

#### 2.2.3.3 Preparation of electrocompetent *L. monocytogenes*

*L. monocytogenes* was cultivated overnight in 50 ml BHI shaking at 37°C and 1 ml of this culture was used for inoculation of 100 ml BHI broth containing 0.1% (w/v) glycine. Cells were grown (37°C, shaking 150 rpm) till $OD_{600}$=0.50 and Penicillin G was added (5 µg/ml) to disrupt the cell-wall. Cells were further cultivated and at

$OD_{600}$=0.65 they were centrifuged in 50 ml Falcon tubes (10 min, 1860 rcf, 4°C). Pellet was washed in 5 ml cold SMHEM (952 mM sucrose [Roth], 3.5 $MgCl_2$ [Roth], 7mM HEPES [Roth]). Centrifugation was repeated as described previously and the pellet was resuspended in 0.5 ml SMHEM. 100 µl aliquots were prepared, shock frozen in liquid nitrogen and stored at -80°C.

#### 2.2.3.4 Trasformation of electrocompetent *L. monocytogenes*

Plasmid DNA (1-5 µg) was added to 100 µl electrocompetent *L. monocytogenes* cells in a cold electroporation cuvette. Cells were electroporated (25 µF, 200 Ω, 12.5 kV/cm) in the Gene Pulser® (Bio-Rad Laboratories GmbH, München, Germany). 1 ml BHI medium was immediately added and the sample was transferred to an Eppendorf tube. Cells were incubated at 30°C shaking for six h to allow formation of the antibiotic resistance encoded on the plasmid. 100 µl of the suspension were plated on BHI plates containing 10 µg/ml of erythromycin (BHI Em10). The remaining cells were pelleted (3 min, 16100 rcf, RT), the supernatant was discarded and the cells were resuspended in the remaining medium and plated on another BHI Em10 plate. The plates were incubated for one to two days at 30°C and the grown colonies were screened by colony PCR to verify the presence of the vector in *L. monocytogenes*.

### 2.2.4 Construction of in-frame deletion mutants in *L. monocytogenes*

Mutagenesis by homologous recombination was the strategy used to create stable in-frame chromosomal deletions in *L. monocytogenes* EGDe (FIG. 3). Primers used for mutant construction are listed in Table 4.

Upstream (primers A and B; ~1000 bp) and downstream (primers C and D; ~1000 bp) flanking regions of the gene to be deleted were amplified using Pfu proof-reading polymerase. The amplified fragments AB and CD were ligated (*Bgl*II or *Kpn*I restriction sites) after purification and digestion and used as template for nested-PCR with the primers nestAB and nestCD via Pfu proof-reading polymerase (~1200 bp). Oligonucleotides (nestAB and nestCD) contained restriction sites at the 5’ ends. After digestion and purification, the nested fragment was used for cloning in the already specifically digested plasmid pLSV101, a temperature-sensitive shuttle vector derived from pLSV1 (Joseph *et al*., 2006). The resulting vector pLSV101-Δ*X* (*X*=gene to be deleted) was transformed into competent *E. coli* DH5α for plasmid amplification (pUC ori). Bacteria were then grown at 37°C on LB Em300 plates to

select positive clones which were subsequentially tested by colony-PCR using pLSV101-specific primers LSV3 and LSV4380rev (Table 4). Plasmids were then isolated from *E. coli* and electroporated in *L. monocytogenes* EGDe cells. Bacteria were cultivated on BHI Em10 plates at 42°C, a non-permissive temperature, to select only bacteria which integrated the vector into the chromosome by homologous recombination. Two possibilities of recombination could occur, via the first or the second flank region, one is shown in FIG. 3, the other not. In Gram-positive bacteria pLSV101 plasmid replicates autonomously only at lower temperatures (30°C) but is lost from the cells after several cell divisions at 42°C due to its temperature-sensitive origin (ori(ts)) . Cells were checked for the correct chromosomal integration of the plasmid by colony-PCR (oligonucleotides A/LSV4380rev and D/LSV3). Positive clones were subcultured in BHI without erythromycin at 30°C to allow excision of the vector by a second recombination event and subsequent loss of the plasmid. The second crossover could occur via the flank used before, then the WT sequence was restored (not shown), or the recombination could occur via the other flank, then the result was the deletion mutant. To verify the deletion of the respective gene, clones erythromycin–sensitive at 30°C were screened by PCR using primers A and D. Furthermore, sequencing was performed to confirm the correct deletion of the gene and to exclude point mutations and frame-shifts.

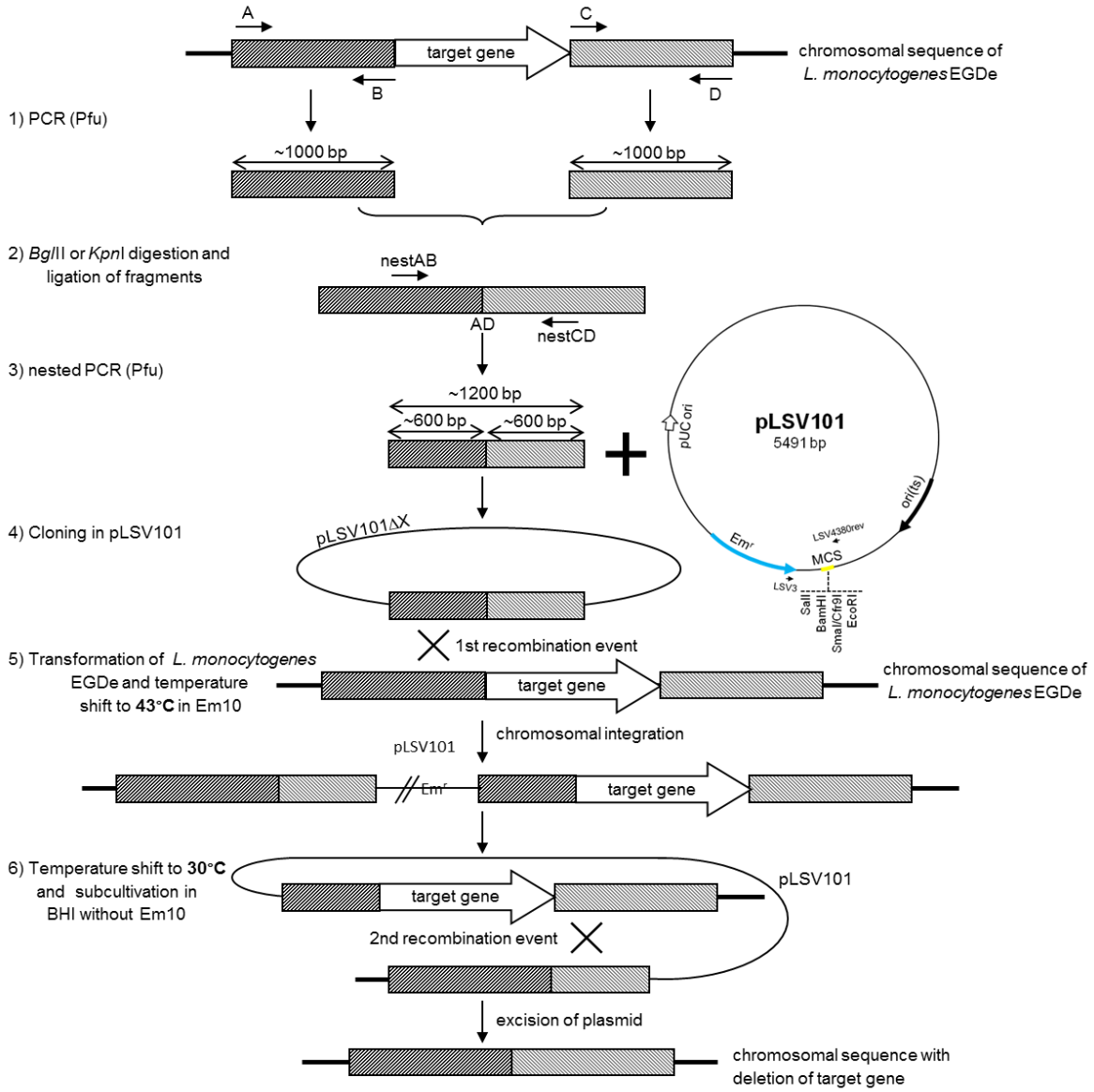



**FIG. 3: Schematic representation of the site-specific mutagenesis by homologous recombination using the shuttle vector pLSV101 to construct *L. monocytogenes* EGDe in frame deletion mutants.** Upstream and downstream flanking regions AB (black) and CD (grey) of the target gene (arrow) are amplified (1), ligated and digested (2). ~1200 bp fragment was amplified by Pfu polymerase (3) and cloned into pLSV101 (4), resulting in pLSV101-Δ*X* (*X*=gene to be deleted). The shuttle vector pLSV101 is a low-copy plasmid which carries erythromycin resistance gene ($Em^r$), a origin for replication in Gram-negative (pUC ori) bacteria and a temperature-sensitive origin (ori(ts)) for replication in Gram-positive bacteria that is active at 30°C, but not above 37°C. Temperature was then switched to the not permissive temperature, 43°C, which allowed a selection of strains in which the plasmid was integrated into the chromosome (5). Integration can occur via recombinantion through the first or the second flank region (not shown). After several passages of growth at 30°C (without erythromycin) the plasmid can be excited via a second cross-over and be lost, what resulted in deletion of the target gene from the chromosome (6). Recombination over the same flank would have resulted in restoring of the WT sequence (not shown).

## 2.3 RNA methods

### 2.3.1 Cell harvesting

*Listeria* cultures (51 ml) were grown, as described before, at the specific experimental temperature aerobically or anaerobically to an $OD_{600}$=0.85-0.95 before cells were harvested by centrifugation (4186 rcf, 8 min, RT). Pellets were shock frozen in liquid nitrogen and stored at -80 °C until further processing.

### 2.3.2 RNA extraction

For RNA isolation, 1 ml TRI Reagent (Sigma-Aldrich) was added to each cell pellet. The suspension was transferred to screw-capped 2 ml tubes containing silica beads (Ø 0.1 mm, Sigma-Aldrich) and incubated at RT for 5 min. The cells were disrupted by bead-beating (FastPrep-24, MP Biomedicals, USA), four times for 45 sec at 6.5 m/s, adding a cooling step for 1 min on ice in between. After centrifugation (3 min, 16100 rcf, RT), the supernatant was transferred to a 2 ml Eppendorf tube. 200 µl chloroform (Roth) were added and the tube was inverted for 15 sec and then incubated for 3 min at RT. Sample was centrifugated (15 min, 12000 rcf, 4°C) to obtain phase separation. The upper aqueous phase, containing RNA, was transferred into a new 1.5 ml Eppendorf tube. RNA was precipitated by adding 500 µl isopropanol, incubating for 10 min at RT and then centrifugating (10 min, 12000 rcf, 4°C). Pellet was washed twice with 1 ml 70% EtOH and, after centrifugation (5 min, 7500 rcf, 4°C), the supernatant was completely removed and the pellet was air-dried for 10-20 min at RT. The RNA was finally dissolved in 80 µl nuclease-free water (Ambion, Thermo Fischer Scientific).

### 2.3.3 DNaseI digestion and 5S rRNA and tRNAs removal

The remaining DNA was digested by adding 10 units RQ1 RNase-Free DNaseI (Promega) and 1x RQ1 DNase Reaction Buffer (Promega) and incubating the samples at 37°C for 45 min. After addition of one volume chloroform and centrifugation at 15000 rcf for 15 min at 4°C, the RNAs were isolated from the aqueous phases and further purified with the RNeasy Mini Kit (Qiagen) following the RNA Cleanup protocol. An additional on-column DNA digest (Qiagen RNase-free DNase, 2 U/µl) was performed according to the manufacturer's instructions before RNA was eluted in a final volume of 30 µl nuclease-free water. The RNeasy technology allows efficient and selective binding of all RNA molecules larger than

200 bp to a silica-based membrane while RNAs smaller than 200 nucleotides are excluded, thus providing an enrichment for the desired mRNA.

### 2.3.4 RNA quality control

RNA quality is specified by quantity, purity and integrity.

Quantity

The concentration of RNA was determined using the Qubit® 2.0 Fluorometer (Thermo Fischer Scientific) which uses highly sensitive and accurate fluorescence-based Qubit™ quantitation assays for the quantitation of DNA, RNA, and protein.

Purity

NanoDrop (Thermo Scientific) was used because the absorbance at 260 nm (A260) is proportional to the RNA concentration (Lambert-Beer law) and the ratios A260/A280 and A260/A230 allow evaluation of RNA purity from protein, phenol or other contaminants (2.0 as optimal).

Integrity

RNA integrity was checked electrophoretically on a 2% agarose gel (2 clear bands showing ribosomal RNAs 16S and 23S indicated integral RNA) and by Bioanalyzer RNA kits (RNA 6000 Nano Kit or RNA 6000 Pico Kit, Agilent Technologies, Santa Clara, CA, USA) which gives a RNA Integrity Number (RIN, 10 as optimal) and the rRNAs ratio (23S/16S) for a reliable and precise integrity check of the sample.

Absence of DNA was verified by control-PCR performed with RNA samples (1 µl) as templates and primers for amplification of a genomic fragment (lmo0355_qRT_F/lmo0355_qRT_R for *L. monocytogenes* and UE46_08610_qRT_F/UE46_08610_qRT_R for *L. weihenstephanensis*.

Chromosomal DNA served as a positive control for the reaction.

### 2.3.5 Quantitative Real-Time PCR (qPCR)

To validate the NGS data, qPCR analyses were performed. RNA was isolated as described before.

#### 2.3.5.1 cDNA synthesis

1 µg of each RNA was transcribed into cDNA (Table 14) by using the qScript™ cDNA SuperMix (Quanta BioSciences, Gaithersburg, USA), which contains all necessary components for the first strand cDNA synthesis except the RNA template

(5x reaction buffer, dNTPs, $MgCl_2$, recombinant RNase inhibitor protein, qScript reverse transcriptase, random primers, oligo(dT) primer and stabilisators).

**Table 14: First strand cDNA Synthesis mix.**

| | |
|---|---|
| 5x qScript cDNA Supermix | 4 µl |
| 100 ng/µl RNA | 10 µl |
| nuclease-free water up to | 20 µl |

Reverse transcription was performed in a thermocycler (0.2 ml PCR tubes) according to the cDNA synthesis (Table 15). cDNA was then diluted 1:5 with 80 µl nuclease-free water and stored at -20°C.

**Table 15: cDNA synthesis program.**

| | |
|---|---|
| 25°C | 5 min |
| 42°C | 30 min |
| 85°C | 5 min |
| 8°C | ∞ |

### 2.3.5.2 qPCR

The ready-to-use PerfeCTa™ SYBR® Green FastMix™ (Quanta Biosciences, Gaithersburg, USA), which contains all necessary components for the qRT PCR reaction (2x Reaction Buffer, $MgCl_2$, dNTPs, AccuFast Taq DNA Polymerase, SYBR Green I dye and stabilisators), was used to perform quantitative real-time PCR. cDNA template (5 µl, corresponding to 50 ng of total RNA) or nuclease-free water (as a negative control) were added to 15 µl master mix (Table 16). Amplification of specific fragments of housekeeping genes were performed in parallel for normalization of the data during the evaluation. When 16S rRNA was used as housekeeping gene, $10^{-3}$ dilutions of the sample were performed prior to the usage due to its high abundancy compared to other RNAs in the cell.

**Table 16: Mastermix qRT PCR.**

| | |
|---|---|
| 2x PerfeCTa™ SYBR® Green FastMix™ | 10 µl |
| Forward Primer (10 pmol/µl) | 1 µl |
| Reverse Primer (10 pmol/µl) | 1 µl |
| DEPC water | 3 µl |

The reagents were mixed thoroughly and collected at the bottom of the reaction tube by short centrifugation and the qPCR reactions were carried out in duplicates for

three biologically independent repetitions in an ICycler (Bio-Rad Laboratories GmbH, München, Germany) or in a SmartCycler® System (Cepheid, CA, USA), according to the following program:

| qRT PCR Program |
|---|
| Initial Denaturation: 95°C, 10 min |
| *40 Cycles* |
| Denaturation: 95°C, 20 sec |
| Annealing: 53°C, 30 sec with open optics |
| Elongation: 72°C, 30 sec |
| Melt Curve: 53°C to 97°C, 0.2°C/sec with open optics |

Oligonucleotides used for qPCR analyses are summarized in Table 5-6.

#### 2.3.5.3 Data evaluation

Data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008). This method allows to analyze the normalized gene expression level. It requires the assignment of a housekeeping, which is assumed to be uniformly and constantly expressed in all samples, and a reference condition. The expression of the other samples is then compared to that in the reference sample. The fold change of the reference condition is set to 100%.

## 2.4 Next Generation Sequencing Methods

### 2.4.1 Genome sequencing of *L. weihenstephanensis*

#### 2.4.1.1 Illumina-in house sequencing

##### 2.4.1.1.1 Isolation of chromosomal DNA (CTAB protocol)

An overnight culture (30°C, 150 rpm) was transferred (3 ml) in a shredder tube, pelleted (16100 rcf, 5 min, RT) and re-suspended in 567 µl 1x TE buffer (Sigma-Aldrich). After addition of shredding beads and vortexing, the sample was homogenized two times at 6.5 m/s for 40 sec. SDS (30 µl of 10% solution [w/v]; Roth) and 3 µl Proteinse K (20 mg/ml; AppliChem, Darmstadt, Germany) were added and the sample was mixed several times by invertion. The sample was then incubated at 37°C for three h to digest the proteins. 100 µl 5 M NaCl (Roth) and 80 µl pre-heated Cetyltrimethyl ammonium bromide (CTAB) solution (Roth) were added and the sample was mixed by vortexing. After incubation for 30 min at 65°C, 780 µl phenol (Roth) were added and the sample was centrifuged 4 min at 15800 rcf. The

upper phase was transferred in a fresh 1.5 ml Eppendorf tube and 700 µl of phenol/chloroform/isoamylalkohol (Roth) were added and mixed. The sample was centrifuged 4 min at 15800 rcf and the upper water phase was transferred to a new 1.5 ml tube. The phenol/chloroform/isoamylalkohol extraction step was repeated. Isopropanol (0.6 volumes; Roth) were added and the sample was mixed by invertion. After incubation for 15 min at 4°C, the sample was centrifuged 2 min at 15800 rcf. 500 µl 70% EtOH (J. T. Baker) were added and the sample was centrifuged 2 min at 15800 rcf. The pellet was air-dried for 30 min at 37°C and then dissolved in 100 µl MQ $H_2O$ and stored overnight at 4°C. 1 µl RNase A (10 mg/ml; Sigma-Aldrich) was added and the sample, after mixing, was incubated 30 min at 37°C. 300 µl MQ $H_2O$ and 400 µl phenol/chloroform/isoamylalkohol (Roth) were added and the sample was centrifuged 4 min at 15800 rcf. The upper water phase was transferred to a new 1.5 ml tube and 3 M sodium acetate pH 5.2 ($1/10^{th}$ of the upper phase volume) and ice-cold 100% EtOH (2.5 volumes) were added and the sample was mixed and incubated 20 min at -20°C. The sample was then centrifuged 4 min at 15800 rcf and the pellet was washed with 600 µl 70% EtOH. After air-drying for 30 min at 37°C, the pellet was dissolved in 100 µl MQ $H_2O$ and stored at 4°C overnight. The isolated DNA was then diluted to 20 ng/µl. 55 µl of this sample were transferred to Snap Cap microTUBE (Covaris, Brighton, United Kingdom) and the DNA was fragmented in the Covaris sonnicator S220 (45 sec, 175 W, 10% duty factor, 200 cycles/Burst). The sample was then stored at -20°C.

#### 2.4.1.1.2 MiSeq run

The *L. weihenstephanensis* genome was sequenced on the MiSeq platform (Illumina, San Diego, USA) using v2 chemistry, 200 cycles, paired-end (Illumina, München, Germany). The library for sequencing was prepared with a modified version of the standard PCR-free TruSeq DNA sample preparation protocol. The PCR step, after adapter ligation, was skipped and the size selection was performed by magnetic beads and not by polyacrylamide gel (C. Huptas, unpublished).

### 2.4.1.2 PacBio sequencing

For sequencing with the Pacific Biosciences PacBio RS II platform, an overnight culture of *L. weihenstephanensis* was diluted 1:100 in BHI medium and grown aerobically at 18°C under constant shaking (150 rpm) to an $OD_{600}$=0.86. The cells

were pelleted by centrifugation (18°C, 8 min, 4186 g). The DNA isolation and sequencing run were performed by GATC Biotech AG.

### 2.4.2 Transcriptional profiling

#### 2.4.2.1 RNA isolation

The RNAs were harvested and extracted as previously described with the only exception that only 60 µg samples were used, after RQ1 DNaseI digestion and chloroform extraction, for the RNeasy Mini Kit to remove RNAs <200bp (like 5s RNAs and tRNAs). This change was applied to avoid the overload of the columns and allow a better removal of this small RNAs. Samples were quality controlled, as described, before and after the RNeasy Mini Kit to ensure optimal quality of the RNAs.

#### 2.4.2.2 rRNAs depletion

After RNA extraction, 16S and 23S rRNAs were still present in the sample. The rRNAs have then been removed, starting from 5 µg (in 5 µl) total RNA (23S/16S ≥1.5, Bioanalyzer), following the MICROBExpress™ Bacterial mRNA Enrichment Kit (Ambion, Thermo Fischer Scientific) protocol (without initial RNA precipitation). 16S and 23S RNAs hybridize to a Capture Oligonucleotide mix which also binds to specific magnetic beads allowing the isolation of rRNAs from the remaining RNA through a magnet. After enrichment, the samples were re-suspended in 25 µl nuclease-free water, quantified (Qubit® 2.0 Fluorometer, RNA Assay Kit, Thermo Fischer Scientific) and qualitatively analyzed (Bioanalyzer, RNA Nano kit) comparing the samples before and after rRNAs depletion. Approximately 90% of the total rRNAs could be removed by this procedure.

#### 2.4.2.3 Sonnication

After rRNA removal, 300 ng of each total RNA were fragmented in 50 µl nuclease-free water (180 sec, 175 W, 10% duty factor and 200 cycles/burst) using the S220 sonicator (Covaris) to obtain fragments with a normal distribution around 150 bp.

#### 2.4.2.4 Dephosphorylation and phosphorylation

To perform stranded RNA sequencing, RNAs were dephosphorylated and re-phosphorylated to ensure RNA molecules with a 5'-phosphate and a 3'-hydroxyl group. The dephosphorylation was carried out using antarctic phosphatase (15 U), 1x antarctic phosphatase buffer (New England BioLabs) with the addition of RNase

inhibitor SUPERase In (65 units, Ambion, Thermo Fischer Scientific) in a final volume of 65.5 µl for one h at 37°C. QIAzol lysis reagent (700 µl) was added to the RNA samples. The RNAs were then purified with the miRNeasy Mini Kit (protocol for purification of total RNA from animal cells, omitting the cell homogenization steps; Qiagen) and eluted in a final volume of 35.5 µl nuclease-free water. For phosphorylation, T4 Polynucleotide Kinase, 1x T4 Polynucleotide Kinase Buffer (New England BioLabs), ATP (1 mM, New England BioLabs), and RNase inhibitor SUPERase In (50 U) were added to the RNA (final volume of 50 µl) and the samples were incubated for one h at 37°C. Afterwards, RNA was purified again using the miRNeasy Mini Kit with a final elution in 30 µl nuclease-free water. After further quality and quantity control (Qubit® 2.0 Fluorometer and Bioanalyzer) 100 ng of the samples were finally concentrated to 20 ng/µl by a Concentrator 5301 (Eppendorf, Hamburg, Germany).

#### 2.4.2.5 Library preparation

The RNA fragments were further treated by ligation of 3´ and 5´adapters, reverse transcription and PCR amplification, following the instructions from the TruSeq® Small RNA Sample Preparation Guide (Illumina, February 2013). The cDNAs derived from the aerobic and anaerobic cultures were mixed before size separation of the amplified cDNA fragments on a 6% Novex TBE polyacrylamide gel (Life Technologies). The fragments were size-selected between 200-350 bp (staining with SYBR® Gold Nucleic Acid Gel Stain, Thermo Fischer Scientific), purified by filtration (0.22 µm Spin-X filter, Corning, USA) and ethanol precipitation. Libraries were checked by Bioanalyzer (High Sensitivity DNA Kit, Agilent, to ensure correct size distributions and calculate its mean) and by Qubit® 2.0 Fluorometer (dsDNA HS [High Sensitivity] Assay Kit, Thermo Fischer Scientific, to check the concentration). The indexed libraries were diluted to a concentration of 2 nM (nM=[(concentration [ng/µl])*$10^6$]/[mean of distribution*650g/mol]) in Buffer EB (Qiagen). For denaturation, 10 µl of 0.1 M NaOH were added to 10 µl of the 2 nM library and the sample was incubated for 5 min at RT. Finally, the library was diluted with pre-chilled HT1 buffer (Illumina) to a concentration of 10 pM in 0.5 mM NaOH.

#### 2.4.2.6 RNA sequencing

The libraries were sequenced on the MiSeq sequencer (Illumina, MiSeq® System User Guide) using a MiSeq Reagent Kit v2 (50 cycles, Illumina) resulting in 50 bp single-end reads.

## 2.5 Bioinformatics

### 2.5.1 Genome sequencing of *L. weihenstephanensis*

#### 2.5.1.1 Genome assembly

For the in-house genome sequencing by MiSeq, read trimming and filtering was performed with the NGS QC Toolkit (v2.2.3) (Patel and Jain, 2012) using automatic FASTQ variant detection and option 2 to remove adapter-contaminated reads. Raw reads passed quality filtering if at least 80% of their nucleotides had a Phred quality score ≥ 30. Both reads of a pair had to pass quality filtering to be further considered. After filtering, reads were trimmed 10 nts from 5' end and 1 nt from 3' end. Finally, a high quality read data set comprising 189 nts paired-end reads was obtained and used to generate an assembly by ABySS (v1.3.3) (Simpson *et al.*, 2009) with standard parameter settings and k-mer size 89.

The raw PacBio sequencing data were assembled with the hierarchical genome-assembly process (Chin *et al.*, 2013) v3 workflow of the SMRT Analysis software, v2.3. Then, Pilon (Walker *et al.*, 2014) was used to improve the assembly further by incorporating the Illumina sequencing data. The final contig was circularized manually. An unusual high coverage region was further analyzed. This region contains a prophage. The attachment sites of this prophage were determined and the sequence was extracted from the chromosomal sequence and circularized. Afterwards, sequencing reads were mapped against the bacterial chromosomal integration site and the circularized phage chromosome to verify the correct breakpoint sequences.

#### 2.5.1.2 Genome annotation

Both sequences were submitted to GenBank and structurally annotated by the NCBI Prokaryotic Annotation Pipeline (PGAP) (Tatusova *et al.*, 2013). Functional annotation was performed by the RAST server (Overbeek *et al.*, 2014) and the PEDANT system to get additional functional annotation like Clusters of Orthologous Groups (COGs) (Galperin *et al.*, 2015) assignment or annotation of genes not

characterized by PGAP. The GenBank file (*.gbk) of *L. weihenstephanensis* was obtained from PGAP (NCBI) and converted by gbk2ptt (A. Villegas, Public Health Ontario) to an NCBI Protein Table (*.ptt) file.

### 2.5.2 Transcriptional profiling

#### 2.5.2.1 Galaxy

The Illumina output files (FASTQ files) were mapped to the specific FASTA file (accession number CP011102 for *L. weihenstephanensis* and accession number NC_003210 for *L. monocytogenes* EGDe) using.Bowtie for Illumina implemented in Galaxy (Goecks *et al.*, 2010; Blankenberg *et al.*, 2010; Giardine *et al.*, 2005). For this step the commonly used settings have been applied (2 maximum number of mismatches permitted, seed length 28 nt). The SAM (Sequence Alignment/Map) files generated were then filtered just for mappable reads and at the end converted to BAM (SAM binary) files and indexed using a Java-based command-line (picard-tools; Li *et al.*, 2009) generating BAM.BAI files.

#### 2.5.2.2 Artemis

The number of reads mapping on each gene were visualized and calculated using Artemis (version 15.0.0) (Rutherford *et al.*, 2000, Carver *et al.*, 2012). The specific GenBank file was used as reference. Gene counts (only coding genes) of each library were normalized to the smallest library in the comparison for a more accurate evaluation. Each gene was multiplied for a factor calculated by dividing the library size of the smallest library for the library size of the libraries to normalize (normalization to the smallest library).

#### 2.5.2.3 Data analysis

Differential gene expressions of the counts of all the coding genes were analyzed using the Bioconductor package edgeR (Robinson *et al.*, 2010), as described previously (Mühlig *et al.*, 2014). Genes with less than 1 count per million (cpm) at both conditions were filtered out and library size was further normalized, as suggested, using the TMM (trimmed mean of M-values; Robinson *et al.*, 2010) normalization method. The exact-test function was used to perform differential expression analysis. Dispersion was set as 0.1 as proposed in the edgeR user's guide for genetically identical model organisms (revised version from 8 October 2015). Genes with FDR ≤0.3 and p-value ≤0.05 were at the end selected. Genes

showing a $log_2$FC (logarithm base 2 fold-change) >1 were considered up-regulated compared to the reference condition (anaerobiosis); genes presenting $log_2$FC <-1 were instead considered down-regulated compared to the reference condition.

# IV. RESULTS

During the colonization of the host GI tract *L. monocytogenes* is supposed to be exposed to anaerobic niches. However, although this ability of the bacterium to adapt to hypoxia might be important for a successful colonization of the host, up to now only a few studies exist, which describe the anaerobic adaptation of *L. monocytogenes* and other *Listeria* spp..

In this thesis, species- (*L. weihenstephanesis* and *L. monocytogenes*) and strain- (*L. monocytogenes* EGD, EGDe, ScottA and 1670) specific adaptations to anaerobiosis were analyzed to further elucidate metabolic pathways and/or mechanism that could allow a successful colonization of the host.

## 1. Genome sequencing and temperature-dependent transcriptional profiling of *L. weihenstephanensis*

In the first part of this thesis, the anaerobic adaptation of the environmental species *L. weihenstephanesis*, which has not been associated with human or animal diseases, was studied. A comparison between the transcriptional adaptations to hypoxia of this not pathogenic species to that of *L. monocyto*genes, should help to better understand pathogen specific adaptations. Analyses were performed at 18°C (environmental temperature) and 34°C (highest temperature at which *L. weihenstephanensis* can replicate and at which virulence factors are expressed in the pathogenic *L. monocytogenes*) to further investigate a putative impact of temperature on the adaptation to anaerobiosis, which might be even more relevant for the pathogenic species.

### 1.1 Complete genome sequence of *L. weihenstephanensis*

As key prerequisite for the analysis of transcriptional data via RNA-NGS sequencing, a high quality genome of *L. weihenstephanensis* is needed. At the beginning of this thesis, the genome sequence of *L. weihenstephanensis* was not available. Therefore, an approach was performed to sequence the complete *L. weihenstephanesis* genome.

The genome sequence of *L. weihenstephanensis* was analyzed via in-house genome sequencing (MiSeq) (reviewed in Metzker, 2010; Liu *et al.*, 2012) and 23 contigs were obtained. Several analyses have been performed to reduce the number of contigs and close the genome (data not shown), but these contigs contained repetitive elements which did not allow to close the genome. For this reason, an

additional run using the PacBio RS II platform from Pacific Biosciences was performed. This technology generates an average read length of up to 10 kb and single reads exceeding 50 kb (Lee *et al*., 2014; Rhoads and Au, 2015) so it should be suitable to overcome the repetitive elements difficulty. A single contig assembly was obtained with the PacBio (Table 17).

**Table 17: Comparison between MiSeq and PacBio sequencing of *L. weihenstephanensis*.**

| | number of reads | mean read length | n°contigs | %GC | assembly size (bp) |
|---|---|---|---|---|---|
| **MiSeq (Illumina)** | 1789120 | 189 | 23 | 40 | 3380080 |
| **RS II (PacBio)** | 78900 | 6091 | 1 | 41.51 | 3406292 |

Data were combined and compared between the two outcomes and 18 single point mutations and a 645 nucletides insertion were tested by PCR. The PacBio sequence was identified as the correct sequence in all the cases and was therefore used for the further analyses. At the end the complete circular chromosome was 3406292 bp with 41.51% GC content. 3229 genes were identified, including 3008 coding genes, 131 pseudogenes, 19 rRNAs (5S, 16S, 23S), 70 tRNAs and 10 long non-coding RNAs (>200 bp). Putative operons were predicted based on the analysis of the transcriptional data (see below), resulting in the identification of 647 operons which comprised 1951 genes (Table S1 in the appendix). Moreover, comparing the protein sequences, for 2094 genes a COG category was identified (Table S2). Bioinformatic analyses were performed by Mathias C. Walter.

The genome of *L. weihenstephanensis*, compared to that of the pathogenic *L. monocytogenes*, exhibits interesting peculiarities. The main difference concerns the presence of virulence genes (*hly*A, *plcA*, *plcB*, *actA*, *mpl, inlA, inlB* and others), which are absent in *L. weihenstephanensis* (Table 18). Moreover, the genome of *L. weihenstephanensis* is bigger (3.4 Mb, in-house) than the genome of *L. monocytogenes* (strain EGDe, 2.9 Mb, Glaser *et al.*, 2001) and presents more coding genes. Among these genes, the one involved in nitrate respiration can be found. In particular the genes enconding for all the subunits of nitrate reductase (*UE46_11965*, *UE46_11970*, *UE46_11975*, *UE46_11980*) and nitrite reductase (*UE46_01300*, *UE46_01305*) are present in the genome of *L. weihenstephanensis*

but not in the genome of *L. monocytogenes*, which is not able to use nitrate as alternative electron acceptor for respiration.

**Table 18: Virulence genes absent in *L. weihenstephanensis* compared to *L. monocytogenes.***

| **Virulence factors absent in *L. weihenstephanensis*** |
|---|
| Actin-assembly inducing protein (*actA*) |
| Virulence regulatory factor (*prfA*) |
| Broad-substrate range phospholipase C (*plcB*) |
| Phosphatidylinositol-specific phospholipase C (*plcA*) |
| Zinc metalloproteinase (*mpl*) |
| Pore forming cytolysin listeriolysin O (*hly*) |
| Internalin A, B, C, E, G, H and Internalin-like proteins |
| Bile resistance proteins (*bsh, btlB, bilE, opuC, pva*) |

In cooperation with Mathias C. Walter, based on the above-average of reads in an ~40 kb region (FIG. 4) and on *in silico* analysis using PHAST (Zhou *et al.*, 2011), an active lysogenic phage was predicted to be integrated in the *L. weihenstephanensis* chromosome but also to exists as a standalone circular chromosome phage with a size of 41687 bp (FIG. 5).

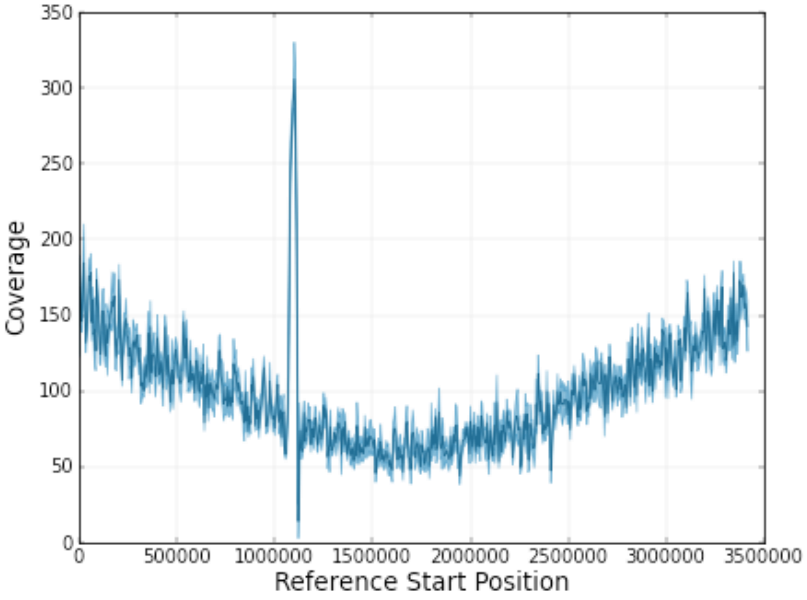



**FIG. 4: Coverage of the PacBio sequencing of *L. weihenstephanensis***. A region of ~40 kb showed an extremely high number of reads followed by a short region with a low number of reads. This region contained phage genes.

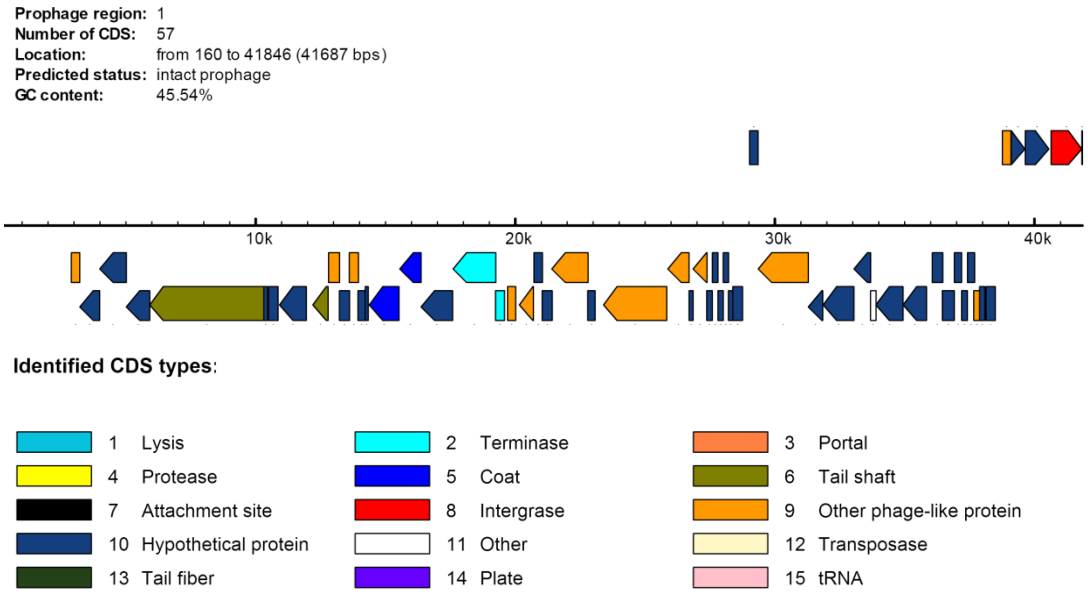



**FIG. 5: Prophage region identified in *L. weihenstephanensis*.** The region was shown as integrated in the *L. weihenstephanensis* genome as well as excised from the bacterial genome. The figure presents the 57 coding genes identified in this region of 41687bp (figure abtained in PHAST, Mathias C. Walter).

The sequencing project was registered in the NCBI BioProject database with accession number PRJNA275474. Genomic raw sequence data was submitted to the sequence read archive (SRA) with accession number SRS845980. The GenBank accession number for the chromosome is CP011102 and for the phage genome CP011103.

### 1.2 Aerobic and anaerobic growth of *L. weihenstephanensis*

Besides the species description (Lang-Halter *et al.*, 2013) and the genome sequence (NCBI Reference Sequence: NZ_AODJ00000000.1 and in this study), nothing is known about *L. weihenstephanensis*. To investigate the oxygen dependent growth of *L. weihenstephanensis*, this environmental species was grown aerobically and anaerobically at 18°C and 34°C (FIG. 6). In general, growth was slower at 18°C (FIG. 6a) compared to growth at 34°C (FIG. 6b). At both temperatures, due to the advantageous oxygen-dependent respiration, aerobic growths reached a higher final $OD_{600}$ compared to the anaerobic condition.

Resazurin controls were added to ensure the oxygen level at the respective conditions. The color of those controls reflected the oxygen condition present in the medium (dark orange=aerobic; yellow=anaerobic). These controls ensured that the

desired conditions were attained within the medium in particular at the time point when the samples were collected for NGS and quantitative PCR (qPCR) experiments (see below).

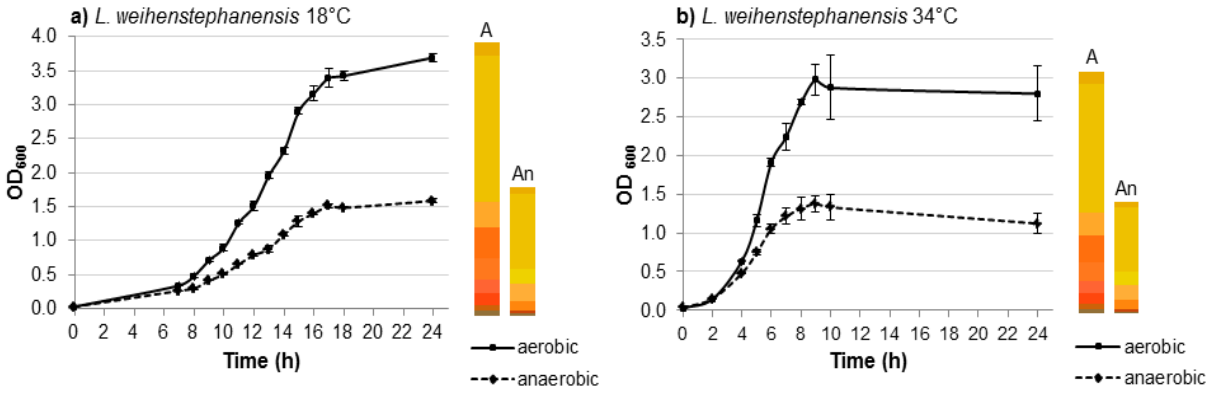



**FIG. 6: Oxygen-dependent growth of *L. weihenstephanensis*.** Depicted are growth curves for *L. weihenstephanensis* grown at 18°C (a) and 34°C (b) aerobically (solid line) and anaerobically (dotted line). $OD_{600}$ was measured every hour. All data points represent means ± standard error from three independent biological replicates. Colored bars indicate the color of a resazurin control ([A] aerobic and [An] anaerobic condition). Resazurin is turning colorless when the culture becomes anaerobic.

### 1.3 Oxygen dependent transcriptional adaptation of *L. weihenstephanensis* at different temperatures

To analyze the oxygen dependent transcription of *L. weihenstephanensis*, transcriptome of bacteria cultivated aerobically and anaerobically at 18°C and 34°C to an $OD_{600}$ = 0.85-0.95 was analyzed via RNA-NGS sequencing. At this $OD_{600}$ resazurin controls indicated high or low oxygen availability (FIG. 6).

The total RNA was isolated and sequenced via NGS. The raw read counts were normalized to smallest library, which in this case was the 18°C anaerobic library. The $log_2$FC (logarithm base 2 fold-change) was calculated in edgeR, which displays the $log_2$ of the expression ratio anaerobic/aerobic.

In total, 104 genes showed an oxygen dependent change in transcription. 42 genes were up-regulated anaerobically, including 16 genes which were up-regulated at 18°C and 34°C, while 19 genes were up-regulated specifically at 18°C and seven genes were up-regulated specifically at 34°C. 62 genes were down-regulated anaerobically, including 22 genes which showed a down-regulation at both temperatures. 22 genes were specifically down-regulated at 18°C and 18 genes at 34°C (Table 19 and 20).

Worth mentioning at this point is the observation that most of the genes that were identified as being regulated depending on the oxygen availability only at one of the investigated temperatures, showed the same tendency of regulation also at the other temperature, but, since they do not fulfill all the filtering criteria applied during the data analysis, they are not regarded as significantly regulated under the alternative growth condition (Table S3a and S3b).

**Table 19: Anaerobically up-regulated genes at 18°C and 34°C of *L. weihenstephanensis*.**

| Gene | $log_2$FC 18°C | $log_2$FC 34°C | Abb. | Product |
|---|---|---|---|---|
| *UE46_00255* | -1.21 | 2.92 | - | PTS cellobiose transporter subunit IIC |
| *UE46_00260* | -1.11 | 2.24 | - | aryl-phospho-beta-D-glucosidase |
| *UE46_00920* | 2.96 | 2.48 | - | ribonucleoside-triphosphate reductase |
| *UE46_00925* | 2.61 | 2.81 | - | ribonucleoside-triphosphate reductase activating protein |
| *UE46_01145* | 2.12 | 0.90 | - | acetate--CoA ligase |
| *UE46_01300* | 8.96 | 7.69 | - | nitrite reductase |
| *UE46_01305* | 12.48 | 8.83 | - | nitrite reductase |
| *UE46_01315* | 5.26 | 4.15 | - | hypothetical protein |
| *UE46_01320* | 5.16 | 4.02 | - | hypothetical protein |
| *UE46_01325* | 5.40 | 4.49 | - | LuxR family transcriptional regulator |
| *UE46_01330* | 2.43 | 2.61 | - | membrane protein |
| *UE46_02500* | 1.51 | 2.14 | - | membrane protein |
| *UE46_03355* | 2.91 | 1.00 | - | alanine dehydrogenase |
| *UE46_03660* | 2.02 | 1.91 | - | hypothetical protein |
| *UE46_03665* | 1.99 | 2.32 | - | hypothetical protein |
| *UE46_03745* | 0.74 | 1.91 | - | PTS system mannose family transporter subunit IID |
| *UE46_04270* | 2.54 | 1.18 | - | hypothetical protein |
| *UE46_04720* | 8.11 | 3.08 | - | hypothetical protein |
| *UE46_04725* | 7.18 | 3.99 | - | multidrug MFS transporter |
| *UE46_04730* | 3.49 | 0.69 | - | Crp/Fnr family transcriptional regulator |
| *UE46_05880* | 2.39 | 1.82 | - | 2-hydroxyglutaryl-CoA dehydratase |
| *UE46_06275* | 2.20 | 1.23 | - | transporter |
| *UE46_08305* | 2.46 | 2.34 | - | pyruvate formate lyase-activating protein |
| *UE46_08310* | 2.77 | 2.28 | - | formate acetyltransferase |

| | | | | |
|---|---|---|---|---|
| *UE46_11095* | 2.46 | 3.20 | - | hypothetical protein |
| *UE46_11925* | 2.31 | 1.47 | - | molybdenum cofactor biosynthesis protein MoeA |
| *UE46_11930* | 2.42 | 1.74 | *moaC* | molybdenum cofactor biosynthesis protein MoaC |
| *UE46_11935* | 2.34 | 1.58 | - | molybdenum cofactor biosynthesis protein D |
| *UE46_11940* | 3.02 | 1.61 | - | molybdenum cofactor biosynthesis protein E |
| *UE46_11945* | 2.38 | 1.44 | - | molybdopterin-guanine dinucleotide biosynthesis protein B |
| *UE46_11950* | 2.55 | 1.30 | - | molybdopterin biosynthesis protein MoeA |
| *UE46_11955* | 2.15 | 1.15 | - | molybdate ABC transporter substrate-binding protein |
| *UE46_11960* | 9.96 | 8.58 | - | MFS transporter |
| *UE46_11965* | 10.88 | 9.32 | - | nitrate reductase |
| *UE46_11970* | 9.68 | 9.34 | - | nitrate reductase |
| *UE46_11975* | 10.26 | 10.04 | *narH* | nitrate reductase |
| *UE46_11980* | 10.90 | 9.27 | *narZ* | nitrate reductase |
| *UE46_12565* | 1.84 | 1.27 | - | 2-nitropropane dioxygenase |
| *UE46_12600* | 2.40 | 1.70 | - | formate/nitrite transporter |
| *UE46_14170* | 3.04 | 1.99 | - | hypothetical protein |
| *UE46_14290* | 0.02 | 2.66 | - | beta-lactamase |
| *UE46_14360* | 1.91 | 1.10 | - | hypothetical protein |

Abb.=abbreviation.
Not statistically relevant data (p-value >0.05 and FDR >0.3) are shown in grey.

**Table 20: Anaerobically down-regulated genes at 18°C and 34°C of *L. weihenstephanensis*.**

| Gene | $log_2$FC 18°C | $log_2$FC 34°C | Abb. | Product |
|---|---|---|---|---|
| *UE46_00135* | -3.68 | -2.01 | - | hypothetical protein |
| *UE46_00145* | -2.26 | -0.64 | *kat* | catalase |
| *UE46_00575* | -2.84 | -0.79 | - | chitinase |
| *UE46_00985* | -3.84 | -2.57 | - | hypothetical protein |
| *UE46_02440* | -2.41 | -1.71 | - | serine hydroxymethyltransferase |
| *UE46_02830* | -0.51 | -2.28 | - | nitrogen fixation protein NifU |
| *UE46_03225* | -2.33 | -1.07 | - | membrane protein |
| *UE46_03370* | -7.71 | -8.47 | - | iron ABC transporter permease |

| | | | | |
|---|---|---|---|---|
| *UE46_03375* | -4.75 | -6.39 | - | iron ABC transporter permease |
| *UE46_03380* | -3.14 | -6.46 | - | iron ABC transporter ATP-binding protein |
| *UE46_03385* | -4.15 | -5.84 | - | ABC transporter |
| *UE46_04105* | -1.95 | -2.50 | - | multidrug ABC transporter permease |
| *UE46_04405* | -1.41 | -5.41 | - | ferrous iron transporter B |
| *UE46_04410* | -1.66 | -4.86 | - | iron transporter FeoA |
| *UE46_04530* | -0.51 | -3.15 | - | membrane protein |
| *UE46_04685* | -3.03 | -1.04 | - | hypothetical protein |
| *UE46_04715* | -2.52 | -0.19 | - | hypothetical protein |
| *UE46_05885* | -3.31 | -2.96 | - | membrane protein |
| *UE46_05890* | -4.05 | -3.34 | - | hypothetical protein |
| *UE46_05895* | -3.73 | -2.86 | - | steroid 5-alpha reductase |
| *UE46_05900* | -2.62 | -1.71 | - | hypothetical protein |
| *UE46_06685* | -0.98 | -3.72 | - | thioredoxin reductase |
| *UE46_06690* | -0.39 | -3.54 | *fecE* | iron-dicitrate transporter ATP-binding subunit |
| *UE46_06695* | -3.70 | -3.94 | - | hypothetical protein |
| *UE46_08435* | -0.87 | -2.31 | - | sulfurtransferase |
| *UE46_08605* | -2.77 | -1.02 | - | glycine dehydrogenase |
| *UE46_08610* | -2.43 | -0.99 | - | glycine dehydrogenase |
| *UE46_08615* | -2.56 | -1.09 | - | hypothetical protein |
| *UE46_10635* | -2.53 | -1.54 | - | rod-share determining protein MreBH |
| *UE46_10730* | -2.15 | -3.69 | - | glutamate synthase |
| *UE46_10735* | -2.04 | -3.84 | - | glutamate synthase |
| *UE46_11885* | -2.43 | -2.36 | - | dihydrolipoamide dehydrogenase |
| *UE46_11890* | -2.40 | -2.46 | - | dihydrolipoamide acetyltransferase |
| *UE46_11895* | -2.30 | -2.40 | - | 2-oxoisovalerate dehydrogenase |
| *UE46_11900* | -2.25 | -2.27 | - | pyruvate dehydrogenase |
| *UE46_12555* | -2.97 | -4.42 | - | argininosuccinate synthase |
| *UE46_12560* | -1.55 | -4.19 | - | argininosuccinate lyase |
| *UE46_12770* | -6.48 | -1.97 | - | oxidoreductase |
| *UE46_13335* | -1.37 | -2.78 | - | ABC transporter permease |
| *UE46_13340* | -1.18 | -2.51 | *glnQ* | glutamine ABC transporter ATP-binding protein |
| *UE46_13400* | -0.92 | -1.93 | - | DNA methyltransferase |
| *UE46_13455* | -0.34 | -6.72 | - | PTS fructose transporter subunit IIC |
| *UE46_13460* | -0.16 | -6.20 | - | phosphofructokinase |
| *UE46_13465* | 0.59 | -6.13 | - | DeoR faimly transcriptional regulator |

| | | | | |
|---|---|---|---|---|
| *UE46_13890* | -2.04 | -6.02 | - | hypothetical protein |
| *UE46_13905* | -2.03 | -3.77 | - | ABC transporter substrate-binding protein |
| *UE46_14300* | -2.20 | -1.59 | - | MarR family transcriptional regulator |
| *UE46_14305* | -4.11 | -3.60 | - | amino acid ABC transporter ATP-binding protein |
| *UE46_14310* | -4.90 | -3.01 | - | cysteine ABC transporter permease |
| *UE46_14315* | -4.66 | -4.05 | - | cysteine ABC transporter permease |
| *UE46_14320* | -5.96 | -3.47 | - | L-cystine-binding protein TcyJ |
| *UE46_14325* | -5.91 | -4.23 | - | GCN5 family acetyltransferase |
| *UE46_14330* | -5.29 | -3.14 | - | monooxygenase |
| *UE46_14335* | -4.14 | -2.63 | - | alkane 1-monooxygenase |
| *UE46_14340* | -6.86 | -2.29 | - | glutaredoxin |
| *UE46_14345* | -7.41 | -2.51 | - | hypothetical protein |
| *UE46_14475* | -2.63 | -0.89 | - | hypothetical protein |
| *UE46_14550* | -7.77 | -1.17 | - | glycosyl hydrolase family 3 |
| *UE46_15265* | -4.49 | -2.10 | - | thiamine-phosphate pyrophosphorylase |
| *UE46_15280* | -3.85 | -2.08 | - | hypothetical protein |
| *UE46_15700* | -4.05 | -0.80 | - | cell surface protein |
| *UE46_16115* | -1.33 | -2.47 | - | porin |

Abb.=abbreviation.
Not statistically relevant data (p-value >0.05 and FDR >0.3) are shown in grey.

For a better overview, the regulated genes were functionally classified according to their COGs (Table S3a and S3b, FIG. 7). Most of the down regulated genes encode for proteins involved in metabolism, in particular in energy production and conversion, amino acid transport and metabolism and inorganic ion transport.

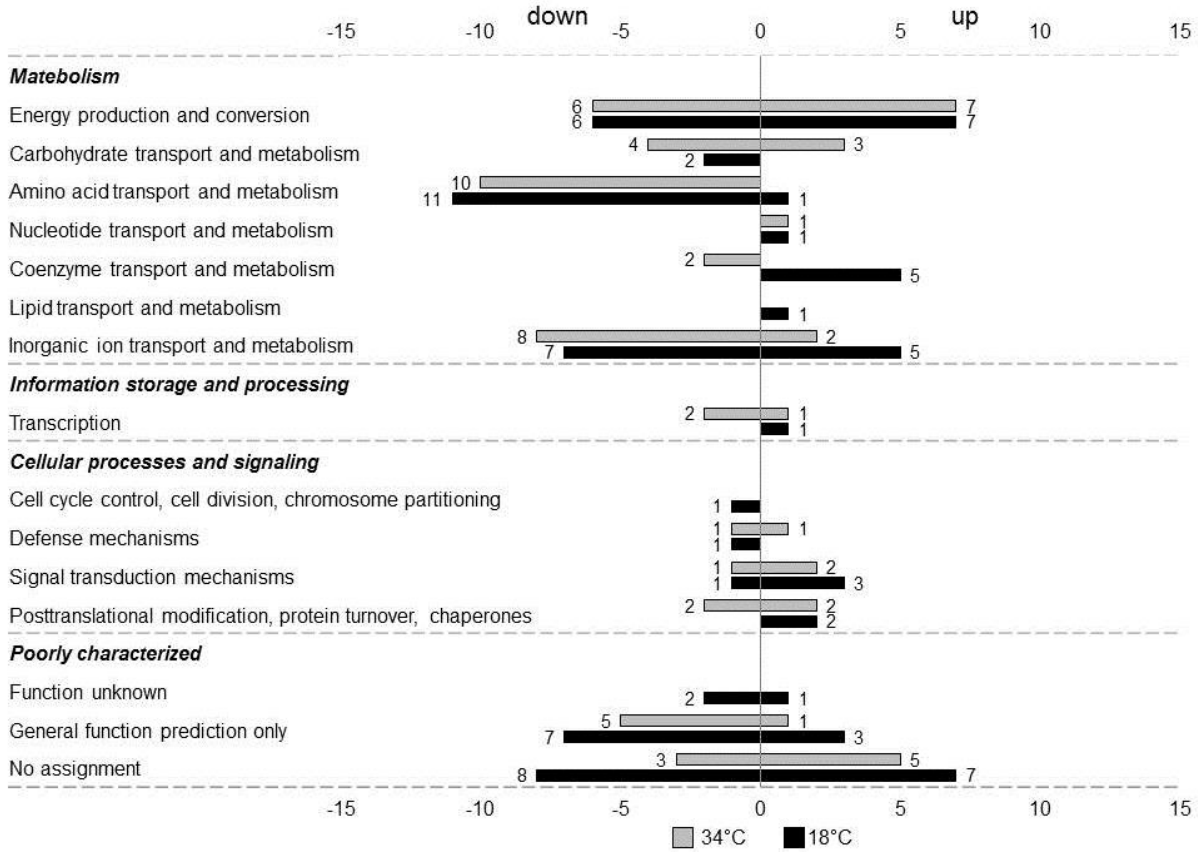



**FIG. 7: Oxygen-dependent regulated genes at 18°C and 34°C in *L. weihenstephanensis* based on their functional category.** Genes up- or down-regulated anaerobically at 18°C (black bars) or 34°C (grey bars) in *L. weihenstephanensis* were grouped according to the NCBI COGs. Bars represent the number of genes up- or down- regulated for each category. Since one gene can belong to several COG categories, summing up the regulated gene of each category, the total number of regulated genes is higher than the absolute number of regulated genes.

Just a few regulated genes were involved in transcription (0.0% for 18°C and 4.8% for 34°C) and in cellular processes and signaling (6.4% for 18°C and 9.5% for 34°C) and many were poorly characterized because their function was still unknown, just predicted or not associated to any GOG class (36.2% for 18°C and 19.1% for 34°C). For the up-regulated genes the situation was similar because, again, the majority of the regulated genes were involved in metabolic pathways (54.1% of the total up-regulated genes for 18°C and 52.0% for 34°C), in particular in energy, carbohydrate, coenzyme and inorganic ion processes. A small number of regulated genes were involved in transcription (2.7% for 18°C and 4.0% for 34°C) and in cellular processes and signaling (13.5% for 18°C and 20% for 34°C). Again, many regulated genes were poorly characterized (29.7% for 18°C and 24.0% for 34°C). These results indicate that the oxygen content is a key driver for the transcriptional regulation.

Under different temperatures, this oxygen-dependent regulation didn't show relevant differences. Indeed, the significantly regulated genes are equally shared between the two temperatures. ~50% of the up- or down-regulated genes are shown to be shared between the two temperatures but most of the remaining ~50% of the regulated genes, as mentioned before, showed the same transcriptional tendency.

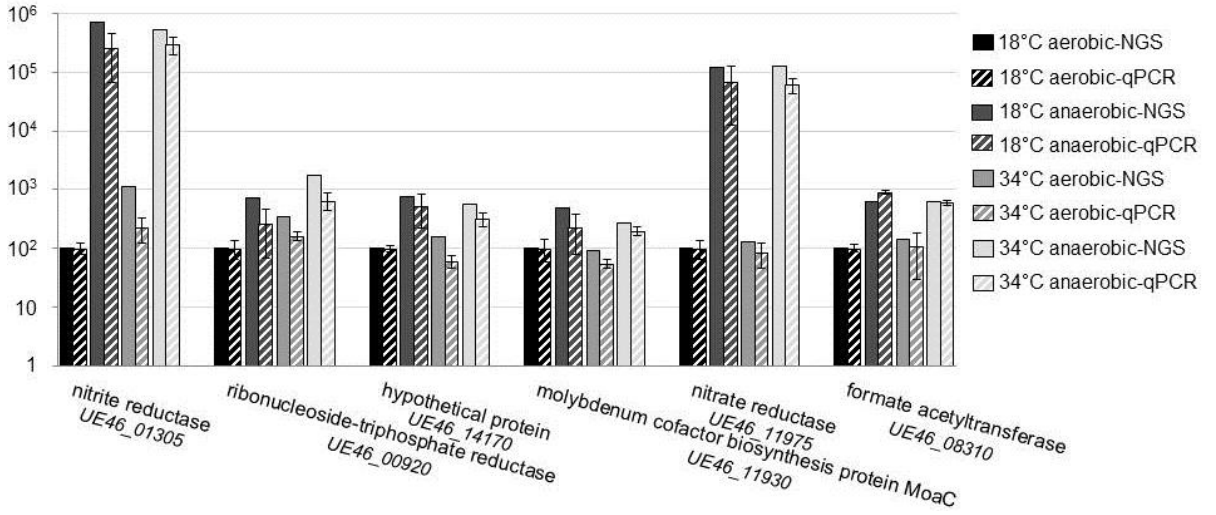
In particular, among the genes stronger transcribed aerobically, gene encoding for proteins involved in the aerobic metabolism were identified. For example catalase (UE46_00145), pyruvate dehydrogenase (UE46_11900), glutamate synthases (UE46_10730, UE46_10735) via glutamine and ABC transporters showed high negative log2FC at both the temperatures. Among the anaerobically up-regulated genes at both temperatures, the genes showing the highest $log_2$FC encoded for the all subunits of nitrate (*UE46_11960*, *UE46_11965*, *UE46_11970*, UE46_*11975*, *UE46_11980*) and nitrite (*UE46_01300*, UE46_*01305*) reductase and their functionality e.g. molybdenum cofactor (*UE46_11925*-*UE46_11955*), indicating that anaerobically *L. weihenstephanensis* performs nitrate respiration to supply energy production via the ETC even in absence of oxygen.

### 1.4 Validation of the RNA-NGS sequencing with qPCR

To validate the transcriptional data, transcription of selected genes was analyzed via qPCR (FIG. 8). This method is a widely used gold standard for assessment of sequencing results (Git *et al.,* 2010). The focus of the selection was the central carbohydrate catabolism pathway in *Listeria monocytogenes* (Romick *et al.*, 1996; Müller-Herbst *et al.*, 2014) and the dissimilatory nitrate reduction to ammonium (DNRA), which uses nitrate or nitrite as terminal electron acceptor for anaerobic respiration. In particular, among the anaerobically up-regulated genes, genes enconding for subunits of the nitrate and nitrite reductases (*UE46_11975*; *UE46_01305*), for molybdenum cofactor biosynthesis protein MoaC (*UE46_11930*) and for the ribonucleoside-triphosphate reductase (*UE46_00920*) were selected. Moreover, a protein annotated as hypothetical by the NCBI (NCBI_PGAP) but annotated as nitric oxide dioxygenase by RAST (*UE46_14170*) and a formate acetyltransferase (*UE46_08310*), which was not described to be regulated in the pathogenic *L. monocytogenes* (Müller-Herbst *et al.*, 2014) were selected. Among the anaerobically down-regulated genes, 2-oxoisovalerate dehydrogenase (*UE46_11895*), glutamate synthase (*UE46_10735*), glycine dehydrogenase

(*UE46_08610*), catalase (*UE46_00145*) and transporters (*UE46_03380*; *UE46_14320*) were selected and additionally a protein annotated as hypothetical (NCBI_PGAP) but described as dihydroxyacetone kinase family protein by RAST (*UE46_05890*) was investigated for validation. r (Pearson product-moment correlation coefficient) between NGS and qPCR results for the tested genes was calculated (0.98) and indicated a nearly perfect correlation between the two methods. Therefore, it can be concluded that the RNA-NGS sequencing data are reliable.

**a)** qPCR analysis of anaerobically up-regulated genes




**b)** qPCR analysis of anaerobically down-regulated genes

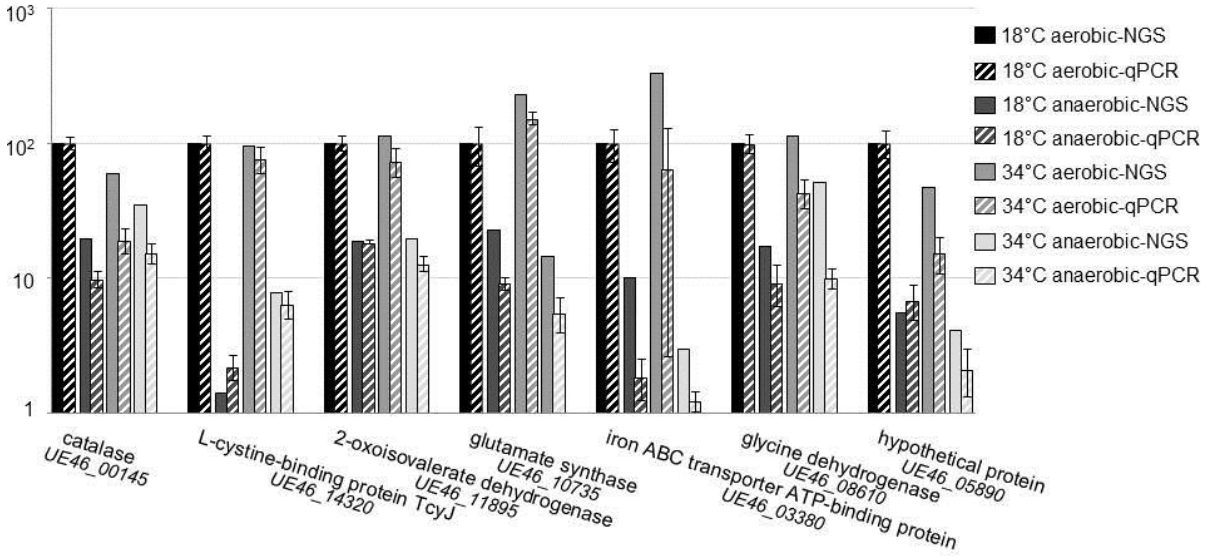


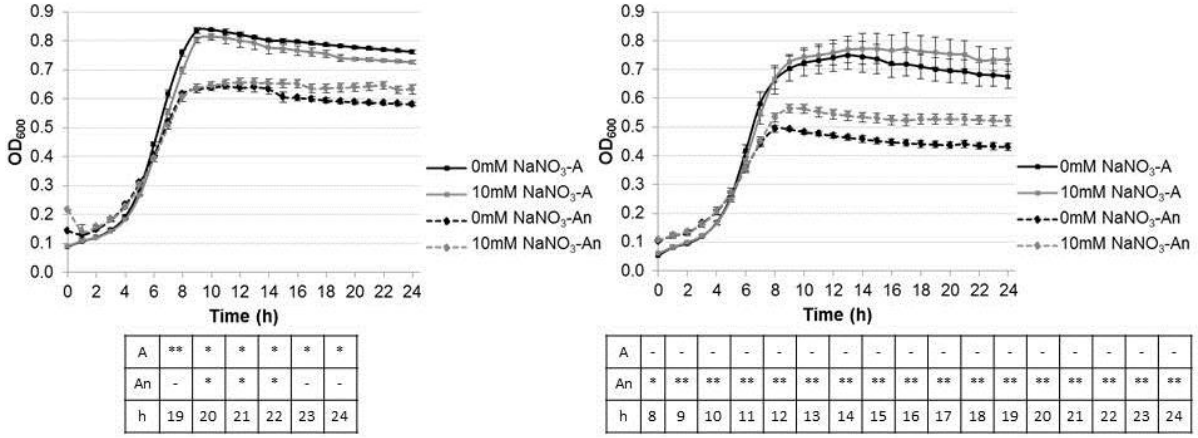
**FIG. 8: qPCR validation of RNA-NGS data for selected differently expressed genes.** RNAs used for the qPCR analyses were isolated from cultures at $OD_{600}$ = 0.85-0.95 under the described conditions. Bars represent the transcriptional level of selected up-regulated (a) or down-regulated (b) genes analyzed by RNA-NGS (solid bars) or qPCR (striped bars). RNA-NGS data were normalized to the smallest library (18°C anaerobic) and bars represent the percentage of transcription of each condition compared to the reference condition (18°C aerobic), set as 100%. qPCR data were analyzed by $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008), using the transcription of *clpX* (encoding the ATP-dependent Clp protease ATP-binding subunit ClpX) transcription for normalization. Transcription at 18°C aerobically was set as reference condition (100%) and bars represent mean values and standard errors from three biologically independent experiments, including duplicates.

## 1.5 Nitrate supports anaerobic growth of *L. weihenstephanensis*

The nitrate and nitrite reductase enzymes are present in *L. weihenstephanensis* and the genes encoding for all their subunits showed the strongest oxygen-dependent transcriptional regulation. To test if nitrate respiration could support the anaerobic growth of *L. weihenstephanensis*, growth analyses were performed at 34°C (Bioscreen C) comparing the growth in the presence and absence of sodium nitrate (10 mM). All the experiments were performed under both aerobic and anaerobic conditions. First, the BHI medium was used (FIG. 9a). Aerobically, starting from 19 h a significant bettergrowth in the absence of sodium nitrate was observed. Anaerobically, a slight difference was seen after 14 h growth, when the death phase of the cultures without sodium nitrate proceeded faster compared to the cultured supplied with $NaNO_3$.

The same experiments were then performed in minimal medium (FIG. 9b) and in this case a clear and very relevant phenotype was observed, showing a support of the anaerobic growth due to the presence of the $NaNO_3$ from eight h till the end of the experiments. Aerobically, in opposite to the BHI experiments, the cultures were dying slower when supplied with sodium nitrate.

**a)** *L. weihenstephanensis* in BHI **b)** *L. weihenstephanensis* in minimal medium




| | | | | | | |
|---|---|---|---|---|---|---|
| A | ** | * | * | * | * | * |
| An | - | * | * | * | - | - |
| h | 19 | 20 | 21 | 22 | 23 | 24 |

| | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| An | * | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** |
| h | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |

**FIG. 9: Oxygen-dependent growth of *L. weihenstephanensis* in the presence and absence of 10 mM sodium nitrate.** Depicted are growth curves for *L. weihenstephanensis* grown at 34°C in BHI (a) and minimal medium (b) aerobically (solid lines) and anaerobically (dotted lines), with 10 mM $NaNO_3$ (grey lines) or without $NaNO_3$ (black lines). $OD_{600}$ was measured every hour. All data points represent means ± standard error from four independent biological replicates for the experiments performed in BHI and seven independent biological replicates for the experiments conducted in minimal medium experiments. A=aerobic; An=anaerobic. Statistical analyses are shown in the table under the respective graphic only for the time points showing a significant difference between the growth with and without 10 mM $NaNO_3$ (*=p-value ≤0.05; **=p-value ≤0.01).

## 2. Temperature-dependent transcriptional profiling of *L. monocytogenes* EGDe

Müller-Herbst *et al*. (2014) have recently described the adaptation of *L. monocytogenes* to anaerobiosis. However the mechanisms driving this adaptation are still not completely clear. Therefore the anaerobic adaptation was further investigated, particularly to study its contribution to the survival in the intestine or promotion of the invasion process into intestinal epithelial cells. Global transcriptional analyses, via RNA-NGS sequencing, were performed aerobically and anaerobically at 18°C and 34°C, to further investigate a putative effect of temperature on the adaptation to anaerobiosis.

### 2.1 Aerobic and anaerobic growth of *L. monocytogenes* EGDe

As described before for the environmental *L. weihenstephanensis*, the pathogen *L. monocytogenes* EGDe was grown aerobically and anaerobically at 18°C (FIG. 10a) and 34°C (FIG. 10b) to examine its oxygen-dependent growth. Growth was more effective aerobically at both temperatures due to the oxygen-related metabolism and

was slower at 18°C compared to the higher temperature. Again, resazurine controls were added to monitor the two different oxygen conditions.

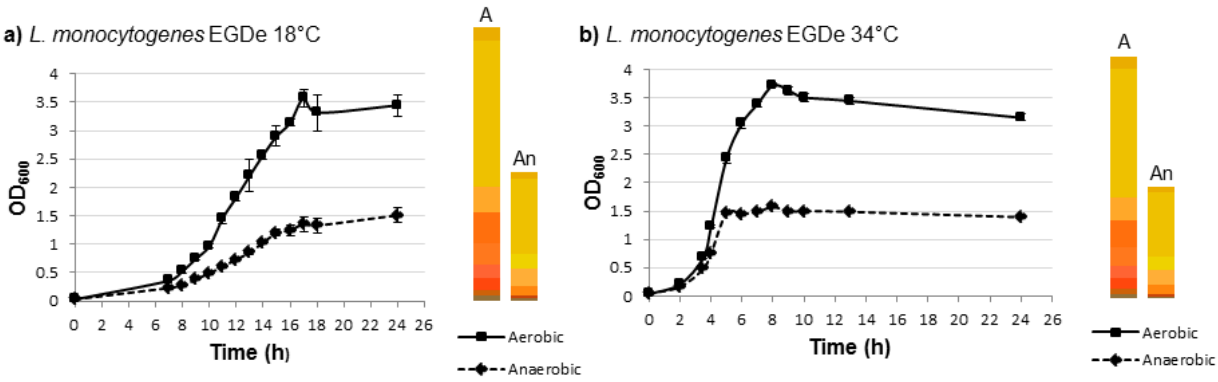



**FIG. 10: Oxygen-dependent growth of *L. monocytogenes* EGDe.** Depicted are growth curves for *L. monocytogenes* EGDe grown at 18°C (a) and 34°C (b) aerobically (solid line) and anaerobically (dotted line). $OD_{600}$ was measured every hour. All data points represent means ± standard error from three independent biological replicates. Colored bars indicate the color of a resazurin control ([A] aerobic and [An] anaerobic condition).

## 2.2 Oxygen dependent transcriptional adaptation of *L. monocytogenes* EGDe at different temperatures

The oxygen-dependent transcription of *L. monocytogenes* EGDe was analyzed to compare the response of this pathogenic *Listeria* species with that of the environmental species *L. weihenstephanensis*. Again, the cultures were cultivated aerobically and anaerobically at 18°C and 34°C till $OD_{600}$ = 0.85-0.95 and in parallel a resazurin control was performed for the validation of the required experimental oxygen conditions. The total RNA was isolated and sequenced via NGS. The raw read counts were normalized to smallest library, which in this case was the 34°C aerobic library. The $log_2$FC was calculated in edgeR, which displays the $log_2$ of the expression ratio anaerobic/aerobic.

In total, 234 genes showed an oxygen-dependent change in transcription. 85 genes were up-regulated anaerobically, including 5 genes that were up-regulated at 18°C and 34°C, while 76 genes were up-regulated specifically at 18°C and 4 specifically at 34°C (Table 21, descriptions are based on from the NCBI database [ftp://ftp.ncbi.nlm.nih.gov/genomes/Bacteria] and on the ListiList database [http://genolist.pasteur.fr/ListiList/]). 149 genes were down-regulated anaerobically, including 35 genes that showed an up-regulation at both temperatures. 101 genes were specifically down-regulated at 18°C and 13 genes at 34°C (Table 22). 56% of the up-regulated genes at 34°C showed up-regulation also at 18°C and 73% of the

down-regulated genes at 34°C were down-regulated also at 18°C. Therefore only 17 genes (in total) showed a specific regulation at 34°C while 176 genes showed a regulation specifically linked to 18°C.

Differently than *L. weihenstephanensis*, in most of the cases, e.g. genes encoding for proteins involved in cobalamin biosynthesis as well as ethanolamine and 1,2-propanediol utilization, the oxygen-dependent regulation was identified only at one of the investigated temperatures while the transcription at the other temperature was very low (Table S4a and S4b and Table S5 in the appendix). These results indicate that, as for *L. weihenstephanensis*, oxygen is a key driver for the anaerobic adaptation. *L. monocytogenes*, additionally, showed also different anaerobic adaptation depending on the cultivation temperature. At 34°C, only 58 genes showed an oxygen-dependent transcriptional adaptation, while 217 genes showed transcriptional regulation at 18°C.

**Table 21: Anaerobically up-regulated genes at 18°C and 34°C in *L. monocytogenes* EGDe.**

| Gene | $log_2$FC 18°C | $log_2$FC 34°C | Abb. | Product |
|---|---|---|---|---|
| *lmo0099* | -0.01 | 1.90 | - | hypothetical protein |
| *lmo0202* | 2.69 | 0.69 | *hly* | listeriolysin O precursor |
| *lmo0279**+* | 5.10 | 3.32 | - | anaerobic ribonucleoside triphosphate reductase |
| *lmo0280**+* | 4.67 | 3.25 | - | anaerobic ribonucleotide reductase activator protein |
| *lmo0302* | 1.77 | 0.40 | - | hypothetical protein |
| *lmo0303* | 1.73 | 0.68 | - | putaive secreted, lysin rich protein |
| *lmo0354* | 1.80 | 0.74 | - | fatty acid CoA ligase |
| *lmo0355**+* | 4.13 | 2.92 | - | fumarate reductase subunit A |
| *lmo0412* | 3.68 | 0.89 | - | hypothetical protein |
| *lmo0450* | 2.69 | 0.95 | - | hypothetical protein |
| *lmo0451* | 2.23 | 1.01 | - | hypothetical protein |
| *lmo0471**+* | 3.48 | 1.57 | - | hypothetical protein |
| *lmo0490* | 3.23 | 1.27 | *aroE* | shikimate 5 dehydrogenase |
| *lmo0491* | 2.25 | 0.95 | *aroD* | 3 dehydroquinate dehydratase |
| *lmo0641** | 5.50 | 6.17 | - | heavy metal transporting ATPase |
| *lmo0661* | 2.81 | -1.96 | - | hypothetical protein |
| *lmo0788* | 1.98 | 1.06 | - | hypothetical protein |

| | | | | |
|---|---|---|---|---|
| *lmo0912** | 3.49 | 1.03 | - | formate transporter |
| *lmo1001* | 1.93 | 1.35 | - | hypothetical protein |
| *lmo1143* | 2.95 | 0.36 | - | PduT protein |
| *lmo1145* | 4.90 | -0.09 | - | PduV protein |
| *lmo1149* | 6.73 | -4.11 | - | alpha ribazole 5' phosphatase |
| *lmo1171* | 4.78 | -3.19 | *pduQ* | NADPH dependent butanol dehydrogenase |
| *lmo1172* | 3.96 | | - | two component response regulator |
| *lmo1173* | 3.09 | 0.58 | - | two component sensor histidine kinase |
| *lmo1174* | 7.64 | | *eutA* | ethanolamine utilization protein EutA |
| *lmo1175* | 7.82 | -0.09 | *eutB* | ethanolamine ammonia lyase large subunit |
| *lmo1176* | 7.91 | 0.83 | *eutC* | ethanolamine ammonia lyase small subunit |
| *lmo1177* | 6.65 | | - | carboxysome structural protein EutL |
| *lmo1178* | 4.34 | 3.10 | - | carboxysome structural protein |
| *lmo1179* | 6.91 | 1.38 | - | alcohol dehydrogenase |
| *lmo1180* | 4.96 | -3.19 | - | carboxysome structural protein |
| *lmo1181* | 6.41 | 0.87 | - | cobalamin adenosyl transferase |
| *lmo1182* | 7.15 | -0.08 | - | PduL protein |
| *lmo1183* | 6.35 | 3.10 | - | hypothetical protein |
| *lmo1184* | 4.80 | | - | carbon dioxide concentrating mechanism protein |
| *lmo1185* | 4.85 | | - | PduT protein |
| *lmo1186* | 3.40 | | - | ethanolamine utilization protein EutH |
| *lmo1187* | 4.41 | -3.19 | - | ethanolamine utilization protein EutQ |
| *lmo1190* | 7.10 | 1.78 | - | hypothetical protein |
| *lmo1192* | 6.75 | 4.02 | *cobD* | cobalamin biosynthesis protein |
| *lmo1193* | 8.88 | 0.83 | *cbiC* | cobalt precorrin 8X methylmutase |
| *lmo1195* | 4.83 | -0.09 | *cbiE* | cobalt precorrin 6Y C(5) methyltransferase |
| *lmo1196* | 7.55 | 4.02 | - | cobalt precorrin 6Y C(15) methyltransferase |
| *lmo1201* | 6.65 | 3.10 | - | uroporphyrinogen III methyltransferase/synthase |
| *lmo1205* | 5.47 | 0.83 | - | cobalamin biosynthesis protein CbiN |
| *lmo1206* | 5.02 | -1.00 | *cbiQ* | cobalamin biosynthesis protein CbiQ |
| *lmo1249* | 10.28 | 6.12 | - | hypothetical protein |
| *lmo1250* | 9.52 | 5.76 | - | antibiotic resistance protein |
| *lmo1251** | 2.76 | 1.91 | - | Fnr/Crp family transcriptional regulator |
| *lmo1308* | 2.28 | 0.82 | - | hypothetical protein |
| *lmo1309* | 2.05 | 1.63 | - | hypothetical protein |
| *lmo1310** | 2.35 | 1.36 | - | hypothetical protein |

| | | | | |
|---|---|---|---|---|
| *lmo1312* | 3.02 | 4.02 | - | hypothetical protein |
| *lmo1406* | 2.64 | 0.52 | *pflB* | pyruvate formate lyase |
| *lmo1407* | 3.28 | 1.12 | *pflC* | pyruvate formate lyase activating enzyme |
| *lmo1634**+* | 4.76 | 0.58 | - | bifunctional acetaldehyde CoA/alcohol dehydrogenase |
| *lmo1659*+* | 3.23 | 2.34 | - | hypothetical protein |
| *lmo1700* | 2.32 | -0.29 | - | hypothetical protein |
| *lmo1717* | 2.67 | 0.66 | - | hypothetical protein |
| *lmo1790* | -0.46 | 2.73 | - | hypothetical protein |
| *lmo1871* | 2.02 | 0.77 | - | phosphoglucomutase |
| *lmo1917* | 4.75 | 1.65 | *pflA* | pyruvate formate lyase |
| *lmo1997* | 1.87 | 4.60 | - | PTS mannose transporter subunit IIA |
| *lmo1998*+* | -0.69 | 2.40 | - | opine catabolism protein |
| *lmo2173* | 1.75 | 0.23 | - | sigma 54 dependent transcriptional regulator |
| *lmo2234* | 2.70 | 1.47 | - | hypothetical protein |
| *lmo2235* | 8.00 | 1.57 | - | NADH oxidase |
| *lmo2236* | 4.54 | 2.27 | - | shikimate 5 dehydrogenase |
| *lmo2237* | 4.72 | 2.52 | - | MFS transporter permease |
| *lmo2238* | 1.92 | 0.60 | - | MFS transporter |
| *lmo2325a* | 4.10 | 2.93 | - | hypothetical protein |
| *lmo2338* | 1.73 | 0.07 | *pepC* | aminopeptidase |
| *lmo2364* | 2.77 | 2.06 | - | hypothetical protein |
| *lmo2365* | 1.84 | 1.37 | - | RofA family transcriptional regulator |
| *lmo2410* | 2.02 | 0.38 | - | hypothetical protein |
| *lmo2447* | 2.68 | 0.62 | - | transcriptional regulator |
| *lmo2467**+* | 2.19 | 1.88 | - | chitin binding protein |
| *lmo2536a* | 2.77 | 1.44 | - | hypothetical protein |
| *lmo2585* | 3.62 | 4.87 | - | hypothetical protein |
| *lmo2637* | 1.57 | -0.17 | - | hypothetical protein |
| *lmo2669** | 3.42 | 1.80 | - | hypothetical protein |
| *lmo2686* | 1.64 | 0.57 | - | hypothetical protein |
| *lmo2792* | 1.59 | 0.01 | - | hypothetical protein |
| *lmo2818* | 3.22 | -0.66 | - | MFS transporter |

Not statistical relevant data (p-value >0.05 and FDR >0.3) are shown in grey.

***** genes previously descrbed to be anaerobically up-regulated regulated in *L. monocytogenes* EGD (Müller-Herbst *et al.*, 2014).

[+] genes previously described to be controlled by the redox sensing transcriptional repressor Rex (Stefanie Wüstner, 2010).

Abb.=abbreviation.

**Table 22: Anaerobically down-regulated genes at 18°C and 34°C in *L. monocytogenes* EGDe.**

| Gene | $log_2$FC 18°C | $log_2$FC 34°C | Abb. | Product |
|---|---|---|---|---|
| *lmo0013** | -3.85 | -1.91 | *qoxA* | aa3-600 quinol oxidase subunit II |
| *lmo0014** | -3.74 | -2.72 | *qoxB* | aa3-600 quinol oxidase subunit I |
| *lmo0015** | -6.37 | -3.93 | *qoxC* | aa3-600 quinol oxidase subunit III |
| *lmo0016** | -4.39 | -3.14 | *qoxD* | aa3-600 quinol oxidase subunit IV |
| *lmo0019* | -3.46 | 1.08 | - | hypothetical protein |
| *lmo0043* | -3.09 | -0.35 | - | arginine deiminase |
| *lmo0133* | -1.81 | 0.07 | - | hypothetical protein |
| *lmo0169* | -3.41 | -0.40 | - | glucose transporter |
| *lmo0263* | -3.58 | -0.21 | *inlH* | internalin H |
| *lmo0265* | -2.41 | 0.68 | - | succinyl diaminopimelate desuccinylase |
| *lmo0321* | -2.82 | 0.02 | - | hypothetical protein |
| *lmo0325* | -1.98 | 0.46 | - | transcriptional regulator |
| *lmo0361* | -7.11 | -5.33 | - | membrane protein |
| *lmo0362* | -6.94 | -9.84 | - | hypothetical protein |
| *lmo0365* | -8.72 | -5.51 | - | hypothetical protein |
| *lmo0366* | -7.89 | -7.72 | - | hypothetical protein |
| *lmo0367* | -7.58 | -5.97 | - | hypothetical protein |
| *lmo0439* | -3.30 | -0.30 | - | hypothetical protein |
| *lmo0445* | -3.67 | 0.07 | - | transcripitonal regulator |
| *lmo0484* | -5.77 | -5.64 | - | heme degrading monooxygenase IsdG |
| *lmo0485* | -8.22 | -7.60 | - | hypothetical protein |
| *lmo0515* | -2.54 | 0.09 | - | hypothetical protein |
| *lmo0519* | -3.24 | -1.89 | - | multidrug resistance protein |
| *lmo0541* | -7.84 | -7.72 | - | ABC transporter substrate binding protein |
| *lmo0554* | -2.08 | -0.18 | - | NADH dependent butanol dehydrogenase |
| *lmo0573* | -3.42 | -2.23 | - | hypothetical protein |

| | | | | |
|---|---|---|---|---|
| *lmo0591* | -3.36 | -1.22 | - | hypothetical protein |
| *lmo0596* | -2.08 | -0.04 | - | hypothetical protein |
| *lmo0597* | -2.74 | -2.86 | - | Crp/Fnr family transcriptional regulator |
| *lmo0609* | -2.07 | -1.48 | - | phage shock protein E |
| *lmo0610* | -3.96 | 0.03 | - | internalin |
| *lmo0611* | -2.85 | -1.83 | *acpD* | azoreductase |
| *lmo0628* | -2.63 | 0.26 | - | hypothetical protein |
| *lmo0629* | -1.91 | 0.06 | - | hypothetical protein |
| *lmo0648* | -2.34 | 0.14 | - | hypothetical protein |
| *lmo0649* | -2.24 | 0.14 | - | transcriptional regulator |
| *lmo0655* | -2.06 | 0.73 | - | phosphoprotein phosphatase |
| *lmo0669* | -3.27 | -0.10 | - | oxidoreductase |
| *lmo0670* | -2.62 | 0.72 | - | hypothetical protein |
| *lmo0722* | -2.32 | 0.85 | - | pyruvate oxidase |
| *lmo0784** | -2.50 | 0.87 | - | PTS mannose transporter subunit IIB |
| *lmo0794* | -2.29 | -0.38 | - | hypothetical protein |
| *lmo0811* | -2.22 | -1.86 | - | carbonic anhydrase |
| *lmo0830* | -2.69 | -2.38 | *fbp* | fructose 1,6 bisphosphatase |
| *lmo0847* | -2.97 | -1.67 | - | glutamine ABC transporter |
| *lmo0848* | -2.39 | -1.64 | - | amino acid ABC transporter ATP binding protein |
| *lmo0880* | -6.46 | -0.37 | - | wall associated protein precursor |
| *lmo0911* | -1.75 | -0.10 | - | hypothetical protein |
| *lmo0913* | -3.20 | 0.29 | - | succinate semialdehyde dehydrogenase |
| *lmo0937* | -3.01 | 0.13 | - | hypothetical protein |
| *lmo0953* | -2.30 | 0.32 | - | hypothetical protein |
| *lmo0994* | -2.63 | -0.17 | - | hypothetical protein |
| *lmo0995* | -2.31 | -0.29 | - | hypothetical protein |
| *lmo1007* | -6.97 | -7.89 | - | hypothetical protein |
| *lmo1052** | -4.06 | -2.80 | *pdhA* | pyruvate dehydrogenase subunit A1 alpha |
| *lmo1053** | -4.10 | -3.12 | *pdhB* | pyruvate dehydrogenase subunit A1 beta |
| *lmo1054** | -5.34 | -4.01 | *pdhC* | dihydrolipoamide acetyltransferase |
| *lmo1055** | -4.00 | -3.93 | *pdhD* | dihydrolipoamide dehydrogenase |
| *lmo1131* | -5.44 | -6.74 | - | ABC transporter ATP binding protein |
| *lmo1132* | -4.10 | -5.30 | - | ABC transporter ATP binding protein |
| *lmo1140* | -1.99 | 0.94 | - | hypothetical protein |

| | | | | |
|---|---|---|---|---|
| *lmo1241* | -1.99 | 0.04 | - | hypothetical protein |
| *lmo1257** | -0.21 | -2.24 | - | hypothetical protein |
| *lmo1266* | -1.83 | 0.55 | - | hypothetical protein |
| *lmo1293* | -2.44 | -0.52 | *glpD* | glycerol 3 phosphate dehydrogenase |
| *lmo1298* | -2.09 | 0.64 | *glnR* | glutamine synthetase repressor |
| *lmo1300* | -2.60 | -0.49 | - | arsenic transporter |
| *lmo1375* | -2.32 | -0.28 | - | aminotripeptidase |
| *lmo1384* | -2.66 | -2.54 | - | hypothetical protein |
| *lmo1433* | -2.05 | 0.13 | - | glutathione reductase |
| *lmo1439* | -2.23 | -1.88 | *sod* | superoxide dismutase |
| *lmo1539** | -2.48 | 0.93 | - | glycerol transporter |
| *lmo1566* | -1.59 | -1.57 | *citC* | isocitrate dehydrogenase |
| *lmo1590* | -0.04 | -2.58 | *argJ* | bifunctional ornithine acetyltransferase/N acetylglutamate synthase |
| *lmo1694* | -2.73 | 0.10 | - | CDP abequose synthase |
| *lmo1830* | -2.65 | 0.37 | - | short chain dehydrogenase |
| *lmo1831* | -2.44 | -1.00 | *pyrE* | orotate phosphoribosyltransferase |
| *lmo1834* | -2.27 | -0.65 | *pyrDII* | dihydroorotate dehydrogenase electron transfer subunit |
| *lmo1835* | -2.76 | -1.04 | *carB* | carbamoyl phosphate synthetase |
| *lmo1839* | -2.61 | -0.61 | *pyrP* | uracil permease |
| *lmo1883* | -4.22 | 0.81 | - | chitinase |
| *lmo1884* | -3.56 | -1.47 | - | xanthine permease |
| *lmo1956* | -1.43 | -1.97 | *fur* | Fur family transcriptional regulator |
| *lmo1957* | -4.31 | -4.74 | *fhuG* | ferrichrome ABC transporter permease |
| *lmo1958* | -6.32 | -6.62 | *fhuB* | ferrichrome ABC transporter permease |
| *lmo1959* | -10.10 | -5.30 | *fhuD* | ferrichrome binding protein |
| *lmo1960* | -6.08 | -6.04 | *fhuC* | ferrichrome ABC transporter ATP binding protein |
| *lmo1961* | -5.40 | -4.65 | - | oxidoreductase |
| *lmo2006** | -0.92 | -1.99 | *alsS* | acetolactate synthase |
| *lmo2057** | -2.66 | -1.11 | *ctaB* | protoheme IX farnesyltransferase |
| *lmo2060* | -1.85 | -0.90 | - | hypothetical protein |
| *lmo2062* | -1.91 | -1.65 | - | copper transporter |
| *lmo2067* | -2.20 | 0.04 | - | bile acid hydrolase |
| *lmo2072* | -1.55 | -1.25 | - | redox sensing transcriptional repressor Rex |

| | | | | |
|---|---|---|---|---|
| *lmo2085* | -3.38 | -0.15 | - | peptidoglycan binding protein |
| *lmo2091* | -0.51 | -2.11 | *argH* | argininosuccinate lyase |
| *lmo2104* | -5.80 | -6.74 | - | hypothetical protein |
| *lmo2104a* | -3.87 | -4.50 | - | ferrous iron transport protein B |
| *lmo2105* | -5.54 | -7.06 | - | hypothetical protein |
| *lmo2132* | -3.59 | 0.13 | - | hypothetical protein |
| *lmo2158** | -1.92 | 0.70 | - | hypothetical protein |
| *lmo2180* | -5.44 | -7.55 | - | hypothetical protein |
| *lmo2181* | -11.13 | -6.79 | - | hypothetical protein |
| *lmo2183* | -6.02 | -5.64 | - | ferrichrome ABC transporter permease |
| *lmo2184* | -7.26 | -7.28 | - | ferrichrome ABC transporter substrate binding protein |
| *lmo2185* | -9.05 | -8.31 | - | hypothetical protein |
| *lmo2186* | -8.43 | -11.36 | - | hypothetical protein |
| *lmo2199* | -1.81 | -0.83 | - | hypothetical protein |
| *lmo2200* | -2.30 | -0.77 | - | MarR family transcriptional regulator |
| *lmo2205* | -1.60 | 0.38 | - | phosphoglyceromutase |
| *lmo2213* | -1.92 | 0.29 | - | hypothetical protein |
| *lmo2230* | -3.63 | -0.15 | - | arsenate reductase |
| *lmo2231* | -3.58 | 0.43 | - | hypothetical protein |
| *lmo2260* | -1.57 | -2.88 | - | hypothetical protein |
| *lmo2261* | -2.34 | -3.56 | - | hypothetical protein |
| *lmo2269* | -6.15 | -1.06 | - | hypothetical protein |
| *lmo2335** | 0.45 | -6.24 | *fruA* | PTS fructose transporter subunit IIABC |
| *lmo2336** | 0.34 | -6.02 | *fruB* | fructose 1 phosphate kinase |
| *lmo2337** | 0.49 | -7.28 | - | DeoR family transcriptional regulator |
| *lmo2343* | -3.95 | -2.85 | - | nitrilotriacetate monooxygenase |
| *lmo2344** | -3.84 | -4.11 | - | hypothetical protein |
| *lmo2345* | -7.34 | 3.10 | - | hypothetical protein |
| *lmo2346* | -3.97 | | - | amino acid ABC transporter ATP binding protein |
| *lmo2347* | -4.17 | -1.62 | - | amino acid ABC transporter permease |
| *lmo2348* | -4.45 | -2.85 | - | amino acid ABC transporter permease |
| *lmo2349* | -4.95 | -1.08 | - | amino acid ABC transporter substrate binding protein |
| *lmo2350* | -4.47 | -2.52 | - | hypothetical protein |

| | | | | |
|---|---|---|---|---|
| *lmo2351* | -4.17 | -2.16 | - | FMN reductase |
| *lmo2352* | -2.05 | -2.21 | - | LysR family transcriptional regulator |
| *lmo2387* | -2.97 | 0.15 | - | hypothetical protein |
| *lmo2389** | -2.63 | -1.54 | - | NADH dehydrogenase |
| *lmo2390* | -2.57 | -1.78 | - | hypothetical thioredoxine reductase |
| *lmo2391* | -2.51 | 0.29 | - | hypothetical protein |
| *lmo2393* | -2.14 | -0.63 | - | hypothetical protein |
| *lmo2411* | -1.68 | -1.19 | - | hypothetical protein |
| *lmo2412* | -2.02 | -1.21 | - | hypothetical protein |
| *lmo2415* | -1.91 | -1.31 | - | ABC transporter ATP binding protein |
| *lmo2423* | -2.20 | 0.29 | - | hypothetical protein |
| *lmo2429* | -2.02 | -1.55 | - | ferrichrome ABC transporter ATP binding protein |
| *lmo2434* | -2.16 | 0.07 | - | glutamate decarboxylase |
| *lmo2463* | -2.74 | -0.13 | - | multidrug transporter |
| *lmo2484* | -1.87 | 0.04 | - | hypothetical protein |
| *lmo2569** | -2.50 | -3.26 | - | peptide ABC transporter substrate binding protein |
| *lmo2571* | -2.88 | 0.73 | - | nicotinamidase |
| *lmo2588* | -2.10 | -0.25 | - | multidrug transporter |
| *lmo2602* | -2.89 | 0.90 | - | hypothetical protein |
| *lmo2673* | -1.60 | 0.45 | - | hypothetical protein |
| *lmo2695** | -2.29 | 0.04 | - | dihydroxyacetone kinase subunit DhaK |
| *lmo2748* | -2.43 | 0.04 | - | hypothetical protein |

Not statistical relevant data (p-value >0.05 and FDR >0.3) are shown in grey.

***** genes previously descrbed to be anaerobically down-regulated regulated in *L. monocytogenes* EGD (Müller-Herbst *et al.*, 2014).

Abb.=abbreviation.

Most of the regulated genes belong to the central metabolic pathway of *L. monocytogenes* (FIG. 2) and many were already described to have oxygen-dependent regulation (Müller-Herbst *et al.*, 2014, Table 21 and 22). These data also indicate that many genes encoding for proteins involved in cobalamin biosynthesis, ethanolamine and 1,2-propanediol utilization (*lmo1143*, *lmo1145*, *lmo1149*, *lmo1171-lmo1187*, *lmo1190*, *lmo1192-1193*, *lmo1195-1196, lmo1201*, *lmo1205-1206*) were stronger transcribed anaerobically at both temperatures, with statistical significance at 18°C. Moreover, the redox sensing transcriptional repressor Rex (*lmo2072*), was

seen to be down-regulated only at 18°C and genes already described to be repressed by this transcriptional regulator (Stefanie Wüstner, 2010) in response showed stronger transcription anaerobically, particularly at 18°C (Table 21). Another regulator already described to be anaerobically induced (Stefanie Wüstner, 2010; Müller-Herbst *et al.*, 2014), the member of the Fnr/Crp superfamily of transcriptional factors (*lmo1251*), was identified as up-regulated anaerobically at 18°C. The only gene discovered to be controlled by this regulator (*lmo1250*) (Krementowski, 2010) was also seen to be up-regulated anaerobically at 18°C.

The regulated genes were functionally classified according to their COGs (FIG. 11).

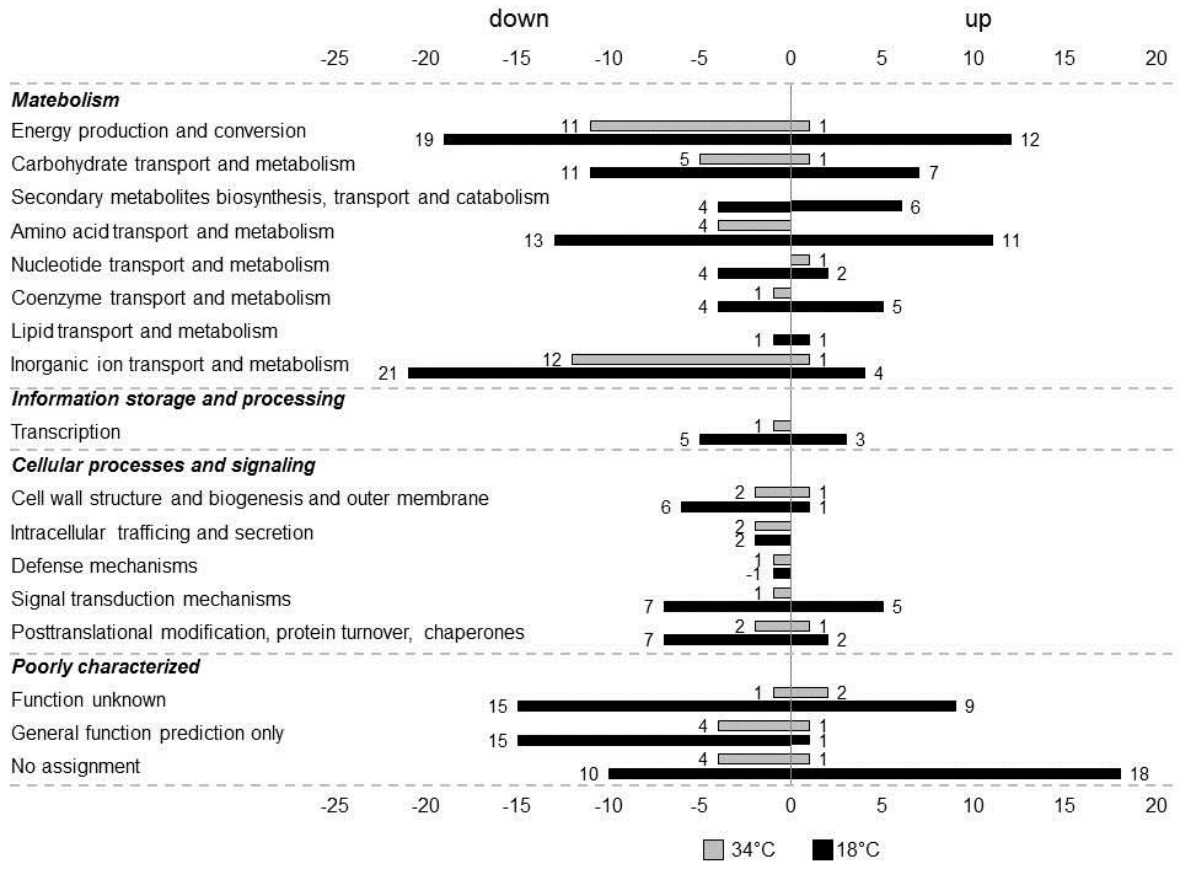



**FIG. 11: Differently regulated genes at 18°C and 34°C in *L. monocytogenes* EGDe based on their functional category.** Genes up- or down-regulated at 18°C (black bars) or 34°C (grey bars) in *L. monocytogenes* EGDe were grouped according to the NCBI COGs. Bars represent the number of genes up- or down-regulated for each category. Since one gene can belong to several COG categories, summing up the regulated gene of each category, the total number of regulated genes is higher than the absolute number of regulated genes.

As described for *L. weihenstephanensis*, most of the down-regulated genes encoded for proteins involved in metabolism, in particular in energy production and conversion, amino acid transport-and-metabolism and inorganic ion processes

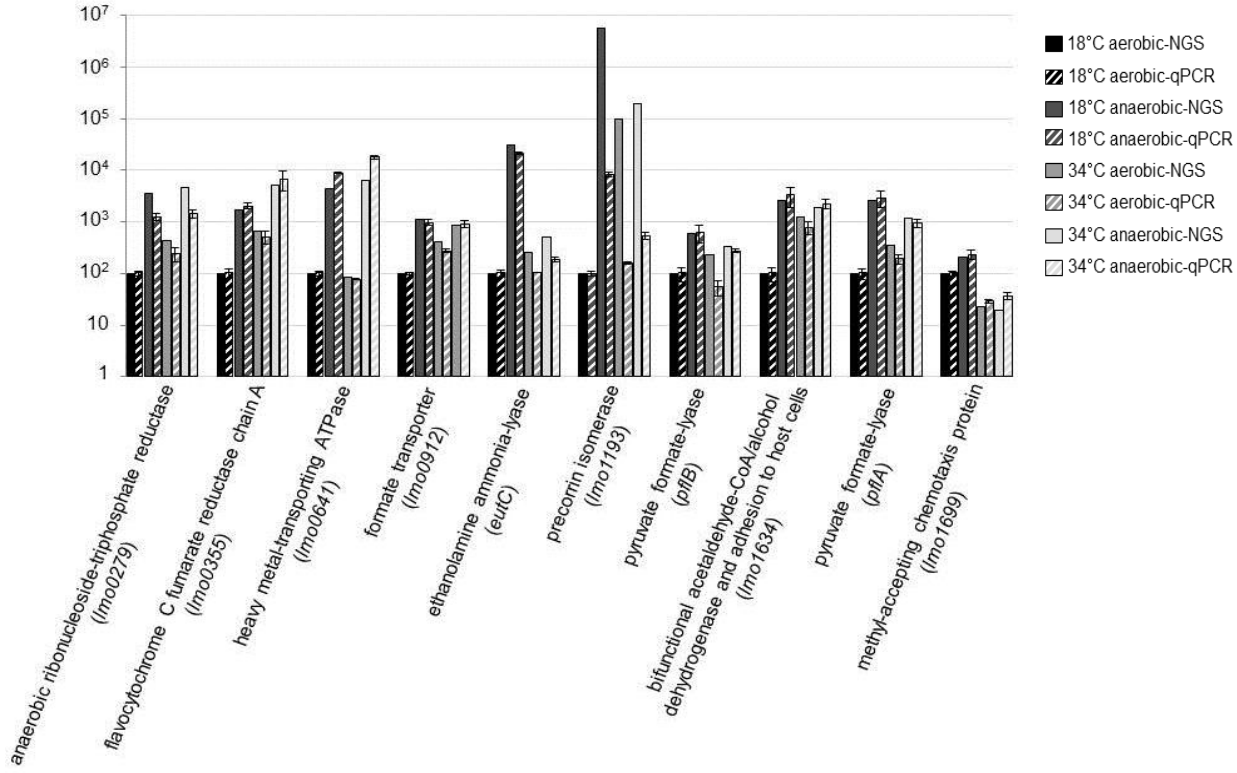
(52.7% for 18°C and 64.7% for 34°C). Just a few regulated genes were involved in transcription and translation (3.4% for 18°C and 2.0% for 34°C) and several regulated genes in cellular processes and signaling (16.5% for 18°C and 15.7% for 34°C). Many genes were poorly characterized because their function was still unknown, just predicted or not associated to any GOG class (27.4% for 18°C and 17.6% for 34°C). A analogous situation was found for the up-regulated genes, since the majority of the regulated genes were involved in metabolic pathways (55.2% of the total up-regulated genes for 18°C and 40.0% for 34°C), in particular in energy, carbohydrate, amino acid and coenzyme processes. Very few genes were involved in transcription and translation (3.5% for 18°C and 0.0% for 34°C) and in cellular processes and signaling (9.2% for 18°C and 20% for 34°C). Again, many regulated genes were poorly characterized (32.1% for 18°C and 40.0% for 34°C).

### 2.3 Validation of the RNA-NGS sequencing with qPCR

For the confirmation of the global transcriptomic data, genes encoding for proteins involved in the central carbohydrate catabolism pathway in *L. monocytogenes* (Romick *et al.*, 1996; Müller-Herbst *et al.*, 2014) were selected to be validated via qPCR, since they showed oxygen- and, in some cases, temperature-dependent transcriptional regulation. In particular among the anaerobically up-regulated genes, the one encoding for an anaerobic ribonucleoside-triphosphate reductase (*lmo0279*), a flavocytochrome C fumarate reductase chain A (*lmo0355*), a heavy metal-transporting ATPase (*lmo0641*), a formate transporter (*lmo0912*), an ethanolamine ammonia-lyase (*eutC*, *lmo1176*), a precorrin isomerase (*lmo1193*), a pyruvate formate-lyase (*pflB*, *lmo1406*), a bifunctional acetaldehyde-CoA/alcohol dehydrogenase and adhesion to host cells (*lmo1634*), a pyruvate formate-lyase (*pflA*, *lmo1917*) and a methyl-accepting chemotaxis protein (*lmo1699*) were selected. Among the anaerobically down-regulated genes, genes encoding for proteins directly involved and active in the aerobic respiration of *Listeria* were validated. aa3-600 quinol oxidase subunit I (*qoxB*, *lmo0014*), pyruvate oxidase (*lmo0722*), pyruvate dehydrogenase E2 subunit (*pdhC*, *lmo1054*), superoxide dismutase (*sod*, *lmo1439*), alpha-acetolactate synthase (*alsS*, *lmo2006*) and redox-sensing transcriptional repressor Rex (*lmo2072*) were selected. r coefficient was again calculated as previously described, resulting in 0.86. NGS data were therefore validated also for *L.*

*monocytogenes* EGDe because a strong correlation occurs between the two methods (FIG. 12).

**a)** qPCR analysis of anaerobically up-regulated genes




**b)** qPCR analysis of anaerobically down-regulated genes

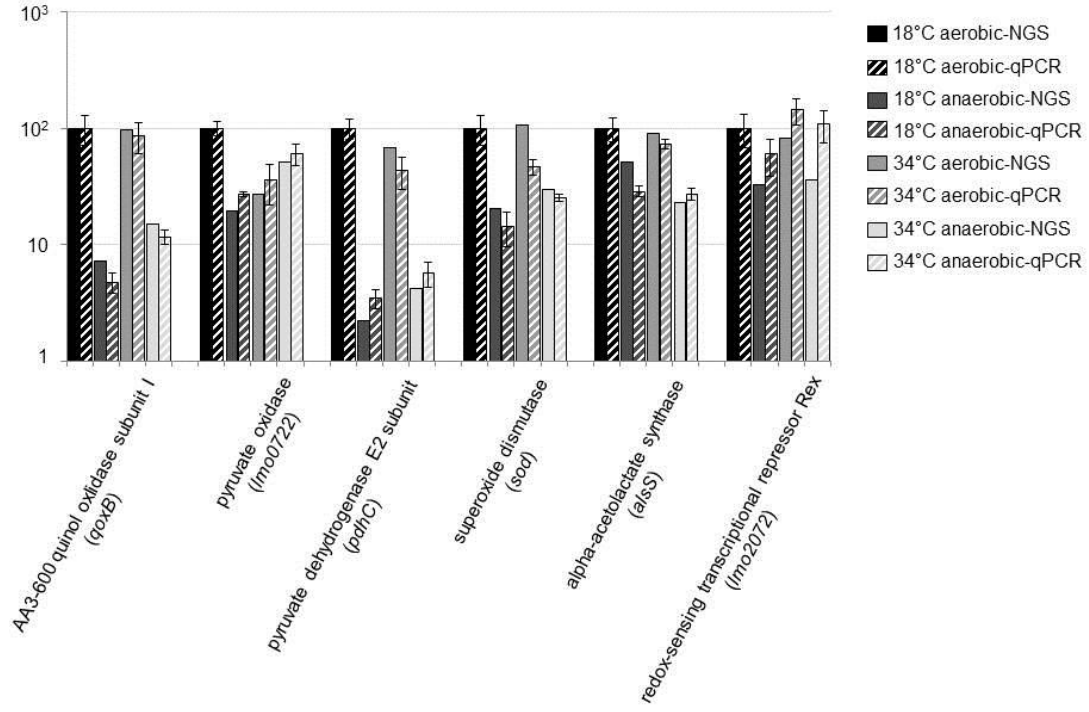


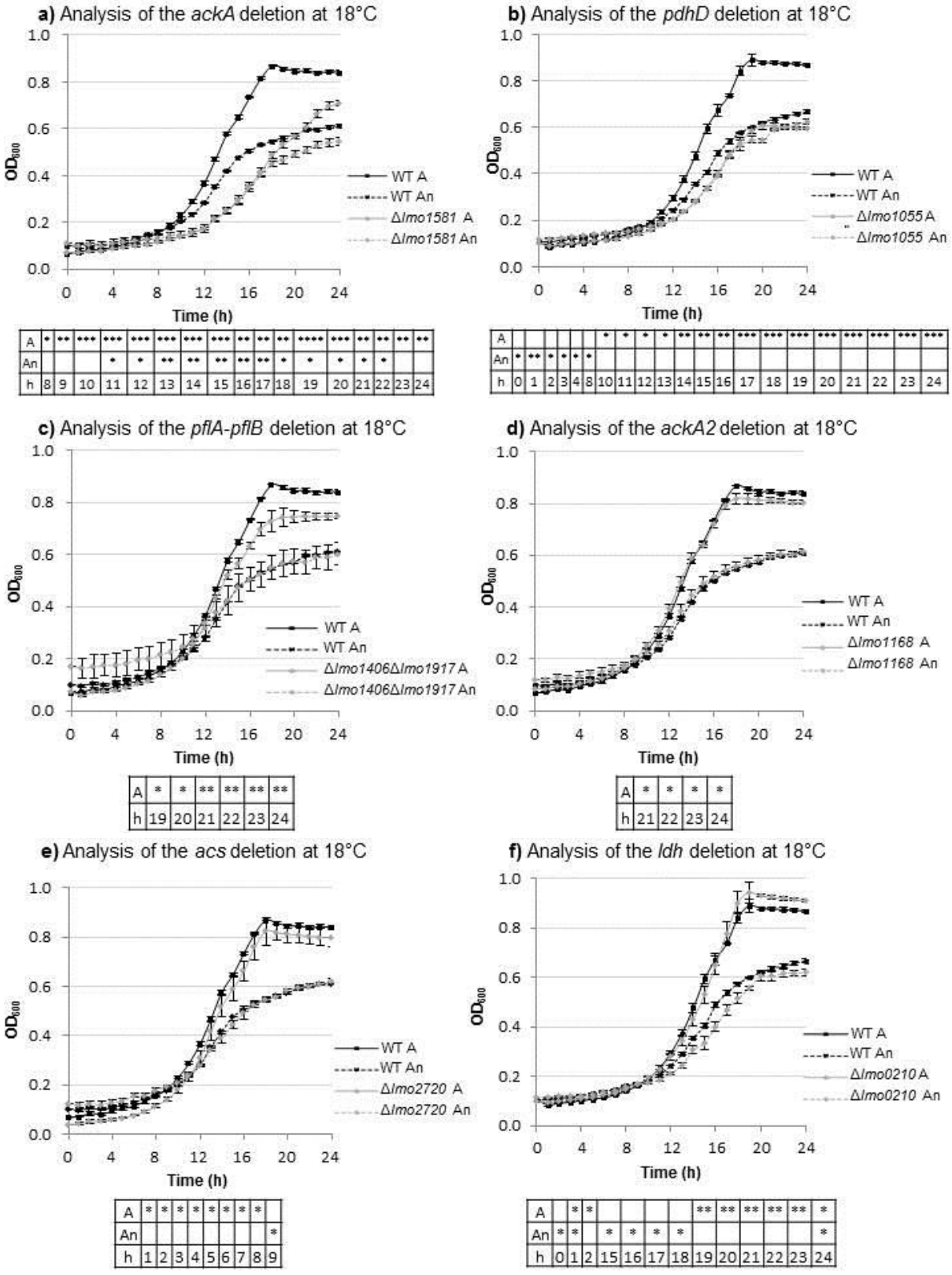
**FIG. 12: qPCR validation of RNA-NGS data for selected differently expressed genes.** RNAs used for the qPCR analyses were isolated from cultures at $OD_{600}$ 0.85-0.95 under the described conditions. Bars represent the transcriptional level of selected up-regulated (a) or down-regulated (b) genes analyzed by RNA-NGS (solid bars) or qPCR (striped bars). NGS data were normalized to the smallest library (34°C aerobic) and bars represent the percentage of transcription of each condition compared to the reference condition (18°C aerobic), set as 100%. qPCR data were analyzed by $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008), using the 16S rRNA transcription for normalization. Transcription at 18°C aerobic was set as reference condition (100%) and bars represent mean values and standard errors from three biologically independent experiments, including duplicates. Analyses performed in collaboration with MSc. Elisabeth Walburga Maria Schwab.

For *lmo1193* the number of normalized reads for the reference condition (18°C aerobic) was very low and this explains the very high percentage of transcription for the NGS results of the other conditions. This gene was not considered for r calculation.

## 2.4 Construction and phenotypic characyerization of *L. monocytogenes* in frame deletion mutants

As mentioned before, most of the regulated genes belong to the central catabolic pathway of *L. monocytogenes* (FIG. 2). Deletion mutants were generated, if not already present in our laboratory, for the most important genes of this pathway (in collaboration with Romy Wecko, Arista Samaras and Matthias Steiger). The mutant *L. monocytogenes* EGDe Δ*lmo0210* (absence of L-lactate dehydrogenase [*ldh*]), *L. monocytogenes* EGDe Δ*lmo0722* (pyruvate oxidase), *L. monocytogenes* EGDe Δ*lmo1055* (E3 subunit of pyruvate dehydrogenase complex [*pdhD*]), *L. monocytogenes* EGDe Δ*lmo1168* (acetate kinase [*ackA2*]), *L. monocytogenes* EGDe Δ*lmo1406* (pyruvate formate-lyase [*pflB*]), *L. monocytogenes* EGDe Δ*lmo1581* (acetate kinase [*ackA*]), *L. monocytogenes* EGDe Δ*lmo1634* (bifunctional acetaldehyde-CoA/alcohol dehydrogenase and adhesion to host cells [*adh*]), *L. monocytogenes* EGDe Δ*lmo1917* for a second pyruvate formate-lyase [*pflA*]), *L. monocytogenes* EGDe Δ*lmo2006* (alpha-acetolactate synthase [*alsS*]), *L. monocytogenes* EGDe Δ*lmo2720* (acetate-CoA ligase [*acs*]) and the double mutant *L. monocytogenes* EGDe Δ*lmo1917*Δ*lmo1406* (pyruvate-formate lyases [*plfA/plfB*]) were produced.

Their growth was compared to that of *L. monocytogenes* EGDe WT at 18°C (FIG. 13) and 34°C (FIG. 14) aerobically and anaerobically. For six mutants the deletion of the specific gene led to a behavioral variation.




| A | * | ** | *** | *** | *** | *** | *** | *** | ** | ** | ** | **** | *** | ** | ** | ** | ** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| An | | | | * | * | ** | ** | ** | ** | ** | * | * | * | * | * | | |
| h | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |

| A | | | | | | * | * | * | * | ** | ** | ** | *** | *** | *** | *** | *** | *** | *** | *** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| An | * | ** | * | * | * | * | | | | | | | | | | | | | | |
| h | 0 | 1 | 2 | 3 | 4 | 8 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |

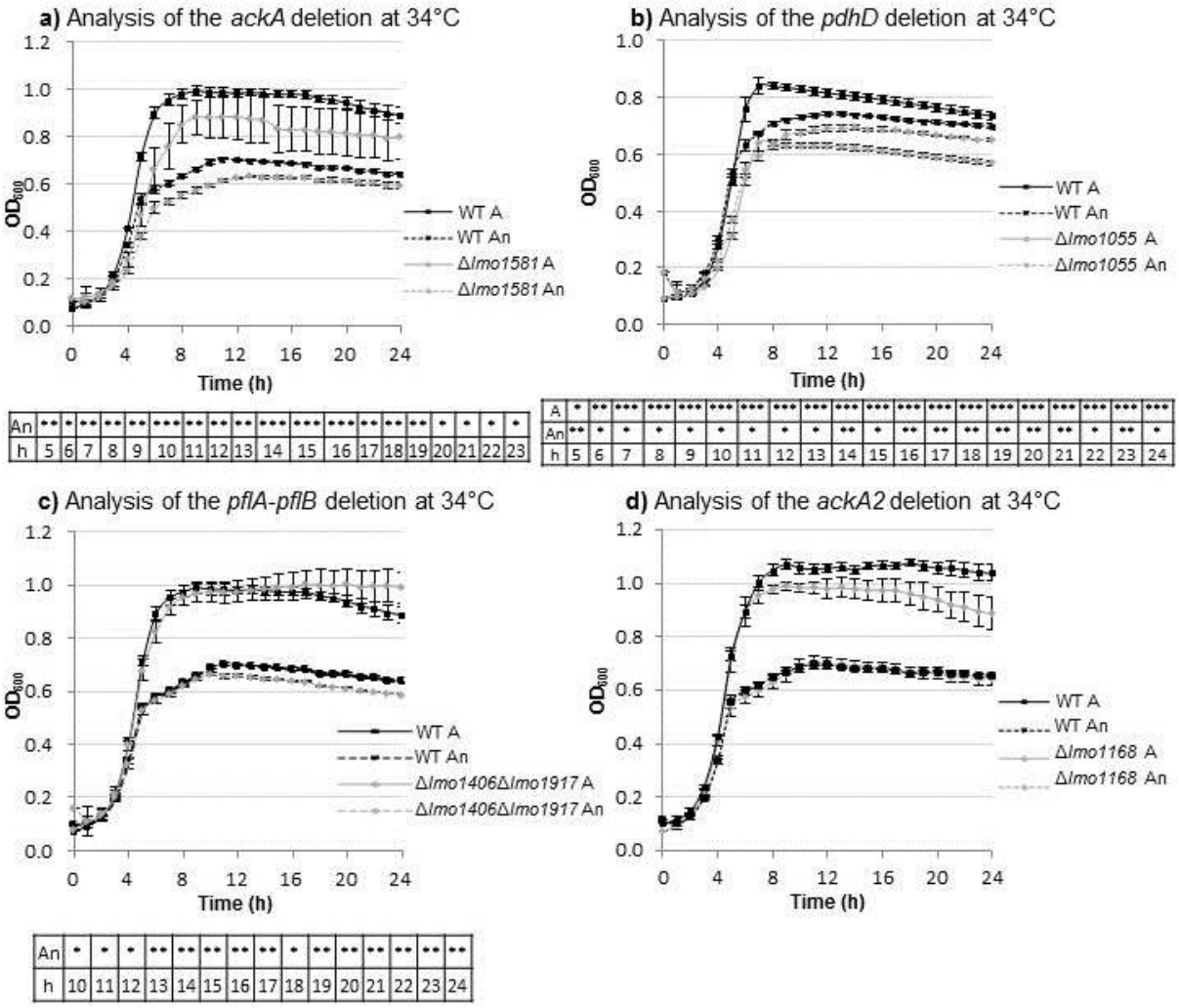
| A | * | * | ** | ** | ** | ** |
|---|---|---|---|---|---|---|
| h | 19 | 20 | 21 | 22 | 23 | 24 |

| A | * | * | * | * | * |
|---|---|---|---|---|---|
| h | 21 | 22 | 23 | 24 | |

| A | * | * | * | * | * | * | * | * | |
|---|---|---|---|---|---|---|---|---|---|
| An | | | | | | | | | * |
| h | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |

| A | | * | * | | | | | ** | ** | ** | ** | ** | * |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| An | * | * | | * | * | * | * | | | | | | * |
| h | 0 | 1 | 2 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |

**FIG: 13: Small scale in *vitro L. monocytogenes* EGDe growth analyses at 18°C.** Growth of *L. monocytogenes* EGDe WT aerobically (A) and anaerobically (An) was specifically compared to the deletion mutant *L. monocytogenes* EGDe Δ*lmo1581* (a), *L. monocytogenes* EGDe Δ*lmo1055* (b), *L. monocytogenes* EGDe Δ*lmo1406*Δ*lmo1917* (c), *L. monocytogenes* EGDe Δ*lmo1168* (d) and *L. monocytogenes* EGDe Δ*lmo2720* (e) at 18°C. Black solid lines (aerobic) and dotted lines (anaerobic) represent the WT while the grey lines represent the respective mutant. $OD_{600}$ was measured every hour. All data points represent means ± standard error from three independent biological replicates. Analyses were performed in collaboration with Matthias Steiger. A=aerobic; An=anaerobic. Statistical analyses are shown in the table under the respective graphic only for the time points showing a significant difference between the growth of the mutant and the WT (*=p-value ≤0.05; **=p-value ≤0.01; ***=p-value ≤0.001).




| An | ** | * | ** | ** | ** | *** | ** | ** | ** | *** | *** | *** | ** | ** | ** | * | * | * | * |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| h | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 |

| A | * | ** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** | *** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| An | ** | * | * | * | * | * | * | * | * | ** | * | ** | ** | ** | ** | ** | ** | * | ** | * | |
| h | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | |

| An | * | * | * | ** | ** | ** | ** | ** | * | ** | ** | ** | ** | ** | ** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| h | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 |

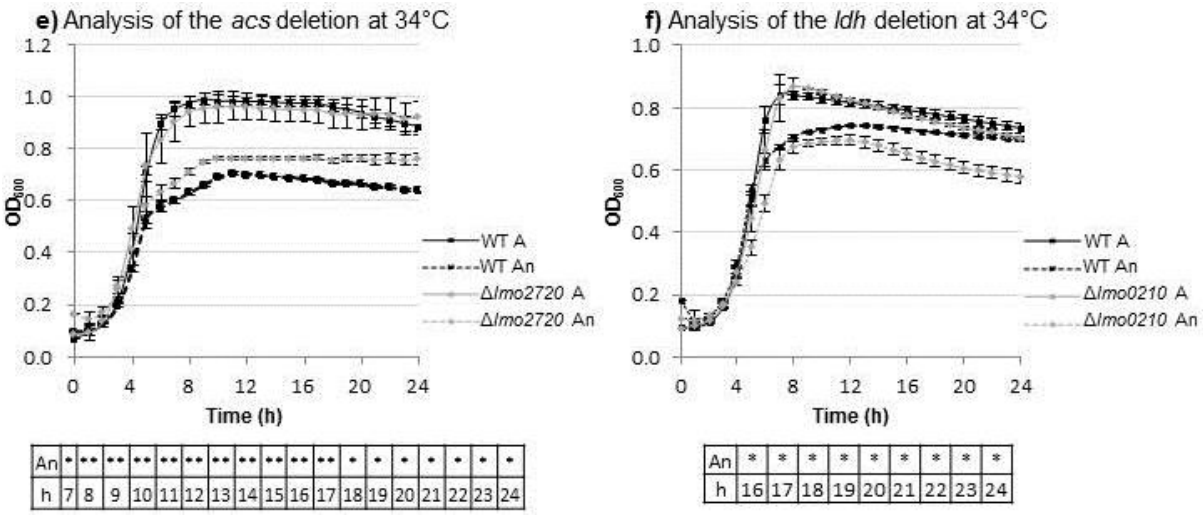



**FIG. 14: Small scale in *vitro L. monocytogenes* EGDe growth analyses at 34°C.** Growth of *L. monocytogenes* EGDe WT aerobically (A) and anaerobically (An) was specifically compared to the deletion mutant *L. monocytogenes* EGDe Δ*lmo1581* (a), *L. monocytogenes* EGDe Δ*lmo1055* (b), *L. monocytogenes* EGDe Δ*lmo1406*Δ*lmo1917* (c), *L. monocytogenes* EGDe Δ*lmo1168* (d) and *L. monocytogenes* EGDe Δ*lmo2720* (e) at 18°C. Black solid lines (aerobic) and dotted lines (anaerobic) represent the WT while the grey lines represent the respective mutant. $OD_{600}$ was measured every hour. All data points represent means ± standard error from three independent biological replicates. Analyses were performed in collaboration with Matthias Steiger. A=aerobic; An=anaerobic. Statistical analyses are shown in the table under the respective graphic only for the time points showing a significant difference between the growth of the mutant and the WT (*=p-value ≤0.05; **=p-value ≤0.01; ***=p-value ≤0.001).

The major difference was found for *L. monocytogenes* EGDe Δ*lmo1581* (*ackA*). The growth was extremely significant reduced aerobically and reduced anaerobically at 18°C (FIG. 13a) and significantly reduced anaerobically at 34°C compared to *L. monocytogenes* EGDe (FIG. 14a). From the NGS results, acetate kinase (*ackA*) was transcribed at high level under all conditions but showed the strongest transcription aerobically at 18°C (Table 23).

**Table 23: Transcriptional results for genes used to generate *L. monocytogenes* in frame deletion mutants.**

| Gene | Product | 18°C aerobic | 18°C anaerobic | 34°C aerobic | 34°C anaerobic |
|---|---|---|---|---|---|
| *lmo0210* | L-lactate dehydrogenase (*ldh*) | 2826.10 | 5347.84 | 4312.00 | 2226.72 |
| ***lmo0722*** | pyruvate oxidase | 318.33 | 61.86 | 87.00 | 161.87 |
| ***lmo1055*** | pyruvate dehydrogenase | 4356.17 | 265.02 | 4063.00 | 274.61 |

| | | | | | |
|---|---|---|---|---|---|
| | (*pdhD*) | | | | |
| *lmo1168* | acetate kinase (*ackA2*) | 10.39 | 25.97 | 16.00 | 17.34 |
| ***lmo1406*** | pyruvate formate-lyase (*pflB*) | 1713.68 | 10405.45 | 3865.00 | 5711.81 |
| *lmo1581* | acetate kinase (*ackA*) | 1621.29 | 1377.06 | 1228.00 | 1246.81 |
| ***lmo1634*** | bifunctional acetaldehyde-CoA/alcohol dehydrogenase (*adh*) | 878.39 | 23173.96 | 11030.00 | 16923.45 |
| ***lmo1917*** | pyruvate formate-lyase (*pflA*) | 428.03 | 11218.09 | 1545.00 | 4974.71 |
| ***lmo2006*** | acetolactate synthase (*alsS*) | 669.38 | 342.93 | 600.00 | 155.13 |
| *lmo2720* | acetate-CoA ligase (*acs*) | 322.18 | 316.96 | 419.00 | 311.22 |

Raw reads normalized to the smallest library (34°C aerobic).

In bold are marked genes showing a statistically relevant regulation at least at one temperature.

*L. monocytogenes* EGDe Δ*lmo1055* (*pdhD*) showed a strong significant reduction in growth aerobically at both temperatures, a reduction anaerobically at 34°C and no significant reduction at 18°C (FIG. 13b and 14b). The RNA-NGS sequencing results, in parallel, indicated anaerobic down-regulation at both temperatures for the gene encoding for this pyruvate dehydrogenase subunit (Table 23).

The transcriptional profiling (Table 23) showed for both the pyruvate formate-lyases (*pflA* and *pflB*) anaerobic up-regulation at both temperatures (significant only at 18°C) but *L. monocytogenes* EGDe Δ*lmo1917* and *L. monocytogenes* EGDe Δ*lmo1406* (*plfA* and *plfB*) individually did not show differences compared to *L. monocytogenes* EGDe (data not shown). The double mutant *L. monocytogenes* EGDe Δ*lmo1917*Δ*lmo1406,* on the other hand, showed a significant reduction of the growth aerobically at 18°C, significant only during the last six h of the experiments, (FIG. 13c) and anaerobically at 34°C (FIG. 14c).

*L. monocytogenes* EGDe Δ*lmo1168* (*ackA2*) showed a slight reduction in growth aerobically compared to *L. monocytogenes* EGDe at both temperatures, but this was observed to be significant only at 18°C for the last four h (FIG. 13d and 14d). These results are in line with the transcriptional results which showed low transcription at both temperatures for acetate kinase (*ackA2*) with a slight increment in transcription anaerobically at 18°C (Table 23).

*L. monocytogenes* EGDe Δ*lmo2720* (*acs*) showed strong significant increase in growth at 34°C anaerobically compared to the WT (FIG. 14e) while the transcriptional data showed stronger transcription aerobically at both temperatures for acetate-CoA ligase (*acs*) (Table 23).
*L. monocytogenes* EGDe Δ*lmo0210* (*ldh*) at 18°C, showed an increase of growth aerobically after 19 h while anaerobically the mutant was growing less then the WT at both temperatures (FIG. 13f and FIG. 14f). By RNA-NGS sequencing, a stronger transcription anaerobically at 18°C was shown for L-lactate dehydrogenase while at 34°C the stronger transcription was observed aerobically (Table 23).
Although a transcriptional adaptation was observed for the genes *alsS*, *lmo1634* and *lmo0722*, the deletion mutants did not show alterations in growth compared to the WT.

### 3. Oxygen dependent transcrptional profiling of *L. monocytogenes* EGD, EGDe, ScottA and 1670.

It has been shown that different *L. monocytogenes* strains exhibit a different virulence potential (Jacquet *et al*., 2004). This is not surprising, since many strain specific differences are described, like strain specific mechanisms to avoid autophagy in host cells (Cemma *et al*., 2015), strain specific survival of the host innate immune system (Yamamoto *et al*., 2012) and strain specific adaptations to specific growth conditions, for example growth in an oxygen limited, $CO_2$-enriched atmosphere (Jydegaard-Axelsen *et al*., 2004).
Of special interest for the present study is the strain specific adaptation to low oxygen conditions, since it has been shown that an anaerobic metabolism and growth under anaerobic conditions enhances the virulence potential of *L. monocytogenes* (Stritzker *et al*., 2005; Bo Andersen *et al*., 2007, reviewed in Lungu *et al.*, 2009). It is therefore tempting to speculate that strain specific adaptations to anaerobiosis could, at least in part, contribute to the observed difference in virulence of different strains. To investigate this hypothesis, the strain specific adaptation to anaerobiosis of four different strains (*L. monocytogenes* EGD, EGDe, ScottA and 1670), which have a different virulence potential (two lab strains [EGD and EGDe], one outbreak strain [ScottA] and one strain isolated from milk, but not associated with an outbreak [1670]), was analyzed.

### 3.1 Aerobic and anaerobic growth of four *L. monocytogenes* species.

Aerobic and anaerobic growth of *L. monocytogenes* EGDe, *L. monocytogenes* EGD, *L. monocytogenes* ScottA and *L. monocytogenes* 1670 was analyzed at 37°C in BHI medium (FIG. 15). All four strains were able to grow under oxygen-deprived conditions, although the growth rates were slower and the maximum $OD_{600}$ reached much lower values than under aerobic growth conditions. Resazurin controls were tested in parallel to monitor the required oxygen conditions.

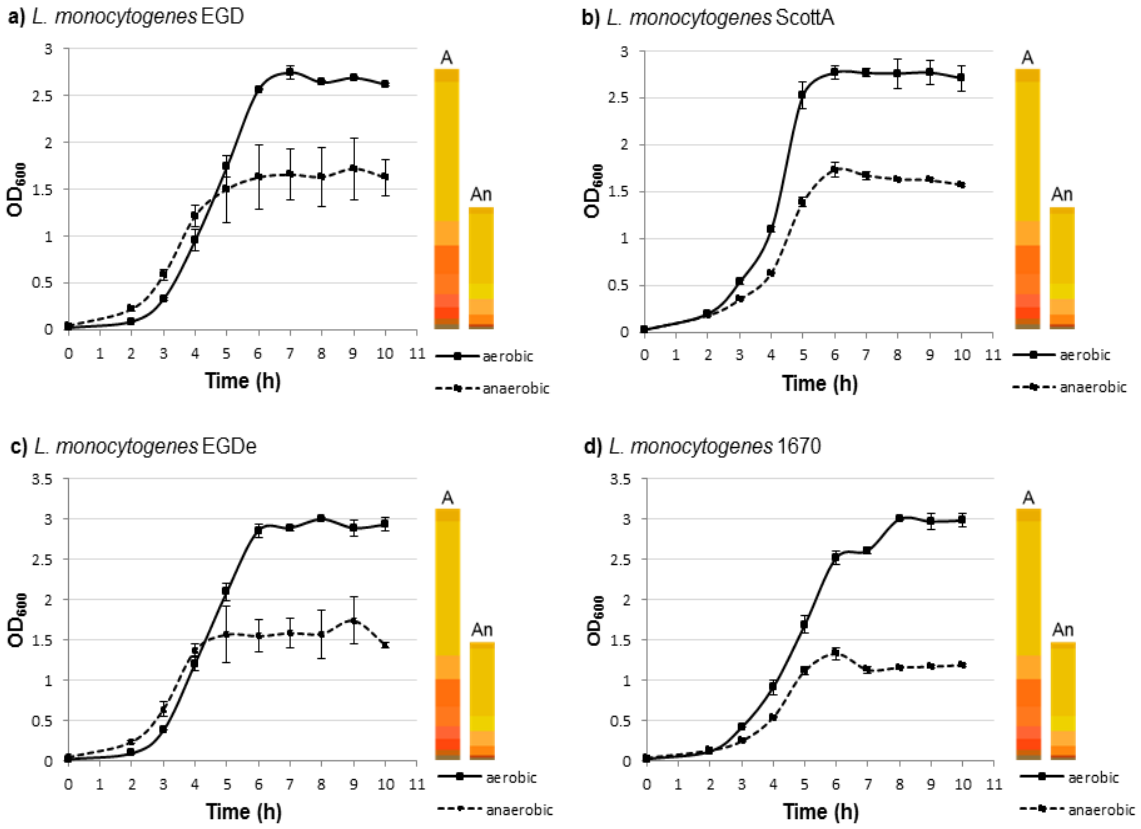



**FIG. 15: Oxygen-dependent growth of selected *L. monocytogenes* species.** *L. monocytogenes* EGD (a), *L. monocytogenes* ScottA (b), *L. monocytogenes* EGDe (c) and *L. monocytogenes* 1670 (d) were grown at 37°C aerobically (solid line) and anaerobically (dotted line). $OD_{600}$ was measured every hour. All data points represent means ± standard error from three independent biological replicates. Colored lines indicate the color of a resazurin control (A=aerobic and An=anaerobic). Analyses were performed in collaboration with MSc. Elisabeth Walburga Maria Schwab.

### 3.2 Oxygen dependent transcriptional adaptation of *L. monocytogenes* strains at 37°C

After comparing the adaptation of the not pathogenic *L. weihenstephanensis* to the oxygen availability to that of the pathogenic *L. monocytogenes* EGDe, the transcriptional profiling of *L. monocytogenes* EGDe, ScottA, 1670 and EGD was

determined via NGS under aerobic and anaerobic conditions at 37°C to better understand the strain-specific anaerobic adaptations in *L. monocytogenes*.

The resulting reads were mapped against the published genome of *L. monocytogenes* EGDe, EGD and ScottA (published in http://www.ncbi.nlm.nih.gov/genome/genomes/159; Table 24). The genome sequence of *L. monocytogenes* 1670 was not available yet. As shown, *L. monocytogenes* EGD, 1670 and EGDe presented the highest number of mapping reads against *L. monocytogenes* EGDe genome. Interestingly, the *L. monocytogenes* strain EGD used in this thesis did not display the highest number of mapping reads against the published sequence for the strain EGD, suggesting a closer relation of the strain EGD used in this thesis to *L. monocytogenes* EGDe than to the published EGD strain. In *L. monocytogenes* ScottA, even if the highest number of mapping reads was identified against its own genome, a high number of reads was mapping against *L. monocytogenes* EGDe genome as well.

To allow further comparisons with the other strains, *L. monocytogenes* EGDe genome was chosen as reference genome for the mapping of all four strains.

**Table 24: Mapping of the four *L. monocytogenes* strains against the available genomes.**

| | | **Reference genome for mapping** | | |
|---|---|---|---|---|
| Strain | Condition | *L. monocytogenes* EGDe | *L. monocytogenes* EGD | *L. monocytogenes* ScottA |
| **1670** | Aerobic | **1.12** | 1.12 | 1.04 |
| | Anaerobic | **0.46** | 0.46 | 0.42 |
| **EGD** | Aerobic | **2.08** | 2.05 | 1.90 |
| | Anaerobic | **3.20** | 3.16 | 2.93 |
| **ScottA** | Aerobic | 4.43 | 4.44 | **4.72** |
| | Anaerobic | 1.16 | 1.17 | **1.25** |
| **EGDe** | Aerobic | **4.16** | 4.11 | 3.84 |
| | Anaerobic | **6.74** | 6.64 | 6.14 |

Total number of mapping reads (Mapped Reads/million) for each strain, aerobically and anaerobically, after mapping against each available genome.

In bold the highest number of total reads for each strain.

For *L. monocytogenes* 1670, the cluster density during the RNA-NGS was much lower than the other strains, resulting in a lower output of reads.

The raw read counts were normalized to smallest library, which in this case was the 1670 anaerobic library. The $log_2$FC was calculated in edgeR, which displays the $log_2$ of the expression ratio anaerobic/aerobic.

Combing all the four strains, 111 genes have shown regulation. In total, 34 genes were up-regulated anaerobically (Table 25, descriptions are based on the .ptt-file taken from the NCBI database [ftp://ftp.ncbi.nlm.nih.gov/genomes/Bacteria] and on the ListiList database [http://genolist.pasteur.fr/ListiList/]; raw read counts are presented in Table S6a). 27 genes showed up-regulation in *L. monocytogenes* 1670, 6 genes in *L. monocytogenes* EGD, 4 in *L. monocytogenes* EGDe and 15 genes in *L. monocytogenes* ScottA. Among these genes, only fumarate reductase (*lmo0355*) and a heavy metal transporting ATPase (*lmo0641*) were up-regulated in all four strains. In total, 77 genes showed down-regulation (Table 26, raw read counts are presented in Table S6b).

61 genes were down-regulated in *L. monocytogenes* 1670, 29 genes in *L. monocytogenes* EGD, 26 genes in *L. monocytogenes* EGDe and 32 genes in *L. monocytogenes* ScottA. Among these genes, 24 genes were down-regulated in at least three strains (15 genes in all four) including genes encoding for proteins involved in the aerobic metabolism, like aa3-600 quinol oxidase subunit I (*lmo0014*), superoxide dismutase (*lmo1439*) and part of the pyruvate dehydrogenase operon (*lmo1053* [*pdhB*] *lmo1054* [*pdhC*] *lmo1055* [*pdhD*]). These results indicate a higher incidence of oxygen-dependent transcriptional regulation in the strains *L. monocytogenes* 1670 and ScottA compared to the strains *L. monocytogenes* EGD and its derivative, EGDe. Moreover many of the regulated genes in at least one strain were already seen to be oxygen-dependent regulated in *L. monocytogenes* EGD at 37°C (Müller-Herbst *et al.*, 2014)

**Table 25: Anaerobically up-regulated genes at 37°C for the four tested *L. monocytogenes* strains.**

| Gene | $log_2$FC 1670 | $log_2$FC EGD | $log_2$FC EGDe | $log_2$FC ScottA | Product |
|---|---|---|---|---|---|
| *lmo0202* | 2.89 | 0.83 | 2.16 | 1.13 | listeriolysin O precursor |
| *lmo0204* | 2.13 | 1.15 | 1.89 | 0.60 | actin assembly inducing protein precursor |
| *lmo0205* | 2.46 | 1.22 | 1.66 | 1.31 | phospholipase C |

| | | | | | |
|---|---|---|---|---|---|
| *lmo0279** | 2.72 | 4.02 | 4.11 | 2.76 | anaerobic ribonucleoside triphosphate reductase |
| *lmo0280** | 3.03 | 4.22 | 3.79 | 1.99 | anaerobic ribonucleotide reductase activator protein |
| *lmo0355** | 3.09 | 2.80 | 3.45 | 3.08 | fumarate reductase subunit A |
| *lmo0398* | 0.20 | -0.01 | 0.05 | 2.64 | PTS sugar transporter subunit IIA |
| *lmo0400* | -0.13 | 0.20 | -0.55 | 2.49 | PTS fructose transporter subunit IIC |
| *lmo0401* | -0.71 | 0.59 | -0.08 | 2.73 | alpha mannosidase |
| *lmo0402* | -0.92 | 0.36 | -0.45 | 2.76 | transcriptional antiterminator BglG |
| *lmo0412* | 1.16 | 0.80 | 0.95 | 2.15 | hypothetical protein |
| *lmo0433* | 2.47 | 1.14 | 1.51 | 2.32 | internalin A |
| *lmo0434** | 4.21 | 0.97 | 2.19 | 2.73 | internalin B |
| *lmo0443* | 1.83 | 1.61 | 1.35 | 0.89 | LytR family transcriptional regulator |
| *lmo0471** | 2.01 | 2.29 | 1.81 | | hypothetical protein |
| *lmo0641** | 6.82 | 4.70 | 6.91 | 7.53 | heavy metal transporting ATPase |
| *lmo0670* | 3.40 | -0.01 | -0.20 | -0.11 | hypothetical protein |
| *lmo0722* | 2.32 | 0.70 | 0.72 | 2.82 | pyruvate oxidase |
| *lmo0788* | 1.95 | 0.70 | 1.38 | 1.38 | hypothetical protein |
| *lmo1310** | 2.94 | 1.96 | 1.19 | 1.63 | hypothetical protein |
| *lmo1407* | 2.25 | 1.23 | 1.67 | 1.94 | pyruvate formate lyase activating enzyme |
| *lmo1425* | 4.41 | 0.90 | 0.86 | 2.90 | glycine/betaine ABC transporter permease |
| *lmo1426* | 3.25 | 0.94 | 0.18 | 3.08 | glycine/betaine ABC transporter substrate binding protein |
| *lmo1659* | 2.73 | 2.82 | 5.12 | | hypothetical protein |
| *lmo1917* | 1.98 | 0.95 | 1.96 | 1.87 | pyruvate formate lyase |
| *lmo2069* | 2.16 | 1.87 | 1.74 | 1.22 | co chaperonin GroES |
| *lmo2156* | 2.97 | 2.53 | 1.60 | 1.31 | hypothetical protein |
| *lmo2158* | 2.32 | -1.84 | -0.04 | -0.49 | hypothetical protein |
| *lmo2288* | | 0.90 | 0.59 | 2.69 | protein GK15 |
| *lmo2292* | | -0.01 | -0.22 | 4.52 | protein GK11 |
| *lmo2363* | 3.00 | -0.01 | -1.47 | 4.03 | glutamate decarboxylase |

| | | | | | |
|---|---|---|---|---|---|
| *lmo2467** | 3.12 | 1.75 | 2.10 | 3.19 | chitin binding protein |
| *lmo2536a* | 2.16 | 1.41 | 1.45 | 2.38 | hypothetical protein |
| *lmo2669** | 3.21 | 1.66 | 1.66 | 2.88 | hypothetical protein |

List of the genes up-regulated in at least one of the tested stains.

Not statistical relevant data (p-value >0.05 and FDR >0.3) are shown in grey.

* genes already described to be anaerobically up-regulated regulated in *L. monocytogenes* EGD (Müller-Herbst *et al.*, 2014) .

**Table 26: Anaerobically down-regulated genes at 37°C for the four tested *L. monocytogenes* strains.**

| Gene | $log_2FC$ 1670 | $log_2FC$ EGD | $log_2FC$ EGDe | $log_2FC$ ScottA | Product |
|---|---|---|---|---|---|
| *lmo0013** | -1.87 | -3.05 | -1.97 | -1.81 | aa3-600 quinol oxidase subunit II |
| *lmo0014** | -2.45 | -4.00 | -3.54 | -1.64 | aa3-600 quinol oxidase subunit I |
| *lmo0016** | -2.34 | -2.94 | -3.30 | -2.95 | aa3-600 quinol oxidase subunit IV |
| *lmo0361* | -5.21 | -6.56 | -6.18 | | membrane protein |
| *lmo0362* | -4.57 | -6.24 | -7.33 | | hypothetical protein |
| *lmo0365* | -5.61 | -6.42 | -6.48 | | hypothetical protein |
| *lmo0366* | -5.49 | -7.17 | -6.61 | | hypothetical protein |
| *lmo0367* | -6.01 | -8.00 | -6.45 | | hypothetical protein |
| *lmo0484* | -3.63 | -5.19 | -5.78 | -4.72 | heme degrading monooxygenase IsdG |
| *lmo0485* | -8.43 | -6.04 | -6.17 | -2.50 | hypothetical protein |
| *lmo0519* | -2.23 | -1.75 | -1.76 | -2.43 | multidrug resistance protein |
| *lmo0541* | -8.00 | -9.58 | -8.04 | -7.08 | ABC transporter substrate binding protein |
| *lmo0573* | -3.58 | -0.41 | -1.92 | -1.65 | hypothetical protein |
| *lmo0597* | -2.94 | -1.71 | -2.78 | -2.86 | Crp/Fnr family transcriptional regulator |
| *lmo0609* | -3.64 | -1.85 | -1.76 | -3.43 | phage shock protein E |
| *lmo0678* | -7.40 | - | -1.47 | -5.42 | flagellar biosynthesis protein FliR |
| *lmo0679* | -3.95 | 3.16 | -1.18 | -3.14 | flagellar biosynthesis protein FlhB |
| *lmo0680* | -3.26 | -0.01 | -1.51 | -2.97 | flagellar biosynthesis protein FlhA |
| *lmo0682* | -3.15 | -0.01 | -1.68 | -1.09 | flagellar basal body rod protein FlgG |
| *lmo0684* | -6.62 | - | -2.19 | -2.38 | hypothetical protein |
| *lmo0686* | -3.04 | -3.18 | -1.10 | -0.84 | flagellar motor rotation MotB |
| *lmo0688* | -2.58 | -0.73 | -0.38 | -2.58 | hypothetical protein |

| | | | | | |
|---|---|---|---|---|---|
| *lmo0689* | -2.40 | -0.01 | -0.77 | -2.74 | chemotaxis protein CheV |
| *lmo0692* | -2.75 | -0.01 | -0.04 | -2.86 | two component sensor histidine kinase CheA |
| *lmo0698* | -3.74 | | -1.45 | -1.99 | flagellar motor switch protein |
| *lmo0701* | -2.56 | -0.57 | -0.89 | -1.99 | hypothetical protein |
| *lmo0705* | -3.00 | -0.93 | -0.50 | -2.51 | flagellar hook associated protein FlgK |
| *lmo0706* | -3.58 | -3.18 | 0.23 | -1.39 | flagellar hook associated protein FlgL |
| *lmo0710* | -6.48 | | -0.91 | -0.11 | flagellar basal body rod protein FlgB |
| *lmo0716* | -2.64 | -0.93 | -0.59 | -2.19 | flagellum specific ATP synthase |
| *lmo0811* | -2.03 | -2.05 | -2.59 | -2.30 | carbonic anhydrase |
| *lmo1007* | -6.32 | -6.03 | -5.93 | -5.42 | hypothetical protein |
| *lmo1052** | -2.48 | -3.79 | -2.20 | -3.40 | pyruvate dehydrogenase subunit A1 alpha |
| *lmo1053** | -2.84 | -4.20 | -2.34 | -3.07 | pyruvate dehydrogenase subunit A1 beta |
| *lmo1054** | -7.13 | -6.03 | -1.83 | -3.29 | dihydrolipoamide acetyltransferase |
| *lmo1055** | -2.84 | -4.93 | -2.50 | -3.18 | dihydrolipoamide dehydrogenase |
| *lmo1132* | -4.66 | -5.35 | -4.55 | -4.21 | ABC transporter ATP binding protein |
| *lmo1257** | -2.13 | -1.94 | -1.77 | -1.64 | hypothetical protein |
| *lmo1293** | -0.38 | -2.01 | -0.81 | -2.44 | glycerol 3 phosphate dehydrogenase |
| *lmo1384* | -2.34 | -2.74 | -2.72 | -3.56 | hypothetical protein |
| *lmo1439* | -1.99 | -2.78 | -1.60 | -1.97 | superoxide dismutase |
| *lmo1538** | 0.01 | -1.15 | -0.52 | -2.35 | glycerol kinase |
| *lmo1539** | -0.95 | -1.71 | -0.49 | -3.94 | glycerol transporter |
| *lmo1595* | -3.90 | -0.88 | -0.59 | -0.12 | hypothetical protein |
| *lmo1641* | -2.46 | -1.83 | -1.76 | -1.17 | aconitate hydratase |
| *lmo1910* | -3.74 | -0.99 | -0.64 | | oxidoreductase |
| *lmo1956* | -2.02 | -3.16 | -1.80 | -2.16 | Fur family transcriptional regulator |
| *lmo1957* | -6.27 | -5.14 | -5.53 | -6.66 | ferrichrome ABC transporter permease |
| *lmo1959* | -5.25 | -5.89 | -5.59 | -8.56 | ferrichrome binding protein |
| *lmo1961* | -4.33 | -5.22 | -4.96 | -4.50 | oxidoreductase |
| *lmo1997* | -2.52 | | -1.03 | -3.63 | PTS mannose transporter subunit IIA |
| *lmo1998** | 1.53 | -0.01 | -0.16 | -4.98 | opine catabolism protein |
| *lmo1999** | 1.58 | -0.01 | -0.04 | -3.32 | hypothetical protein |
| *lmo2000* | 0.66 | -0.01 | 0.04 | -3.79 | PTS mannose transporter subunit IID |

| | | | | | |
|---|---|---|---|---|---|
| *lmo2002* | -2.52 | -3.18 | -1.50 | -5.28 | PTS mannose transporter subunit IIB |
| *lmo2003* | 0.00 | -3.18 | -0.99 | -3.49 | GntR family transcriptional regulator |
| *lmo2006** | -2.19 | -0.70 | -1.85 | -0.63 | acetolactate synthase |
| *lmo2057** | -2.35 | -1.50 | -1.69 | -0.68 | protoheme IX farnesyltransferase |
| *lmo2064* | -1.23 | -1.60 | -2.13 | -0.56 | large conductance mechanosensitive channel protein |
| *lmo2104a* | -3.50 | -7.19 | -4.62 | | ferrous iron transport protein B |
| *lmo2105* | -10.63 | -5.65 | -5.60 | -5.01 | hypothetical protein |
| *lmo2129* | -2.99 | -1.14 | -1.75 | 0.38 | hypothetical protein |
| *lmo2180* | -5.93 | -6.61 | -5.65 | -8.36 | hypothetical protein |
| *lmo2181* | -6.68 | -7.25 | -6.37 | -10.86 | hypothetical protein |
| *lmo2184* | -6.41 | -6.20 | -5.71 | -6.09 | ferrichrome ABC transporter substrate binding protein |
| *lmo2185* | -7.41 | -8.78 | -8.14 | -11.33 | hypothetical protein |
| *lmo2186* | -10.59 | -7.39 | -8.89 | -6.54 | hypothetical protein |
| *lmo2261* | -2.91 | -3.64 | -3.56 | -2.56 | hypothetical protein |
| *lmo2335** | -6.05 | -1.20 | -5.31 | 0.02 | PTS fructose transporter subunit IIABC |
| *lmo2336** | -6.87 | | -5.19 | | fructose 1 phosphate kinase |
| *lmo2337** | -7.40 | 3.16 | -5.11 | | DeoR family transcriptional regulator |
| *lmo2343* | -3.55 | -2.47 | -2.49 | -1.59 | nitrilotriacetate monooxygenase |
| *lmo2390* | -2.01 | -1.75 | -1.75 | -2.24 | hypothetical thioredoxine reductase |
| *lmo2397* | -3.37 | -1.00 | -1.35 | -1.65 | NifU protein |
| *lmo2411* | -1.12 | -2.08 | -0.77 | -1.30 | hypothetical protein |
| *lmo2412* | -1.01 | -2.16 | -0.78 | -1.55 | hypothetical protein |
| *lmo2569** | -2.44 | -1.83 | -2.35 | -1.49 | peptide ABC transporter substrate binding protein |

List of the genes down-regulated in at least one of the tested stains.

Not statistical relevant data (p-value >0.05 and FDR >0.3) are shown in grey.

* genes already described to be anaerobically down-regulated regulated in *L. monocytogenes* EGD (Müller-Herbst *et al.*, 2014) .

Moreover, ten flagella genes (*lmo0678-0680*; *lmo0682*; *lmo0686*; *lmo0698*; *lmo0705*; *lmo0706*; *lmo0710*; *lmo0716*) showed significant down-regulation only in *L. monocytogenes* 1670. The raw read normalized to the smallest library (1670 anaerobic) indicated that these genes were transcribed only in *L. monocytogenes*

1670 and only slightly in *L. monocytogenes* ScottA (Table 27), even mapping the reads against the ScottA genome (data not shown).

**Table 27: Normalized read counts for the cluster of flagella genes**.

| Gene | 1670 A | 1670 An | EGD A | EGD An | EGDe A | EGDe An | ScottA A | ScottA An | Product |
|---|---|---|---|---|---|---|---|---|---|
| *lmo0678* | 21.16 | 0.00 | 0.00 | 0.35 | 0.80 | 0.15 | 4.60 | 0.00 | fla. biosynthesis protein FliR |
| *lmo0679* | 33.50 | 2.00 | 0.00 | 0.69 | 1.11 | 0.39 | 8.58 | 0.82 | fla. biosynthesis protein FlhB |
| *lmo0680* | 49.96 | 5.00 | 1.12 | 0.52 | 1.75 | 0.54 | 8.37 | 0.82 | fla. biosynthesis protein FlhA |
| *lmo0682* | 46.43 | 5.00 | 0.56 | 0.86 | 1.59 | 0.39 | 5.86 | 3.26 | fla. basal body rod protein FlgG |
| *lmo0686* | 59.95 | 7.00 | 1.12 | 0.35 | 1.75 | 0.70 | 5.23 | 3.26 | fla. motor rotation MotB |
| *lmo0698* | 15.28 | 1.00 | 0.00 | 0.00 | 0.48 | 0.08 | 3.98 | 0.82 | fla. motor switch protein |
| *lmo0705* | 66.41 | 8.00 | 1.68 | 0.86 | 2.07 | 1.39 | 10.88 | 2.45 | fla. hook associated protein FlgK |
| *lmo0706* | 25.86 | 2.00 | 0.56 | 0.35 | 0.64 | 0.70 | 5.02 | 1.63 | fla. hook associated protein FlgL |
| *lmo0710* | 11.17 | 0.00 | 0.00 | 0.17 | 0.48 | 0.15 | 0.84 | 0.82 | fla. basal body rod protein FlgB |
| *lmo0716* | 45.25 | 7.00 | 1.68 | 1.38 | 2.70 | 1.70 | 13.39 | 3.26 | flagellum specific ATP synthase |

NGS raw data normalized to the smallest library (1670 anaerobic).

A=aerobic, An=anaerobic.

fla.=flagellar

### 3.3 Validation of the RNA-NGS sequencing with qPCR

qPCR analyses were performed to validate the RNA-NGS results and genes which were regulated in a oxygen-dependent manner were selected for this purpose. In particular the oxygen-dependent genes encoding for the aa3-600 quinol oxidase subunit I (*qoxB*, *lmo0014*) (FIG. 16a) and the fumarate reductase (*lmo0355*) (FIG. 16b), were tested. The qPCR results for these two genes were correlating perfectly with the RNA-NGS data and the previously published results (Muller-Herbst *et al.,* 2014).

Moreover, a flagellum component of *L. monocytogenes* (*lmo0682*) (FIG. 16c), as representative for the cluster of flagella genes described before, was selected. This gene was statistically down-regulated only in *L. monocytogenes* 1670 under anaerobic conditions (Table 26) while NGS results revealed a very low transcription in *L. monocytogenes* EGD and EGDe independently on the oxygen content (Table 27). As said, from the NGS results no significant read counts were observed for this gene (<2 anaerobically and <1 aerobically) and this, in logarithmic scale, could generate unrealistic sceneries and therefore clarifies the variations observed between the NGS and qPCR results. Genes encoding for the glutamine synthetase repressor (*glnR*, *lmo1298*) (FIG. 16d) and catalase (*kat*, *lmo2785*) (FIG. 16e) were selected for the validation of genes not statistically significant oxygen-dependent regulated. Catalase showed stronger transcription aerobically in the strain EGD but was not included amoung the anaerobically down-regulated genes because of the strict setting (p-value ≤0.05; FDR ≤0.3) used for the filtering (*kat* FDR=0.53).

r=0.97 (calculated as explained before but using *L. monocytogenes* 1670 anaerobic library for NGS raw reads normalization) specified also in this case a very strong correlation between the two methods, and so validation of the NGS data.

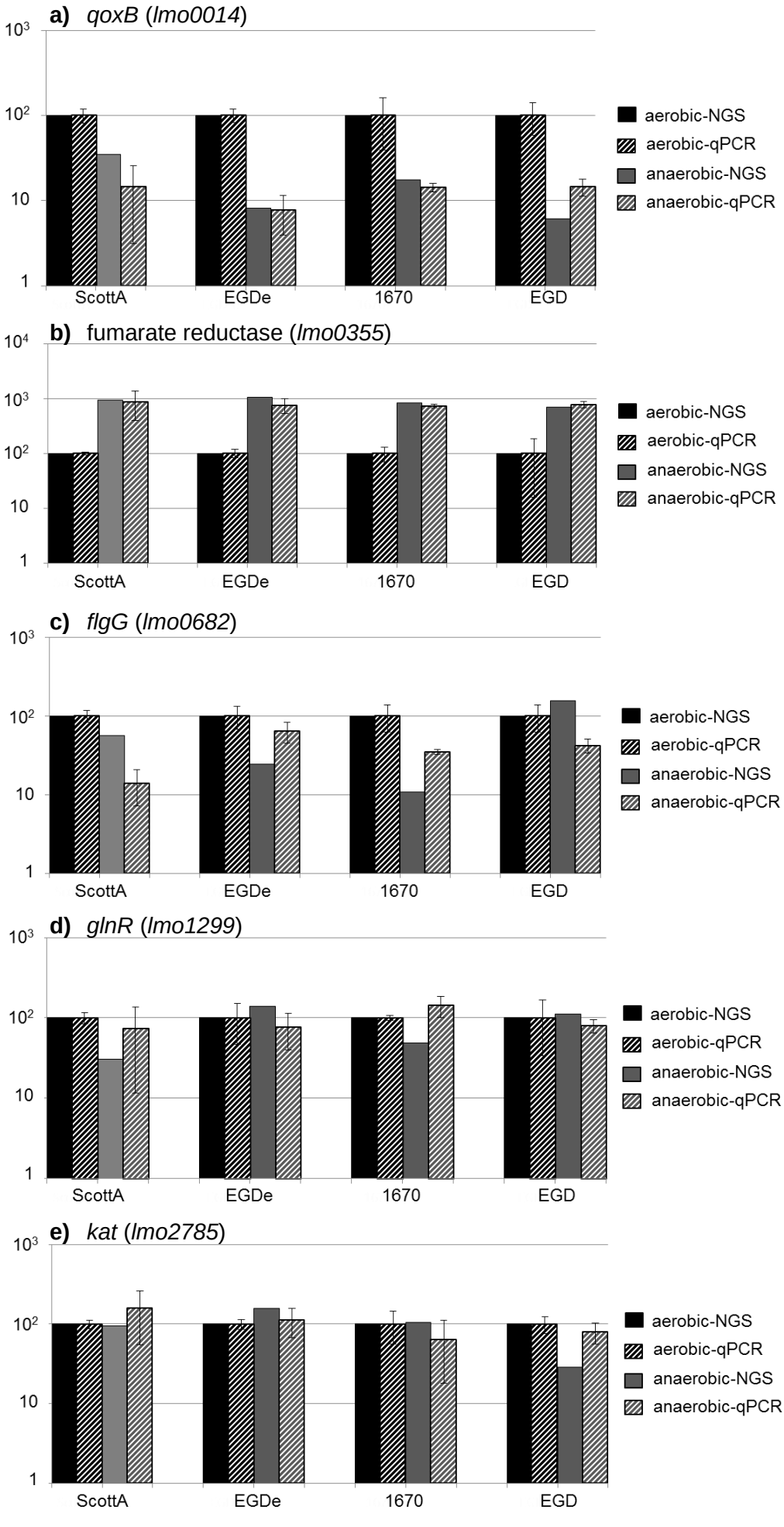

a) qoxB (lmo0014)
10³
10²
10
1
ScottA
EGDe
1670
EGD
aerobic-NGS
aerobic-qPCR
anaerobic-NGS
anaerobic-qPCR
b) fumarate reductase (lmo0355)
10⁴
10³
10²
10
1
ScottA
EGDe
1670
EGD
aerobic-NGS
aerobic-qPCR
anaerobic-NGS
anaerobic-qPCR
c) flgG (lmo0682)
10³
10²
10
1
ScottA
EGDe
1670
EGD
aerobic-NGS
aerobic-qPCR
anaerobic-NGS
anaerobic-qPCR
d) glnR (lmo1299)
10³
10²
10
1
ScottA
EGDe
1670
EGD
aerobic-NGS
aerobic-qPCR
anaerobic-NGS
anaerobic-qPCR
e) kat (lmo2785)
10³
10²
10
1
ScottA
EGDe
1670
EGD
aerobic-NGS
aerobic-qPCR
anaerobic-NGS
anaerobic-qPCR

**FIG. 16: qPCR validation of RNA-NGS data for selected differently expressed genes in different *L. monocytogenes* strains.** RNAs used for the qPCR analyses were isolated from cultures at $OD_{600}$ 0.85-0.95 under the described conditions. Bars represent the transcriptional level of selected genes analyzed by NGS (solid bars) or qPCR (striped bars) encoding for a) aa3-600 quinol oxidase subunit I (*qoxB*, *lmo0014*), b) flavocytochrome C fumarate reductase chain A (*lmo0355*), c) flagellar hook-basal body protein FlgG (*flgG*, *lmo0682*), d) glutamine synthetase repressor (*glnR*, *lmo1298*) and e) catalase (*kat*, *lmo2785*). RNA-NGS data were normalized to the smallest library (*L. monocytogenes* 1670 anaerobic) and bars represent the percentage of transcription of each condition compared to the reference condition (aerobic), set as 100%. qPCR data were analyzed by the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak, 2008), using the 16S rRNA transcription for normalization. The aerobic condition was set as reference (100%) and bars represent mean values and standard errors from three independent experiments, including duplicates. Analyses were performed by MSc. Elisabeth Walburga Maria Schwab.

## 3.4 Motility experiments

As shown from the RNA-NGS results, the four analyzed *L. monocytogenes* strains exhibited different regulation of the flagella genes. In particular, at 37°C, ten flagella genes were transcribed only in *L. monocytogenes* ScottA and 1670, with stronger transcription aerobically (Table 27). To further analyze this finding, motility assays were performed for all the four strains at 24°C, an expected motile temperature, and 37°C, the human body and expected not motile temperature (Peel *et al*., 1988). Strains were grown overnight at 24°C (FIG. 17) or 37°C (FIG. 18), inoculated on 0.25% BHI agar and then cultivated aerobically and anaerobically at 24°C and 37°C for 24 (data not shown) and 48 h.

Starting from overnight cultures incubated at 24°C, the motility behavior of the *L. monocytogenes* strains incubated at 24°C, after 48 h, showed aerobic motility for all the strains (FIG. 17a) and anaerobic clear swarming only in *L. monocytogenes* ScottA and 1670 (FIG. 17b). After incubation at 37°C, aerobically *L. monocytogenes* ScottA showed swarming while the strain 1670 displayed a marginal effect and *L. monocytogenes* EGD and EGDe showed a slight motility (FIG. 17c). Anaerobically, *L. monocytogenes* EGD, EGDe and 1670 did not show motility. In contrast, *L. monocytogenes* ScottA developed a wide, translucent aura (FIG. 17d).

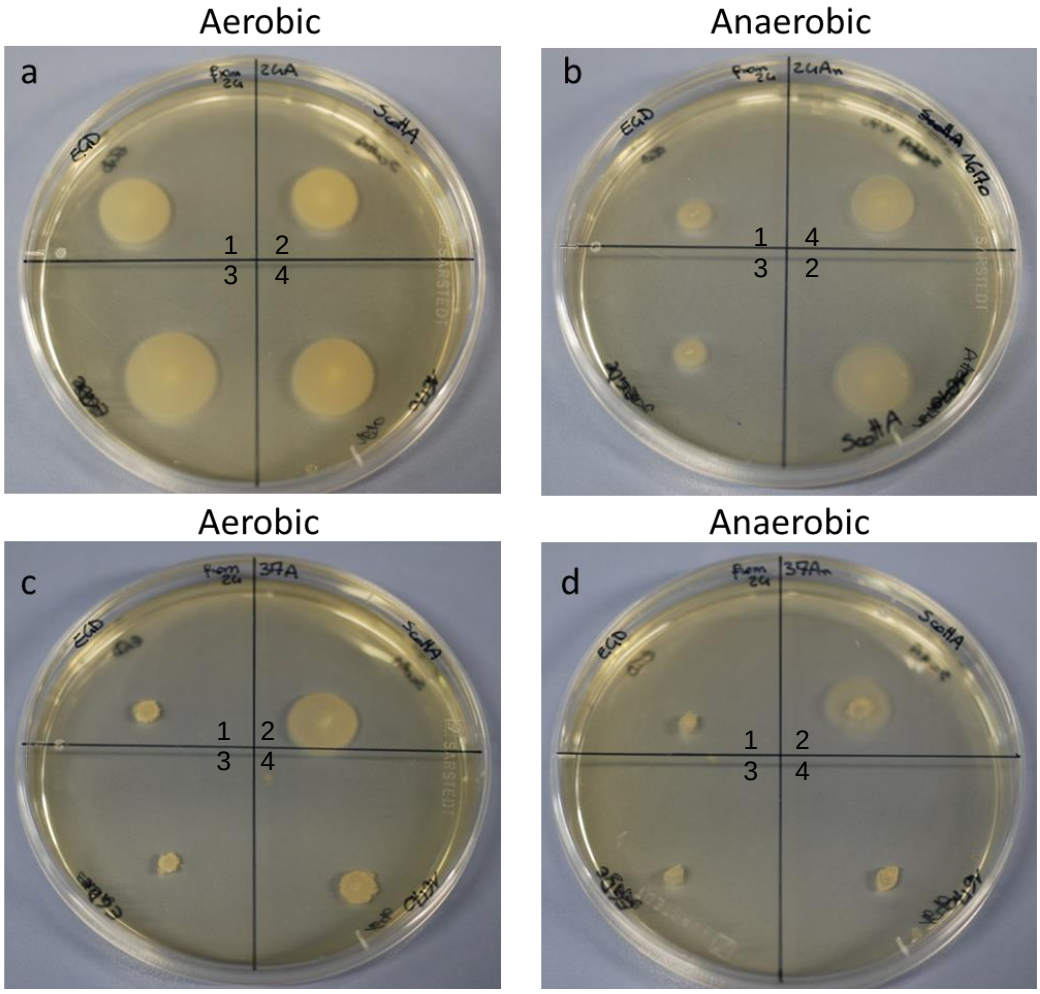



**FIG. 17: Motility of *L. monocytogenes* strains at 24°C and 37°C from overnight cultures incubated at 24°C.** *L. monocytogenes* EGD (1), ScottA (2), EGDe (3) and 1670 (4) were inoculated from an overnight culture grown at 24°C on 0.25% BHI agar and incubated aerobically and anaerobically for 48 h at 24°C (a and b) or 37°C (c and d). Three repetitions of the experiment were performed with consistent results. Representative pictures for each condition are shown.

Starting from overnight cultures incubated at 37°C, the motility pattern after 48 h incubation at 24°C reflected the pattern shown starting from overnight cultures incubated at 24°C (see above) (FIG. 18a and b). After incubating the plates at 37°C, no motility was detected for all the strains at both conditions, only growth (FIG. 18c and d).

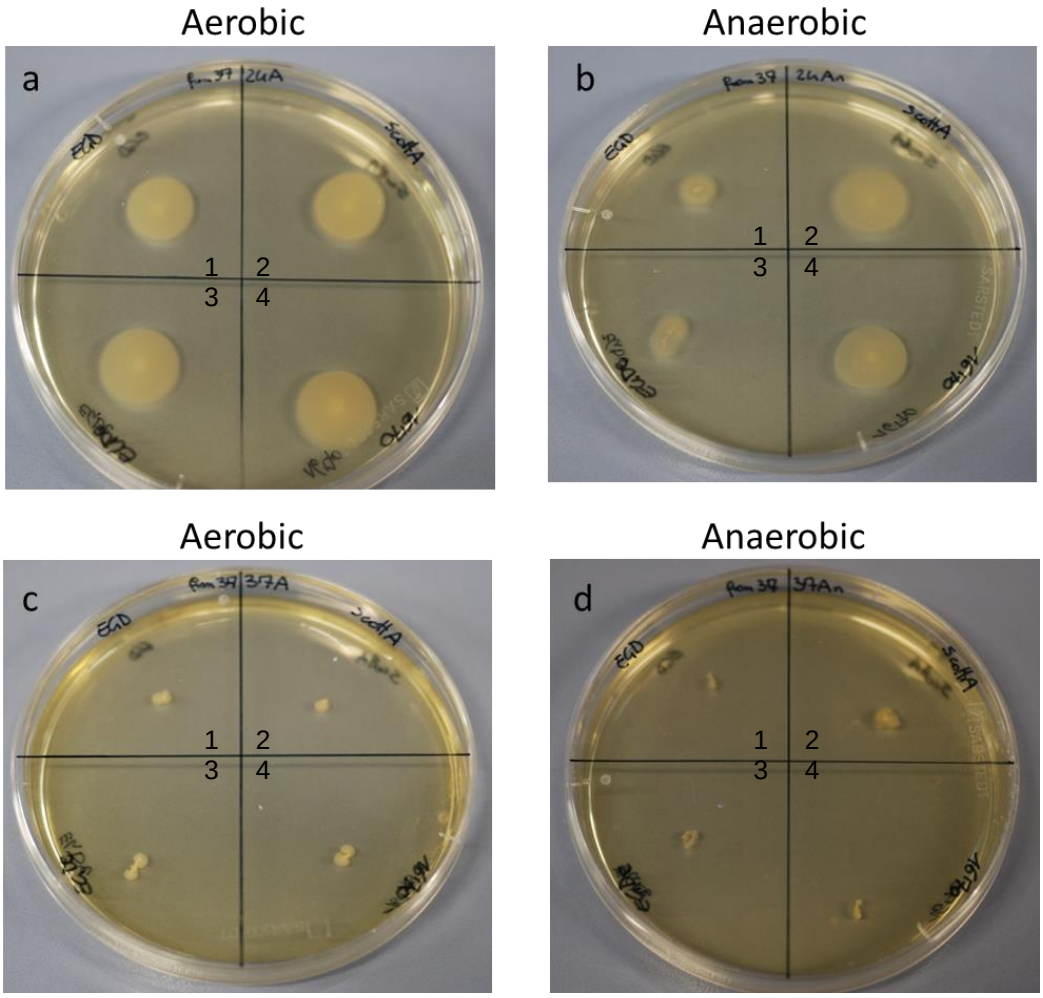



**FIG. 18: Motility of *L. monocytogenes* strains at 24°C and 37°C from overnight cultures incubated at 37°C**. *L. monocytogenes* EGD (1), ScottA (2), EGDe (3) and 1670 (4) EGD were inoculated from an overnight culture grown at 37°C on 0.25% BHI agar and incubated aerobically and anaerobically for 48 h at 24°C (a and b) or 37°C (c and d). Three repetitions of the experiment were performed with consistent results. Representative pictures for each condition are shown.

At first, these motility assays supported the previous described inability of *L. monocytogenes* to swarm when grown constantly at 37°C (Peel *et al.*, 1988). Moreover they indicated a higher motility for the strains 1670 and ScottA compared to the strains EGD and EGDe, expecially when grown at 37°C after incubation at 24°C. In particular, the behavoiur of *L. monocytogenes* ScottA under oxygen-deprived conditions suggested an advantage for this outbreak strain during the initial stages of infection of the GI tract.

# V. DISCUSSION

## 1. Adaptation of *L. weihenstephanensis* to anaerobiosis

### 1.1 Complete genome sequence

*L. weihenstephanensis* is a recently discovered environmental species of *Listeria* (Lang-Halter *et al.*, 2013). The complete genome sequence is not available and only an assembly of a shotgun sequence was published in January 2014 (Cornell University, GenBank: AODJ00000000.1), and annotated in 2015 (NCBI Reference Sequence: NZ_AODJ00000000.1). In this project the genome sequence of *L. weihenstephanensis* was successfully closed. The already published assembly presented high similarity to the complete genome sequence. However the complete genome sequence identified 28 additional coding sequences, seven additional rRNAs and one additional tRNA.

Moreover, the complete genome sequence revealed the presence of a temperate phage in *L. weihenstephanensis.* A region of ~40 kb was eye-catching within the assembly (FIG. 4), showing an initial high coverage (increment of 470%) followed by a low coverage (decrement of 85%). After using PHAST (Zhou *et. al*, 2011), this region was recognized to contain phage genes and a well-defined attachment site. This phage integrates site-specifically into the chromosome between *attP* and *attB,* the plasmid and chromosomal attachment sites, as described for other known *Listeria* phages (Zink and Loessner, 1992; Loessner *et al.*, 1994a and 1994b; Dorscht *et al.*, 2009; Mandali *et al.*, 2013). We observed all three states in the PacBio data set: *L. weihenstephanensis* genome without phage, lytic and lysogenic cycle. This phage presents similarity to the two *Staphylococcus aureus* (*S. aureus*) dsDNA phages StauST398-2 and LH1 belonging to the family *Siphoviridae*. To our knowledge, it is the first time that these three cases were observed in a single sequence data set without pre-treatment of the sample.

Analyzing the complete genome sequence, it was shown that the main virulence genes present in the pathogenic *L. monocytogenes* (*hly*A, *plcA*, *plcB*, *actA*, *mpl, inlA* and *inlB*) (reviewed in Bonazzi *et al*., 2009; Chakraborty *et al.*, 2000) are totally absent in *L. weihenstephanensis*, therefore the genome sequence explains the lack of pathogenicity in this species.

Moreover, genes enconding for all the subunits of nitrate reductase (*UE46_11965*, *UE46_11970*, *UE46_11975*, *UE46_11980*) and nitrite reductase (*UE46_01300*,

*UE46_01305*) were identified in the genome sequence of *L. weihenstephanensis,* suggesting that, under anaerobic conditions, this bacterium is able to perform nitrate respiration. The pathogenic *L. monocytogenes*, on the contrary, does not posses these genes and therefore is not able to use nitrate as alternative electron acceptor in during respiration.

It is interesting to notice that the clade representing *L. monocytogenes*, *L. marthii*, *L. innocua*, *L. welshimeri*, *L. seeligeri* and *L. ivanovii*, which is refered as *Listeria sensu stricto* (den Bakker *et al.,* 2013), together with the recently described species *L. floridensis*, do not present the genes for nitrate and nitrite reductase enzymes (FIG. 19).

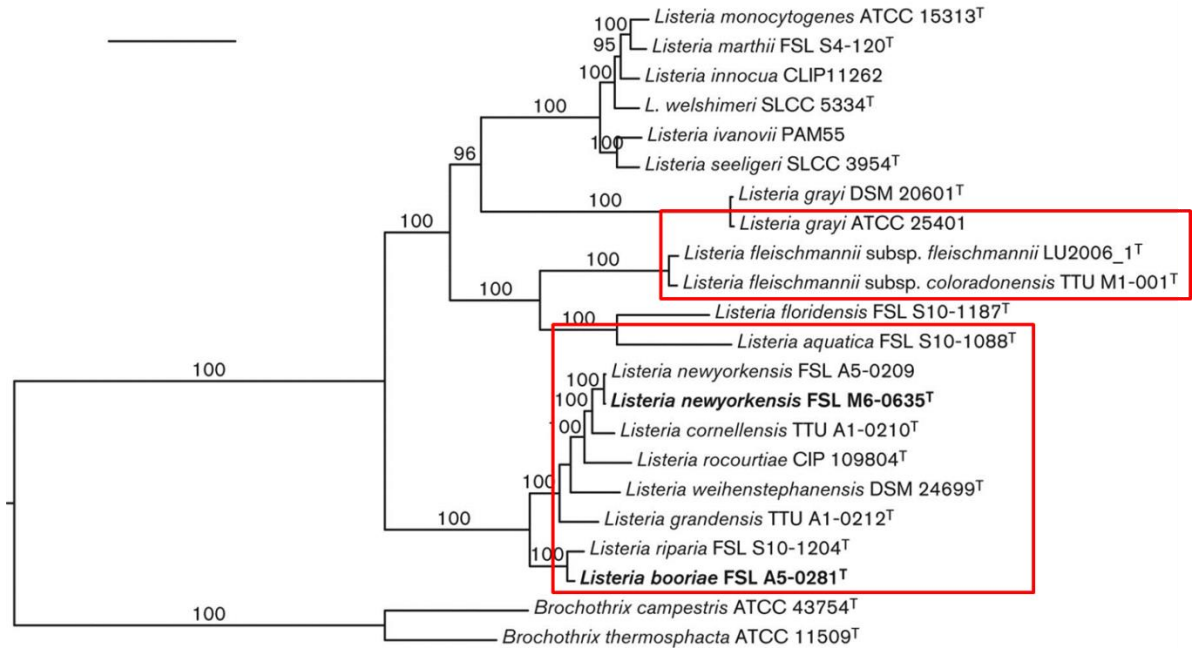



**FIG. 19: Maximum likelihood phylogeny published by Weller *et al.* (2015) based on concatenated amino acid sequences of 325 single copy core genes of members of the genus *Listeria*.** Values on branches represent bootstrap values based on 250 bootstrap replicates; bootstrap values < 70% are not displayed. The most recently discovered species of *Listeria* are shown in bold. Bar, 0.1 amino acid substitutions per site. *Listeria* spp. which contain all the subunits of nitrate and nitrite reductases are marked as contained in the red squares.

Moreover, among all the *Listeria* species, *L. weihenstephanensis*, *L. grandensis*, *L. rocourtiae*, *L. booriae*, *L. newyorkensis L. cornellensis, L. riparia*, *L. aquatica* and *L. fleischmannii*, subsp. *fleischmannii* and *coloradonensis*, contain the genes for all the subunits of nitrate (α, β, δ, γ subunits) and nitrite reductase (small and large subunits). In *L. grayi* subsp. *grayi* only the nitrite subunits could be identified, while in *L. grayi* subsp. *murrayi* all the subunits of the two enzymes were found

(supplemental material, Table S7). den Bakker *et al*., 2014 have shown that *L. weihenstephanensis* is supposed to reside in the *L. cornellensis*, *L. rocourtiae*, *L. grandensis* and *L. riparia* clade. As shown in Weller *et al.*, 2015, the new discovered species *L. newyorkensis* and *L. booriae* are also belonging to this clade which is far related to the *Listeria sensu stricto* clade, *L. grayi* and the clade composed by *L. fleischmannii*, *L. floridensis* and *L. aquatica*. These phylogenetic relationships partially reflect the ability of nitrate respiration, by nitrate and nitrite reduction. These finding suggest an ancestral evolution of the nitrate respiration capability, which was then lost by the common ancestor of the *Listeria sensu stricto* and by *L. floridensis*.

### 1.2 Transcriptional profiling of *L. weihenstephanensis*

The complete genome sequence described before was used as reference for the transcriptional profiling of *L. weihenstephanensis*. RNA-NGS revealed a clear oxygen-dependent transcriptional regulation but no significant differences between the two temperatures. The strict settings used for the data analysis (p-value ≤0.05 and FDR ≤0.3) showed a ~50% difference in gene regulation between the two temperatures, but, comparing the tendency of the regulation, it was shown that the majority of the genes showed the same regulation.

In particular, among the genes showing anaerobically less transcription, genes encoding for important proteins for the aerobic metabolism, like catalase, pyruvate dehydrogenase and many iron transporters (e.g. *fecE*), are shown. The first is important to get rid of the reactive oxygen species produced during aerobic respiration (reviewed in Cabiscol *et al.*, 2000). Pyruvate dehydrogenase is a key enzyme to produce acetyl-CoA, the fuel for the TCA cycle, and carbon dioxide from pyruvate (Berg *et al.*, 2002). Moreover, iron is contained in many oxidoreductive proteins of the respiratory chain, therefore iron transporters are stronger transcribed aerobically (Berg *et al.*, 2002).

On the other side, the most interesting genes stronger transcribed anaerobically, encoded for formate acetyltransferase (pyruvate-formate lyase) and pyruvate-formate lyase-activating protein already seen to be important for anaerobic growth of *E. coli* and *S. aureus* (Pope and Cole, 1982, Sawers and Böck, 1988; Leibig *et al*., 2011) since this enzyme converts pyruvate to Acetyl-CoA with production of formate, as alternative to the oxygen-dependent enzyme pyruvate dehydrogenase. Formate is an electron donor for the anaerobic reduction of nitrate or nitrite (Pope and Cole,

1982). In accordance, *L. weihenstephanensis*, anaerobically, was shown to induce nitrate and nitrite reductases. These two outcomes suggested activation of anaerobic respiration by using nitrogen as alternative electron acceptor. Nitrate respiration is less effective than aerobic respiration in sense of energy production, but anyway it is more effective compared to fermentation (Nelson and Cox, 2008), which is is not shown to be anaerobically induced in *L. weihenstephanensis*.

Nitrogen is essential for the organisms being a key component for proteins and nucleic acids productions; moreover it is one of most important elements on Earth. The main step for the nitrate reduction to nitrites is catalyzed by nitrate reductase (NRase). Prokaryotic nitrate reductases can be respiratory membrane-bound nitrate reductases (Nar), periplasmic nitrate reductases (Nap) and cytoplasmic assimilatory nitrate reductases (Nas) (Richardson *et al.*, 2001; González *et al.*, 2006; Morozkia and Zvyagilskaya, 2007). In contrast, the dissimilatory nitrogen metabolism is the anaerobic respiration by using nitrate or nitrite as terminal electron acceptor. The two dissimilatory processes are denitrification (*Pseudomonas* species, for example) and dissimilatory $NO_3^-$ reduction to ammonium (DNRA, for example *Bacillus subtilis*, *Salmonella*, *E. coli*, *S. aureus*) (reviewed in Moreno-Vivián *et al.*, 1999 and by Zumft, 1997; Morozkia and Zvyagilskaya, 2007; Rütting *et al.*, 2011). The Gram-positive *L. weihenstephanensis*, under anaerobic condition, is supposed to follow the DNRA. Nitrate is reduced to nitrite via the nitrate reductase (NarG, membrane bound) and is then reduced to ammonium via a membrane-bound Cytochrome-C nitrite reductase (NiR). Nitrate and nitrite are used as alternative electron acceptors and the anaerobic respiration is established (Morozkia and Zvyagilskaya, 2007; Richardson, 2000; Kraft *et al.*, 2011).

To further investigate this anaerobis respiration, the support of nitrogen to the anaerobic growth by nitrate was studied. The results showed a clear growth support under anaerobiosis, underlining once again the hypothesis that *L. weihenstephanensis* is able to reduce nitrate and so support its anaerobic adaptation.

Nitrate respiration produces high quantity of ammonium which is then used by *L. weihenstephanensis* to produce glutamate, via glutamate dehydrogenease (GDH), incorporating one ammonium to one mole 2-oxoglutarate. Another pathway for the production of glutamate via the glutamine synthetase (GS, to produce glutamine from ammonium and glutamate) - glutamate synthase (GOGAT, to produce 2 mole

glutamate from 2-oxoglutarate and glutamine) is present in *L. weihenstephanensis*. This pathway has a higher affinity for ammonium than the GDH (Kanamori *et al*., 1989) and so it is more active when the ammonium level in the cell is lower. Aerobically, the production of ammonium is lower than anaerobically because nitrate and nitrite reductases are shown to be down-regualated, therefore, under this condition, the GS-GOGAT pathway is preferred. This hypothesis is further strengthened by the fact that the gene encoding for GOGAT was shown to be down-regulated under anaerobic condition.

In conclusion, oxygen influences the metabolism of *L. weihenstephanensis* by mediating the switch from aerobic to anaerobic respiration. This mechanism is not possible in the pathogenic species, like *L. monocytogenes*, because the genes involved in nitrate respiration are absent. Moreover this anaerobic adaptation was shown to be temperature-independent because no big differences were shown between the genes regulated at 18°C and 34°C.

### 2. Anaerobic adaptation of *L. monocytogenes* EGDe

The transcriptional profiling results indicated strong oxygen-dependent transcriptional regulation and revealed differences between the two investigated temperatures in *L. monocytogenes* EGDe. Only 17 genes showed specific regulation at 34°C, while 176 genes showed differences in regulation at 18°C.

Among the genes showing stronger transcription anaerobically only at 18°C, many encoded for proteins involved in ethanolamine and 1,2-propanediol utilization and cobalamin biosynthesis. Cobalamin (coenzyme B12) supports fermentation of small molecules in many bacteria and its biosynthesis and transport was seen to be closely related to ethanolamine and 1,2-propanediol utilization (Roth *et al*., 1996; Joseph *et al*., 2006; Tang *et al*., 2015).

Ethanolamine is part of phosphotidylethanolamine, which is an abundant phospholipid in both bacterial and mammalian cell membranes and can be used by *L. monocytogenes* as carbon and nitrogen source (Joseph *et al*., 2006).

1,2-propanediol is produced during bacterial anaerobic break down of rhamnose and fucose and is supposed to be an alternative carbon source in *L. monocytogenes* (Staib and Fuchs, 2014).

In the present study, a 55-kb gene cluster ranging from *lmo1142* (*pduS*) to *lmo1208* (*cbiP*), which encodes for enzymes of the cobalamin synthesis and degradation of 1,2-propanediol and ethanolamine, was shown to be stronger transcribed anaerobically at both temperatures, with statistical significance only at 18°C. This cluster was already described to be up-regulated in intracellularly growing *Listeria* to support the replication and so the pathogenicity of *L. monocytogenes* in mammalian cells (Joseph *et al*., 2006). It is known that in *S. enterica* ethanolamine and 1,2-propanediol utilization support the growth in foods (Goudeau *et al.*, 2013). These cobalamin-dependent degradations could also support the environmental fitness of *L. monocytogenes*, explaining the major up-regulation of the cluster at 18°C. Moreover these results support the previous hypothesis of a role of ethanolamine and 1,2-propanediol utilization for the proliferation of *L. monocytogenes* in both, food and host environments (Joseph *et al*., 2006; Tang *et al*., 2015).

Another interesting finding was a stronger anaerobic transcription (significant only at 18°C) of the gene for the redox sensing transcriptional repressor Rex (*lmo2072*). Rexmodulates the transcription in response to changes in cellular NADH/$NAD^+$ redox state. NADH/$NAD^+$ ratio is low aerobically due to the rapid reoxidation of NADH produced during glycolysis and TCA cycle by the ETC (reviewed in Haddock and Jones, 1977; Berg *et al.*, 2002). Anaerobically, since no alternative electron acceptors are present in *L. monocytogenes* (Glaser *et al.*, 2001), only fermentation can reduce pyruvate to various compounds and reoxidatize NADH to $NAD^+$ to allow the glycolysis to continue (Nelson and Cox, 2008), therefore the NADH/$NAD^+$ ratio is higher. It was shown that in *S. coelicolor*, *B. subtilis*, and *Thermus aquaticus* Rex binds either oxidized or reduced pyridine dinucleotides (Brekasis and Paget, 2003), with a strong preference for reduced NADH (Gyan *et al.*, 2006). When NADH binds to Rex, Rex cannot generate the complex with the DNA target site, the Rex operator and act as gene repressor (Wang *et al.*, 2008). The *L. monocytogenes* Rex regulon is not published yet, but Stefanie Wüstner (2010) identified genes specifically controlled by this repressor during her master thesis. These genes encode some of the proteins previously described to be involved in the oxygen-dependent adaptation of *L. monocytogens* (Müller-Herbst *et al*., 2014), for example the bifunctional acetaldehyde CoA/alcohol dehydrogenase (*lmo1634)* and fumarate reductase (*lmo0355)*. In the present study these two genes were shown to be stronger

transcribed anaerobically, in particular at 18°C, when the repressor was less transcribed than aerobically Interesting to notice is that neither at 34°C (this study) nor at 37°C (Stefanie Wüstner, 2010) the transcription of *lmo2072*repressor was shown to be significantly regulated dependent on the oxygen-availability, only at 18°C. This indicates a stronger influence of this redox sensor at environmental temperature.

Glaser *et al*. (2001) identified 15 members of the CRP/FNR superfamily in *L. monocytogenes*. Among these, the virulence genes regulator PrfA is the most important and well studied. Another member of the CRP/FNR superfamily of transcriptional factors, with similarities to FNR (fumarate and nitrate reductase regulator protein) of *B. subtilis* (27% identity), is encoded by *lmo1251* and was already described to be anaerobically up-regulated (Stefanie Wüstner, 2010; Müller-Herbst *et al.*, 2014). FNR is a transcriptional activator, active only when oxygen is absent. The NGS data in this study supported this result showing a stronger transcription of the gene anaerobically, in particular at 18°C, and in parallel up-regulation of the only gene (*lmo1250*) controlled by this regulator which encodes an antibiotic resistance protein (Krementowski, 2010).
In *E. coli*, FNR activity is regulated by the oxygen content. Anaerobically, FNR contains a $[4Fe\text{-}4S]^{2+}$ cluster which supports dimerization of FNR allowing DNA binding and transcriptional induction of specific genes. Aerobically, $[4Fe\text{-}4S]^{2+}$ is converted to $[2Fe\text{-}2S]^{2+}$, leading to disassembly of dimeric FNR, loss of the DNA binding and so loss of transcriptional activation (reviewed in Kiley and Beinert, 1998). The [Fe-S] cluster is bound to FNR through interaction of $Fe^{2+}$ with four cysteines presents in the N-terminal domain of FNR (Spiro and Guest, 1988; Kiley and Reznikoff, 1991). In *L. monocytogenes* the protein is smaller (219 aa compared to the 250 aa in *E. coli*) and the four cysteine residues present in the N-terminal domain in *E. coli* are not conserved. This might be the reason why FNR is not a central oxygen-dependent transcriptional regulator and why only *lmo1250* is controlled by this regulator.

Not many genes showed oxygen-dependent transcriptional regulation at 34°C, but it was shown that the gene encoding for the acetolactate synthase protein, AlsS, (*lmo2006*, *alsS*) and ferrichrome ABC transporters for iron-uptake (*fuhBCDG*) were down-regulated anaerobically.

AlsS is an oxygen-dependent enzyme involved in acetoin generation and the gene encoding for this protein was already shown to be stronger transcribed aerobically at 37°C (Müller-Herbst *et al.*, 2014). This enzyme is important aerobically to get rid of the pyruvate and allow the glycolysis to continue due to the split TCA cycle in *L. monocytogenes* (Trivett and Meyer, 1971; Glaser *et al.*, 2001).

Iron is a key molecule for many enzymes; therefore its availability is essential for *L. monocytogenes* survival (reviewed in Andrews *et al*., 2003). In particular, the ability to acquire iron is very important during respiration since this is a key cofactor for cytochromes and respiratory chain proteins (reviewed in Litwin *et al*., 1993 and in Lechowicz J. and Krawczyk-Balska, 2015). Anaerobically, the respiratory chain is not active, since *L. monocytogenes* is only able to use oxygen as electron acceptor. Therefore the iron uptake can be reduced. The RNA-NGS data showed a down-regulation of *fhuG* (*lmo1957*)*, fhuB* (*lmo1958*)*, fhuD* (*lmo1959*) and *fhuC* (*lmo1960*). This operon encodes for an ABC transporter responsible for the transfer of iron from the environment into the bacterial cytosol (Jin *et al*., 2006).
On the contrary, it was observed an up-regulation anaerobically, especially at 34°C, of the gene encoding for the heavy metal transporting ATPase, Fur-regulated virulence factor A (FrvA, encoded by *lmo0641*). FrvA is an ortholog of the *B. subtilis* Fur- and PerR-regulated Fe(II) efflux $P_{1B4}$ -type ATPase PfeTand it was suggested to protect against iron toxicity (Pi *et al*., 2016). In correlation with the anaerobic down-regulation of the iron uptake system described before, an up-regulation anaerobically of this efflux system was shown. This system was described to be required for systemic infection of mice with *L. monocytogenes* (McLaughlin *et al*., 2012, reviewed in Lechowicz J. and Krawczyk-Balska, 2015), therefore these results, taken together, showed a very interesting correlation between oxygen-dependent adaptation and virulence.

Most of the regulated genes at both temperatures encoded for proteins involved in the central metabolic pathway of *L. monocytogenes*. Among the genes up-regulated anaerobically, genes encoding for enzymes involved in anaerobic metabolism, e.g. fumarate reductase (*lmo0355*) and acetaldehyde-CoA/alcohol dehydrogenase (*adh*, *lmo1634*), were identified. The last one was shown to be significantly up-regulated anaerobically only at 18°C even if it showed stronger transcription at 34°C. This enzyme catalyzes the reduction of Acetyl-CoA to generate ethanol and was also

described as being involved in adhesion to host epithelial cells and as being induced anaerobically (Burkholder *et al.*, 2009; Burkholder and Bhunia, 2010). Genes encoding for key enzymes for the aerobic pathways like quinol oxidase (*qoxA*, *qoxB*, *qoxC*, *qoxD*) and pyruvate dehydrogenase (*pdhA*, *pdhB*, *pdhC*, *pdhD*) were shown, on the contrary, to be down-regulated anaerobically. Most of them were already described to have oxygen-dependent regulation by Müller-Herbst *et al.* (2014). Therefore, the NGS results presented in this studty convalidated the previous findings.

*L. monocytogenes*, as described before, possesses a non-cyclic TCA cycle due to the lack of 2-oxoglutarate dehydrogenase (Trivett and Meyer, 1971; Glaser *et al.*, 2001). Thus, the TCA cycle is separated in an oxidative and a reductive branch terminating at 2-oxoglutarate and succinate, respectively (Eisenreich *et al.*, 2006) (FIG. 1). For this reason, during aerobic growth, pyruvate is not completely oxidized to carbon dioxide and the cells produce a lower amount of NADH, no $FADH_2$ and consequently less ATP compared to cells with a complete TCA cycle. Therefore, pyruvate needs to be channeled to other pathways to let the glycolysis to continue and to supply the energy production deficiency. Romick *et al.* (1996) described that *L. monocytogenes* is able to performed mixed acid fermentation to supply the lack of a complete TCA cycle and obtain a higher amount of ATP by constantly remaining glycolysis active. This means that this bacterium produces anaerobically lactate (79%), acetate (2%), formate (5.4%), ethanol (7.8%) and carbon dioxide (2.3%) while aerobically, combined with aerobic respiration, fermentative end products are also produced. In particular, lactate (28%), acetate (23%), and acetoin (26%) are generated under aerobic condition. Therefore, there are five fermentative pathways, production of acetoin (via acetolactate synthase), production of lactate (via L-lactate dehydrogenase), production of formate (via pyruvate formate lyases), production of acetate (via pyruvate oxidase, pyruvate dehydrogenase and acetate kinases) and production of ethanol (via the bifunctional acetaldehyde-CoA/alcohol dehydrogenase). From the RNA-NGS results, several genes encoding for these central carbon metabolic enzymes (FIG. 2) showed oxygen-dependent regulation in dependency of the temperature.

The transcriptional level is not always corresponding to the amount of protein produced and actively functional, thus to further investigate the impact of the

temperature on the oxygen-dependent regulation of the carbohydrate catabolism of *L. monocytogenes*, construction and phenotypic characterization of specific mutants under the same transcriptional experimental conditions was performed. In particular, *L. monocytogenes* EGDe Δ*lmo1581* and *L. monocytogenes* EGDe Δ*lmo1168* (deletion *ackA* and *ackA2,* encoding for acetate kinases), *L. monocytogenes* EGDe Δ*lmo1055* (*pdhD,* E3 subunit of pyruvate dehydrogenase), *L. monocytogenes* EGDe Δ*lmo2720* (*acs*, acetate-CoA ligase), *L. monocytogenes* EGDe Δ*lmo0210* (*ldh,* L-lactate dehydrogenase), *L. monocytogenes* EGDe Δ*lmo1917* (*pflA* pyruvate formate-lyase), *L. monocytogenes* EGDe Δ*lmo1406* (*pflB,* pyruvate formate-lyase), *L. monocytogenes* EGDe Δ*lmo0722* (pyruvate oxidase), *L. monocytogenes* EGDe Δ*lmo1634* (*adh*, bifunctional acetaldehyde-CoA/alcohol dehydrogenase and adhesion to host cells), *L. monocytogenes* EGDe Δ*lmo2006* (*alsS*, alpha-acetolactate synthase acetolactate synthase) and the double muntant *L. monocytogenes* EGDe Δ*lmo1917*Δ*lmo1406* (*plfA/plfB*, pyruvate-formate lyases) were generated.

At 18°C, it was observed that, under aerobic condition, *L. monocytogenes* EGDe Δ*lmo1581* and *L. monocytogenes* EGDe Δ*lmo1168* (*ackA* and *ackA2*) and *L. monocytogenes* EGDe Δ*lmo1055* (*pdhD*) showed an extremely reduced growth of the mutants compared to *L. monocytogenes* EGDe WT. The NGS data are in total accordance with the growth results for these three genes. Pyruvate dehydrogenase is involved in the transformation of pyruvate to acetyl-coenzyme A (acetyl-CoA), important for fatty acid synthesis and for the TCA cycle and so for energy production via aerobic respiration (Rose et al., 1954; Glaser et al., 2001; Müller-Herbst et al. 2014). The reaction catalyzed by this enzyme generates NADH, which is essential for the respiration, especially when the TCA cycle it not properly working as in *L. monocytogenes*. The deletion of the gene encoding for pyruvate dehydrogenase could not be compensated by other genes encoding for alternative enzymes. Therefore this result suggested that this pathway might be the most important in pyruvate turnover aerobically. Acetyl-CoA can be then converted to first to Acetyl-P (via phosphotransacetylase [*pta*]) and then to acetate by the acetate kinases. This last reaction is important for production of ATP and the lack of acetate kinase (*ackA*) brought a clear disadvantage to the cells probably due to a lower production of energy and so a lower compensation of the incomplete TCA cycle. The other acetate

kinase (*ackA2*) is suggested to have a secondary activity compared to *ackA* because its lack led only to a slight reduction of growth compared to the WT. Anyway, it would be interesting to generate a double mutant for acetate kinases to avoid the functional compensation and therefore study the effect of a complete lack of this enzyme on the growth.

The single mutants *L. monocytogenes* EGDe Δ*lmo1917* and *L. monocytogenes* EGDe Δ*lmo1406* (*pflA* and *pflB*) did not show differences in growth compared to the WT under all the conditions, while the *pflA* and *pflB* showed up-regulation at both temperatures, significantly relevant just at 18°C (*pflB*). This may be due to the fact that the two genes transcribe for enzymes with the same function so a functional compensation could explain the single-mutants results (Bhattacharjee *et al.*, 2013). The double mutant *L. monocytogenes* EGDe Δ*lmo1917*Δ*lmo1406,* on the other hand, showed a significant reduction of the growth aerobically at 18°C. Aerobically the production of acetyl-CoA is mainly controlled by the pyruvate dehydrogenase complex, as described before, but the pyruvate formate-lyases contribute to its generation with consequent increment of energy production. Moreover, formate can then be converted to carbon dioxide by formate dehydrogenase (*fdh*) under aerobic condition, with production of NADH to support the aerobic respiration.

*L. monocytogenes* EGDe Δ*lmo0210* (*ldh*) showed an increase of growth after 19 h aerobically at 18°C. This enzyme is a key protein for production of lactate from pyruvate during fermentation under both the oxygen conditions (Romick *et al*., 1996). Lactic acid production could alterate the pH of the medium and could generate a stress environment. It was hypothesized that the increase of growth detected for the lactate dehydrogenase mutant could be due to the toxicity of lactate for the cells. However, pH of the culture medium did not change significantly neither for the mutant nor for the WT culture (data not shown), meaning that the observed increase of growth was due to other reasons. A possible alternative explanation could be that, in absence of L-lactate dehydrogenase, more pyruvate could be channeled to the pyruvate dehydrogenase→phosphotransacetylase→acetate kinase pathway leading to a higher production of ATP compared to the WT and so to an increment of growth aerobically.

Anaerobically, on the other hands, the *L. monocytogenes* EGDe Δ*lmo0210* (*ldh*) showed a decrease of growth at 18°C. From the NGS, the gene for this enzyme was seen to be stronger transcribed anaerobically at 18°C. This enzyme presents more

activity under anaerobic condition due to the reoxidation of NADH to $NAD^+$ to let the glycolysis continue when aerobic respiration is not possible (Romick *et al.*, 1996; Nelson and Cox, 2008; Müller-Herbst *et al.*, 2014), explaining the reduced growth of the mutant under anaerobiosis. Moreover, it can be assumed that this pathway is more effective with respect to the recycling of NADH than the ethanol production via the bifunctional acetaldehyde-CoA/alcohol dehydrogenase (*adh*). In fact, *L. monocytogenes* EGDe Δ*lmo1634 (adh*) did not shown any effect on the growth of the mutant compared to the WT, meaning that its lack could be compensated by the lactate pathway.

*L. monocytogenes* EGDe Δ*lmo1168* (*ackA*), which showed reduction of growth aerobically, exhibited an extreme reduced growth compared to the WT also anaerobically, supporting once again its clear importance as energy supplier in *L. monocytogenes*, under all the oxygen conditions. Anaerobically the phenotype was even more accentuated because, under this condition, the respiration is not performed and therefore the ATP produced by this enzyme is absolutely essential for the cell survival.

In conclusion, at 18°C, it was shown that the deletion of central metabolic pathways, in particular the acetate production via *pdhD-pta-ackA*/*ackA2*, resulted in an extreme reduced fitness of *L. monocytogenes* EGDe, which could not be compensated by other pathways.

At 34°C the results were different. Aerobically only *L. monocytogenes* EGDe Δ*lmo1055* (*pdhD*) presented significant reduction in growth, correlating with the RNA-NGS data, compared to the WT due to the reasons explained above. *L. monocytogenes* EGDe Δ*lmo1581* and *L. monocytogenes* EGDe Δ*lmo1168* at this temperature did not display a significant reduction of growth, suggesting that at 34°C the two acetate kinases could compensate each other. These results indicated, differently than at 18°C, a high flexibility of the metabolism.

Anaerobically, *L. monocytogenes* EGDe Δ*lmo1168* (*ackA*) showed an extremely significant reduced growth of the mutant anaerobically compared to *L. monocytogenes* EGDe WT, most probably due to its relevance in energy production, as described earlier. Moreover the lack of the enzymes involved in acetyl-CoA production, *L. monocytogenes* EGDe Δ*lmo1055* (*pdhD*) and *L. monocytogenes*

EGDe Δ*lmo1917*Δ*lmo1406* (*pflA*/*pflB*), exhibited in both cases a reduction of growth anaerobically at 34°C.

*pdhD* is more transcribed aerobically and it was already shown to be more active in the presence of oxygen (Müller-Herbst *et al.* 2014) but here it was shown to be relevant also for the anaerobic growth during anaerobiosis at 34°C. One explanation could be that at this high temperature the bacteria are growing faster and therefore need to get rid of the pyruvate more actively to let the glycolysis proceed and therefore supply the cells with enough energy. Moreover, acetyl-CoA is then channeled to the acetate pathway via production of additional ATP. In accordance, the genes encoding for the two pyruvate formate-lyases, were up-regulated anaerobically (Leibig *et al.*, 2011; Müller-Herbst *et al.* 2014) and the deletion of the double mutant led to a reduction of growth anaerobically.

The gene for acetate-CoA ligase (*acs*) was slightly stronger transcribed aerobically, especially at 34°C, and *L. monocytogenes* EGDe Δ*lmo2720* showed a strong significant increase in growth at 34°C anaerobically. This enzyme is involved in acetate metabolism. In *E. coli* is known to be active only anaerobically, using ATP to produce acetyl-CoA from acetate (Kumari *et al*., 2000), but its function in *L. monocytogenes* is not completely clear and the effective direction of the reaction is not known. Its deletion led to an increase of growth probably due to the canalization of acetyl-CoA to the alternative acetyl-CoA→acetyl-P→acetate pathway, in case it functions in the direction of production of acetate, which produces ATP and which was seen to be important aerobically and, even more, anaerobically for energy production. In case that its function is only in the direction of production of acetyl-CoA, then its deletion could be favorable anaerobically due to its utilization of ATP to catalyze the anabolic reaction. This increase of growth of the *acs* mutant compared to the WT was observed only at 34°C probably due to the need of more energy for the faster growth compared to 18°C (see above).

Moreover, *L. monocytogenes* EGDe Δ*lmo0210* (*ldh*) was also growing less then the WT probably because it is a key enzyme to recycle the NADH via lactate production, as explained above at 18°C, and to get rid of the pyruvate directly.

The results at 34°C anaerobically showed once again that, at higher temperature, the metabolism is flexible since the observed reduction of growth of the mutants were not extreme compared to the *L. monocytognenes* EGDe, but just minor.

*L. monocytogenes* EGDe Δ*lmo0722* (pyruvate oxidase), *L. monocytogenes* EGDe Δ*lmo1634* (*adh*) and *L. monocytogenes* EGDe Δ*lmo2006* (*alsS*) did not show relevant difference in growth compared to *L. monocytogenes* EGDe WT, under all the conditions, even if the NGS data showed different regulation depending on oxygen or temperature or both. This means that their pathways can be compensated by other pathways, as explained before.

In summary, at 18°C, as non-optimal environmental temperature, bacteria might activate a strong oxygen-dependent regulation in order to adapt to different and wide conditions in nature and to save energy by reducing the transcription of genes not required. Many microorganisms showed different environment-dependent regulation, when the conditions are not optimal, to conserve energy (reviewed in Cotter and Miller, 1998 and in López-Maury *et al*., 2008). White-Ziegler *et al*. (2008) identified a stronger expression of stress-response genes in *E. coli* at 23°C compared to 37°C, suggesting a temperature-dependent stress adaptation. In this study a temperature-dependent anaerobic adaptation in *L. monocytogenes* is suggested. Additionally, the observed less flexibility of the metabolism at this temperature could also explain the need of a higher regulation.
On the other hand, 34°C is a favorable temperature for the bacteria and it is close to the mammalian gut temperature. Therefore at 34°C a unique adaptation to the clear gut condition might occur, explaining the differences between the two temperatures. Moreover, a more flexible metabolism requires less regulation thanks to functional compensation between functional-related pathways. Anyway it is hard to suggest if one pathway might be especially important for host colonization since this high flexibility led to compensation of deletions by other pathways. Further *in vivo* studies are needed to solve this question.

Comparing the RNA-sequencing results between the environmental species *L. weihenstephanensis* and the pathogenic *L. monocytogenes* EGDe, the second showed a higher oxygen-dependent regulation. 223 total genes have been shown to be regulated in *L. monocytogenes* EGDe and only 104 in *L. weihenstephanensis*. This is not due to the genome size because genome of *L. weihenstephanensis* (3.4 Mb, in-house) is bigger than *L. monocytogenes* EGDe (2,9 Mb, Glaser *et al.*, 2001). *L. weihenstephanensis* possess a total of 3077 coding genes and *L. monocytogenes* EGDe 2867. The major regulation of *L. monocytogenes* EGDe at 18°C might be due

to the incapacity of this species to perform nitrate respiration (Glaser *et al.*, 2001), leading to activation of other strategies to adapt to anaerobiosis.

It's known that temperature is a trigger for metabolic changes in many bacteria (reviewed in Barria *et al*., 2013). For example, *in E. coli* temperature-dependent changes in energy metabolism were described. In particular, genes encoding for enzymes involved in glycolysis, amino acid biosynthesis and transport and encoding for TCA cycle enzymes showed differential transcriptional regulation in dependency of the temperature (Gadgil *et al*., 2005).

As described before, also *L. monocytogenes* regulates the expression of genes involved in central metabolic pathways, like nitrogen metabolism (Kaspar *et al*., 2014) and carbon metabolism (Grubmüller *et al*., 2014), in a temperature-dependent manner.

Here it was demonstrated that *L. monocytogenes* activates a temperature-dependent adaptation to anaerobiosis, suggesting an optimal adaptation to its current ecological niche, host vs. environment. *L. weihenstephanensis*, on the other hand, is an environmental species which is not known to colonize a host and so it is not necessary to differentiate between environment and host conditions.

### 3. Strain-specific adaptation to anaerobiosis in *L. monocytogenes*

It is known that adaptation to restricted oxygen condition positively influences the virulence of *L. monocytogenes* (Stritzker *et al*., 2005, Bo Andersen *et al.*, 2007; reviewed in Lungu *et al.*, 2009) and strain-specific differences in *L. monocytogenes* suggested an interaction between the bacterial stress response and virulence gene expresiion (Jydegaard-Axelen *et al.*, 2004; Yamamoto *et al.*, 2012; Cemma *et al.*, 2015).

To investigate a possible contribution of the strain-specific adaptation to anaerobiosis to difference in virulence, different *L. monocytogenes* strains with different virulence potential were selected and their transcriptional profiling was determined via RNA-NGS under aerobic and anaerobic conditions at 37°C. The very well studied *L. monocytogenes* EGD and its derivative EGDe (EGD European), which stands for strain EGD after passage through mice to maintain virulence (Bécavin *et al.*, 2014), were specifically selected. Additionally, the outbreak strain *L.*

*monocytogenes* ScottA and the environmental isolate *L. monocytogenes* 1670 (isolated from milk) were also investigated.

The genome sequence of *L. monocytogenes* EGDe was used as reference for the mapping of the RNA-NGS reads of all the strains to allow comparisons. However, even if *L. monocytogenes* 1670 reads could be mapped against *L. monocytogenes* EGDe genome, the number of mapping reads was very low compared to the other strains. This could be due to the lower cluster density during the RNA-NGS for this strain, resulting in a lower output of reads.

Interestingly, *L. monocytogenes* EGD used in this thesis did not display the highest number of mapping reads against the sequence of the published strain EGD (Bècavin *et al.*, 2014, NCBI accession number HG421741), suggesting a more closely relation to *L. monocytogenes* EGDe than to the published strain EGD.

The strain EGD used in this study was kindely provided to our depertment by Prof. Dr. Dagmar Beier from the Department of Microbiology, Universität Würzburg (Williams *et al*., 2005a). Previously this strain was provided to Universität Würzburg by Prof. Dr. Kaufmann from Max Planck Institute for Infection Biology, Berlin (Kaufmann, 1984) who obtained the strain from Prof. Dr. Helmuth Hahn who obtained it from the Trudeau Institute (Kaufmann *et al*., 1979). The strain was also given from Prof. Dr. Helmuth Hahn to Prof. Dr. Trinad Chakraborty (Institute for Medical Microbiology Justus-Liebig-University Faculty of Medicine, Giessen) who retested the strain EGD for its virulence in mice, and re-named it EGDe, and collaborated for the sequencing of the strain EGDe (Glaser et al., 2001).

Bécavin *et al.*, 2014 sequenced the strain EGD wich was provided by Prof. Dr. Patrick Berche who obtained the strain from the Trudeau Institute, as Prof. Dr. Helmuth Hahn.

Therefore the strain used in this study was obtained from the Trudeau Institute, as the one sequenced by the Institut Pasteur, France (Bécavin *et al.*, 2014). Hovewer, from the NGS data, it was suggested that the strain used in this study is closer related to the strain EGDe than the published EGD. Therefore it might be possible that, during the passages of the strain EGD from different Institutes, a mistake happened and that the published strain EGD is not the strain EGD provided to our department. It is thus recommended to sequence the genome of the *L. monocytogenes* EGD strain used in this work to point out the differences to the published one and repeat the mapping of the RNA-NGS reads.

A repetition of the mapping using the specific strain genome is also suggested for *L. monocytogenes* ScottA to avoid an underestimation of the results. In fact, even if the mapping against the *L. monocytogenes* EGDe genome revealed good results, the best genome for mapping was its own genome (Briers et al., 2011, NCBI accession number AFGI00000000). Moreover it would be optimal to sequence the genome of *L. monocytogenes* 1670 and use this for mapping of the reads for a complete overview of the oxygen-dependent regulation.

The transcriptional profiling of the four strains at 37°C using the EGDe genome as reference showed anyway interesting results. A total of 111 genes showed regulation in at least one of the four strains; 34 genes were up-regulated and 77 down-regulated anaerobically. The genes encoding for the well described anaerobic protein fumarate reductase (*lmo0355*) and a heavy metal transporting ATPase (*lmo0641*) (Müller-Herbst *et al.*, 2014) were anaerobically up-regulated in all four strains. On the other side, genes for emblematic aerobic enzymes like aa3-600 quinol oxidase subunit I (*lmo0014*), superoxide dismutase (*lmo1439*) and part of the pyruvate dehydrogenase operon (*lmo1053* [*PdhB*] *lmo1054* [*pdhC*] *lmo1055* [*PdhD*]), were down-regulated in at least three strains.

Moreover, *L. monocytogenes* 1670 exhibited anaerobic down-regulation of ten flagella genes genes (*lmo*0678; *lmo0679*; *lmo0680*; *lmo0682*; *lmo0686*; *lmo0698*; *lmo0705*; *lmo0706*; *lmo0710*; *lmo0716*). These genes encode for proteins involved in flagellar biosynthesis which are organized in two operons (*lmo0675–lmo0689)* and (*lmo0691–lmo0718)* and flank the *flaA* gene (*lmo0690*) (Williams *et al.*, 2005b).

These genes were not transcribed at all in *L. monocytogenes* EGD and EGDe at 34°C, supporting the idea of a temperature-dependent motility in *L. monocytogenes*. Previus studied already described that the number of flagella is temperature-dependent (Griffin and Robbins, 1944) with best motility detected at 20°C since the gene for the key subunit of flagella FlaA (*flaA),* and the chemotaxis proteins CheY (*lmo0691)* and CheA (*lmo0692)* are optimally transcribed at 22°C-24°C but very poorly at 37°C (Leifson and Palen, 1955; Peel *et al.*, 1988; Dons *et al.*, 1992; Dons *et al.*, 2004; Gründling *et al*., 2004; Williams *et al.*, 2005b).

In *L. monocytogenes* ScottA, these ten flagella genes showed a stronger transcription aerobically (Table 27). Synthesis and rotation of bacterial flagella is driven by the proton motive force (PMF) (Manson *et al.*, 1977). Aerobically, the aerobic respiration generates the PMF across the membrane which is used by the

membrane ATPase for ATP synthesis (reviewed in Haddock and Jones, 1977; Berg *et al.*, 2002). Anaerobically this process is not possible since *L. monocytogenes* is not able to establish anaerobic respiration and the PMF generation would need high energy consumation by ATPase-driven ATP hydrolysis (Maloney *et al.*, 1974). This justifies the anaerobic down-regulation of these flagella genes in *L. monocytogenes* 1670 and ScottA.

Motility experiments confirmed the NGS data and supported the previous findings for EGD and EGDe since bacteria were moderately motile only at 24°C, in particular aerobically.

From the NGS results, *L. monocytogenes* 1670 was seen to transcribe actively the flagella genes aerobically, but the phenotype observed by motility experiments did not correlate perfectly. At 37°C this strain was slightly motile aerobically and not motile at all anaerobically, when starting from overnight cultures incubated at 24°C. No motility was observed under all conditions when starting from cultures cultivated at 37°C. Anyway, the strain 1670 was shown to be more motile compared to EGD and EGDe at 24°C anaerobically, starting from cultures cultivated at both temperatures.

The *L. monocytogenes* outbreak strain, ScottA, showed stronger transcription aerobically for the flagella genes, even if in a lower rate than the strain 1670. Motility experiments showed that this strain was the most motile and, even if it showed no motility anaerobically at 37°C when starting from cultures cultivated at 37°, at 24°C, surprisingly, a wide, translucent swarming aura was detected. In *L. monocytogenes* the anaerobic down-regulation of *flaA* during *in vivo* infection was proposed as an adaptive mechanism to avoid the activation of innate immune responses (Dons *et al.*, 2004; Way *et al.*, 2004) since it functions as pattern recognition molecules for Toll-like receptor 5-mediated signaling (Hayashi *et al.*, 2001; McSorley *et al.*, 2002). However, at the same time, it was suggested that *L. monocytogenes* uses components of bacterial flagella to increase the efficiency of epithelial cell invasion (Dons *et al.*, 2004; Bigot *et al*., 2005). It was shown that flagella do not act as adhesins for the host cells but motile bacteria were advantaged during the early stage of colonization (O'Neil and Marquis, 2006). In fact, even if the bacteria can spread cell-to-cell during its invasion, for the initial infection it is necessary to bind the receptors on the epithelial cells and to approach this receptor the flagella could be involved. This could explain the *L. monocytogenes* ScottA results because only

when the culture was cultivated at 24°C and then incubated at 37°C, bacteria presented a wide, translucent swarming aura under anaerobic condition which could help the initial attach to the host cells. Since this event was not detected when the culture was cultivated and incubated at 37°C, this study pointed out a possible relation between the temperature upshift and infectivity. Therefore, this hypothesis should be further analyzed.

## VI. LITERATURE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## VII. APPENDIX

The supplementary Table S1-S7 can be visualized in the attached CD-ROM.

### 1. Abbreviations

| | |
|---|---|
| °C | degree Celsius |
| µ | micro |
| 3' | three prime; third carbon of sugar deoxyribose |
| 5' | five prime; fifth carbon of sugar deoxyribose |
| A | adenine |
| ABC | ATP-binding cassette |
| *ackA1*/*ackA2* | genes encoding for acetate kinases |
| *acs* | gene encoding for acetyl-CoA synthetase |
| ActA | actin Assembly-Inducing Protein |
| *adh* | acetaldehyde-CoA/ alcohol dehydrogenase |
| *adh* | gene encoding for bifunctional acetaldehyde-CoA/ alcohol dehydrogenase |
| ADP | adenosine diphosphate |
| *alsS* | acetolactate synthase |
| ATP | adenosine triphosphate |
| ATR | acid tolerance respons |
| BetL | glycine betaine uptake system |
| BHI | brain heart infusion |
| BLAST | Basic Local Alignment Search Tool |
| bp | base pair |
| bsh | bile salt hydrolase activity |
| C | cytosine |
| cDNA | complementary DNA |
| CheA | two-component sensor histidine kinase CheA |
| CheY | chemotaxis response regulator CheY |
| $CO_2$ | carbon dioxide |
| CoA | coenzyme A |
| COGs | clusters of Orthologous Groups |
| CTAB | cetyltrimethyl ammonium bromide |
| DNA | deoxyribonucleic acid |
| DNRA | dissimilatory nitrate reduction to ammonium |
| dNTP | desoxynucleotide triphosphate |
| dsDNA | double stranded DNA |
| *E. coli* | Escherichia coli |
| e.g. | for example |
| EDTA | ethylenediaminetetraacetic acid |
| Em10 | erythromycin 10 µg/ml |
| Em300 | erythromycin 300 µg/ml |
| Emr | erythromycin resistance |

| | |
|---|---|
| *et al.* | and others |
| ETC | electron transport chain |
| EtOH | ethanol |
| F | farad |
| FAD | flavine adenine dinucleotide |
| $FADH_2$ | flavine adenine dinucleotide reduced |
| FC | fold change |
| *fdh* | gene encoding for formate dehydrogenase |
| FDR | false discovery rate |
| FIG. | figure |
| FlaA | flagellin protein |
| FNR | fumarate and nitrate reductase regulator protein |
| Fur | ferric uptake regulator |
| G | guanosine |
| GABA | γ-Aminobutyric acid |
| GAD | glutamate decarboxylases |
| Gbu | glycine betaine transport system |
| GDH | glutamate dehydrogenease |
| GI | gastro intestinal |
| GlnR | glutamine synthetase repressor |
| GOGAT | glutamine-2-oxoglutarate aminotransferase |
| GS | glutamine synthetase |
| h | hours |
| H | hydrogen |
| $H^+$ ions | protons |
| HEPES | 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid |
| Inl | internalin |
| Kat | catalase |
| kb | kilo base pairs |
| kDa | kilodalton |
| *L. aquatica* | Listeria aquatica |
| *L. booriae* | Listeria booriae |
| *L. cornellensis* | Listeria cornellensis |
| *L. fleischmanii* | Listeria fleischmanii |
| *L. floridensis* | Listeria floridensis |
| *L. grandensis* | Listeria grandensis |
| *L. grayi* | Listeria grayi |
| *L. innocua* | Listeria innocua |
| *L. ivanovii* | Listeria ivanovii |
| *L. marthii* | Listeria marthii |
| *L. newyorkensis* | Listeria newyorkensis |
| *L. riparia* | Listeria riparia |
| *L. rocourtiae* | Listeria rocourtiae |

| | |
|---|---|
| *L. seeligeri* | Listeria seeligeri |
| *L. weihenstephanensis* | Listeria weihenstephanensis |
| *L. welshimeri* | Listeria welshimeri |
| *L. monocytogenes* | Listeria monocytogenes |
| LB | Luria-Bertani |
| LDH | lactate dehydrogenase |
| *ldh* | gene encoding for lactate dehydrogenase |
| LLO | lysteriolysin O |
| *lmo* | L. monocytogenes gene |
| m | milli |
| M | molar |
| Mb | mega base pairs |
| min | minute |
| mol | mole |
| Mpl | zinc metalloprotease |
| MQ | Milli-Q |
| mRNA | messenger RNA |
| n | nano |
| $NAD^+$ | nicotinamide adenine dinucleotide |
| NADH | nicotinamide adenine dinucleotide reduced |
| Nap | periplasmic dissimilatory NRase |
| Nar | membrane-bound respiratory NRase |
| Nas | cytoplasmic assimilatory NRase |
| NGS | Next Generation Sequencing |
| NiR | nitrite reductase |
| NRase | nitrate reductase |
| $O_2$ | molecular oxygen |
| $OD_{600}$ | optical density at 600 nm |
| OpuC | ABC transporter for carnitine uptake |
| ori | origin of replication |
| p | pico |
| PC-PLC | phosphatidylcholine-phospholipases C |
| PCR | polymerase chain reaction |
| *pdhABCD* | pyruvate dehydrogenase |
| *pflA*/*pflB* | genes encoding for pyruvate formate lyases |
| pfu | proof-reading |
| PGAP | Prokaryotic Annotation Pipeline |
| pH | potential of hydrogen |
| PI-PLC | phosphatidylinositol-phospholipase C |
| PMF | proton motive force |
| PrfA | listeriolysin positive regulatory protein |
| *pta* | gene encoding for phosphotransacetylase |
| PTS | phosphotransferase system |

| | |
|---|---|
| *qox* | gene encoding for quinol oxidase |
| qPCR | quantitative PCR |
| r | Pearson product moment correlation coefficient |
| rcf | relative centrifugal force |
| rev | reverse |
| RNA | ribonucleic acid |
| rpm | revolutions per minute |
| rRNA | ribosomal RNA |
| RT | room temperature |
| RTE | ready to eat |
| *S. aureus* | Staphylococcus aureus |
| SAP | Shrimp Alkaline Phosphatase |
| SDS | sodium dodecyl sulfate |
| sec | second |
| SigB | Sigma B |
| spp. | species |
| subsp. | subspecies |
| TAE | Tris-acetate-EDTA |
| Taq | Thermus aquaticus |
| TBE | Tris/Borate/EDTA |
| TCA | tricarboxylic acid |
| TE | Tris-EDTA |
| Tris | tris(hydroxymethyl)aminomethane |
| tRNA | transfer RNA |
| U | enzyme unit |
| V | Volt |
| v/v | volume per volume |
| W | watt |
| w/v | weight per volume |
| WT | wild-type |
| α | alpha |
| β | beta |
| γ | gamma |
| Δ | delta |
| σ | sigma |
| Ω | ohm, electrical resistance |

## 2. List of figures

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## 4. CurriculumVitae

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## PUBLICATIONS

- Ferrari E., Walter M. C., Huptas C., Scherer S., Müller-Herbst S.. Adaptation of *Listeria weihenstephanensis* to anaerobiosis. (In preparation)

- Ferrari E., Scherer S., Müller-Herbst S. Adaptation of *Listeria monocytogenes* to different temperatures. (In preparation)

## POSTER PRESENTATIONS

- Poster presentation at 6th Congress of European Microbiologists, 7. – 11. Juli 2015. Maastricht (Netherlands): Ferrari E., Scherer S., Müller-Herbst S.: “Adaptation of Listeria weihenstephanensis to anaerobiosis”.

- Poster presentation at 4. Joint Conference of the Association for General and Applied Microbiology (VAAM) and the Society of Hygiene and Microbiology (DGHM), 5.-8. October 2014. Dresden (Germany): Ferrari E., Scherer S., Müller-Herbst S.: “Adaptation of *Listeria weihenstephanensis* to anaerobiosis”.

- Poster presentation at Nestlé and École Polytechnique Fédérale de Lausanne (EPFL), 26.-28. March 2014. Lausanne (Switzerland): Ferrari E., Haller D., Scherer S., Müller-Herbst S.: “Impact of the adaption of *Listeria monocytogenes* to anaerobic niches on the colonization of the intestine”.

- Poster presentation at 5th Congress of European Microbiologists, 21. – 25. Juli 2013. Leipzig (Germany): Ferrari E., Huptas C., Scherer S., Müller-Herbst, S.: “Genome sequence of *Listeria weihenstephanensis* sp. nov.“.

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