Molecular characterization of the interaction of lactobacilli with food environments and enterohemorrhagic *Escherichia coli* O157:H7

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vorgelegt von

Eric Hüfner
aus Hanau am Main

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Dekan:	Prof. Dr. H. Breer
Berichterstatter, 1. Prüfer:	PD Dr. C. Hertel
Mitberichterstatter, 2. Prüfer:	Prof. Dr. H. Schmidt
3. Prüfer:	Prof. Dr. R. Kölling-Paternoga

meiner Familie

Contents

Chapter I

Scope and outline

Scope

Lactic acid bacteria of the genus *Lactobacillus* are key organisms in food fermentation and are used in starter cultures for the industrial production of fermented meats, vegetables and dairy products as well as sourdough bread. Besides their biopreservative properties that contribute to the hygienic safety of the products, the metabolic activity of lactobacilli determines the development of favorable product properties like flavor and texture. The commercial use of lactobacilli has promoted the investigation of the organisms' adaptation to the food environment, which is responsible for the high ecological competitiveness in food fermentation. The availability of the steadily increasing number of *Lactobacillus* genome sequences greatly facilitates the exploration of genetic features affecting their bioprocessive and metabolic capabilities on a whole-genome scale. The knowledge obtained by using molecular methodologies to explore the microbial diversity and gene expression in the food fermentation environment will deeply influence the selection and optimization of new potential starter organisms.

The first part of this thesis focuses on the investigation of the gene expression of *Lactobacillus sakei* and *Lactobacillus reuteri* in food fermentation using *in vivo* expression technology (IVET) and DNA microarray hybridization analysis, respectively. Both

technologies allow the identification of regulated genes in a specific environment, which are likely to contribute to the ecological performance of the organism. Thus, the obtained results provide a basis for the development of new strategies to improve the fermentation process, as it was demonstrated by the development of an efficient method for the improvement of sausage fermentation using *L. sakei*.

To obtain hygienically safe products, the function of starter cultures mostly relies on the ability to acidify and produce other antimicrobial principles. However, it was recently demonstrated that the interaction with pathogens also can take place on another level, apart from killing or growth inhibition. Lactobacilli have been shown to influence the virulence gene expression of enterohemorrhagic *Escherichia coli* (EHEC) via the bacterial communication system termed quorum sensing. The second part of the thesis explores the impact of quorum sensing between *Lactobacillus reuteri* strains and EHEC O157:H7 on EHEC virulence gene expression. By using a green fluorescent protein reporter gene assay, it was demonstrated for the first time that the transcription of the *ler* virulence regulator gene is significantly reduced by secreted substances of *L. reuteri* in a strain- and quorum sensing-dependent manner.

Outline of the thesis

Chapter II provides an overview of the *Lactobacillus* biota of food fermentations with a special focus on sausage and sourdough fermentation. The species composition as well as the role of lactobacilli in these habitats are comprehensively discussed. In particular, the present knowledge of the impact of metabolic activity on the quality of the fermented food as well as genetic factors that contribute to the competitiveness of certain *Lactobacillus* species during fermentation is presented. Lastly, bacterial communication via signal molecules, termed quorum sensing, is described with emphasis on quorum sensing-regulated virulence gene expression of enterohemorrhagic *Escherichia coli*, and the inhibitory impact of lactobacilli hereon.

Chapter III describes the application of *in vivo* expression technology (IVET) to investigate the gene expression of *Lactobacillus sakei* 23K during sausage fermentation. A genomic library of *L. sakei* 23K was established in an IVET vector bearing promoterless reporter genes mediating beta-glucuronidase activity and erythromycin resistance, thus allowing the detection of inducible promoter activity in sausage fermentation compared to *in vitro* growth conditions. Fifteen *in carne*-induced promoters were identified, and for several corresponding genes, an essential role could be demonstrated by the construction and use of isogenic mutants. This study establishes a molecular basis which allows to investigate bacterial properties that are likely to contribute to the ecological performance of the organism and to influence the final outcome of sausage fermentation.

This chapter has been published in APPLIED AND ENVIRONMENTAL MICRO-BIOLOGY: Eric Hüfner, Tobias Markieton, Stéphane Chaillou, Anne-Marie Crutz-Le Coq, Monique Zagorec, and Christian Hertel. 2007. Identification of *Lactobacillus sakei* genes induced during meat fermentation and their role in survival and growth. Appl. Environ. Microbiol. 73(8):2522-2531. The original publication is available at http://aem.asm.org under doi:10.1128/AEM.02396-06.

Chapter IV demonstrates the successful approach to improve sausage fermentation through exploitation of insights obtained by the application of IVET. Pre-inoculation treatments of *L. sakei* 23K with defined sublethal stresses led to accelerated acidification activity during fermentation, which is a critical parameter to ensure the hygienic safety and sensory quality of the final product. The highest acidification activity was observed for cold-stressed cells (4°C). The use of pre-inoculation stress treatments is thus a promising way to improve the effectiveness of meat starter lactobacilli.

This chapter was published in CURRENT MICROBIOLOGY: Eric Hüfner and Christian Hertel. 2008. Improvement of raw sausage fermentation by stress-conditioning of the starter organism *Lactobacillus sakei*. Curr. Microbiol. 57:490–496. The original publication is available at http://www.springerlink.com under doi:10.1007/s00284-008-9274-x.

In **Chapter V** the transcriptional profile of *L. reuteri* ATCC 55730 in type II sourdough fermentation is presented. The use of whole-genome microarrays allowed the investigation of global gene expression in the sourdough habitat in relation to that under *in vitro* conditions (growth in a chemically defined medium). The obtained results as well as the use of isogenic mutant strains provide fundamental insights into the niche-specific regulation of gene expression of this species, which is closely associated with cereal fermentations as well as the gastrointestinal tract of animals and humans.

This chapter was published in SYSTEMATIC AND APPLIED MICROBIOLOGY: Eric Hüfner, Robert A. Britton, Stefan Roos, Hans Jonsson and Christian Hertel**.** 2008. Global transcriptional response of *Lactobacillus reuteri* to the sourdough environment. Syst. Appl. Microbiol. 31:323–338. The original publication is available at http://www.sciencedirect.com under doi:10.1016/j.syapm.2008.06.005.

Chapter VI describes the investigation of quorum-sensing mediated impact of probiotic strain *L. reuteri* ATCC 55730 and rodent isolate *L. reuteri* 100-23C on the virulence gene expression of enterohemorrhagic *Escherichia coli* O157:H7. A significant repressive effect of secreted substances of *L. reuteri* ATCC 55730, but not of *L. reuteri* 100-23C, on the transcription of the virulence regulator gene *ler* was documented by the use of a green fluorescent protein reporter gene assay. In particular, this repression was for the first time shown to be dependent on the activity of the LuxS synthase, a key quorum sensing enzyme, by using LuxS-negative isogenic mutant strains.

This chapter was published in APPLIED AND ENVIRONMENTAL MICROBIOLOGY: Ivan Jelčić, Eric Hüfner, Herbert Schmidt, and Christian Hertel. 2008. Repression of the LEE-encoded regulator gene transcription of *Escherichia coli* O157:H7 by culture supernatants of *Lactobacillus reuteri* is LuxS and strain dependent. Appl. Environ. Microbiol. 74(10):3310–3314. The original publication is available at http://aem.asm.org under doi:10.1128/ AEM.00072-08.

Co-authors

This dissertation comprises studies that were carried out in collaboration with several researchers. All studies of this dissertation were supervised by PD Dr. Christian Hertel.

Chapter III: Tobias Markieton assisted in the construction of the IVET genomic library, the conduction of the fermentation experiments and the identification of induced genes. Dr. Anne-Marie Crutz-Le Coq constructed the plasmid pRV601 and provided helpful advice regarding the cloning strategy. Dr. Stéphane Chaillou assisted by providing the genomic information of *Lactobacillus sakei* 23K, conducted bioinformatic analyses and contributed much helpful information. Prof. Dr. Monique Zagorec contributed to the interpretation and discussion of the results.

Chapter V: Dr. Robert A. Britton conducted the basic statistical data analysis and provided substantial advice on the design and execution of the microarray experiments, as well as the interpretation of the obtained results. Dr. Stefan Roos and Dr. Hans Jonsson provided the genomic information of *Lactobacillus reuteri* ATCC 55730 and assisted in the interpretation of the results and the discussion of the manuscript.

Chapter VI: Eric Hüfner and Ivan Jelčić contributed equally to this study. Ivan collaborated in the design of the study, the construction of the reporter gene vector and the conduction of experiments. Prof. Dr. Herbert Schmidt contributed to the interpretation of the results and the discussion of the manuscript.

Chapter II

Introduction

The genus *Lactobacillus*

Lactobacilli are virtually ubiquitous bacteria that are found on plant and other environmental material and in food fermentation, but are also commensal constituents of the microbiota of the human and animal body. They represent one of the most important bacterial taxa for human nutrition and health (comprehensively reviewed in (60)). Lactobacilli are characterized as Gram-positive, non-spore-forming and non-motile rods, which are strictly fermentative and anaerobic to microaerophilic. The genomic DNA is characterized by average to low GC content, generally below 50 mol%. Furthermore, they are highly fastidious regarding the nutritional requirements for carbohydrates, amino acids and vitamins (60). Together with the genera *Streptococcus*, *Enterococcus*, *Oenococcus*, *Carnobacterium*, *Pediococcus* and *Leuconostoc*, lactobacilli belong to the heterogenous group of lactic acid bacteria (LAB), which share the ability to produce large amounts of organic acids, mainly lactic acid, by fermentation of carbohydrates. The acid production, which inhibits the growth of many concomitant microorganisms, combined with their aciduric or acidophilic nature, accounts for the remarkable competitiveness of LAB in a wide range of habitats. Furthermore, these characteristics are fundamental to the preservation of food and thus makes LAB the most technologically important bacteria for food production.

The genus *Lactobacillus* is grouped in the family *Lactobacillaceae*, order *Lactobacillales*, class *Bacilli* and phylum *Firmicutes* (42), and to date comprises more than 170 species including subspecies (http://www.dsmz.de/microorganisms/main.php?contentleft_id=14 and http://www.bacterio.cict.fr). The taxonomy of lactobacilli still proves to be difficult due to their great genetic and phenotypic heterogeneity, and remains a constant matter of debate for more than a century. Orla-Jensen provided the first classification in 1919, defining the groups Thermobacteria, Streptobacteria and Betabacteria on the basis of characteristics like growth temperature and biochemical reactions (107). With the advent of molecular genetics and the feasibility of DNA sequence comparison, the so-called polyphasic taxonomic approach led to reclassification and more exact definition of phylogenetic relationship of lactobacilli (150). A multitude of phylogenetic markers are frequently used to investigate the evolutionary connection of species (150). Both genotypic characteristics such as 16S and 23S ribosomal RNA (rRNA) gene sequences, genomic DNA homology, and genome GC content (89), and phenotypic features, e.g., morphology, fermentation pattern, the type of isomer of lactic acid produced, or the composition of peptidoglycan of the cell wall are combined. The availability of an increasing number of *Lactobacillus* genome sequences establishes the basis for comparative phylogenetic analyses, termed 'phylogenetics' (reviewed in (91)), which aim to identify conserved and variable genes and pathways on the genus, species and even strain level. The determination and analysis of sets of orthologs, which are genes derived from the same gene in the last common ancestor of the compared species (79), of the core gene set of a taxon, or of genetic events like horizontal gene transfer are crucial for the understanding of evolutionary relationships as well as adaptation to ecological niches. Recent studies, for example, identified the gene encoding the molecular chaperone GroEL as phylogenetic marker superior to the 16S rRNA gene (19), and investigated the intraspecies genomic diversity among *Lactobacillus sakei* isolates (16).

The ongoing isolation and description of new species results in constant need of species re-grouping. For example, Hammes and Hertel (2003) defined 7 goups (59), whereas the most recent classification of Felis and Dellaglio (42) distinguishes 12 main groups: *L. delbrueckii* group, *L. salivarius* group, *L. reuteri* group, *L. buchneri* group, *L. alimentarius*-*L. farciminis* group, *L. casei* group, *L. sakei* group, *L. fructivorans* group, *L. coryniformis* group, *L. plantarum* group, *L. perolens* group and *L. brevis* group.

Lactobacilli are closely associated with the human and animal body, in particular mucosal surfaces. A great variety of species has been isolated from the oral cavity, the gastrointestinal and the genital tract (60). While certain species are frequently found in the respective body part and persist over long periods of time, many species appear to be only transiently present. The first group, which contains species that apparently are well adapted to the respective habitat and have stably occupied the ecological niche, are called 'autochthonous', or true resident, lactobacilli, whereas temporary species are called 'allochthonous' (154, 164). The borderline between the two groups has been difficult to establish due to the great species and cell count variability among hosts species and individual organisms/subjects. For example, the number of lactobacilli in the gastrointestinal tract of humans ranges from none to $<10^9$ colony-forming units (CFU) per gram faeces (100), with a total of 17 species detected (154). However, only few species are regarded as true autochthonous intestinal lactobacilli – i.e., *L. gasseri*, *L. crispatus*, *L. reuteri*, *L. salivarius*, and *L. ruminis* (153, 154). Since the gastrointestinal tract is an open ecosystem, lactobacilli are constantly introduced in great numbers via ingestion of fermented foods or plant material, but also originate from the oral cavity, which accounts for the great heterogeneity of isolated species (151, 154). The role of lactobacilli in intestinal ecosystems has received much attention, especially with respect to their beneficial effect on human and animal health. The so-called 'probiotic' effects of certain *Lactobacillus* strains include: stimulation/modulation of mucosal immunity (78, 148), reduction of inflammatory or allergic reactions (71, 85), reduction of blood cholesterol (84), anti-carcinogenic activity (73), exclusion of pathogens (45, 117) (for reviews see (24, 50, 92)). However, many of these potential health promoting effects have only been demonstrated *in vitro*, and extensive clinical studies are needed to assess the actual *in vivo* efficacy. Moreover, the mechanisms responsible for eliciting the probiotic effects largely remain to be investigated. Nevertheless, probiotic lactobacilli and other bacteria are commercially available and used as dietary and therapeutic adjuncts for humans and animal nutrition since many years (43).

The specialized sugar metabolism of lactobacilli is fundamental to the oldest food processing technique used by mankind: the fermentation of foods (12, 123). Since ancient times, food of animal and plant origin is treated and stored in a specific manner, which results in spontaneous fermentation of available carbohydrates and accumulation of organic acids by the

endogenous LAB biota of the raw materials or the environment. Fermentation does not only extent the shelf life, but also improves the sensory characteristics of the food, e.g., sourdough, wine, cheese or fermented sausage. Table 1 provides an overview of the key microbiota of important fermented foods.

Product	Substrate	Associated microorganisms	
		Lactobacilli	**other**
Raw sausage	Pork and/or beef	*L. sakei, L. curvatus, L. plantarum*	LAB (*Pediococcus* spp., *Leuconostoc* spp., *Enterococcus* spp.) Coagulase-negative cocci (mostly *Kocuria varians Staphylococcus carnosus, S. xylosus*) Yeasts and/or moulds
Bread/sourdough	Grain(s) (mostly wheat or rye)	*L. sanfranciscensis, L. pontis, L. reuteri*	Yeasts (*Candida humilis, Saccharomyces cerevisiae*)
Cheese	Milk	*L. helveticus, L. delbrueckii*	*Lactococcus lactis* *S. thermophilus*
Yogurt	Milk	*L. delbrueckii* subsp. *bulgaricus*	*Streptococcus thermophilus*
Sauerkraut/Pickles	Cabbage/Cucumbers	*L. brevis, L. plantarum, L. curvatus, L. sakei*	*Leuconostoc mesenteroides, Pediococcus* spp.
Soy sauce	Soybeans and wheat		Moulds *(Aspergillus oryzae, A. soyae*) Yeasts (*Zygosaccharomyces rouxii*)
Wine, beer	Grapes, malt		Yeasts (*Saccharomyces cerevisiae* *Oenococcus oeni*
Olives	Olives	*L. pentosus*	*L. mesenteroides*

Table 1. Fermented foods and key fermentation microorganisms (Sources of information: (12, 123, 135)

Based on the accumulated fermentation end-products, homo- and heterofermentative lactobacilli are distinguished. According to the generally accepted definition given by Hammes and Vogel (64), obligately homofermentative lactobacilli ferment hexoses almost exclusively to lactic acid by the Embden–Meyerhof–Parnas (EMP) pathway, whereas pentoses and gluconate are not fermented due to a lack of phosphoketolase. Obligately heterofermentative lactobacilli utilize hexoses and pentoses via the pentose phosphate pathway producing lactic acid, ethanol, acetic acid and carbon dioxide; and facultatively heterofermentative lactobacilli produce lactic acid from hexoses via the EMP pathway and are also able to degrade pentoses and often gluconate as they possess both aldolase and phosphoketolase. Organic acids produced during fermentation are present in their undissociated form due to the low pH of the fermented food, which results in higher membrane permeability due to reduced polarity (28). The organic acid molecules diffuse into the bacterial cells, and dissociate in the more alkaline cytosol, resulting in disturbance of the intracellular pH homeostasis. While the majority of bacteria is very susceptible to acidity, lactobacilli and other Gram-positive bacteria possess diverse acid resistance systems, which protect them against the acid produced (25). Examples are the F_1F_0-ATPase, glutamate decarboxylase reaction or the arginine deiminase **(**ADI) system. Since many decades, selected *Lactobacillus* strains have been utilized as starter organisms due to their superior properties in food fermentation (10, 66). Hammes and Hertel (61) give the following definition:

> "Starter cultures are preparations which contain living or resting forms of microorganisms that develop in the fermentation substrate the desired metabolic activity. In the rule, but not necessarily, the organisms grow (multiply) in this substrate."

In particular industrial food production requires reliable and stable processes, which ensure a reproducible outcome of the fermentation of raw materials. The hygienic quality, that is absence of pathogenic and spoilage microorganisms, and sensory quality such as flavor and texture, are decisively determined by the starter organisms. The antagonistic activities of starter organisms against spoilage and pathogenic organisms are an integral element of the hurdle technology (82). Furthermore, the addition of probiotic strains may positively affect the health of the consumers. Thus, selection criteria have been defined to identify strains that

are especially suitable for the diverse types of food fermentation (10, 66). First of all, the capacity to quickly and reliably acidify the raw material is of capital importance. Depending on the produced food, proteolytic and lipolytic activities, production of antimicrobial compounds such as bacteriocins, and polysaccharide biosynthesis are also important attributes of starters which contribute to the quality of fermented foods. The prerequisite for the development of these favorable metabolic activities is the ecological competitiveness of starter organisms, i.e., the ability to grow and dominate the accompanying microbiota. The selection and combination of suitable strains represents the oldest and most obvious way of improving food fermentation. However, during the last decades, food-associated micro-organisms were increasingly manipulated in order to maximize their usefulness (81). For example, metabolic engineering by means of genetic manipulation has been successfully used to confer catalase and lysostaphin activity (13, 68), to enhance autolysis (127) or the ability to produce bacteriocins (127). Although the genetic manipulation is now possible on a food-grade level, which matches or even exceeds the safety level of natural screening and selection (66), there are no industrial applications of genetically modified organisms to date due to consumer inacceptance.

Comparative genomics reveal the genetic differences of species and strains isolated from different habitats, and thus provides a basis for the identification of 'life-style islands' (91, 101), i.e., genes that are important for the survival in different habitats such as different foods or the gastrointestinal tract. In addition, the availability of genomic information facilitates the investigation of global gene expression of lactobacilli in response to diverse environments. For example, *in vivo* expression technology (IVET), a promoter-trap technology using reporter gene fusions, has been utilized to identify induced genes of *L. reuteri* (155) and *L. plantarum* in the gastrointestinal tract of mice (8). Moreover, Dal Bello and co-workers applied IVET to analyze induced genes of *L. reuteri* in sourdough fermentation (27). During the last years, the application of whole-genome microarrays in the investigation of gene expression provided more differentiated knowledge of transcriptional responses. The transcriptional profile of *L. johnsonii* during *in vitro* growth and in the murine gut was investigated (32), as well as analysis of *L. helveticus* in cheese fermentation (131). Such novel approaches are fundamental to understand the ecological adaptation of lactobacilli to diverse

ecological niches, and will establish opportunities to improve technological and health-promoting features of lactobacilli (75, 142).

Sausage fermentation

Raw meat is one of the most perishable foodstuffs, along with poultry and seafood. It is prone to expeditious decay by endogenous enzymes (67, 105) and provides ideal growth conditions for microbial spoilage organisms such as *Enterobacteriaceae* or *Pseudomonas* spp., and also for pathogenic bacteria like *Salmonella* spp., *Listeria monocytogenes* and enterohemorrhagic *Escherichia coli* (7, 105). Fermentation of meat has a long tradition as an effective method of preservation, for instance, there is evidence that in acient Egypt cured meat was manufactured (33). Fermentation does not only extent the shelf life, but also improves the sensory characteristics of meat products, foremost fermented sausages (87, 121). A great variety of fermented sausage types are produced throughout the world, with Europe and North America as the main producers. Two basic types can be distinguished: semi-dry and dry fermented sausages, which differ in the process parameters that are applied during production. Therefore the products posess different characteristics such as water content, fermentation biota and pH values. Summer sausage, Cervelat, Lebanon Bologna, Mortadella and Thüringer are examples for the first category, whereas Chorizo, Salami, Salchichón and Pepperoni are dry sausages. A fermented sausage is defined as a mixture of comminuted fat and meat (mostly pork and/or beef), salt, nitrate and/or nitrite, sugar and spices, which is stuffed into casings, subjected to fermentation and then allowed to dry (70). Hence, the fermentation process is generally divided into three well-defined phases that vary depending on the sausage produced: mixing of ingredients, fermentation and drying or ripening. The fermentation stage, generally 2-3 days, constitutes the "critical control point" due to complex biological and physio-chemical changes of the sausage batter that are the result of microbiological activity. The duration of drying is variable (up to several weeks) and determines the final water content of the sausages. Semi-dry sausages generally have a water activity (a_w) of 0.90-0.95, whereas dry sausages are characterized by a_w values as low as 0.85 (12, 102). The drying phase determines the texture of the sausage as well as the formation of aroma components.

Besides meat and other ingredients used, the fermentation microbiota is the determinative factor for the final quality of the sausage produced. LAB of the genera *Lactobacillus, Pediococcus* and *Lactococcus*, and non-pathogenic coagulase-negative cocci (CNC) like *Staphylococcus* spp. and micrococci like *Kocuria* spp. constitute the two major groups of bacteria that are considered technologically important for the fermentation and ripening of sausages (56, 120). In addition, molds (e.g., *Penicillium* spp. and *Mucor* spp.) and yeasts such as *Debaryomyces hansenii* and *Candida* spp. play important roles as surface biota in certain types of sausage fermentation (61, 87). Lactobacilli are by far the predominant LAB with species *L. sakei* and *L. curvatus* being the most frequently isolated, followed by other species like *L. plantarum* (60, 70). Furthermore, *Enterococcus* spp. have repeatedly been demonstrated to constitute a part of the fermentation microbiota of certain sausage types (3, 114, 126). In industrial production, selected starter organisms are added to the meat batter, whereas traditional artisanal production relies on spontaneous fermentation by environmental microbiota that contaminates the meat during slaughtering and manufacturing (140). The microbial composition, growth and succession depends greatly on the type of fermentation, but there are characteristics that most types share (86, 88): Firstly, LAB rapidly dominate the fermentation during the initial phase, typically 2-4 days, and generally reach cell counts of more than 10^8 CFU g^{-1}. The number of LAB stays relatively constant during the whole ripening process. Secondly, CNC also proliferate during the first days predominantly close to the surface.

During fermentation, the diverse microbiological activities contribute to the quality of the product:

- accumulation of organic acids, most notably lactic acid
- reduction of nitrate and nitrite
- secretion of antimicrobial and other compounds
- lipolysis and proteolysis

The drop of pH from approximately 5.8-6.2 to values of 5.0 or below during the initial phase of fermentation is caused by LAB, which convert available sugars to lactic acid. Low pH acts as a hurdle for unwanted bacteria of the genera *Salmonella*, *Pseudomonas* or *Clostridium* by disturbing the cellular pH homeostasis, and thus promotes the growth of acid-tolerant

bacteria. Furthermore, low pH facilitates gelation of myofibrillar proteins and reduction of the water holding capacity of the meat, which declines when the pH approaches the isolelectric point of meat proteins (pH~5.0) (55). This favors the drying and consequently the weight loss of the sausage, which result in firm texture and sliceability of the end product (10). Another crucial process is the reduction of nitrate/nitrite by CNC and also to a lesser extent by LAB. This contributes to the formation of the typical red colour and aroma of cured meat products (56, 162). Nitrite is reduced to nitric oxide, which in turn reacts with metmyoglobin (Fe^{3+}) to form bright red nitrosomyoglobin (Fe^{2+}) (69). These chemical reactions are favored by low pH, low redox potential and addition of ascorbic acid. Moreover, nitrosyl compounds elicit bacteriostatic effects on undesirable bacteria such as *Clostridium botulinum* via multiple mechanisms (11). In addition, some bacterial members of the fermentation biota are able to directly impede the growth of competing bacteria via production of antimicrobial substances. Besides the production of lactic acid, acetic acid and hydrogen peroxide, many LAB species produce small peptides, so-called bacteriocins, that elicit inhibitory effects on accompanying bacteria. Bacteriocins are selectively active towards foodborne Gram–positive pathogens such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium perfringens* and *Bacillus cereus*, but also towards other LAB, thus contributing to the competitiveness of the producing strain (34). Examples for bacteriocins that act as effective antimicrobials in sausage fermentation are sakacin K of *L. sakei* (83) or pediocin of *Pediococcus acidilactici* (44). Microbial proteolysis and lipolysis have an impact on the sensory quality of sausages, although the extent remains a matter of debate. Primarily CNC, especially *Staphylococcus xylosus*, are able to degrade protein and fat during ripening, but also several strains of LAB have been shown to display similar activity. (106). For example, *L. sakei*, *L. curvatus* and *L. plantarum* have been demonstrated to degrade sarcoplasmic and myofibrillar proteins as source of amino acids (39, 40). Moreover, yeasts like *Debaryomyces hansenii* and *Yarrowia lipolytica* as well as moulds diplay great lipolytic and proteolytic effect in some types of surface-inoculated dry sausages (72, 111).

The industrial production of fermented meat products requires means to assure quality and safety of products. The use of selected starter cultures as inoculants has considerably improved the standardization of meat fermentation during the last decades. Strains of virtually every microbial species found in fermented meat products, including LAB, CNC, moulds and

yeasts, have been utilized as starter organisms (see Table 2) (61). The selection of suitable strains is based on characteristics that are considered important for the fermentation process and product properties (10). For example, LAB should exhibit quick acidification and bacteriocin production – two features that are important for the hurdle-concept of food preservation (82). CNC on the other hand should possess high enzymatic activity for the production of aroma components like amino acids or fatty acids. For an overview of selection criteria see Table 2.

Table 2. Important species used as starter organisms in sausage fermentation and relevant properties (after (61, 62, 88))

Group	Species	Selection criteria		
		Important trait(s)	Impact on	
			sensory quality	safety
LAB	*Lactobacillus sakei*,	Decrease of pH / acid production	+	+
	L. curvatus, *L. plantarum*,	Production of antimicrobials	-	(+)
	Pediococcus acidilactici,	Nitrate/nitrite reduction	+	+
	P. pentosaceus,	No production of biogenic amines	-	+
	Lactococcus lactis			
CNC	*Staphylococcus xylosus*,	Nitrate/nitrite reduction	+	+
	S. carnosus,	O_2 consumption	-	+
	Kocuria varians	H_2O_2 degradation	-	+
		Proteolysis + lipolysis	+	-
		Delaying rancidity	+	-
Fungi	*Penicillium nalgiovense*,	Proteolysis + lipolysis	+	-
	P. chrysogenum,	Secretion of metabolites	+	-
	P. camemberti	Prevention of discoloration / rancidity	+	-
		No toxinogenic / pathogenic potential	-	+
Yeasts	*Debaryomyces hansenii*,	Proteolysis + lipolysis	+	-
	Candida famata			

+, property has impact; -, property does not have impact; (+), strain-dependent property.

The basic requirement for the suitability of starter organisms for sausage fermentation is their ecological competitiveness. Only if organisms are able to compete with the accompanying microbiota under the prevailing environmental conditions, they are able to exert beneficial effects. In comparison to other fermentation types, the conditions during sausage fermentation can be considered harsh, and several stresses act on the microorganisms involved. Surprisingly, very few studies exist that deal with the investigation of the physiologic and genetic basis of adaptation and survival mechanisms of meat starter cultures (61). The major stress factors are moderate to high salt concentrations of about 2% (a_w = 0.94–0.98) in the batter and up to 15% (a_w = 0.85–0.86) in the final product, the low pH values of < 5.0 and the rather low process temperatures during mixing (4-7°C), fermentation (18-24°C) and drying/ripening (12-15°C), which constitute limiting factors for the performance of microorganisms (88, 102). As a very competitive species during sausage fermentation, *L. sakei* has repeatedly been used as model organism for the study of the adaptation of LAB to the meat and sausage fermentation environment, especially to stress conditions and thus its advantage over other bacteria (17). For example, Marceau and co-workers demonstrated that the long-term survival of *L. sakei* is enhanced by growth at low temperatures with added salt (94), and also that several proteins impact the survival of *L. sakei* at low temperatures and moderate salt concentration (93). Furthermore, the long-term survival of *L. sakei* could be enhanced by an arginine aminopeptidase showing optimal activity at low pH and in the presence of sodium chloride (128). Besides the nutritional value, the accumulation of free arginine can be used to produce ATP and ammonium via the arginine deiminase (ADI) pathway and therefore permits buffering of intracellular pH, although it has been demonstrated that survival due to arginine catabolism is mainly due to energy generation by ATP generation rather than a rise in pH (18). In addition, the *dnaK* operon of *L. sakei* has been shown to be regulated by temperature, salt and ethanol stress (130), and the inactivation of several two-component systems (TCS) leads to reduced tolerance towards stress stimuli like temperature, aerobiosis and pH (103).

The sequencing and publication of the annotated whole genome sequence of *L. sakei* 23K by the Institute de la Recherche Agronomique (INRA), France, constituted a key progress in providing fundamental insights into the genetic endowment of this specialized bacterium (15). On the basis of genome characteristics like presence and absence of genes and metabolic

pathways, conclusions on the adaptation to the meat environment and on the genetic fundamentals of the specific phenotypic traits of *L. sakei* can be drawn (15, 38). Chaillou and co-workers reported a genome size of 1.88 Megabases (Mb), which is small compared to other lactobacilli, and a specialized metabolic repertoire, which seems unusual for lactobacilli. The auxotrophy for all amino acids, except glutamic and aspartic acid, and the absence of proteolytic enzymes indicated that *L. sakei* has adjusted to the overabundance of free amino acids in the meat substrate by disposal of redundant pathways. The carbohydrates glucose/mannose, N-acetylglucosamine, fructose, sucrose, trehalose, cellobiose, melibiose, gluconate, galactose, arabinose, ribose, citrate, malate and glycerol can be utilized as indicated by the presence of phosphotransferase systems and catabolic pathways, but there also exist multiple genes involved in nucleoside salvage pathways. This might provide the organism with the possibility to use nucleosides as alternative energy source when the scarce meat carbohydrates are depleted. Furthermore, the presence of a putative second ADI pathway corroborates the importance of scavenging arginine, an amino acid abundant in meat, for ATP production. Other genetic elements might explain exceptional phenotypic features of *L. sakei* like tolerance to high salt concentrations (up to 9%, (17)) and low temperatures. For example, *L. sakei* harbors a similar set of uptake systems for compatible solutes like betaine and carnitine, which possess osmo- and cryoprotective properties. The genome of *L. sakei* also harbors more cold shock protein (CSP) genes than other lactobacilli, comparable to *Listeria monocytogenes*. The high tolerance of *L. sakei* to changing redox conditions and deleterious oxygen by-products can be explained with an abundance of hypothetical proteins coping with peroxides (diverse peroxidases, a heme-dependent catalase) or with restoring protein functionality (methionine sulfoxide reductases, thioredoxin/glutaredoxin systems). Furthermore, the presence of many putative oxidoreductases suggests that *L. sakei* maintains redox balance through tight control of the $NADH/NAD^+$ ratio and use of wide range of electron acceptors. The organism´s ability to effectively colonize meat surfaces might be due to specialized cell surface proteins mediating adhesion or cellular aggregation. A large number of gene products with no homologues in other lactobacilli were identified, featuring cell-wall binding LysM domains, homologies to aggregation-promoting factors domains involved in binding of lipoteichoic acid and collagen. Moreover, two gene clusters potentially involved in production of cell-surface-linked polysaccharides, more precisely sugar-modification of teichoic acid, were identified. Although strain *L. sakei* 23K does not produce

relevant amounts of exopolysaccharide (EPS), these surface-bound polysaccharides might be involved in the attachment to the meat matrix.
In spite of knowledge of the genome sequence of *L. sakei* 23K, the contribution of the majority of gene products to its superior performance in sausage fermentation remains to be investigated. On the basis of the available data, further studies must focus on the identification of regulatory mechanisms and pathways which enable the organism to elicit its technological and biopreservative effects.

Sourdough fermentation

Sourdough bread is produced and consumed worldwide since ancient times (125). Per definition, sourdough is the mixture of cereal flour and water that undergoes fermentation, resulting in the accumulation of gas and acid. It is generally not consumed directly, but utilized as intermediate product in the manufacturing of bakery products, mostly leavened bread. Its addition improves the nutritional, technological, organoleptical properties and the shelf-life of the manufactured foods (58). Three types of sourdough fermentation can be distinguished by means of technological and microbiological characteristics (6). Type I sourdoughs are produced with traditional techniques by continuous (daily) propagation at ambient temperatures (20-30°C). All traditional sourdough products such as San Francisco French bread, Panettone and three-stage sourdough rye breads belong to this category. Type II doughs are characterized by higher incubation temperatures (up to 40°C), higher water content to permit pumping of the dough and longer fermentation times (up to 5 days). They are mainly utilized as dough acidifiers. Type III sourdoughs are dried doughs which are used as acidifier supplements and aroma carriers. Both type II and III doughs require the addition of baker's yeast for leavening.

Sourdough contains metabolically active microorganisms, which are responsible for the development of the desired characteristics. The composition of the sourdough microbiota is dependent on both endogenous and exogenous factors (31, 58). The microbial and chemical composition of the flour are key endogenous factors, whereas process parameters like

fermentation temperature, number of propagation steps, dough yield (a_w), redox potential or composition of the starter are the exogenous parameters influencing the dough ecology. At the beginning of fermentation, the microbiota resembles that of the flour used, consisting of bacteria, yeasts and molds at cell counts ranging from 10^4 to 10^6 CFU per gram (136). Gram-negative enteric bacteria are dominant at the start of fermentation, but during the initial fermentation phase and after several rounds of propagation, LAB of the genus *Lactobacillus*, and to a much lesser extent *Pediococcus*, *Leuconostoc* and *Weissella*, constitute the dominant organisms, usually reaching cell counts of 1×10^9 to 3×10^9 CFU per gram dough (57). Yeasts of the genera *Saccharomyces* and *Candida* are also frequently present in significant numbers (up to 5×10^7 CFU g^{-1}) (57, 124), but are generally outnumbered by lactobacilli by several orders of magnitude (98, 108). Dependent on the type of fermentation and the region of origin, a multitude of different *Lactobacillus* species have been shown to be especially competitive, and more than 50 species have been identified to date (2). In contrast to many other types of food fermentation that are characterized by a predominance of homofermentative LAB, obligately heterofermentative strains are dominating in sourdough. *Lactobacillus sanfranciscensis*, *Lactobacillus pontis*, *Lactobacillus panis* and *Lactobacillus reuteri* are examples of frequently isolated species, but the exploration of the sourdough ecosystem leads to the continuing identification and description of new species like *Lactobacillus secaliphilus* (36) or *Lactobacillus nantensis* (147) (for recent reviews see (23, 31)).

Sourdough decisively influences the quality of the final baked goods due to the metabolic activities of its microbiota. Yeasts mainly produce CO_2 necessary for leavening, and acetate which affects the sensory qualities and improves the shelf life of bread (58, 63). Lactobacilli are responsible for a multiplicity of events during fermentation. Firstly, the production of lactic and acetic acid results in a reduction in pH, leading to pH values of 3.5 to 4.3 for ripe doughs (2). The acidification impacts the technological properties of the dough by influencing flour components like enzymes, gluten, starch and arabinoxylan. For example, water uptake and consistency are increased by organic acids/low pH, whereas the mixing time and firmness is decreased (90, 159). The reduction of structural firmness is ascribed to an increase of gluten solubility and enzymatic degradation (20). Also, the solubilization of arabinoxylans at low pH might reduce bread staling as pentosans have been postulated to prevent starch-gluten

interactions responsible for staling (53, 58). Secondly, sourdough lactobacilli are responsible for the production of amylases and proteases during fermentation. In particular, proteolytic activity and amino acid metabolism contribute to the sensory properties of baked goods (48). The flavor is strongly influenced by accumulation and bacterial modification of amino acids, or the thermal degradation during baking, and leads to the generation of aroma compounds (65). Conversion reactions like deamination and decarboxylation generate ketoacids, amines, aldehydes, acids and alcohols. For example, ornithine, which is produced by the ADI pathway of several *Lactobacillus* species (29), is converted during baking to 2-acetyl-1-pyrroline, a key flavour compound of the wheat bread crust (143). However, the mentioned enzymatic activities are highly strain-specific and the main proteolytic action on gluten protein is exerted by endogenous flour enzymes (144). Thirdly, lactobacilli produce exopolysaccharides (EPS), which recently have attracted attention as components contributing towards shelf-life and textural properties of baked goods (reviewed in (30, 145)). Bacterial EPS, either added to the dough or produced *in situ*, has great technological potential as replacement of plant-derived polysaccharides/hydrocolloids. Another interesting potential of EPS is its possible prebiotic effect. For example, Dal Bello and co-workers demonstrated a bifidogenic effect of a levan-type EPS of *L. sanfranciscensis* (26), which also possesses acid- and heat-resistant characteristics, making it suitable for technological application (80).

During the last decades, the investigation of sourdough lactobacilli, predominantly of key species *L. sanfranciscensis*, *L. pontis*, *L. plantarum* and *L. reuteri*, has revealed various physiologic features that are fundamental for the organisms´ superior competitiveness in this particular ecological niche (reviewed in (48, 51)). First demonstrated for *L. sanfranciscensis*, the preferential use of maltose, delivered by flour amylases or by *L. amylovorus* in type II doughs, as energy source combined with the use of alternative electron acceptors are evidence for the highly adapted carbohydrate and energy metabolism (137). Two maltose phosphorylase enzymes, one being constitutively expressed and the other maltose-inducible, catalyze the cleavage of maltose to glucose-1-phosphate and glucose (37). Glucose-1-phosphate is metabolized via the pentose phosphate pathway, and glucose is excreted and utilized in a strain-dependent manner. The use of fructose allows for the disposal of electrons and NAD^+ regeneration with concomitant production of mannitol, but also citrate and malate can be used as electron acceptors. Fructose may be supplied by cleavage of sucrose by yeasts

like *Candida humilis*, which commonly accompanies lactobacilli in type I fermentations, or by certain *Lactobacillus* species. Thus, acetate production is favored to the energetically disadvantageous formation of ethanol as fermentation end-product. Additionally, acetate can be formed by NADH oxidase reaction via use of oxygen as electron acceptor, as demonstrated for *L. sanfranciscensis* (137). The increased acetate production itself alters the lactate/acetate ratio which affects sensorial and baking properties of the sourdough bread (95, 122). The competitiveness of sourdough LAB also depends on the production of antimicrobial substances (99). Besides the production of compounds eliciting unspecific inhibition of a broad range of microorganisms like organic acids, hydrogen peroxide, diacetyl or reuterin, lactobacilli are also affecting closely related bacteria with bacteriocins, which are short peptides, in a highly strain-dependent manner. Examples include plantaricin ST31 of *L. plantarum* ST31 (146) and BLIS C57 of *L. sanfranciscensis* (22). Recently, Gänzle and co-workers described a non-bacteriocin compound produced by strains of *L. reuteri* that displayed activity against several Gram-positive spoilage and pathogenic organisms as *Staphylococcus aureus*, *Enterococcus faecalis*, *Listeria monocytogenes* and *Bacillus cereus*. The cyclic tetramic acid derivative reutericyclin is produced in relevant amounts in sourdough (47). The production of antimicrobial compounds and activity against closely related bacteria is generally viewed as competitive advantage for the producing strains in the ecosystem sourdough and could explain the stable persistence of certain strains in industrial sourdoughs over many years of propagation (23, 49). Thus, production of bacteriocins and antimicrobial substances is viewed as an important selection criterion for novel competitive starter organisms. Taken together, important physiologic traits have been identified, which are crucial for the ecological performance of sourdough lactobacilli. However, information on the genetic basis and regulation involved in the adaptation to this specific environment is still scarce. To date, there are several genomes of strains of sourdough-related *Lactobacillus* species published, although all are isolates from other environments: the draft genome sequence of *L. reuteri* ATCC 55730 (human mother´s milk isolate) (5), complete annotated sequences of *Lactobacillus brevis* ATCC 367 (silage isolate) (91), *L. plantarum* WCFS1 (human saliva isolate) (76), *Lactobacillus johnsonii* NCC 533 (human intestinal isolate) (113), *Lactobacillus acidophilus* NCFM (human intestinal isolate) (1) and the *Lactobacillus delbrueckii* subsp. *bulgaricus* strains ATCC 11842 (yogurt isolate) (149) and ATCC BAA-365 (cheese isolate) (91). In the course of the continuing sequencing and publication of

Lactobacillus genome sequences, the investigation of global cellular responses to sourdough fermentation becomes feasible, and will provide fundamental insights into the environmental adaptation and competitiveness of sourdough lactobacilli.

Quorum sensing: The role of LuxS synthase, autoinducers AI-2 and AI-3 in interspecies communication

The term quorum sensing (QS) refers to the ability of bacteria to communicate via signaling molecules (for reviews see (74, 158, 160)). Many Gram-positive and -negative bacteria produce a variety of molecules, which can influence the expression of target genes if the concentrations exceed a certain threshold level. By using these signal-response systems, bacteria are able to adjust and synchronize cellular functions and the concerted development of specific phenotypes in a cell density-dependent way. This autoregulation is often compared with that of simple multicellular organisms. Although the ability to communicate is evidently a fundamental characteristic of bacteria, the utilized signaling molecules, called autoinducers (AI) or pheromones, and signal transduction mechanisms are very diverse. To date, three major groups of autoinducers are distinguished. Firstly, acyl homoserine lactones (AHL), also called autoinducer-1 (AI-1), are predominantly used by Gram-negative bacteria (46). Secondly, many Gram-positive bacteria utilize small autoinducing peptides (AIP) (35, 77). Thirdly, a group of furanosyl borate diester compounds are referred to as autoinducer-2 (AI-2), which are used by both Gram-positive and -negative bacteria, thus representing a signal for interspecies communication (152).

The first QS system was described for the marine bioluminescent bacterium *Vibrio fischeri* (104), and is still viewed as paradigm of Gram-negative AHL QS. The expression of the bioluminescent protein luciferase, encoded by the *luxICDABE* operon, is regulated by an AHL autoinducer, which binds to the regulator protein LuxR (134). When the AHL-LuxR complex activates transcription of the *luxICDABE* operon upon reaching a threshold level, AHL production is concomitantly increased by heightened expression of the AHL synthase LuxI also located on the operon. This positive feedback loop leads to a sudden explosive

increase of luciferase and thus light production by cell density-dependent autoinduction. A distinct feature of AHL QS is the absence of membrane-bound signal-transduction systems. The AHL signals are able to pass the cell membrane by diffusion, and directly activate the cognate transcriptional regulators intracellularly. To date, there are many LuxI/R systems described, characterized by high specificity between the LuxR proteins and their cognate AHL signals, which suggests roles in intraspecies communication. Several important bacterial phenotypes have been shown to be dependent on QS regulation: For example, *Pseudomonas aeruginosa* uses the LasR/I and RhlR/I systems to regulate virulence gene expression and colonization and persistence within the host (110), and the plant pathogen *Erwinia carotovora* regulates virulence gene expression and antibiotic production by utilizing the CarR/I system (96).

In the case of Gram-positive bacteria, many phenotypes are regulated by cell density-dependent communication. Virulence (*Staphylococcus aureus agr* system (9)), competence (*Streptococcus pneumoniae*/*Bacillus subtilis com* systems (21, 54)) and peptide bacteriocin production (for example the nisin system (34)) are modulated by AIP-QS systems, which all comprise similar functional elements. In contrast to AHL QS, the AIP is actively exported into the environment by an ATP binding cassette transporter. A dedicated histidine kinase of a TCS senses the AIP and the cognate response regulator regulates gene expression of target genes.

The LuxS/AI-2 system, discovered by Bassler and co-workers investigating the bio-luminescence regulation of *Vibrio harveyi* (4), constitutes an exception among QS systems, since it permits not only intraspecies, but also interspecies communication (152). The AI-2 signal molecules are a group of furanosyl borate diesters, displaying minor modifications depending on the producing organism, and are the products of the LuxS synthase. This enzyme is involved in the activated methyl cycle of S-adenosyl methionine (SAM) metabolism. In a two-step enzymatic reaction, the toxic by-product of methyl transfer reactions S-adenosyl homocysteine (SAH) is first converted to S-ribosyl homocysteine (RH) by the SAH nucleosidase enzyme, also called Pfs (112). RH is subsequently cleaved by LuxS into homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) (129, 161). DPD is an unstable compound that spontaneously cyclizes to form several furanones including AI-2. To date,

highly conserved homologues of *luxS* genes have been identified in more than 30 bacterial species both Gram-positive and –negative, corroborating the hypothesis of a universal language signal (138). According to recent investigations, *luxS* homologues are widespread among members of the phyla *Firmicutes*, gamma- and epsilon-Proteobacteria, but rarely present in alpha-Proteobacteria and Actinobacteria. Bacteria without LuxS, for example *Pseudomonas* spp. or *Mycobacterium* spp., are using an alternative pathway of SAH detoxification in which SAH is directly converted to homocysteine by the enzyme SAH hydrolase SahH (161). Several important bacterial phenotypes such as biofilm formation, motility and virulence have been discussed to be LuxS/AI-2 regulated based on mutagenesis studies (41). However, studies using *luxS* mutant strains should be carefully examined, because the inactivation of this important metabolic enzyme will lead to pleiotropic effects as a result of, e.g., a defective methylation activity or detoxification of SAH. In contrast to inactivation, the addition of purified AI-2 is a more promising approach to investigate AI-2-mediated signaling (118). Besides *V. harveyi*, LuxS-related QS is best studied for the human enteric pathogens enterohemorrhagic *Escherichia coli* (EHEC) and *Salmonella enterica*. Only for these bacteria, the mechanism of AI-2 uptake has been described. An ABC transporter termed Lsr (LuxS-regulated) is responsible for the internalization and phosphorylation of AI-2, which in turn affects target gene expression via binding to the transcription regulator LsrR (139, 163). The regulation of EHEC virulence genes such as genes of the type III secretion system (TTSS) located on the pathogenicity island locus of enterocyte effacement (LEE), and the flagella regulon has initially been attributed to AI-2. The study of Sperandio and co-workers (132) soon disproved this assumption, and identified yet another LuxS-associated autoinducer, AI-3, as the molecule responsible for virulence gene regulation. Additionally, AI-3 production was demonstrated to be independent from LuxS activity, as the addition of aspartate and expression of an aromatic amino acid transporter, as well as a tyrosine-specific transporter, restored AI-3-dependent phenotypes in an EHEC *luxS* mutant (156). Interestingly, AI-3 and the eukaryotic catecholamine hormones L-epinephrine and L-norepinephrine act agonistically towards induction of the LEE operon (157). Thus, 'interkingdom' cross-talk is also possible between bacteria and the eukaryotic host. The TCS QseBC and QseEF, the first bacterial adrenergic receptors described, are responsible for the detection of the signals and the subsequent signal transduction (119, 133). Interestingly, cellular responses to both signals can be blocked by alpha- and beta-adrenergic antagonists like phentolamine and propranolol,

an effect providing good prospects for anti-infective strategies (132). In recent years the possibility of attenuating virulence gene expression of human pathogens by interuption of QS signaling has gained considerable research interest as an alternative to antibiotic approaches (14, 52, 115). For AI-1/AHL and AIP QS, several promising strategies have been published, for example inhibition of *Pseudomonas aeruginosa* or *Staphylococcus aureus* infections by QS-interference (109, 116). Recently, Medellin-Peña and co-workers demonstrated an inhibitory effect of secreted molecules present in fractionated stationary (OD_{600} of 1.6) culture supernatants of the probiotic strain *Lactobacillus acidophilus* La-5 on EHEC O157:H7 virulence gene expression (97). Although the fractions displaying maximum inhibitory effect on LEE gene expression also repressed AI-2 detection in an *V. harveyi* bioassay, no conclusion could be drawn if the compounds are related to LuxS activity. In contrast to these findings, stationary supernatants of the rodent gut isolate *L. reuteri* 100-23C were found to induce LEE gene expression (141). QS-mediated interaction of probiotic LAB with EHEC obviously bears considerable potential for the development of anti-infective treatments, but the exact molecular mechanisms and *in vivo* efficacy remain to be thoroughly investigated.

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Chapter III

Identification of *Lactobacillus sakei* genes induced in meat fermentation and their role in survival and growth

Eric Hüfner[1], Tobias Markieton[1], Stéphane Chaillou[2], Anne-Marie Crutz-Le Coq[2], Monique Zagorec[2] and Christian Hertel[1]

[1] *Institute of Food Science and Biotechnology, Section Food Microbiology, University of Hohenheim, Stuttgart, Germany*

[2] *Unité Flore Lactique et Environnement Carné, Institut National de la Recherche Agronomique, Domaine de Vilvert, Jouy en Josas, France*

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Summary

Lactobacillus sakei is a lactic acid bacterium that is ubiquitous in the food environment and is one of the most important constituents of commercial meat starter cultures. In this study, *in vivo* expression technology (IVET) was applied to investigate gene expression of *L. sakei* 23K during meat fermentation. The IVET vector used (pEH100) contained promoterless and transcriptionally fused reporter genes mediating β-glucuronidase activity and erythromycin resistance. A genomic library of *L. sakei* 23K was established and the clones were subjected to fermentation in a raw sausage model. Fifteen *in carne*-induced fusions were identified. Several genes encoded proteins, which are likely to contribute to stress-related functions. One of these genes was involved in acquisition of ammonia from amino acids, and the remaining were either part of functionally unrelated pathways or encoded hypothetical proteins. The construction and use of isogenic mutants in the sausage model suggested that four genes have an impact on the performance of *L. sakei* during raw sausage fermentation. Inactivation of the heat shock regulator gene *ctsR* resulted in increased growth, whereas knockout of the genes *asnA2*, LSA1065 and LSA1194 resulted in attenuated performance when compared to the wild-type strain. The results of our study are first to provide an insight into the transcriptional response of *L. sakei* when growing in the meat environment. In addition, this study establishes a molecular basis, which allows to investigate bacterial properties that are likely to contribute to the ecological performance of the organism and to influence the final outcome of sausage fermentation.

Introduction

Lactobacillus sakei is a ubiquitous lactic acid bacterium (LAB) and is commonly associated with the food environment. Although the organism can be isolated from various plant fermentations, e.g. sauerkraut and silage fermentation (32, 63), it is mostly isolated from the meat environment (14, 27). *L. sakei* is recognized as one of the most important components of starter cultures used for production of fermented meat products, most notably raw fermented sausages, in Western Europe (28, 40). Recently, it was shown that this species is also a

transient member of the human gastrointestinal microbiota (15). In addition to the fact that *L. sakei* occurs ubiquitously, it also displays notable differences in physiological and biochemical properties when compared to other lactobacilli (4, 13). For example, *L. sakei* is exceptionally adaptable to changing environmental redox conditions due to its heme-dependent catalase KatA (33) and other enzymes that allow the organism to cope with deleterious oxygen by-products (13). In addition, *L. sakei* is able to proliferate at refrigeration temperatures and in the presence of high salt concentrations (up to 9% sodium chloride) (13). Both low temperature and high salt tolerance play a key role in meat processing in many meat manufacturing environments (14). The special status of *L. sakei* amongst lactobacilli is also highlighted by 16S rRNA gene sequence-based phylogenetic analysis, which shows that *L. sakei* belongs to the deepest branch within the genus *Lactobacillus* (27).

The prevalence of *L. sakei* in a variety of habitats indicates its potential to adapt to and/or to compete in different ecosystems. Recently, the 1.88 Mb genome sequence of the sausage isolate *L. sakei* 23K (14) was published (13), providing fundamental information on the genetic endowment of this organism. The genome analysis revealed potential survival strategies, as well as metabolic properties that enable *L. sakei* to effectively compete in the raw meat environment. The existence of such unique features can be viewed as evolutionary adaptation to the meat environment (13). For example, genes involved in exogenous nucleoside salvage pathways (alternative energy source) and ABC transporters for osmo- and cryo-protective substances are present. In contrast, little is known about the regulation of gene expression of *L. sakei* in various environments. It has been argued, that genes showing greater expression in a particular ecosystem ('niche-specific genes') are more likely to contribute towards ecological fitness than genes expressed equally across a range of environments (53). If this is true, then only a combined knowledge of genome features and specific gene expression is required for understanding the adaptive mechanisms of *L. sakei* to the meat environment.

In vivo expression technology (IVET) has proved to be a valuable tool for the identification of genes that contribute to the performance of an organism in specific environments (for reviews see (53, 54)). IVET permits the detection of promoters that are selectively induced in a particular habitat, and has been successfully used to identify lactobacilli genes that are

induced during sourdough fermentation and colonization of the murine gut (10, 16, 65). Recently, it has been shown using mutant studies, that several of the *in vivo*-induced *Lactobacillus* genes are essential for the fitness of the organism in a particular ecosystem (64). This supports the hypothesis of Rainey and Preston (53), that specific gene expression is an essential tool for bacterial adaptation.

In this paper, we describe the application of IVET to elucidate specific gene expression of *L. sakei* 23K during raw sausage fermentation. Eight of 15 genes induced during fermentation (*in carne* induced –´*ici*´) were selected for the construction of isogenic mutants. Four mutants exhibited altered growth during fermentation, indicating that the *ici* genes contribute to the ecological performance of *L. sakei* in raw sausage fermentation.

Materials and methods

Bacterial strains and culture conditions.

The bacterial strains used in this study are listed in Table 1. *L. sakei* was cultured micro-aerobically (2% O_2, 10% CO_2, 88% N_2) at 30°C in modified MRS (mMRS) medium containing (g L^{-1}) Bacto tryptone (Becton Dickinson, USA) 10.0, Difco beef extract (Becton Dickinson, USA) 8.0, Bacto yeast extract (Becton Dickinson, USA) 4.0, glucose 20.0, Tween-80 1.0, K_2HPO_4 x $3H_2O$ 2.0, diammonium citrate 2.0, $MgSO_4$ x $7H_2O$ 0.2, and $MnSO_4$ x H_2O 0.05 (pH 6.3). *Lactobacillus gasseri* was grown microaerobically at 37°C in Difco MRS medium (Becton Dickinson, USA). *Escherichia coli* was cultured aerobically at 37°C in LB or SOB medium (56). When required, antibiotics were added at the following concentrations: chloramphenicol 7 μg mL^{-1} (lactobacilli) and 20 μg mL^{-1} (*E. coli*), erythromycin 10 μg mL^{-1} (lactobacilli) and 400 μg mL^{-1} (*E. coli*), ampicillin 100 μg mL^{-1}. To screen clones for active promoters *in vitro* (active β-glucuronidase), the mMRS medium was supplemented with 100 μg mL^{-1} X-Glu (5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid) and 7 μg mL^{-1} chlor-amphenicol.

Genetic techniques.

Recombinant DNA techniques and agarose gel electrophoresis were carried out by using standard protocols (56). Plasmid DNA from *E. coli* and lactobacilli were isolated using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich, USA) with the following modifications: for lactobacilli, cells of an overnight culture (5 mL) were harvested by centrifugation (9,000 × *g*, 3 min) and washed once with 1 mL of phosphate buffered saline (PBS; (56)). Cells were resuspended in 200 µL of resuspension buffer from the GenElute Plasmid Miniprep Kit containing lysozyme (20 mg mL^{-1}) (Serva Electrophoresis GmbH, Germany) and mutanolysin (250 U mL^{-1}, Sigma-Aldrich, USA) and incubated at 37°C for 1 h. Further steps were performed according to the supplier's recommendations. Genomic DNA of *L. sakei* was isolated as described previously (57). Purification of PCR products and plasmid DNA was carried out with the NucleoSpin II Kit (Macherey-Nagel, Germany) according to the supplied protocol. Recombinant DNA molecules were introduced into *E. coli* and lactobacilli by electro-transformation (6, 20).

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristic(s)[a]	Reference
Strains		
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI*^{q}*ZΔM15* Tn*10*], Tet^r	Stratagene
L. sakei LTH667	Raw sausage isolate, source of *ldhL* promoter	(26)
L. sakei 23K	Meat isolate, plasmid-cured strain	(14)
L. sakei EH100	Strain 23K harbouring pEH100, Em^s, Cm^r, GusA$^-$.	This study
L. sakei EH200	Strain 23K harbouring pEH200, Em^r, Cm^r, GusA$^+$.	This study
L. sakei Blue1	*L. sakei* 23K harbouring pEH100 containing an insert with *in vitro* promoter activity, Em^r, Cm^r, GusA$^+$	This study
L. sakei White1	*L. sakei* 23K harbouring pEH100 containing an insert without *in vitro* promoter activity, Em^s, Cm^r, GusA$^-$.	This study
L. sakei 23K (pLPV111)	*L. sakei* 23K harbouring pLPV111, Em^r	This study
L. sakei RVRR3	*L. sakei rrp-3* mutant, *rrp-3*::pRV300, Em^r	(49)
L. sakei RVASP	*L. sakei asnA2* mutant, *asnA2*::pRV300, Em^r	This study
L. sakei RVCLPC	*L. sakei clpC* mutant, *clpC*::pRV300, Em^r	This study
L. sakei RVCTSR	*L. sakei ctsR* mutant, *ctsR*::pRV300, Em^r	This study
L. sakei RVNOD	*L. sakei* LSA1194 mutant, LSA1194::pRV300, Em^r	This study
L. sakei RVBETA	*L. sakei* LSA1065 mutant, LSA1065::pRV300, Em^r	This study
L. sakei RVTERC	*L. sakei* LSA1637 mutant, LSA1637::pRV300, Em^r	This study
L. sakei RVERFK	*L. sakei* LSA1649 mutant, LSA1649::pRV300, Em^r	This study
L. gasseri ADH	Source of the promoterless *gusA* gene	(55)
Plasmids		
pRV566	Derivative of the indigenous plasmid pRV500 of *L. sakei* RV332, monocopy *E. coli-Lactobacillus* shuttle vector, Em^r, Ap^r, 7.29 kb	(1)
pRV601	Derivative of pRV566, Ap^r, Em^r, 4.76 kb	This study
pRVcat	Derivative of pRV601, replacement of *bla* and *ermAM* with *cat-194*, Cm^r, 3.47 kb	This study
pFX3	Source of chloramphenicol resistance gene *cat-194*, Cm^r, 4.3 kb	(70)
p29TIVET	Source of the IVET cassette, Ap^r, 4.8 kb	(65)
p29TIVETgus	Derivative of p29TIVET, replacement of ´*bglM* with ´*gusA*, Ap^r, 5.3 kb	This study
pEH100	Promoter trap vector, Cm^r, 6.83 kb	This study
pEH200	pEH100 with *ldhL* promoter inserted upstream of *gusA*, Em^r, Cm^r, 7.0 kb	This study
pLPV111	*E. coli-L. plantarum-L. sakei* shuttle vector, Em^r, 4.2 kb	(5)
pRV300	Nonreplicative delivery vector, Ap^r, Em^r, 3.55 kb	(39)

[a] Ap^r, ampicillin resistant; Em^r, erythromycin resistant; Cm^r, chloramphenicol resistant; Tet^r, tetracycline resistant.

Construction of the IVET vector.

All oligonucleotides used are listed in Table 2 and a scheme depicting cloning steps in the construction of IVET vector pEH100 is given in Figure 1. The *E. coli-Lactobacillus* shuttle vector pRV601 was constructed as follows. The relevant *repA*-containing fragment was amplified from template plasmid pRV566 (1) using primers AML005 and AML006 and digested with ApaI and EcoRV. The PCR product was ligated with pRV300 cut with the same enzymes, generating pRV601 (4.76 kb). The erythromycin (*ermAM*) and ampicillin (*bla*) resistance genes of plasmid pRV601 were then removed by digesting with PagI, blunt-ended with Klenow polymerase, and then digested with KpnI. The chloramphenicol resistance gene *cat-194* from plasmid pFX3 was amplified by using primers catKpnF2 and catBspR2. The 1.1 kb-PCR product was digested with KpnI and ligated with the 1.1 kb pRV601 fragment, resulting in vector pRVcat (3.47 kb). The IVET cassette was designed on the basis of pJW100 (65), by replacing the β-glucanase gene *bglM* with the β-glucuronidase gene *gusA* of *L. gasseri* ADH (55). To do this, vector p29TIVET (65) was amplified by inverse PCR with primers pJWinversFor and pJWinversRev containing restriction enzyme recognition sites for XmaJI and PstI respectively. The 4.1 kb PCR product was digested with PstI and XmaJI. The gene *gusA* was amplified by using primers gusAFor and gusARev containing recognition sites for the restriction enzymes PstI and XmaJI respectively. The 1.8 kb PCR product containing the promoterless *gusA* and the dedicated ribosome binding site was digested with PstI and XmaJI and ligated with the 4.1 kb PCR product of p29TIVET to create plasmid p29TIVETgus. To construct the promoter trap vector pEH100, the resulting 3.4 kb IVET cassette was amplified with primers IVET-EclF and IVET-EclR, both containing the recognition site for SacI. The PCR product was digested with SacI and ligated with the dephosphorylated SacI-digested basic vector pRVcat, resulting in pEH100 (6.83 kb).

To test the functionality and stability of pEH100, the promoter of the lactate dehydrogenase gene *ldhL* (47) of *L. sakei* LTH667 (26) was amplified by using primers LDHL1 and LDHL2. The resulting PCR product was inserted into the dephosphorylated SmaI site of pEH100, generating pEH200. After transformation, the Em^r and β-glucuronidase-positive phenotype of the resulting strain *L. sakei* EH200 was confirmed by cultivation on selective agar plates containing X-Glu. The minimal inhibitory concentration (MIC) of erythromycin for *L. sakei* strains was determined by using dilution series in mMRS broth in microtiter plates and

concentrations up to 1 mg mL^{-1} were tested. Experiments to determine the stability of plasmids maintained in *L. sakei* were performed as described previously for *Lactobacillus reuteri* (29).

Construction of the genomic library.

Chromosomal DNA of *L. sakei* 23K was partially digested with MseI to obtain fragments ranging from approximately 0.1 to 1.5 kb in size. Plasmid pEH100 was digested with NdeI, dephosphorylated with alkaline phosphatase and ligated with the genomic DNA fragments. Heat-inactivated (65°C, 10 min) ligation mixtures were used to transform *E. coli* XL1-Blue. Transformants were plated on LB agar with chloramphenicol. More than 2.5 x 10^5 colonies were recovered by flooding the agar plates with sterile PBS. Pooled suspensions containing the transformants were subjected to plasmid DNA extraction. *L. sakei* 23K was transformed with 1 to 5 µg of the plasmid DNA. Transformants were screened for *in vitro* active promoters (blue colonies) by plating on mMRS agar containing X-Glu and chloramphenicol. To cleanse the IVET library of constitutive and *in vitro* active promoters, more than 25,000 colonies exhibiting undetectable or very weak promoter activity (white or light blue colonies) were recovered by flooding the agar plates with sterile PBS. Plasmid DNA was isolated from pooled cell suspensions and used to transform *L. sakei* 23K. To determine the average insert size, DNA from 30 randomly chosen colonies of *E. coli* and *L. sakei* 23K clones was isolated and subjected to PCR with primers IVETFor and IVETgusRev.

Meat fermentation model.

To simulate the ecological conditions in fermenting raw sausages, a meat fermentation model was designed. Frozen meat (beef and pork, 40% of each) and back fat (20%) were cut, minced and stored at -20°C. Upon thawing, 6 g kg^{-1} glucose, 0.5 g kg^{-1} sodium ascorbate, 28 g kg^{-1} curing salt (sodium chloride with 0.5% sodium nitrite) and 10 mg kg^{-1} erythromycin were added to the meat under constant mixing in a KitchenAid food processor (KitchenAid, USA). To start the fermentation, 20 g of the meat mixture was inoculated with either *L. sakei* 23K containing the genomic library (10^8 cells), control strains EH100 or White (10^7 cells), EH200 or Blue1 (10^3 cells); or the mutants strains (10^6 cells). The mixtures were incubated microaerobically at 26°C in sterile plastic bags for 24 h. The fermented meat was propagated by back-slopping and further incubated for 24 h. A 20 g sample was then mixed with 80 mL

of PBS using a stomacher. A 1mL aliquot of the suspension was used to inoculate a fresh meat mixture (20 g).

Recovery of *ici* clones and identification of promoter sequences.

From the sausages fermented with the IVET library, clones were recovered by growing on mMRS agar with X-Glu and chloramphenicol. Clones with no GusA activity were subcultured and stored at -85°C. The *in vitro* susceptibility to erythromycin was confirmed by comparing the growth on mMRS medium, supplemented with chloramphenicol (5 μg mL^{-1}) and erythromycin (10 μg mL^{-1}), with that of the control strains EH100 and EH200. The *in carne* induction of promoters of putative *ici* clones was confirmed by sausage fermentation by inoculating 10^4 cells as described above. Plasmid DNA of the *ici* clones was isolated and subjected to PCR with primers IVETFor and IVETgusRev. The PCR products were purified and nucleotide sequences were determined using primers IVETFor and IVETgusRev. DNA sequencing was executed using the Dye Terminator Cycle Sequencing Quick Start Kit and CEQ 8000 Genetic Analysis System, both supplied by Beckman Coulter Inc. (Fullerton, USA). Sequences were compared to the genome of *L. sakei* 23K using the BLAST algorithm with a local version of the BLASTN program (at http://genome.jouy.inra.fr/soft/sakei/blast.html) and also to sequences in the GenBank database (http://www.ncbi.nlm.nih.gov/BLAST) (2).

Construction of *L. sakei* mutants.

Isogenic mutants of *ici* genes of *L. sakei* 23K were constructed by using pRV300 for insertional inactivation via single-crossover integration as described previously (39). Internal sequences of the *ici* genes *asnA2*, *ctsR*, *clpC*, LSA1194, LSA1065, LSA1637 and LSA1649 were amplified (primers are listed in Table. 2) and the PCR products were digested with the restriction enzymes PstI and SalI. The fragments were ligated to the PstI/SalI-digested vector pRV300 and the ligation mixtures were used to transform *E. coli* XL1-Blue. Plasmids were used to transform *L. sakei* 23K and their correct integration was checked by PCR using chromosomal DNA of Em^r transformants and a primer targeted against the flanking sequence of the inactivated gene in combination with a primer targeted against the cloning vector (DH05, Table 2).

Table 2. Oligonucleotides used in this study.

Name	Sequence (5´ → 3´)[a]	Created restriction site	Application
AML005	ATGGGCCCGTTTTGATCTCGTACAGTG	ApaI	Construction of pRV601
AML006	ATGATATCAATTCATCAGCAAGTTCTC	EcoRV	Construction of pRV601
catKpnF2	TGACTGGTACCGTTACCCTTATTATCAAGAT	KpnI	Amplification of *cat-194*
catBspR2	TGACTTTCGAATTGATCTGGAGCTGTAATAT		Amplification of *cat-194*
pJWinversFor	TAATACCTAGGCAGCTGGGCATGATGGAT	XmaJI	Amplification of p29TIVET
pJWinversRev	ACACCCTGCAGATGACTAGCTATCTATGATCAC	PstI	Amplification of p29TIVET
gusAFor	GAGAACTGCAGACTAGAAAGGAAAATCATCT	PstI	Amplification of *'gusA*
gusARev	AAAAACCTAGGTATTAATTTAATTGTTGCCA	XmaJI	Amplification of *'gusA*
IVET-EclF	TGACTGAGCTCGAGCTTCTGTTTTGGCG	SacI	Amplification of IVETgus cassette
IVET-EclR	TGACTGAGCTCCAAGATTGTTTAAGCAAAATAAG	SacI	Amplification of IVETgus cassette
ldhL1	AGTCGACGTTTGTTATTGC		Amplification of *ldhL* promoter
ldhL2	CAATTCTTCATTTCGAAAAC		Amplification of *ldhL* promoter
IVETFor	CATTCAAATATGTATCCGCTC		Sequencing primer
IVETgusRev	AGTGCCATTCATTAAAGTGT		Sequencing primer
asnFor	GATCTGCAGTAACGAATGGCTTATGAAGGAT	PstI	Creation of *L. sakei asnA2* mutant
asnRev	GATGTCGAC TCATCAAATCTTCGCCTAAA	SalI	Creation of *L. sakei asnA2* mutant
asn_controlFor	ACGCTTTTTTATTTTGGAAA		Control of *L. sakei asnA2* mutant
clpCFor	GATCTGCAGTAAGGAACGCACTACGTGAACT	PstI	Creation of *L. sakei clpC* mutant

Name	Sequence (5´ → 3´)[a]	Created restriction site	Application
clpCRev	GATGTCGACTCAAAGTCTTGTGCACCAAC	SalI	Creation of *L. sakei clpC* mutant
clpC_controlFor	ATTTACACCAAGTGCCAAAA		Control of *L. sakei clpC* mutant
ctsRFor	GATCTGCAGTAAATTGAAATTCGACGCTCCG	PstI	Creation of *L. sakei ctsR* mutant
ctsRRev	GATGTCGACAATACCAAATTCCCCTCTTTTT	SalI	Creation of *L. sakei ctsR* mutant
ctsR_controlFor	TGCAAAGTCAGAATATCTCGG		Control of *L. sakei ctsR* mutant
terCFor	GATCTGCAGTAAGTTATTCGTTCTTCTGAAGA	PstI	Creation of *L. sakei* LSA1637 mutant
terCRev	GATGTCGACGCTAAGACGGAATCAATCGAG	SalI	Creation of *L. sakei* LSA1637 mutant
terC_controlFor	CCTTTCTTTGAATTAAATAACTGGC		Control of *L. sakei* LSA1637 mutant
lactamFor	GATCTGCAGTAAGTTGTGGAAGTTAACGACG	PstI	Creation of *L. sakei* LSA1065 mutant
lactamRev	GATGTCGACGCCTGCGCGATAAATCAT	SalI	Creation of *L. sakei* LSA1065 mutant
lactam_controlFor	ATGCCACTTGGTGGTGTACGC		Control of *L. sakei* LSA1065 mutant
erfKFor	GATCTGCAGTAAAAAATGAAAAAAAGCCCTT	PstI	Creation of *L. sakei* LSA1649 mutant
erfKRev	GATGTCGACTTAAAGAAGGCTTCACCGTG	SalI	Creation of *L. sakei* LSA1649 mutant
erfK_controlFor	GCGATTACCGCAAGCTATCC		Control of *L. sakei* LSA1649 mutant
nodFor	GATCTGCAGTAAATTAACGTCATGCGAGCCA	PstI	Creation of *L. sakei* LSA1194 mutant
nodRev	GATGTCGACCATAAGGATTTGTGAAGGCGT	SalI	Creation of *L. sakei* LSA1194 mutant
nod_controlFor	TCGGTTGAAGTATTACAAATCGT		Control of *L. sakei* LSA1194 mutant
DH05	ACGACGTTGTAAAACGACGGCCAG		Control of *L. sakei* mutants

[a] Recognition sites for restriction endonucleases are underlined.

Results

Development of a plasmid-based IVET system for *L. sakei* 23K and construction of the genomic library.

To identify *L. sakei* genes that are specifically induced during raw sausage fermentation, the promoter-trap vector pEH100 was constructed based on the endogenous monocopy *L. sakei* plasmid pRV500 (1) and the IVET cassette developed for *L. reuteri* 100-23 (65) (see Fig. 1). Genomic DNA fragments of *L. sakei* 23K were inserted upstream of two transcriptionally fused, promoterless reporter genes. The first gene *´ermGT* confers resistance to macrolide antibiotics and the second *´gusA* encodes the β-glucuronidase of *L. gasseri* ADH (55). Expression of *´ermGT* is essential for growth under the selective pressure of erythromycin. The *´gusA* gene allows to differentiate on mMRS agar plates between clones with and without *in vitro* promoter activity. The functionality of both reporter genes was confirmed by insertion of the constitutive *ldhL* promoter (47) in pEH100. The resulting strain EH200 was resistant to erythromycin (MIC, >1000 μg mL^{-1}) and formed dark blue colonies due to a strong GusA activity. In contrast, strain EH100 was highly susceptible to erythromycin (MIC, approx. 1.0 μg mL^{-1}) and showed no visible GusA activity (white colonies). Strains EH100 (Em^s, $GusA^-$) and EH200 (Em^r, $GusA^+$) served as negative and positive controls, respectively. *E. coli* was used as an intermediate cloning host to establish the IVET library in *L. sakei*. From the resulting clone pool, two randomly picked clones were chosen as control strains. Strain Blue1 showed strong GusA activity and high erythromycin resistance (MIC, > 1000 μg mL^{-1}), whereas strain White1 had no visible GusA activity and was erythromycin sensitive (MIC, approx. 1.0 μg mL^{-1}). As clones with constitutive promoters might outnumber the *ici* clones during sausage fermentation, the clone pool was cleansed of clones showing *in vitro* promoter activity. Determination of the plasmid insert sizes of clones revealed that the size ranges from 150 to 1,800 bp, with an average size of 400 bp. Additionally, the segregational and structural stability of plasmids pEH100 and pEH200 were determined *in vitro*. In case of plasmid pEH100, approximately 88% of the cells still contained the intact plasmid after 20 generations without antibiotics. Plasmid pEH200 displayed a lower stability, as approximately 80% of the cells harboured the plasmid after 20 generations. This number of generations was sufficient for selecting the clones in the IVET experiment. After 64 generations, approximately 59% and 17% of the cells contained plasmids pEH100 and pEH200, respectively.

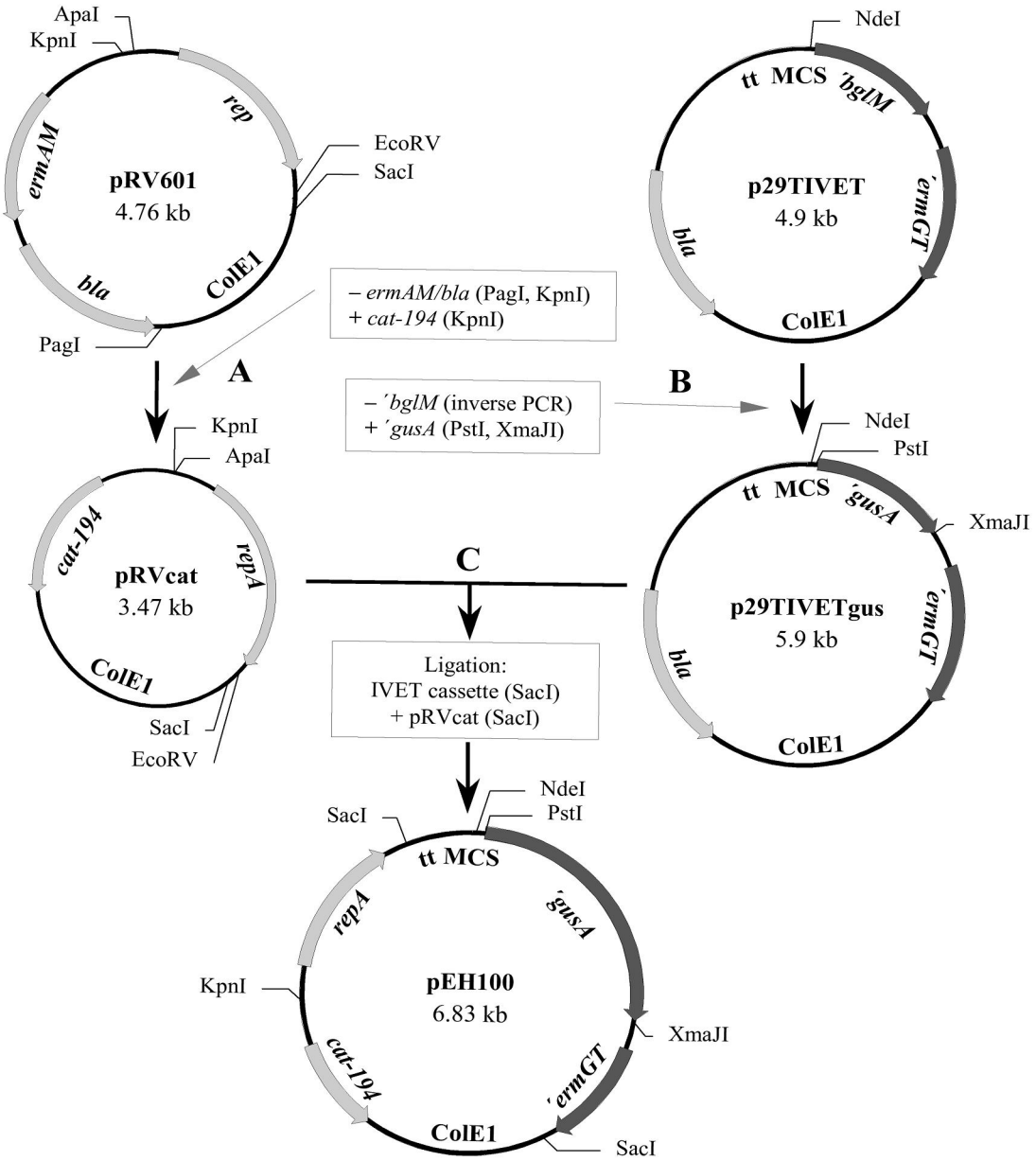

Figure 1. Schematic illustration showing the cloning strategy for the construction of the IVET vector pEH100. The IVET cassette containing promoterless reporter genes *´gusA* and *´ermGT* was designed based on the previously published IVET vector pJW100 (65). ORFs are represented as dark gray arrows (genes of the IVET cassette) or light gray arrows (other genes). Abbreviations: MCS, multiple cloning site; tt, transcriptional terminator *rrn*T1T2 from *E. coli*; ColE1, *E. coli* replication region; *repA*, *Lactobacillus* replication gene; *cat-194*, chloramphenicol resistance gene; *ermAM*, erythromycin resistance gene; *bla*, ampicillin resistance gene; *´ermGT*, promoterless erythromycin resistance gene; *´gusA*, promoterless beta-glucuronidase gene from *L. gasseri*; *´bglM*, promoterless beta-glucanase gene.

In vivo selection and _in vitro_ screening of _L. sakei_ up-regulated genes during sausage fermentation.

A meat fermentation model was designed which reflects the ecological conditions prevailing during raw sausage fermentation. In this model, the initial phase of fermentation was simulated by continuous back-slopping of the meat mixture every 24 h. The applicability of the model in the IVET study was evaluated by inoculating the control strains EH100, EH200, Blue1 or White1 in the meat mixture. As shown in Fig. 2, after 72 h of fermentation the negative control strains EH100 and White1 could not be detected any longer, whereas strains EH200 and Blue1 containing *in vitro* active promoters grew to cell counts of $>10^{10}$ CFU g^{-1} sausage. In addition, each fermentation cycle was characterised by a drop of the pH from approximately 5.9 to 5.5 (data not shown). These results indicated that the ecological conditions were suitable for growth of the *L. sakei* clones in the meat mixture, and that the erythromycin concentration was sufficient for the selection of clones containing an active promoter. Thus, a batch of meat mixture was inoculated with 50,000 *L. sakei* transformants containing the IVET library. After 48 h and 72 h of fermentation, lactobacilli were screened for putative *ici* clones on mMRS agar plates. Approximately 1,000 clones exhibited no or very weak GusA activity, but only 114 clones were sensitive to erythromycin. Amplification and restriction digestion of the plasmid inserts from the erythromycin-sensitive clones revealed 15 different chromosomal DNA fragments which occurred with varying incidence. To confirm the *in vivo* promoter activity of the inserts, the clones were subjected to meat fermentation for 24 h in the presence of erythromycin. The clones differed in their ability to grow in the meat mixture, reaching cell counts ranging from $>10^6$ to 10^8 CFU g^{-1} sausage (data not shown).

Identification of _ici_ genes.

Sequence analysis of the 15 chromosomal DNA fragments (*ici* fragments) allowed characterization of fifteen different putative promoter sequences. Several *ici* fragments consisted of multiple fusions of diverse chromosomal fragments. However, in all fifteen cases one unambiguous putative promoter region and its corresponding open reading frame(s) could be identified. This was accomplished by comparing the nucleotide sequence with that of the genome of *L. sakei* 23K, and by considering the promoter orientation relative to the reporter genes *´ermGT* and *´gusA* (Figure 3). Most sequences contained a putative *Lactobacillus*

promoter signal (–35 region, **TT**GAC**A**; –10 region, **TA**TAA**T**; (48)) and a ribosome binding site (AG**GAGG**). The ORFs were sorted according to the Clusters of Orthologous Groups (COG) classification (58) and the results are listed in Table 3.

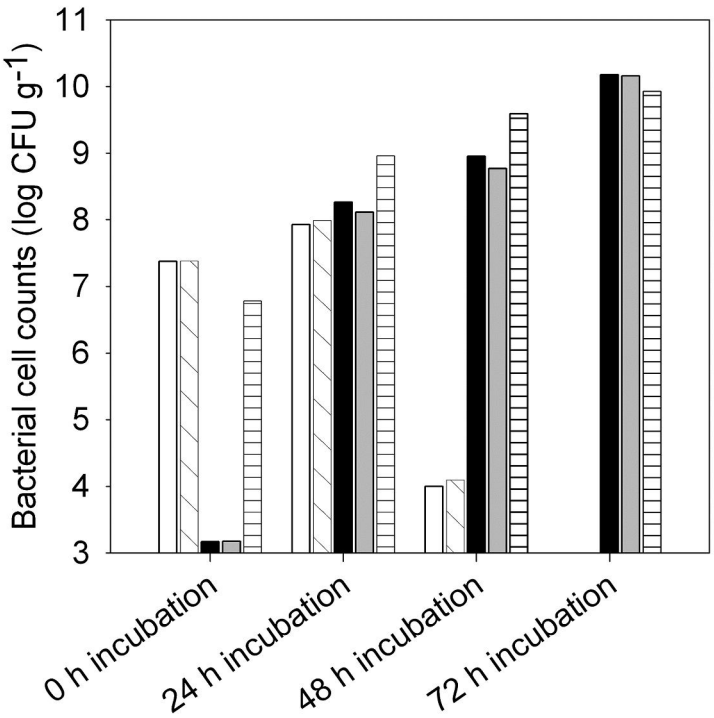

Figure 2. *Lactobacillus* populations during raw sausage fermentation in the presence of erythromycin. Batches were inoculated with the negative control strains EH100 (empty bars) or White1 (diagonally striped bars), or the positive control strains EH200 (black bars) or Blue1 (grey bars), or the IVET library (horizontally striped bars). Bacterial cell counts were determined at the beginning of fermentation (0 h incubation), after 24 hours (24 h incubation), and then 24 hours after the first back-slopping (48 h incubation) and 24 hours after the second back-slopping (72 h incubation) by plating on mMRS agar supplemented with chlor-amphenicol.

Table 3. *L. sakei* 23K genes that were induced during raw sausage fermentation.

Clusters of Orthologous Groups (COG)	***ici clone***	**Redun -dancy**	**Product or function [gene]**	**GeneBank accession nr. of gene product**	**Mutant strain**	**Genomic localization of *ici* fragment[a]**
Amino Acid Transport and Metabolism	*ici05*	1	L-asparaginase2 [*asnA2*, LSA1693]	YP_396306	RVASP	1675301-1675454
Carbohydrate Transport and Metabolism	*ici06*	60	Phosphoglucomutase [*pgm*, LSA0521]	YP_395133		536970-537333
Lipid Metabolism	*ici19*	23	Undecaprenyl pyrophosphate synthetase [*uppS*, LSA1260]	YP_395871		1242610-1242876
Translation, Ribosomal Structure and Biogenesis	*ici04*	1	Asparaginyl-tRNA synthase [*asnS*, LSA0914]	YP_395525		904640-904886
DNA Replication, Recombination and Repair	*ici23*	1	Transposase of IS1520 *orfA* [*tnpA1*-IS1520, LSA0145]	YP_394754		140625-140698
Posttranslational Modifications, Protein Turnover, Chaperones	*ici11*	2	Regulator of class III heat shock genes [*ctsR*, LSA1780]	YP_396393	RVCTSR	1757863-1758224
Signal Transduction Mechanisms	*ici12*	1	Response regulator, two-component system [*rrp-3*, LSA0077]	YP_394688	RVRR3	72282-72393
General Function Predicted Only	*ici09*	2	Hypothetical metallo-β-lactamase [LSA1065]	YP_395675	RVBETA	1061711-1061893
	ici15	3	Hypothetical cell surface protein, ErfK family [LSA1649]	YP_396262	RVERFK	1634364-1634458

Clusters of Orthologous Groups (COG)	*ici clone*	Redun -dancy	Product or function [gene]	GeneBank accession nr. of gene product	Mutant strain	Genomic localization of *ici* fragment[a]
General Function Predicted Only	*ici20*	2	Hypothetical transcription regulator, Xre family [LSA1717]	YP_396330		1700921-1701165
	ici17	2	Hypothetical integral membran protein, TerC family [LSA1637]	YP_396250	RVTERC	1622922-1623152
Function unknown	*ici10*	5	Hypothetical small protein [LSA1714]	YP_396327		1698813-1698907
	ici03	2	Hypothetical small peptide [LSA0121]	YP_394731		112773-113780
	ici14	5	Hypothetical protein [LSA0945]	YP_395556		932126-932667
	ici18	4	Hypothetical membrane protein, nodulin 21-like/DUF125 family [LSA1194]	YP_395806	RVNOD	1175881-1175993

[a] Localization of *ici* fragments on the genome of *L. sakei* 23K (GeneBank accession no. NC_007576).

Performance of *L. sakei* 23K mutants in raw sausage fermentation.

Isogenic mutants of *L. sakei* 23K were used to identify genes that are essential for the ecological performance of the organism during raw sausage fermentation. Six *ici* genes were selected for mutagenesis (Table 3) on the basis of the following criteria. Genes coding for apparent essential functions (*uppS*, *pgm*), as well as genes of insufficient length for mutagenesis by homologous recombination (LSA1714, LSA0945, LSA1717, LSA0145, LSA0121, see Fig. 3 and Table 3) were excluded. Six mutants of *ici* genes were constructed by insertional inactivation using the nonreplicative vector pRV300 (39). In addition, a mutant of gene *clpC* (LSA1779) was constructed, as it forms a transcriptional unit together with the *ici* gene *ctsR* (LSA1780). Mutant RVRR3 of the *ici* gene *rrp-3* (Table 3) has previously been constructed (49). To investigate the effect of gene inactivation on the overall performance, the growth of the mutants in mMRS medium was investigated in comparison to the wild-type strain *L. sakei* 23K (pLPV111), harboring plasmid pLPV111 to confer erythromycin resistance. All mutants except RVERFK grew similar to the wild-type strain 23K (pLPV111) and only mutant RVBETA showed a slightly prolonged lag phase (Fig. 4A). Mutant RVERFK could not grow in mMRS medium resulting in cell counts less than 10^5 CFU mL^{-1} after 30 h of incubation. It was thus excluded from further studies, as the inactivated gene appeared to be essential for effective growth.The remaining seven mutants were tested for their ecological performance in raw sausage fermentation and were compared to the performance of strain 23K (pLPV111). In contrast to the results obtained in mMRS medium, four mutants showed differences in growth during sausage fermentation (Fig. 4B). Compared to the wild-type strain, growth of mutants RVASP, RVNOD and RVBETA was impaired to varying extents. Mutant RVNOD showed the strongest growth restriction, reaching cell counts of only 10^7 CFU g^{-1} after 47 h of fermentation. In addition, a prolonged lag phase (mutant RVBETA) and reduced exponential growth (mutant RVASP) was also observed. On the other hand, during the first 12 hours of incubation mutant RVCTSR showed increased growth with no apparent lag phase when compared to strain 23K (pLPV111). Finally, mutants RVRR3, RVCLPC and RVTERC showed growth patterns similar to the wild-type strain (data not shown).

Discussion

By applying the IVET to *L. sakei* we identified 15 genes and/or operons which demonstrated induced expression during raw sausage fermentation (Table 3, Fig. 3). For some of these, a role in the adaptation of the organism to the fermenting raw sausage environment could be deduced. The initial phase of fermentation, as simulated in our raw sausage model, is characterized by rapid growth of lactobacilli, associated with production of organic acids (most notably lactic) and a related rapid decrease in pH. A low pH (<5.0 after the first few days of fermentation), together with a high osmolarity due to the addition of curing salt, is responsible for the inhibition of undesirable microorganisms (37, 38). Such harsh ecological conditions may also influence the growth of the starter organism *L. sakei*. This view is consistent with our finding of several up-regulated *ici* genes that might be involved in the stress response of *L. sakei* to the ecological conditions prevailing in the initial phase of fermentation.

Gene *ctsR* (clone *ici*11) is the first gene in an operon in which *clpC* is also located. CtsR is a highly conserved transcriptional regulator of class three heat shock genes in Gram-positive bacteria (19). It negatively regulates the expression of Clp proteins, some of which function as chaperones while others exhibit ATPase activity (e.g. ClpC) and form the so-called Clp proteolytic complex together with a structurally unrelated ClpP peptidase (24, 66). This complex specifically degrades misfolded proteins, an event which is crucial for the survival of bacteria under stress conditions (24, 34, 60). Although CtsR acts as a repressor of Clp expression, induction of CtsR in the course of stress response leads to subsequent degradation of the repressor itself, resulting in increased levels of Clp proteins (35). Therefore, elevated expression of *ctsR* and presumably also of the downstream located *clpC* could be part of the stress response of *L. sakei* to the high osmolarity in the meat mixture. This assumption is consistent with the enhanced growth of the *ctsR* mutant in the raw sausage model observed in this study (Fig. 4B).

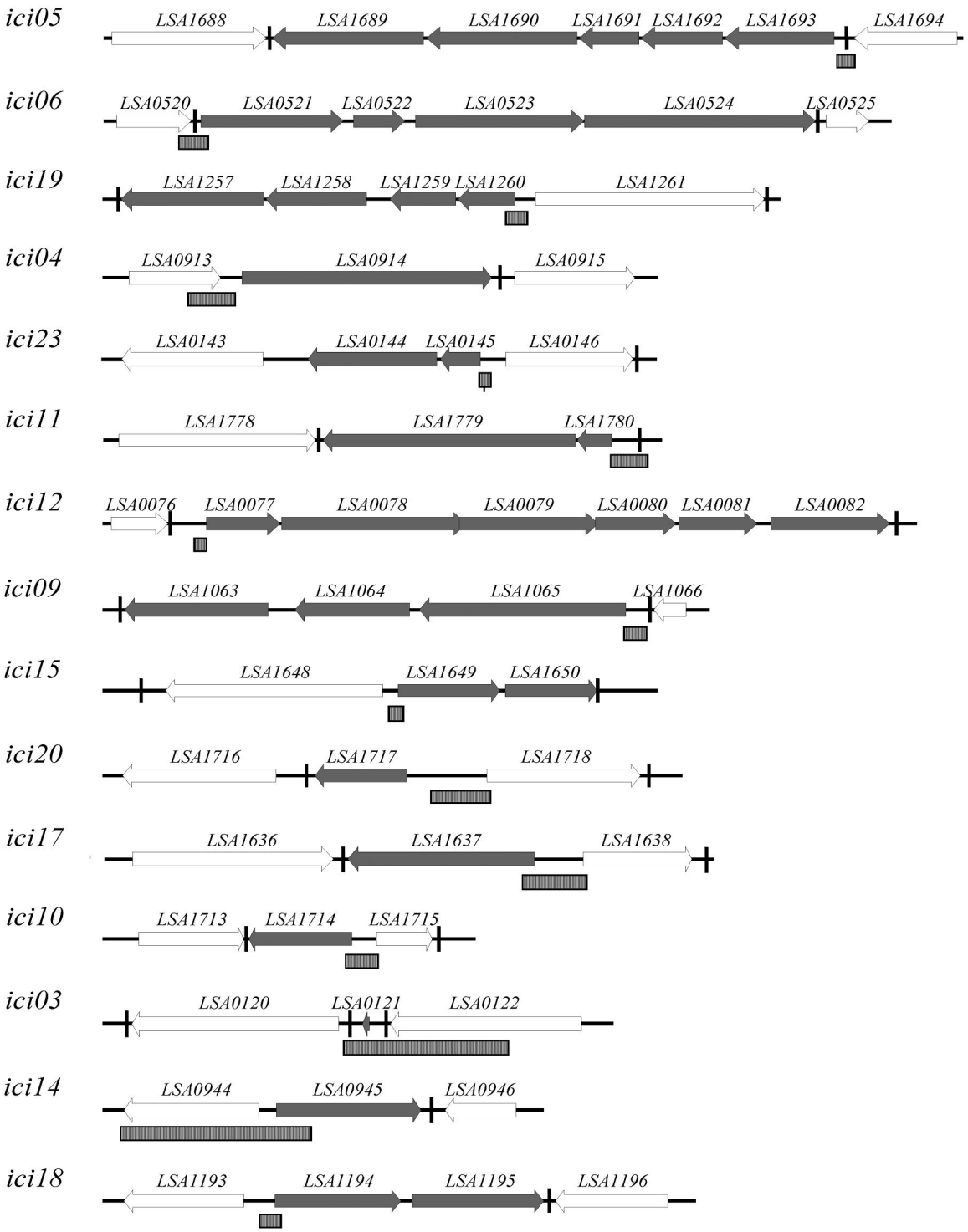

Figure 3. Schematic representation of *ici* fragment localizations (dark boxes) in the genome of *L. sakei* 23K. *Ici* genes and downstream located ORFs that might be part of a putative operon are displayed as gray arrows, adjacent ORFs as white arrows. Vertical black lines denote transcriptional terminators according to the genome annotation (GeneBank accession no. NC_007576).

In addition, the involvement of class three heat shock genes in the osmotic stress response has already been demonstrated in other Gram-positive bacteria, including LAB (60). For example, *Bacillus subtilis* (51) showed induction of *ctsR* and *Lactococcus lactis* (30) displayed increased production of class three heat shock proteins, when exposed to high sodium chloride concentrations. Moreover, it was recently shown that *clpC* is induced during the passage of *L. plantarum* through the murine gastrointestinal tract (10). As the murine gut has been identified as stressful environment for lactobacilli (9, 65), it is tempting to speculate that the class three heat shock genes of lactobacilli are also involved in the general stress response.

Clone *ici06* contained the promoter of the *ici* gene *pgm* coding for a phosphoglucomutase. Pgm catalyzes the interconversion of glucose-6-phosphate and α-glucose-1-phosphate and represents the branching point between the glycolytic and the Leloir pathway. It has been shown that in *L. sakei* high Pgm activity is associated with high exopolysaccharide (EPS) production (17). In addition, *pgm* was shown to be induced under stress conditions, e.g. in *Streptococcus mutans* when growing under acidic conditions (68), as well as in *Lactococcus lactis* after cold shock (69). This indicates that *L. sakei* might respond to the harsh conditions during sausage fermentation by EPS production, a response that was already observed for several LAB confronted with adverse environmental conditions (31, 42, 44). However, unlike other *L. sakei* strains, 23K does not produce EPS, although some genetic information involved in EPS production, for example cluster LSA1510 to LSA1513 (13), is present in the genome.

The *uppS* gene (clone *ici19*) encoding an undecaprenyl pyrophosphate synthase (UppS) is a central enzyme of bacterial cell wall synthesis, and gene LSA1645 (clone *ici15*) may also be involved in this activity. UppS catalyses the consecutive condensation of farnesyl pyrophosphate with eight isopentenyl pyrophosphates to form undecaprenyl pyrophosphate (C_{55} UPP), a lipid carrier for peptidoglycan precursors and also for activated nucleotide sugars used for EPS production (61, 67). LSA1645 codes for a hypothetical protein containing a conserved domain belonging to the ErfK-YbiS-YhnG protein family (pfam accession number PF03734). This domain has been shown to be essential for a peptidoglycan cross-linking enzyme of *Enterococcus faecium* which is involved in an alternate transpeptidation pathway of cell wall synthesis (7, 46). Induced expression of *uppS* and LSA1645 suggests that modifications in the cell wall and/or membrane composition are important for *L. sakei* to

adapt to the adverse environmental conditions in raw sausages. This hypothesis is supported by the observation that lactobacilli respond to environmental stresses with alterations of the cell wall or cytoplasmatic membrane, e.g. changes of the ratio of saturated/unsaturated fatty acids or incorporation of certain glycolipids into the membrane (3, 22, 45, 52, 59). Interestingly, the *uppS* gene was also specifically induced during sourdough fermentation of *L. reuteri* (16), indicating an important function of this gene for the growth of lactobacilli in fermenting foodstuffs.

Sequence analysis of gene *asnA2* (*ici05*), coding for L-asparaginase, revealed a moderate homology (38 % amino acid identities) to N4-(beta-N-acetylglucosaminyl)-L-asparaginase (aspartylglucosaminidase, AGA) of *Flavobacterium meningosepticum* (pfam accession number PF01112). Besides generating ammonium from asparagine due to its general asparaginase activity, AGA plays a pivotal role in the degradation of N-glycans/N-linked glycoproteins by cleaving the Asn-GlcNAc linkage that joins the sugar moiety to the protein (43). Elevated expression of *asnA2* during raw sausage fermentation could be explained in two ways. Firstly, *L. sakei* may require ammonium, because the meat substrate is in general a poor source of free nitrogen (50). However, strain 23K harbours another L-asparaginase (AsnA1) belonging to the asparaginase/amidohydrolase family (pfam accession number PF00710) which may contribute to the acquisition of ammonium. Secondly, *L. sakei* may improve its performance during fermentation by metabolizing the sugar moiety of glycoproteins occurring in the raw meat (62). Inactivation of gene *asnA2* resulted in reduced growth of mutant strain RVASP in the meat mixture. Thus, it is tempting to speculate that the degradation of N-glycans contributes to the ecological performance of *L. sakei* in raw sausage fermentation. However, this would require an extracellular activity, but no signal peptide could be identified by *in silico* analysis, indicating an intracellular localization of the enzyme. Additionally, both the wild-type and the mutant RVASP are unable to grow on glycoproteins like ovalbumin, apo-transferrin and fibrinogen as the sole carbon source (data not shown). Thus, it is more likely that AsnA2 plays a role in nitrogen acquisition rather than N-glycan degradation.

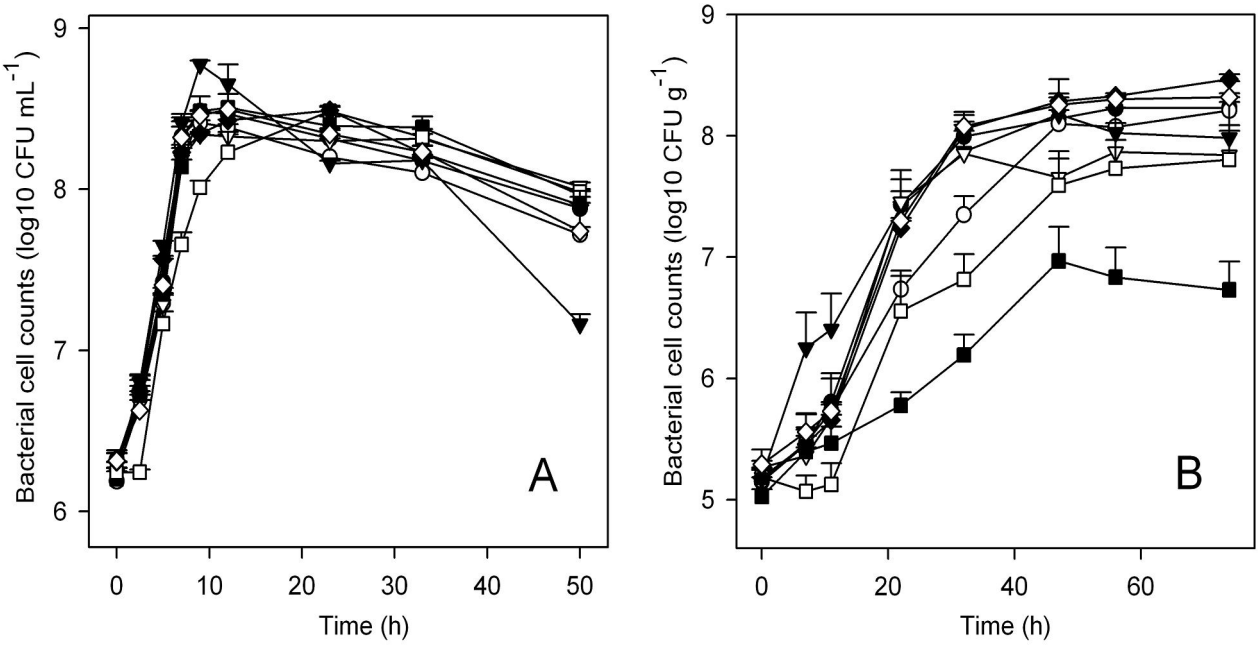

Figure 4AB. Growth of *L. sakei* strains 23K (pLPV111) (●), RVASP (○), RVCTSR (▼), RVCLPC (▽), RVNOD (■), RVBETA (□), RVTERC (◆) and RVRR3 (◇) in mMRS medium (A) and during raw sausage fermentation (B). Bacterial cell counts were determined by plating on mMRS agar supplemented with erythromycin. Values are means of two independent experiments.

In bacteria, environmental signals are sensed and linked with cellular processes via two-component systems (TCS), which consist of a protein histidine kinase as the sensing unit and a response regulator as transcriptionally regulatory element (for reviews see (for survey see 8, 12)). Gene *rrp-3* (clone *ici12*) codes for the response regulator Rrp-3 (49) and is the first of five genes occurring in an operon. It is followed by the gene coding for the cognate protein histidine kinase Hpk-3 and three genes encoding hypothetical proteins. The Rrp-3/Hpk-3 system shows high homology to an essential TCS family (VicRK or CovRS), whose members have been shown to be important for bacteria to react to environmental stimuli, e.g., high osmolarity, extracellular Mg^{2+} concentrations for streptococci (25, 41) and cold-shock induction in *L. lactis* (69). Rrp-3/Hpk-3 might therefore possess a similar function in *L. sakei*. However, the *rrp-3* mutant was not impaired in its performance during sausage fermentation. This is consistent with the previous finding that the Rrp-3/Hpk-3 system of *L. sakei* is not

involved in responses to acid, temperature and oxidative stress (49), as well as to high osmolarity in mMRS medium (data not shown). A sequence homology search revealed that gene LSA1065 (clone *ici09*) possesses the β-CASP functional domain, a characteristic domain for a new family of RNA metabolising metallo-β-lactamases (pfam accession number PF07521) (11). In *B. subtilis*, the members RnjA (YkqC) and RnjB (YmfA) of this enzyme family act as endoribonucleases that appear to be implicated in regulatory processing and maturation of specific mRNAs, and may therefore be important for a fast adaptation of the organism to nutritional and environmental changes (21). LSA1065 could be an ortholog of this family in *L. sakei*, playing a role in posttranscriptional regulation. As mutant RVBETA exhibited a prolonged lag phase during sausage fermentation, it is tempting to speculate that expression of LSA1065 is part of the adaptive response of *L. sakei* to the ecological conditions in the meat fermentation.

The hypothetical product of gene LSA1194 (clone *ici18*) showed weak homology to members of the nodulin-21-like/DUF125 family of integral membrane proteins (pfam accession number PF01988). Representatives are nodulin-21, a plant nodule-specific protein that may be involved in symbiotic nitrogen fixation (18), and CCC1, a yeast vacuole transmembrane protein that plays a role in Ca^{2+} homeostasis and functions as an iron and manganese transporter (23, 36). Orthologs are widespread among pro- and eukaryotic organisms with frequent occurrence of multiple copies per genome, pointing to a conserved function for these membrane associated proteins. *L. sakei* 23K harbors two additional ORFs that show the nodulin-21 like domain (LSA1524 and LSA1195). Interestingly, the inactivation of LSA1194 leads to a reduced performance of *L. sakei* during raw sausage fermentation (Fig. 4B), however no conclusions can be drawn about the function of this hypothetical protein.

The use of IVET facilitated a first insight into the transcriptional response of *L. sakei* during raw sausage fermentation. The results of this study are of importance in several ways. Firstly, they contribute to the knowledge of properties required for the ecological adaptation of *L. sakei* to the meat environment. Secondly, the information can be used for the selection and development of improved starter organisms. As the sausage model used in this study has been designed to simulate the initial fermentation phase, the results may also give an indication as to which genes need to be expressed in order to maximize the organism's competitiveness.

This information may be of importance for production of starter cultures, for example by pre-conditioning the cells at the end of growth in the fermenter to obtain expression of genes that are required in the initial phase of meat fermentation.

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Chapter IV

Improvement of raw sausage fermentation by stress-conditioning of the starter organism *Lactobacillus sakei*

Eric Hüfner[1] and Christian Hertel[2]

[1] *Institute of Food Science and Biotechnology, Section Food Microbiology, University of Hohenheim, Garbenstraße 28, D-70599 Stuttgart, Germany.*

[2] *German Institute of Food Technology (DIL e.V.), Professor-von-Klitzing-Straße 7, D-49610 Quakenbrück, Germany.*

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Abstract

Effective growth and high acidification activity during meat fermentation are key characteristics of starter lactobacilli to ensure hygienic safety and sensory quality of the product. In this study, we demonstrated that the performance of *Lactobacillus sakei* in sausage fermentation can be improved by pre-inoculation treatments with sublethal heat, cold and salt stress. Sausages were produced and inoculated with stress-treated cells of *L. sakei* 23K (pLPV111) and the isogenic mutant of the class III heat shock repressor CtsR that has previously been shown to exhibit improved growth in fermenting sausages. The pH values of sausages fermented with stressed cells attained defined threshold values in distinctly shorter time than those inoculated with unstressed cells. In particular, the cold-stressed cells (4°C) reduced the pH to 5.0 within approximately 40 h as compared to approximately 70 h for untreated cells. This enhanced acidification activity of the cold-stressed cells was consistent with an increased growth rate. Growth studies in culture medium showed that stress-treated cells with improved performance did not exhibit this advantage when exposed to curing salt, one of the major stressors at the beginning of sausage fermentation. Pre-inoculation stress treatment is a promising way to improve the effectiveness of meat starter lactobacilli.

Introduction

Fermentation is an ancient method for biopreservation of the highly perishable food meat and results in storable products, mainly fermented sausages. The microbiota of fermenting sausages consists of lactic acid bacteria (LAB), most notably lactobacilli, and coagulase-negative cocci of the genera *Staphylococcus* and *Kocuria* [18, 31]. The predominating bacteria have been isolated from spontaneously fermented artisan meat products and during the last decades, selected strains have been used in starter cultures to ensure an optimal fermentation process and a reproducible quality of the products [20, 29]. Several principles are fundamental to the production of fermented sausages. First, the rapid decrease of the relatively high pH of raw meat in the initial fermentation phase due to formation of organic acids, mainly lactic acid, by LAB [19, 30]. Second, the reduction of water activity (a_W) during

ripening due to addition of salt and drying [27, 31]. Furthermore, adjuvants like potassium or sodium nitrite and/or nitrate are mostly added to optimise the fermentation process [31, 35]. The combination and timing of these ecological factors is generally referred to as hurdle technology, which is crucial to assure the overall product hygiene, first of all the inhibition of pathogenic and spoilage organisms by disturbing the cellular homeostasis [26]. Strains used in starter cultures have to tolerate the prevailing stress conditions and exhibit a high ecological performance in the stressful food environment [19].

Lactobacillus sakei is a species frequently used in meat starter cultures in Western Europe due to its high competitiveness in sausage fermentation [20, 29]. Analysis of the genome sequence of strain *L. sakei* 23K [4] revealed the genetic basis of survival strategies that could contribute to the superior performance of this species in meat fermentation [10]. Moreover, fifteen genes of *L. sakei* that were specifically induced during raw sausage fermentation were recently identified by using *in vivo* expression technology (IVET) [21]. One gene exhibiting elevated expression in the meat mixture was *ctsR* (LSA1780), coding for a class III heat shock repressor associated with the response of Gram-positive bacteria to environmental stresses, e.g. high osmolarity and temperature shifts [6, 44]. The *ctsR* mutant strain *L. sakei* RVCTSR displayed increased growth during the first 2 days of raw sausage fermentation as compared to the wild-type strain [21]. Thus, it is tempting to speculate that this increased growth resulted from an enhanced tolerance against the stresses prevailing in the sausage environment. The improved stress tolerance could be attributed to increased expression of CtsR-dependent Clp chaperones due to the absence of the CtsR repressor, which has already been shown for diverse Gram-positive bacteria [24, 25].

Based on the presumption that adaptation to stress is improving the performance of *L. sakei* during sausage fermentation, a pre-inoculation treatment of *L. sakei* by exposure to sublethal stresses prior to inoculation of the meat mixture was designed. The aim of this study was the improvement of the performance of *L. sakei* during meat fermentation and thus to allow for manufacturing safer raw meat products.

Materials and Methods

Bacterial strains and culture conditions. *Lactobacillus sakei* 23K (pLPV111) [21] and *L. sakei* RVCTSR [21] were routinely cultured microaerobically (2% O_2, 10% CO_2, 88% N_2) at 30°C in modified MRS (mMRS) medium containing (g L^{-1}) Bacto tryptone (BBL) 10.0, beef extract (Difco) 8.0, Bacto yeast extract (BBL) 4.0, glucose 20.0, Tween-80 1.0, K_2HPO_4 x $3H_2O$ 2.0, diammonium citrate 2.0, $MgSO_4$ x $7H_2O$ 0.2, and $MnSO_4$ x H_2O 0.05 (pH 6.3), supplemented with erythromycin to a final concentration of 10 µg mL^{-1}. When needed, mMRS was supplemented with salts to the following final concentrations: 140 or 250 ppm sodium nitrite, 2.8 or 5.0% curing salt containing 0.5% sodium nitrite, and 2.8 or 5.0% sodium chloride.

Stress conditioning of *L. sakei* cultures. Heat, cold and salt shock treatments of *L. sakei* 23K (pLPV111) cultures were performed basically as described before [32, 41], with several modifications. Briefly, over night cultures of *L. sakei* 23K (pLPV111) were diluted 1:100 in fresh, prewarmed mMRS broth containing 10 µg mL^{-1} erythromycin and incubated at 26°C. When the cultures reached the exponential growth phase (OD_{600nm} of 0.2–0.3), cells were subjected to stress treatment. An aliquot (5 mL) of the culture was incubated for 20 min at 42°C in a water bath (heat shock, HS) or quickly cooled on ice and further incubated for 20 min at 4°C (cold shock, CS). For salt shock treatment (SS), the cells of 25 mL of the culture were harvested by centrifugation (3,000 x g, 3 min, room temperature) and resuspended in 25 mL of mMRS broth containing 6% sodium chloride. After 20 min of incubation at 26°C, cells were harvested by centrifugation and resuspended in 25 mL of mMRS medium. The salt-, heat- and cold treated cells were subsequently used to inoculate mMRS medium or sausage batter.

Meat fermentation. A salami-style meat fermentation model was used to simulate sausage fermentation [21]. Briefly, frozen meat (beef and pork, 40% of each) and back fat (20%) were cut, minced and stored at -20°C. Upon thawing, 6 g kg^{-1} glucose, 0.5 g kg^{-1} sodium ascorbate, 28 g kg^{-1} curing salt (sodium chloride with 0.5% sodium nitrite) and erythromycin 10 mg kg^{-1} were added to the meat under constant mixing in a KitchenAid food processor (KitchenAid, USA). To start the fermentation, 25 g batches of sausage batter were inoculated with

untreated *L. sakei* 23K (pLPV111), HS, SS and CS cultures of *L. sakei* 23K (pLPV111) or the mutant strain RVCTSR (approximately 5×10^6 cells). Fermentations were carried out microaerobically (2% O_2, 10% CO_2, 88% N_2) at 26°C in sterile plastic bags for 96 h. The pH was determined directly in the sausage batter and in mMRS medium by means of an Inlab 410 device (Inlab GmbH, Germany). Homogenised and diluted samples of sausages (1 g) were subjected to microbial analysis by plating on mMRS agar supplemented with 10 $\mu g\, mL^{-1}$ erythromycin.

Modelling the kinetics of cell growth and pH development. Growth was monitored by determination of viable cell count by plating on mMRS agar or by measuring the optical density at 600 nm. The maximum growth rates (μ_{max}) of cultures were obtained by fitting the mean data from duplicate measurements to the Gompertz equation [48] with a minimum correlation coefficient (r^2) of 0.991. The development of pH was determined by fitting the mean data of duplicate measurements to a 4 parameter logistic model [43] with a minimum r^2 of 0.993. SigmaPlot version 8.02 software (SPSS Inc., USA) was used for all curve fit routines.

Results

Influence of stress treatments on meat fermentations. The effect of pre-inoculation treatments using sublethal stresses on the performance of *L. sakei* 23K (pLPV111) in sausage fermentation was investigated and compared to that of the untreated strain *L. sakei* 23K (pLPV111) and the untreated isogenic *ctsR* mutant strain *L. sakei* RVCTSR [21]. To do this, *L. sakei* 23K harbouring plasmid pLPV111 [1], conferring erythromycin resistance, was used to ensure comparable growth conditions for the wild-type strain 23K (pLPV111) and the mutant strain RVCTSR (Em^R). Based on data showing the conditions necessary to induce a stress response in *L. sakei* 23K [32, 41], the following sublethal stress treatments were used: shift to high (42°C) and low (4°C) temperatures and to high osmolarity (6% sodium chloride), resulting in heat-stressed (HS), cold-stressed (CS), and salt-stressed (SS) cells. Exponentially growing cells of strain 23K (pLPV111) were subjected to the various stress treatments and in

the control, cells of strains 23K (pLPV111) and RVCTSR remained untreated. Five batches of the salami-style meat fermentation model, all supplemented with 10 mg kg¯1 erythromycin, were inoculated with the stress-treated or untreated cultures to obtain a final cell count of 2×10^5 CFU g^{-1}. The growth and acidification of strains during sausage fermentation was monitored by viable cell counting and pH measurements. No erythromycin-resistant background microbiota could be detected in the batches. For comparison of the pH developments in the various meat matrices, two threshold pH values of 5.3 and 5.0 were chosen. The values are of hygienic and technological importance, since growth and enterotoxin production of *Staphylococcus aureus* is inhibited below pH 5.3 [33] and drying of sausages is favoured below pH 5.0 [2]. The fermentation time needed to obtain those values was designated 'threshold fermentation time'. After fermentation for 96 h at 26°C, the cell growth and pH development were modelled using appropriate regression equations.

The maximum growth rate of CS *L. sakei* 23K (pLPV111) and untreated mutant RVCTSR were increased compared to those of the untreated *L. sakei* 23K (pLPV111), whereas HS and SS cells exhibited only marginally increased growth rates (Table 1, Fig. 1A). For all batches, the final cell counts after 96 h of fermentation were nearly identical. As compared to the control with untreated cells, marked differences in acidification were observed for the sausages inoculated with the stress-treated cells (Table 1, Fig. 1B), correlating with however minor differences in the growth rates. The pH values of all fermented sausages fell below the threshold pH values of pH 5.3 and 5.0 in distinctly shorter time than that of the untreated *L. sakei* 23K (pLPV111). The fastest acidification was observed for CS cells, reducing the pH 16 h and 29 h faster than the untreated cells to pH 5.3 and 5.0, respectively. The mutant strain *L. sakei* RVCTSR displayed similar acidification as CS cells, whereas pH development of sausages inoculated with SS cells was intermediate. Acidification by HS cells was slower than that of untreated cells during the first 30 h, but it subsequently increased strongly to surpass that of untreated *L. sakei* 23K (pLPV111).

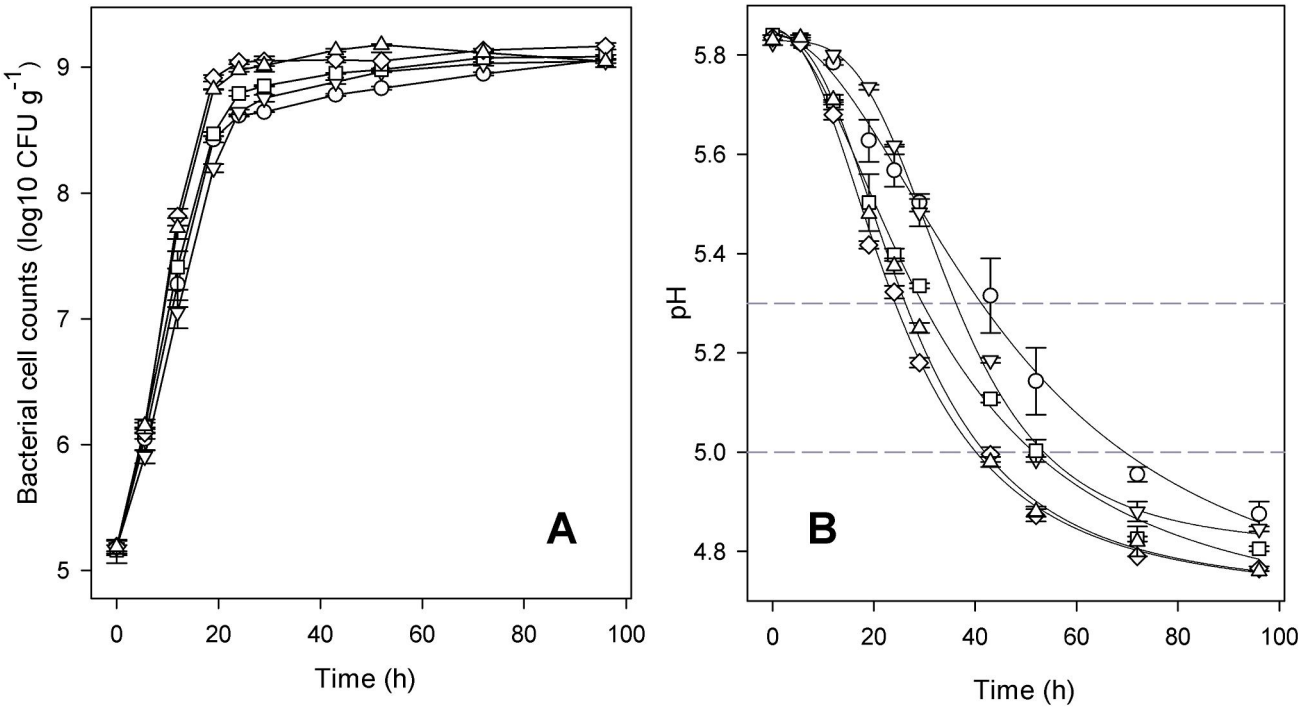

Figure 1. Growth of *L. sakei* strains during sausage fermentation (A) and pH development of sausages (B). *L. sakei* RVCTSR (△), *L. sakei* 23K (pLPV111) untreated (○), heat-stressed (▽), cold-stressed (◇) and salt-stressed (□). Dashed lines indicate threshold pH values (B). Bacterial cell counts were determined by plating on mMRS agar supplemented with erythromycin. Values are means of two independent experiments with one standard deviation.

Discussion

In this study, we demonstrated that the performance of the starter organism *L. sakei* in sausage fermentation can be improved by "conditioning" the cells with sublethal stress treatments prior to inoculation into the meat matrix. Pre-inoculation treatment of cells with heat, cold or salt stress resulted in increased growth and acid production of *L. sakei* in the fermenting sausage (Table 1, Fig. 1). Thus, it is likely that the induction of a stress response permits *L. sakei* to adapt faster to the harsh conditions prevailing in the fermenting sausage. This hypothesis is supported by the finding that the sausage environment exerts stress on *L. sakei* and leads to the induction of stress-related genes like *ctsR* [21]. In contrast, commercial *Lactobacillus* starter organisms, which are generally supplied as freeze- or spray-dried cultures [3], can also be considered stressed especially CS but they do not exhibit such improved performance. These starter culture cells are rather characterised by a decrease of growth and acidification activity [11, 39]. Thus, the modulation of fermentative properties by exerting stress appears to be strongly dependent on the stress conditions used.

There is little information available on the stress response mechanisms of *L. sakei* and its regulation, thus we can only speculate about the mechanism responsible for this adaptation. CtsR is part of the class III heat shock mechanism and negatively regulates the expression of Clp proteins. These proteins are involved in the specific degradation of misfolded proteins, an event which is crucial for the survival of bacteria under stress conditions [14, 44]. Although CtsR acts as a repressor of Clp expression, induction of CtsR in the course of stress response leads to subsequent degradation of the repressor itself, resulting in increased levels of Clp proteins [25], correlated with enhanced bacterial stress tolerance [12, 24, 44]. The involvement of CtsR/Clp proteins in the tolerance against stresses like high or low temperature as well as high osmolarity has already been demonstrated for various LAB [15, 34, 45]. Therefore, class III heat shock response might be the underlying principle standing behind the improved performance of the stress-conditioned *L. sakei* cells. Moreover, genome analysis of strain 23K has uncovered many genes putatively involved in stress adaptation, e.g., osmo- and cryoprotectant transporters and diverse cold shock proteins (Csp), which might play an important role in sausage fermentation [4, 10].

Table 1. Growth of *L. sakei* strains and pH of sausages during sausage fermentations.

Culture	Maximum growth rate μ_{max} (h^{-1}) [a]	Final cell counts (CFU g^{-1}) after 96 h of incubation [b]	Threshold fermentation time (h) [c]		Final pH after 96 h of incubation [b]
			pH 5.3	pH 5.0	
L. sakei 23K (pLPV111) untreated	0.51 ± 0.036	$1.1 \times 10^9 \pm 6.0 \times 10^6$	40.1	69.5	4.88 ± 0.025
L. sakei 23K (pLPV111) heat-treated	0.48 ± 0.017	$1.1 \times 10^9 \pm 1.3 \times 10^7$	36.2	53.5	4.85 ± 0.005
L. sakei 23K (pLPV111) cold-treated	0.72 ± 0.036	$1.5 \times 10^9 \pm 8.5 \times 10^7$	24.2	40.1	4.77 ± 0.004
L. sakei 23K (pLPV111) salt-treated	0.53 ± 0.003	$1.2 \times 10^9 \pm 4.0 \times 10^7$	29.6	51.5	4.81 ± 0.005
L. sakei RVCTSR (Δ*ctsR* mutant)	0.65 ± 0.033	$1.1 \times 10^9 \pm 3.0 \times 10^7$	26.2	41.9	4.77 ± 0.005

[a] The maximal growth rate μ_{max} was calculated by fitting the mean values of two experiments to a 3-parameter Gompertz model [48]
with minimum r^2 of 0.991. The mean values and standard deviations of two independent experiments are displayed.
[b] The mean values and standard deviations of two independent experiments are displayed.
[c] The threshold fermentation times were calculated by using of mean pH values [43] of two experiments with minimum r^2 of 0.993.

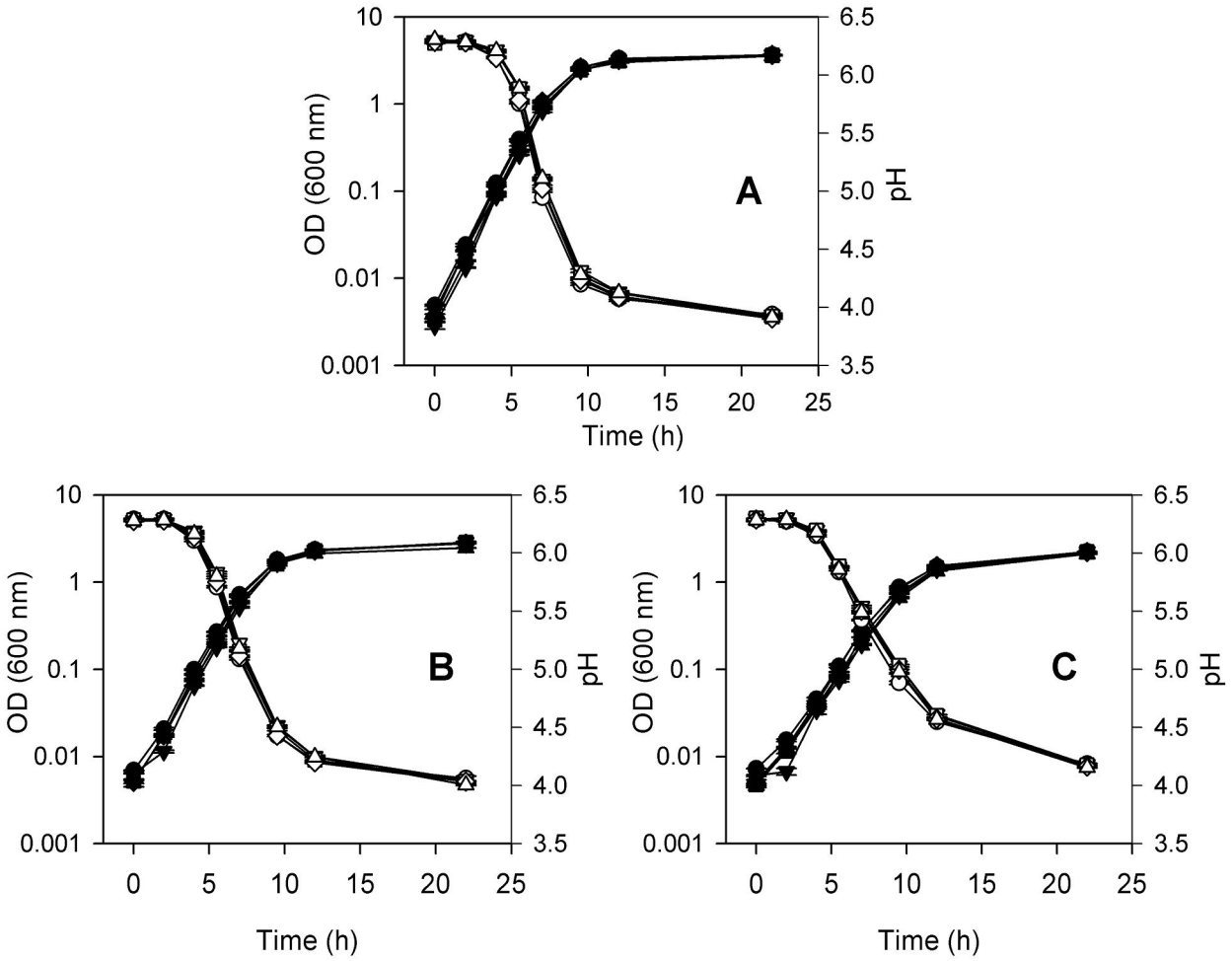

Figure 2. Growth of *L. sakei* strains (filled symbols) in mMRS broth containing erythromycin and supplemented with salts, and pH development of cultures (empty symbols). *L. sakei* strains RVCTSR (▲/△) as well as *L. sakei* 23K (pLPV111) untreated (●/○), heat-stressed (▼/▽), cold-stressed (◆/◇)and salt-stressed (■/□) were grown in mMRS broth without the addition of salt (A), with 2.8% (B) and 5 % curing salt (C). Similar results were obtained with mMRS broth supplemented with 2.8 % and 5 % sodium chloride, and 140ppm and 250 ppm sodium nitrite (not shown). Values represent means of duplicate experiments with one standard deviation.

Thus, the involvement of different stress response mechanisms acting in combination or providing cross-protection cannot be excluded. In addition, lactobacilli exposed to stresses were found to change the membrane composition, e.g., increased amount of short or unsaturated fatty acids as response to high salt or temperature stresses [16, 37]. Such an adaptation might also be of benefit to *L. sakei* during sausage fermentation.

The presence of curing salt is regarded as one of the major hurdles in the initial phase of sausage fermentation. As nitrite was found to be the effective part for growth inhibition of pathogens, we assumed that nitrite is a stressor for *L. sakei* and that the observed improved performance of stress-treated cells might be traced back to an increased tolerance against this stressor. Interestingly, our *in vitro* studies showed that neither an increased growth nor an increased acidification activity could be observed for the CS, SS or HS cells as well as for mutant strain RVCTSR, compared to the untreated *L. sakei* cells (Fig. 2). Thus, an increased tolerance against curing salt seemed not to be the reason for the improved performance of stress-treated *L. sakei* cells in sausage fermentation. However, it should be considered that the experiments were conducted in culture medium. Thus, the results reflected the performance of *L. sakei* in a liquid environment and not in a semi-solid matrix like meat. Nevertheless, media based on MRS medium are frequently used to simulate the conditions prevailing in meat fermentation [9, 28, 36].

As no particular stressor could be identified, it is tempting to speculate that the improved performance is based on changes in the metabolism of *L. sakei*. It has already been shown that exposure of other LAB to stress can induce changes in metabolic activities in a food environment [5, 8, 44]. Such metabolic changes in *L. sakei* might result in enhanced exploitation of available nutrients or increased activity of glycolytic enzymes, leading to the observed accelerated production of lactic acid by stress-treated *L. sakei* cells. For example, cold induction of genes of the glycolytic pathway that results in increased acidification rates have already been demonstrated for *Lactococcus lactis* [46]. Nevertheless, this assumption is not supported by our *in vitro* growth experiments in the presence of curing salt using stress-treated *L. sakei*, because for these cells no faster acidification was found (Fig. 2). Furthermore, a repression of phosphofructokinase has recently been demonstrated for *L. sakei*

exposed to low temperature and high osmolarity, suggesting a decreased flux through the glycolytic pathway [32].

With this study, we identify sublethal stress conditions for pre-inoculation treatments of the starter organism *L. sakei* as an alternative way of improving sausage fermentation. The improvement of performance of LAB in food fermentation has been subject of numerous studies, concerning for example wine or sourdough fermentation [7, 13, 38, 42]. However, only a few papers deal with meat fermentation. Pre-inoculation treatments with manganese and magnesium were demonstrated to increase acidification and bacteriocin production of *Lactococcus lactis* [40], and *Pediococcus acidilactici* reduced cell counts of food-borne pathogens like *E. coli* O157:H7, *Listeria monocytogenes*, and *S. aureus* when stimulated with manganese [23]. However, use of stress treatments to modulate the technological properties of meat bacteria has not been demonstrated up to now. Such data is for example available for *Oenococcus oeni* used in wine fermentation, showing that sublethal stress treatment improves the *in vitro* tolerance to diverse stresses [17, 22, 47]. In our study we could demonstrate for the first time that such a pre-inoculation treatment with sublethal stresses contributes to improve the sausage fermentation process. Further experiments are necessary to understand the molecular mechanisms of the adaptational responses of *L. sakei* to be able to draw conclusions on the basic principles of its superior performance in sausage fermentation.

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Chapter V

Global transcriptional response of *Lactobacillus reuteri* to the sourdough environment

Eric Hüfner[1], Robert A. Britton[2], Stefan Roos[3], Hans Jonsson[3] and Christian Hertel[1]*

[1] *Institute of Food Science and Biotechnology, Section Food Microbiology, University of Hohenheim, Stuttgart, Germany*

[2] *Department of Microbiology and Molecular Genetics, Michigan State University, East Lansing, Michigan 48824, USA*

[3] *Department of Microbiology, Swedish University of Agricultural Sciences, Box 7025, SE-750 07 Uppsala, Sweden*

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Summary

Lactobacillus reuteri is a lactic acid bacterium that is highly adapted to the sourdough environment. It is a dominant member of industrial type II sourdoughs, and is as well able to colonize the intestinal tract of mammals, including humans, and birds. In this study, we investigated the transcriptional response of *L. reuteri* ATCC 55730 during sourdough fermentation by using whole-genome microarrays. Significant changes of mRNA levels were found for 101 genes involved in diverse cellular processes, e.g., carbohydrate and energy metabolism, cell envelope biosynthesis, exopolysaccharide production, stress responses, signal transduction and cobalamin biosynthesis. Our results evidence extensive changes of the organism's gene expression to the growth in sourdough as compared to the growth in chemically defined medium, and thus allowed us to uncover pathways involved in the adaptation of *L. reuteri* to the ecological niche of sourdough. The utilization of starch and non-starch carbohydrates, the remodeling of the cell wall characterized by reduced D-alanylation and increased amounts of cell wall-associated polysaccharides, and regulatory function of two-component systems for cell wall biogenesis and metabolism are suggested by the gene expression data as being important for growth in sourdough. An impact of several genes of *L. reuteri* for effective growth in sourdough was shown by implementation of mutant strains in sourdough fermentation. This study contributes to the understanding of the molecular fundamentals of *L. reuteri*'s ecological competitiveness, and provides a basis for further exploration of genetic traits involved in adaptation to the food environment.

Introduction

Sourdough results from the fermentation of cereal such as wheat or rye and has been used for centuries in the manufacturing of numerous baked goods, foremost bread. It is responsible for the acidification of dough, development of aroma precursors and texture, and extension of shelf-life of products [36]. The beneficial properties of sourdough are determined by the metabolic activity of the sourdough microbiota, mainly consisting of lactobacilli associated with yeasts [19, 31, 89]. Dependent on the type of fermentation, numerous *Lactobacillus*

species have been shown to be highly competitive in sourdough [19]. The predominant species of type II sourdough fermentations, which are characterized by higher temperatures, longer fermentation times and higher water contents than type I doughs [88], are thermophilic and acid-tolerant lactobacilli such as *Lactobacillus pontis*, *Lactobacillus reuteri*, and *Lactobacillus amylovorus* [20, 89]. In particular, strains of *L. reuteri* are highly competitive, persist in industrial fermentation processes over several years of continuous propagation, and are constituents of industrial sourdough starter cultures [28, 55]. However, *L. reuteri* is not only adapted to the food fermentation environment, but also a resident (autochtonous) member of the intestinal microbiota of animals and humans, capable of eliciting beneficial, i.e., probiotic effects for the host organism [73, 83].

A multitude of physiological features have been characterized that are responsible for the competitiveness of *L. reuteri* and other sourdough lactobacilli (reviewed in [27, 32]). Most notably, the highly adapted carbohydrate and energy metabolism enables efficient exploitation of cereal carbohydrates, foremost maltose and sucrose, with a concomitant increase of energy yield through the use of external electron acceptors like fructose or oxygen [76, 77]. Furthermore, the arginine deiminase (ADI) pathway permits protection against acidity by intracellular NH_3 production as well as extra ATP generation [18]. In addition, the secretion of antimicrobial substances such as organic acids and reutericyclin can provide competitive advantage over the accompanying microbiota [26, 56]. However, the genetic background responsible for the ecological performance of *L. reuteri* in sourdough fermentation is poorly understood. Recently, the draft genome sequences of *L. reuteri* ATCC 55730 [4], a strain isolated from human mother's milk, was determined, providing fundamental information on the genetic endowment of this species. On the basis of the genome information, we identified 38 conditionally expressed genes of *L. reuteri* LTH5531, mainly involved in cellular processes like stress response and metabolism of amino acids and nucleotides, growing in a type II rye bran sourdough by applying *in vivo* expression technology (IVET) [15].

To gain further insight into the specific gene expression of *L. reuteri* and to complement the findings obtained with IVET, we investigated in the present study the transcriptomes of *L. reuteri* ATCC 55730 cells growing in sourdough and in chemically defined medium

(CDML) by using whole-genome DNA microarrays. Strain *L. reuteri* ATCC 55730 was chosen as object of study because of the availability of the genome sequence and its ability to dominate rye sourdough fermentation (E. H., personal observation), although it was not originally isolated from this environment. Our results shed light on the cellular mechanisms beyond the well-investigated metabolic characteristics of lactobacilli and establish a basis for further research on the genetic fundamentals of the adaptation to the ecological niche of sourdough.

Material and Methods

Bacterial strains, media and culture conditions. Strains used in this study are listed in Table 1. *L. reuteri* strains were cultured microaerobically (2% O_2, 10% CO_2, 88% N_2) in MRS medium (Oxoid) at 37 °C or in the chemically defined medium CDML at 40 °C. CDML was developed on the basis of a previously published synthetic medium for lactobacilli [24] with following modifications of the recipe (g L^{-1}): glucose 20, $FeSO_4$ x $7H_2O$ 0.01, $Co(SO_4)_2$ x $7H_2O$ 0.01, calcium citrate 0.05, folic acid 0.0005, p-aminobenzoate 0.0006, D-biotin 0.001, calcium panthothenate 0.003, riboflavin 0.003, nicotinic acid 0.003, thiamin 0.005, cyanocobalamin 0.005, myo-inositol 0.005, pyridoxine HCl 0.01, adenine 0.1, guanine 0.1, uracil 0.1, xanthine 0.1, inosine 0.1. Calcium lactate and sodium acetate were omitted from the CDML recipe. To prepare CDML, salts were resuspended in distilled water and autoclaved. Amino acids, vitamins and glucose were each dissolved in distilled water with optional pH adjustment to facilitate complete solubility. The solutions were mixed, the pH was adjusted to 6.3 and the medium was then filter-sterilized. *Escherichia coli* strains were cultivated aerobically at 37 °C in Luria-Bertani (LB) medium [70]. To determine cell counts of the indigenous lactobacilli biota, MRS5 agar [55] containing 0.1 g cycloheximide L^{-1} was used and the plates were incubated microaerobically at 30 as well as 37 °C for 48 h. For counting of yeasts, YGC agar [54, 55] was used and the plates were incubated aerobically at 25 °C for 48 h When required, erythromycin or chloramphenicol was added to media to final concentrations of 10 or 5 µg mL^{-1}, respectively.

Table 1. Bacterial strains and plasmids used in this study.

Strain or plasmid	Relevant characteristic(s)*	Reference or source
Strains		
E. coli XL1-Blue	cloning strain, Tet^r	Stratagene
E. coli EC1000	cloning strain, harboring a copy of the *repA* gene of pVW01 in the chromosome, Km^r	[50]
L. reuteri ATCC 55730	*L. reuteri* ATCC 55730 harboring pERY, Em^r	This study
L. reuteri D0849	*L. reuteri* ATCC 55730 Δlr0849 mutant, lr0849::pORI28, Em^r	This study
L. reuteri lr0677/*rr2* mutant	*L. reuteri* ATCC 55730 Δlr0677 mutant, lr0677::pORI28, Em^r	(T. Wall, E. Hüfner, H. Jonsson, C. Hertel and S. Roos, submitted for publication)
L. reuteri D1056	*L. reuteri* ATCC 55730 Δlr1056 mutant, lr1056::pORI28, Em^r	This study
L. reuteri D1057	*L. reuteri* ATCC 55730 Δlr1057 mutant, lr1057::pORI28, Em^r	This study
L. reuteri D1610	*L. reuteri* ATCC 55730 Δlr1610 mutant, lr1610::pORI28, Em^r	This study
Plasmids		
p29cat232	*E. coli-Lactobacillus* shuttle vector, Cm^r, 5.6 kb	[93]
pRV601	monocopy *E. coli-Lactobacillus* shuttle vector, derivative of pRV566, Ap^r, Em^r, 4.76 kb	[40]
pERY	Derivative of p29cat232 harboring *ermAM*, Em^r, 5.58 kb	This study
pORI28	*repA*-negative derivative of pWV01, Em^r	[49]
pVE6007	*repA*-positive temperature-sensitive derivative of pWV01, Cm^r	[51]

* Tet^r, tetracycline resistant; Km^r, kanamycin resistant; Em^r, erythromycin resistant; Cm^r, chloramphenicol resistant.

Sourdough fermentation. Type II sourdough fermentation was performed as described previously [55] with modifications. Briefly, batches of dough were prepared from rye bran (8 g) and sterile tap water (21.36 ml) providing a dough yield of 367 (mass of dough / mass of bran x 100). Erythromycin was added at a concentration of 10 µg g^{-1} dough. Fermentation was started by the addition of 100 µL of inoculum (about 5 x 10^7 *L. reuteri* cells) followed by incubation in 50 ml plastic tubes at 40 °C in a water bath. After 24 h of incubation, the dough was propagated by back-slopping of 1% of ripe dough and incubation for further 24 h. Back-slopping was carried out three times. Samples of 0.1 g were subjected to microbial counting by plating on MRS agar plates supplemented with 10 µg L^{-1}erythromycin followed by microaerobic incubation overnight at 37 °C.

Genetic techniques. Recombinant DNA techniques and agarose gel electrophoresis were carried out by using standard protocols [70]. Plasmid DNA from *E. coli* and lactobacilli were isolated using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich) according to the manufacturer's instruction with the following modifications. For lactobacilli, cells of an overnight culture (5 mL) were harvested by centrifugation (9,000 *g*, 3 min) and washed once with 1 ml of PBS buffer (pH 7.4). Cells were suspended in 200 µL of resuspension buffer from the GenElute Plasmid Miniprep Kit containing 20 mg lysozyme mL^{-1} (SERVA) and 250 U ml^{-1} mutanolysin (Sigma-Aldrich) and were incubated at 37 °C for 1 h. Purification of PCR products and plasmid DNA was carried out by using the NucleoSpin II Kit (Macherey-Nagel) according to the supplied protocol. Restriction enzymes and T4 DNA ligase were purchased from Fermentas Life Sciences and used according to the supplier's recommendation. Recombinant DNA molecules were introduced into *E. coli* and lactobacilli by electro-transformation [1, 21].

Construction of pERY. To construct pERY, the erythromycin resistance gene *ermAM* from *E. coli-Lactobacillus* shuttle vector pRV601 [40] was inserted into p29cat232 [93], harboring the origin of replication and *repA* gene of *L. reuteri*, by replacing the chloramphenicol resistance gene *cat-194*. To do this, pRV601 was digested with NsbI and the *ermAM*-containing fragment (1.77 kb) was cloned into the NsbI/Eco147I-digested p29cat232 (3.82 kb), yielding pERY (5.58 kb). For all cloning steps, *E. coli* XL1-Blue was used as host organism. The correct insertion was confirmed by restriction digest and agarose gel

electrophoresis, and pERY was then introduced into *L. reuteri* ATCC 55730 by electro-transformation.

Experimental procedures and RNA isolation. Type II sourdoughs were propagated by back-slopping for three days. On the day of RNA isolation, a new batch was inoculated by back-slopping. After 3.5 h of incubation, samples of 5 g were mixed immediately with 50 mL of PBS buffer (pH 7.4, 4 °C). The supernatant (25 mL) containing cells was decanted and mixed immediately with an equal volume of cold methanol (-20 °C) followed by incubation at room temperature for 2 minutes [8]. Cells were harvested by centrifugation and pellets were resuspended in a mixture of two volumes of RNAprotect reagent (Qiagen, Germany) and one volume of PBS according to the manufacturer's instructions. After 10 min of incubation at room temperature, aliquots of the cell suspensions were harvested by centrifugation and subjected immediately to total RNA isolation or stored at –85 °C. Correspondingly, *L. reuteri* was subcultured in CDML medium at 40 °C for three consecutive days, diluted 1:100 into 50 ml of fresh CDML medium every 24 h. On the day of RNA isolation, 50 mL of fresh CDML supplemented with 10 μg L^{-1} erythromycin was inoculated with 500 μL of culture and incubated at 40 °C in a water bath. When the culture reached an optical density (600 nm) of approximately 0.3 (exponential growth phase), 25 ml were mixed immediately with methanol and treated with RNAprotect reagent as described above for sourdough samples. Cells were harvested by centrifugation and stored at –85 °C or subjected immediately to total RNA isolation. Growth and pH development of sourdough fermentation and CDML cultures was measured for up to 33 h. Three independent experiments were conducted (biological replicates).

Total RNA from *L. reuteri* was isolated using the Gentra Purescript RNA Isolation Kit (Gentra Systems) with modifications. *L. reuteri* cells (approximately 1×10^9 cells) were resuspended in 200 μL TE buffer containing 20 mg ml^{-1} lysozyme (SERVA) and 500 U mL^{-1} mutanolysin (Sigma-Aldrich) and incubated for 30 minutes at 37 °C. The cell suspensions were transferred to 2.0 mL screw cap tubes containing 50 mg of 0.1-mm zirconia beads (Carl Roth), and 200 μL Cell Lysis Solution (Gentra) was added. The cells were disrupted at 5,000 rpm for 5 min using the Mini-Beadbeater (BioSpec Products). 100 μL DNA/Protein Precipitation Solution (Gentra Systems) was added to the lysate and the mixture was

incubated at 65 °C for 5 min. After centrifugation, the clear supernatant containing the RNA was added to 300 µL isopropanol and total RNA was harvested by centrifugation. After two washes with 70% ethanol the RNA was resuspended in RNAse-free deionized water and storage at –85 °C. The samples were treated with DNAse I (Roche Applied Science) and subsequently purified with RNeasy Mini Kit (Qiagen), both according to the manufacturers' instructions. The concentration and purity of RNA samples were determined by standard spectrophotometer measurements (Ultrospec 3300 *pro*, GE Healthcare) and agarose gel electrophoresis.

cDNA preparation and hybridization. cDNA for microarray hybridization were prepared as described previously [90]. Briefly, aminoallyl-cDNA was synthesized from identical amounts (15-25 µg) of DNase I-treated RNA isolated from sourdough or CDML samples using random hexamer primers (Qiagen), aminoallyl-dUTP (Ambion) and Superscript II reverse transcriptase (Invitrogen) followed by fluorescence labeling with Cy3 or Cy5 esters (CyDye Postlabeling Reactive Dye Pack, Amersham Pharmacia Biotech). For every biological replicate, a reverse labeling (flip dye) hybridization was performed as described before [23, 90]. The *L. reuteri* whole-genome microarrays [90] comprised 60-mer oligonucleotide probes for 1864 of approximately 2100 open reading frames of a draft genome sequence of *L. reuteri* ATCC 55730 [4] as well as 15 open reading frames encoding known extracellular proteins from *L. reuteri* DSM 20016 [91] (Information regarding the microarray platform can be found at NCBIs Gene Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/) under GEO platform number GPL6366). The arrays were hybridized with combined Cy5- and Cy3-labeled cDNA for 16 h at 42 °C in a water bath using CMT hybridization chambers (Corning), and scanned immediately or stored dry and dark. Finally, 12 replicates for every spot (gene) were obtained (three independent experiments, two flip dye hybridizations per experiment, and duplicate genomes on each glass slide).

Image acquisition and data analysis. Finished slides were scanned using the GenePix 4200 microarray scanner (Molecular Devices Corporation) and stored in the dark at room temperature. GenePix 6.0 software (Molecular Devices) was used for all data analysis procedures. Spots were only considered valid and included in the following data analysis if at least 40% of the pixels had a fluorescence value more than 1 standard deviation above the

local background in at least one of the channels. The remaining fluorescence signals were normalized by adjusting the total signal values in each of the channels (535 or 632 nm wavelength). Then, expression ratios of each gene were calculated by dividing fluorescence values of sourdough samples by the values of CDML samples. Only genes that displayed valid values in at least two of the three biological replicates were used for further analysis. The geometric mean of the expression ratios and standard deviations were calculated and log2-transformed. For each gene, the ratio was generated using the average of three independent experiments (independently grown and prepared samples) with two replicate hybridizations. Genes with M-values (mean log2 ratio RNA_{dough} / RNA_{CDML}) smaller than –1 (<0.5-fold expression) were considered repressed and genes with values greater that +1 (>2-fold expression) were considered induced in sourdough as compared to CDML medium. To determine which genes differed statistically from the mean, we utilized iterative outlier analysis [8], in which three successive rounds of analysis are done to find genes with M-values greater than 2.5 standard deviations from the mean. Confidence limits of the data were calculated, including a standard error factor (distance from gene's average value to the nearest 2.5 standard deviation cut-off) and the number of standard deviations separating the spot from the 2.5 standard deviation cut-off. The M-values of 121 outliers ranged from 5.08 (33.7-fold change) to -3.75 (0.07-fold change). Furthermore, all genes belonging to a putative operon were considered for analysis if at least one gene of the operon showed significant changes in the expression, and the remaining genes showed trends toward that expression. The complete microarray data was deposited at NCBIs Gene expression omnibus (GEO, http://www.ncbi. nlm.nih.gov/geo/) under GEO Series accession number GSE10495.

Functions of to date uncharacterized gene products were predicted by protein homology and conserved domain searches using the BLASTp/BLASTx algorithm at the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/blast) and the InterProScan algorithm at the European Bioinformatics Institute (EMBL-EBI) (http://www.ebi.ac.uk/ InterProScan/). ARTEMIS 9 (Wellcome Trust Sanger Institute, UK) and Vector NTI 10 software (Invitrogen) software was used to edit nucleotide and protein sequences. The differentially regulated genes were grouped into functional categories according to the Clusters of Orthologous Groups classification [80].

Table 2. Oligonucleotides used in this study.

Name	Sequence (5´ → 3´)*	Application
For cloning purposes		
lr1056-f2	TGACTGGATCCTAAATTCTTTCCCTCACAACTGATCG (BamHI)	Creation of *L. reuteri* Δlr1056 mutant
lr1056-r	TGACTGAATTCAGCTTGACGTAATTGGCAAAG (EcoRI)	Creation of *L. reuteri* Δlr1056 mutant
lr1056-cf	TGGCCCATATGAGCAGATTT	Control of *L. reuteri* Δlr1056 mutant
lr1056-cr	TTTGCATTTGTAATATAATCCTTTTCC	Control of *L. reuteri* Δlr1056 mutant
lr1057-f2	TGACTGGATCCTAAGCGATGACAGCGAGTGGTTAT (BamHI)	Creation of *L. reuteri* Δlr1057 mutant
lr1057-r	TGACTGAATTCGGCTTAGTTAAATAATCGTCAGCA (EcoRI)	Creation of *L. reuteri* Δlr1057 mutant
lr1057-cf	TGCGAATCCTTGTAGCAGAA	Control of *L. reuteri* Δlr1057 mutant
lr1057-cr	AGAATCATCTCGGCGTTGAC	Control of *L. reuteri* Δlr1057 mutant
lr0849-f	TGACTGGATCCTAAAGACGTAAAGCGATCTTCAATCAA (BamHI)	Creation of *L. reuteri* Δlr0849 mutant
lr0849-r	TGACTGAATTCTTCCACACTTGTTAGCTTATCCATT (EcoRI)	Creation of *L. reuteri* Δlr0849 mutant
lr0849-cf	GCTTAGCCCATACGAAGTGGTT	Control of *L. reuteri* Δlr0849 mutant
lr0849-cr	TGACTACTTTGCCACCGCTTT	Control of *L. reuteri* Δlr0849 mutant
lr1610-f	TGACTGGATCCTAAGCTTCAACTCAAACCCCAAC (BamHI)	Creation of *L. reuteri* Δlr1610 mutant
lr1610-r	TGACTGAATTCAGCGGACCGAAGAAACATTA (EcoRI)	Creation of *L. reuteri* Δlr1610 mutant
lr1610-cf	TAGTAGCAGTGGCAGCAGGA	Control of *L. reuteri* Δlr1610 mutant
lr1610-cr	TTGTCGGGTACCAAAAATCG	Control of *L. reuteri* Δlr1610 mutant

Name	Sequence (5´ → 3´)*	Application
For real-time RT-PCR		
lr0677F	CCAGAAATCGATGGAATGGATG	Relative quantification of lr0677 transcripts
lr0677R	CATATCTGCCGGCTTAGCTGAA	Relative quantification of lr0677 transcripts
lr1056F	AAATCCAGAGCGAATTGGTCG	Relative quantification of lr1056 transcripts
lr1056R	AATTGCCGGATTGTTTGCATT	Relative quantification of lr1056 transcripts
lr1057F	TAAGTTCAGCGATGACAGCGAGT	Relative quantification of lr1057 transcripts
lr1057R	GGCATTTTCTTTCGCCTGCT	Relative quantification of lr1057 transcripts
lr1610F	TTCAACTCAAACCCCAACTGTTG	Relative quantification of lr1610 transcripts
lr1610R	ATCAGTTGCCAAATTAGGTGCCT	Relative quantification of lr1610 transcripts
lr1971F	AAAGAACGAACCGGCAATTCA	Relative quantification of lr1971 transcripts
lr1971R	TTGCGTGCTCCAATTCGTG	Relative quantification of lr1971 transcripts
lr2108F	TGCCGATGTTGGTGAAAAGC	Relative quantification of lr2108 transcripts
lr2108R	CAGCCTTAAAAATTTCTGGAGCG	Relative quantification of lr2108 transcripts
16SF	CTTGCACCTGATTGACGATGG	Amplification of 16S rRNA reference gene used for relative transcript quantification
16SR	TCCAAATGTTATCCCCCGCT	Amplification of 16S rRNA reference gene used for relative transcript quantification

* The restriction sites used for cloning are underlined and indicated in parentheses.

Real-time RT-PCR. Real-time-RT PCR was performed by using an iQ5 Real-Time PCR Detection System (Biorad Laboratories, USA) with SYBR Green detection of PCR products (iQ SYBR Green Supermix, Biorad) according to the manufacturer's recommendations. cDNA was synthesized from 500 ng of RNA by using the QuantiTect Reverse Transcription Kit (Qiagen) according to the supplier´s instruction. The same RNA samples used for the microarray hybridizations were utilized to generate cDNA as template for the real-time RT-PCR. Primers targeted against genes lr0677, lr0956, lr1057, lr1127, lr1971, lr2108 and 16S rRNA were chosen by using Primer Express software version 1.0 (Applied Biosystems, USA) (Table 2). Real-time PCR reactions contained 50 ng of cDNA template and final concentrations of 0.4 µM of sense and antisense primers and 1-fold iQ SYBR Green Supermix. The following temperature program was used to amplify all cDNA templates: Initial denaturation step of 95 °C for 3 min followed by 35 cycles of 95ºC for 15 s, 60ºC for 20 s and 68 °C for 20 s. PCR efficiencies of amplifications were determined using serial dilutions of cDNA as template and ranged from 93.8 to 101.8% for the primers used. Gene expression ratios were calculated in relation to relative 16S mRNA abundance using the efficiency-corrected relative quantification model according to Pfaffl [62]. Determination of expression ratios was conducted in duplicate for all three biological replicates with appropriate controls.

Gene inactivation and sourdough fermentation using mutant strains. Isogenic mutant strains of *L. reuteri* ATCC 55730 were constructed as described before [92]. Insertional mutagenesis of genes was achieved by site-specific integration of plasmid pORI28 into the *L. reuteri* ATCC55730 chromosome using the temperature-sensitive plasmid pVE6007 [51] as the helper plasmid. Briefly, internal fragments of the target genes lr0677, lr1056, lr1057, lr1610 and lr0849 were amplified by PCR (using the primers in Table 2) and inserted into pORI28 [49] by directional cloning using *E. coli* EC1000 as cloning host [69]. The absence of plasmids was confirmed by plasmid extractions and agarose gel electrophoresis. The integration of the pORI28-based plasmids into target genes was checked by PCR using primers flanking the target region (test primers in Table 2, data not shown). The *L. reuteri* mutant strains were tested for the ability to grow in sourdough. To start fermentation, batches of dough were inoculated with 10^6 *L. reuteri* cells. Growth of *L. reuteri* strains in sourdough fermentation was monitored up to 36 h as described above.

Results and Discussion

Setup of experimental design for the transcriptome analysis in sourdough. In order to identify genes of *L. reuteri* ATCC 55730 regulated in sourdough, we compared the transcriptome of cells growing in a type II sourdough with that of cells growing in rye bran dough and the chemically defined medium CDML. Such synthetic media are commonly used in physiological and genetic studies using microarrays [25, 48, 53, 72] to increase the reproducibility and interpretability of the experimental data. CDML permitted growth of *L. reuteri* ATCC 55730 comparable to that in standard lactobacilli MRS medium, indicating non-limiting nutrient availability and optimal growth conditions (data not shown). Moreover, as the indigenous *Lactobacillus* biota of rye bran may compete with strain ATCC 55730 and thus may affect the transcriptome analysis in sourdough, the sourdough fermentation was designed to be performed under antibiotic selection (erythromycin). Determination of cell counts of the indigenous rye bran microbiota revealed minor numbers of lactobacilli (10 to 100 CFU per gram) and no yeasts. Addition of erythromycin (10 μg g^{-1}) to freshly prepared doughs and incubation for 24 h at 40 °C showed no growth of erythromycin resistant lactobacilli. Thus, strain *L. reuteri* ATCC 55730 was endowed with plasmid pERY to confer erythromycin resistance. The resulting strain *L. reuteri* ATCC 55730 (pERY) was used in all microarray experiments.

Growth of *L. reuteri* in sourdough and chemically defined medium. To ensure adaptation of *L. reuteri* to both environments, sourdoughs were propagated by back-slopping and CDML cultures were subcultured, both three times before starting the microarray experiments. The growth and acidification of the adapted *L. reuteri* ATCC 55730 (pERY) were monitored in sourdough and CDML in the presence of 10 μg mL^{-1} erythromycin (Fig. 1). *L. reuteri* displayed more rapid growth and acidification in sourdough than in CDML medium, but the final pH of CDML cultures was lower than that of the doughs. *L. reuteri* reached maximum cell counts of 5.7 ± 0.3 x 10^9 CFU g^{-1} after 24 h of sourdough fermentation, whereas CDML cultures reached maximum cell counts of 1.5 ± 0.4 x 10^9 CFU mL^{-1}. Correspondingly, the reduction of pH during 24 h of incubation was 6.56 ± 0.005 to 4.15 ± 0.003 (sourdough) and 6.24 ± 0.01 to 3.72 ± 0.005 (CDML). Cell counts and pH values at the time of sampling for RNA isolation were 8.3 ± 0.9 x 10^8 CFU g^{-1} and 5.32 ± 0.07 for sourdough samples, and 1.1 ±

0.2×10^8 CFU mL^{-1} and 5.47 ± 0.11 for CDML samples. Thus, the fermentation parameters pH and temperature of sourdough were comparable to the growth conditions in CDML at the time of sampling for RNA isolation.

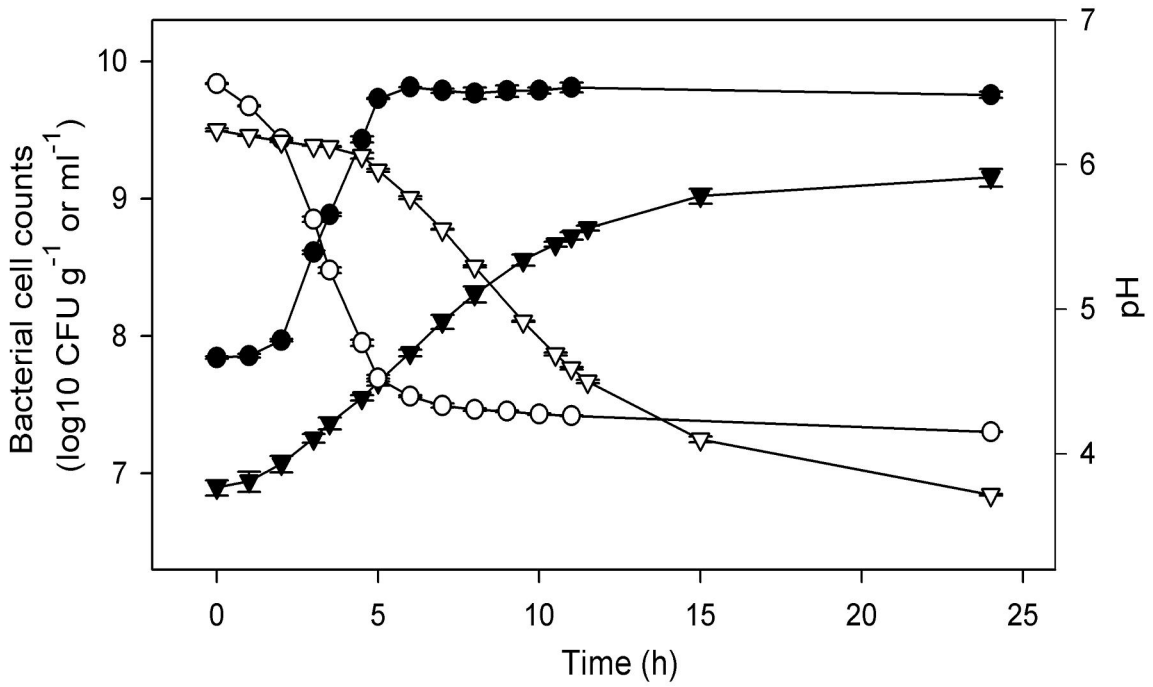

Figure 1. Growth of *Lactobacillus reuteri* ATCC 55730 (pERY) in CDML medium (▼) and during type II sourdough fermentation using rye bran (●), and changes of pH occurring during growth in CDML (▽) and sourdough (○) in the presence of 10 μg g^{-1} or ml^{-1} erythromycin. Values are means of duplicate experiments with one standard deviation displayed.

We chose to investigate the impact of sourdough-regulated genes of *L. reuteri* ATCC 55730 involved in two-component signalling and cell wall biosynthesis on the organism's growth in sourdough. Genes lr1057 (two component response regulator gene rr5), lr1056 (putative GTP pyrophosphokinase gene) and lr1610 (hypothetical cell-wall peptidase gene) were inactivated

by insertion of suicide plasmid pORI28 [92]. The resulting mutant strains *L. reuteri* D1056, D1057 and D1610 (Table 1), as well as an already constructed lr0677 (two-component response regulator *rr2*) mutant strain (T. Wall, E. Hüfner, H. Jonsson, C. Hertel and S. Roos, submitted for publication) were chosen for growth experiments. To create an erythromycin resistant 'wild-type' control strain of *L. reuteri* ATCC 55730 with chromosomally located pORI28 to be used in the growth experiments, the transposase gene lr0849 was insertionally inactivated (strain D0849). The *in vitro* growth in CDML medium of all used *L. reuteri* strains was not affected by the gene inactivations (data not shown). As shown in Figure 4, all *L. reuteri* ATCC 55730 mutants except D1610 displayed reduced final cell counts in sourdough as compared to the respective wild-type strains, but did not display attenuation during exponential growth. The differences in cell counts were relatively small (maximum reduction of 4-fold). However, it has been debated that bacterial physiological versatility might often compensate the defects caused by gene inactivation, leading to only small phenotypic changes (Rediers, 2005). Furthermore, ecological performance has to be viewed as result of coordinated expression of multiple genes rather than being determined by single genes, rendering drastic changes of phenotype by single gene-inactivation unlikely. Thus, we conclude that although the individual effects of the tested genes on the growth of *L. reuteri* in sourdough are minor, but cumulative effects of genes might account for the ecological performance of *L. reuteri*. The *L. reuteri* mutant strains are discussed in the appropriate paragraphs below.

Global transcriptional profile of *L. reuteri* during growth in sourdough. Total RNA was isolated from exponentially growing *L. reuteri* cells obtained from both environments (RNA_{dough} and RNA_{CDML}) and the differentially labeled cDNA was hybridized against whole-genome microarrays of *L. reuteri* ATCC 55730. 84 repressed genes (M-values < –1 (<0.5-fold repression)) and 121 induced genes (M-values > +1 (>2-fold induction)) were subjected to iterative outlier analysis. In total, 101 genes (5.4% of the total number of genes on the array) were significantly regulated in sourdough including 58 genes displaying elevated expression and 43 genes being repressed as detected by iterative outlier method (Fig. 2, Table 3). Several additional genes that displayed regulation, although not significant, were also considered for analysis. Proteins encoded by induced genes were mainly involved in energy and carbohydrate metabolism, coenzyme biosynthesis and stress responses, whereas gene

products of genes displaying reduced expression could be assigned to cellular processes like lipid metabolism and cell wall biosynthesis.

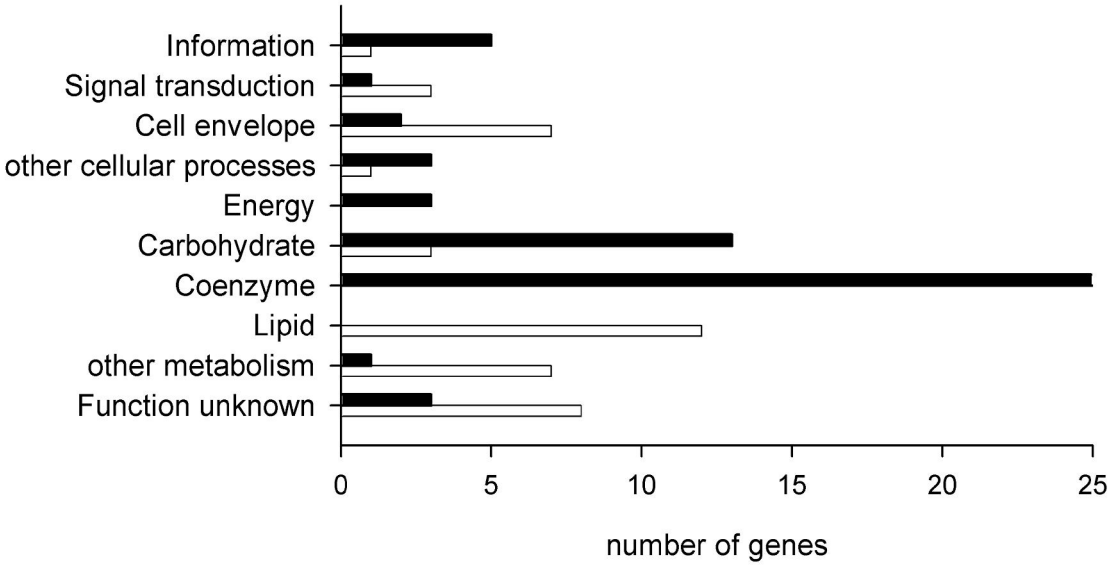

Figure 2. Number of *L. reuteri* ATCC 55730 genes, grouped in functional categories, that were differentially expressed during growth in sourdough relative to growth in CDML medium as determined by iterative outlier analysis [8]. Black bars denote induced expression in sourdough, white bars induction in CDML. Abbreviated Clusters of Orthologous Groups (COG) [80] functional categories are as follows: Information, Information storage and processing; Signal transduction, Signal transduction mechanisms; Cell envelope, Cell wall/membrane/envelope biogenesis; Energy, Energy production and conversion; Carbohydrate, Carbohydrate transport and metabolism; Coenzyme, Coenzyme transport and metabolism; Lipid, Lipid transport and metabolism.

Validation of microarray data using real time RT-PCR analysis. To verify the microarray data, we measured relative mRNA abundance of 6 genes of interest involved in two-component signaling (response regulator genes lr0677, lr1057), capsular polysaccharide biosynthesis (tyrosine-protein kinase gene lr0956), stress response (heat shock regulator gene *hrcA* lr1127, general stress protein Gls24 gene lr2108), and cobalamin biosynthesis (*cbiG*

gene lr1971) by real time RT-PCR analysis. We chose genes that showed a great range of expression ratios in microarray hybridizations and also several genes not displaying significant regulation. The expression ratios obtained by real time PCR were -1.47 ± 0.42 (lr0677), 1.74 ± 0.67 (lr1057), 2.82 ± 0.33 (lr0956), 2.22 ± 0.48 (lr1127), 2.8 ± 1.1 (lr1971) and -1.24 ± 0.46 (lr2108), and correlated well with the microarray results (Fig. 3). Despite differing quantitative values, the level of correlation was good (r^2=0.93), with regard to the different techniques applied. For all genes except lr2108, stronger regulation was determined by RT-PCR, which could be explained by the greater sensitivity of PCR compared to microarray hybridizations [16].

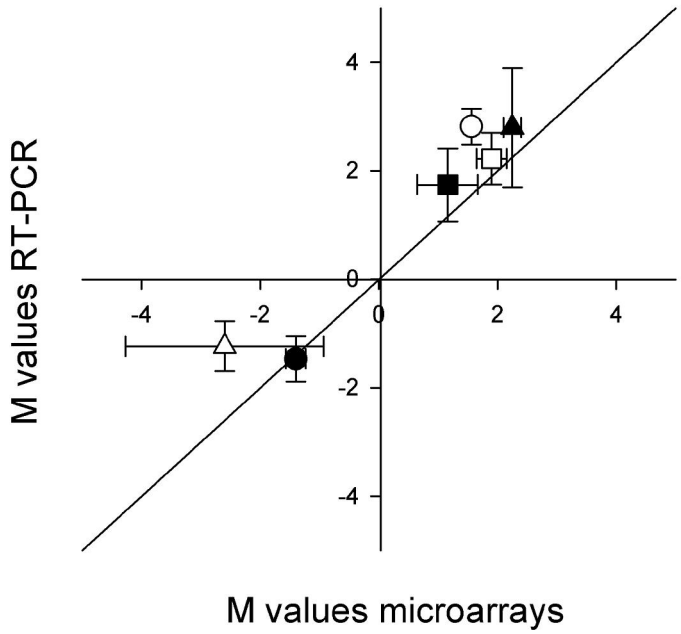

Figure 3. Validation of microarray data by real-time RT-PCR. M values (log2-transformed expression ratios) of genes lr0677 (●), lr0956 (○), lr1057 (■), lr1127 (□), lr1971 (▲) and lr2108 (△) obtained from microarray experiments and RT-PCR are shown. Error bars denote one standard deviation from three biological replicates tested in duplicate.

Table 3. *L. reuteri* ATCC 55730 genes that are regulated during growth in sourdough compared to growth in CDML.

Classification by Cluster of Orthologous Groups (COG)	Description of gene product*	Gene ID	GeneBank accession no.	M value†	SD
UP-REGULATED GENES:					
Information storage and processing					
Transcription	Galactoside utilization regulator GalR	lr0752	*EU153356*	1.94	0.33
	Heat-inducible transcription repressor HrcA	lr1127	EF421896	1.90	0.25
	Transcriptional regulator, TetR family	lr1347	EU038244	1.82	0.16
	Transcriptional regulator, TetR/AcrR family	lr0365	EU153353	1.67	0.52
	Arsenical resistance operon repressor	lr1999	DQ857855	1.32	0.12
Cellular processes and signalling					
Signal transduction mechanisms	Tyrosine-protein kinase	lr0956	EU153361	1.56	0.08
	GTP pyrophosphokinase (EC 2.7.6.5)	lr1056	DQ233684	0.97‡	0.38
	Two component histidine kinase Hpk5	lr1058	DQ219933	1.22‡	0.27
	Two-component response regulator Rr5	lr1057	DQ219940	1.16‡	0.51
Cell envelope biogenesis	Cell wall-associated hydrolases, LysM domain	lr1712	DQ074925	2.47	0.48
	Hypothetical cell-wall peptidase, NlpC/P60 superfamily	lr1610	DQ074910	2.15	0.18
	FlgJ Muramidase, LysM domain	lr1822	DQ074939	1.55‡	0.25
	Nucleoside-diphosphate-sugar epimerases	lr0452	EU153354	1.39‡	0.06
	Putative cell wall biosynthesis glycosyl transferase	lr0953	EU153360	1.39‡	0.36
	Undecaprenyl-phosphate galactose phosphotransferase (EC 2.7.8.6)	lr0954	DQ074861	1.26‡	0.15
	Glycosyltransferase (EC 2.4.-.-)	lr0740	DQ233678	1.14‡	0.40
	N-acetylglucosaminyl-transferase (EC 2.4.1.-)	lr1096	DQ074878	1.14‡	0.15
	dTDP-glucose 4,6-dehydratase (EC 4.2.1.46)	lr0955	DQ857772	1.10‡	0.18
Posttranslational modification, protein turnover, chaperones	GrpE protein	lr1126	DQ074880	1.76	0.13
	Chaperone protein DnaK	lr1125	DQ074879	1.67	0.35
	Chaperone protein DnaJ	lr1123	DQ074961	1.36‡	0.19
Metabolism					
Energy production and conversion	Pyruvate dehydrogenase E1 component beta subunit (EC 1.2.4.1)	lr0840	EF421877	1.67	0.42
	Pyruvate dehydrogenase E1 component alpha subunit (EC 1.2.4.1)	lr0839	DQ857767	1.58	0.35
	Dihydrolipoamide acetyltransferase component of pyruvate dehydrogenase complex (EC 2.3.1.12)	lr0842	EF421878	1.49‡	0.61

Classification by Cluster of Orthologous Groups (COG)	Description of gene product*	Gene ID	GeneBank accession no.	M value†	SD
Carbohydrate transport and metabolism	Arabinose/xylose symporter	lr0053	EU153351	3.71	0.94
	L-ribulose-5-phosphate 4-epimerase (EC 5.1.3.4)	lr0658	DQ857765	3.29	1.36
	L-arabinose isomerase AraA (EC 5.3.1.4)	lr0799	EF421876	2.82	0.26
	Sucrose permease	lr0848	EU038267	2.80	1.16
	Sucrose permease	lr0128	EU038220	2.72	0.87
	Beta-phosphoglucomutase (EC 5.4.2.6)	lr1054	DQ466579	2.57	0.91
	L-ribulokinase (EC 2.7.1.16)	lr0657	EU153355	2.32	1.02
	Phosphoglycerate mutase (EC 5.4.2.1)	lr1029	DQ074870	2.23	1.14
	Maltose phosphorylase (EC 2.4.1.8)	lr1053	EU153362	2.15	0.96
	Na+/melibiose/xyloside related symporter, PTS system IIA subunit	lr0346	DQ470845	1.87	0.36
	Galactokinase (EC 2.7.1.6)	lr0947	EU153359	1.82	1.08
	Maltose O-acetyltransferase (EC 2.3.1.79), truncated ORF	lr0798	EU153357	1.72	2.13
	Alpha-glucosidase (EC 3.2.1.20)	lr1335	DQ857783	1.56	0.65
	Alpha-galactosidase, melibiase (EC 3.2.1.22)	lr0910	DQ857879	1.48‡	0.43
	Beta-glucanase (EC 3.2.1.73), licheninase	lr1095	DQ074877	1.29‡	0.20
	Sucrose phosphorylase (EC 2.4.1.7)	lr0321	DQ466578	1.16‡	0.77
	Phosphopentomutase (EC 5.4.2.7)	lr0675	DQ857878	1.07‡	0.99
Amino acid transport and metabolism	Dipeptidase (EC 3.4.-.-), PepD like	lr2006	EU153364	1.07‡	0.10
Nucleotide transport and metabolism	Dihydropyrimidine dehydrogenase [NADP+] beta subunit (EC 1.3.1.2)	lr0907	EU153358	1.65	0.44
Coenzyme transport and metabolism	Cobyric acid synthase (EC 3.-.-.-)	lr1961	DQ857830	3.11	0.19
	Precorrin-2 oxidase	lr1960	DQ857829	2.90	0.07
	Glutamyl-tRNA reductase (EC 1.2.1.-)	lr1959	DQ857828	2.76	0.12
	Precorrin-3B C17-methyltransferase (EC 2.1.1.131)	lr1970	DQ857839	2.76	0.27
	CbiM protein	lr1965	DQ857834	2.74	0.50
	Precorrin-6X reductase (EC 1.3.1.54)	lr1969	DQ857838	2.74	0.18
	Precorrin-2 methyltransferase	lr1966	DQ857835	2.73	0.18
	Cobalt transport ATP-binding protein CbiO	lr1962	DQ857831	2.69	0.29
	Cobalt chelatase (EC 4.99.1.-)	lr1967	DQ857836	2.66	0.28
	Delta-aminolevulinic acid dehydratase (EC 4.2.1.24)	lr1957	DQ857826	2.52	0.29
	Cobalt transport protein CbiN	lr1964	DQ857833	2.49	0.28
	Precorrin-4 C11-methyltransferase (EC 2.1.1.133)	lr1972	DQ857841	2.49	0.38
	Precorrin-6Y C5,15-methyltransferase subunit CbiT (EC 2.1.1.132)	lr1973	DQ857842	2.42	0.39
	Porphobilinogen deaminase (EC 4.3.1.8)	lr1958	DQ857827	2.42	0.39
	Glutamate-1-semialdehyde 2,1-aminomutase (EC 5.4.3.8)	lr1956	DQ857825	2.38	0.45
	Uroporphyrin-III C-methyltransferase (EC 2.1.1.107)	lr1968	DQ857837	2.29	0.09
	CbiG protein	lr1971	DQ857840	2.25	0.15
	CbiD protein	lr1975	DQ857844	2.21	0.43

Classification by Cluster of Orthologous Groups (COG)	Description of gene product*	Gene ID	GeneBank accession no.	M value†	SD
	Uroporphyrinogen-III synthase	lr1952	DQ857821	2.12	0.35
	Precorrin-8X methylmutase (EC 5.4.1.2)	lr1976	DQ857845	2.08	0.37
	Precorrin methyltransferase subunit CbiE (EC 2.1.1.132)	lr1974	DQ857843	2.03	0.48
	Cobalt transport protein CbiQ	lr1963	DQ857832	2.00	0.17
	Alpha-ribazole-5'-phosphate phosphatase (EC 3.1.3.-)	lr1953	DQ857822	1.93	0.32
	Cobalamin biosynthesis protein CobD	lr1977	DQ857846	1.92	0.57
	Cobalamin adenosyltransferase	lr1980	DQ857849	1.84	0.28
	Nicotinate-nucleotide--dimethylbenzimidazole phosphoribosyltransferase (EC 2.4.2.21)	lr1951	DQ857820	1.83	0.33
	Cobinamide kinase (EC 2.7.1.-)	lr1955	DQ857824	1.59	0.17
	Cobyrinic acid a,c-diamide synthase (EC 3.-.-.-)	lr1978	DQ857847	1.54	1.26
Lipid transport and metabolism	2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase	lr2138	EU153365	1.17‡	0.08
	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase (EC 2.7.1.148)	lr1903	EU153363	1.12‡	0.17
Function unknown	Phage infection protein	lr1348	DQ074898	2.50	0.24
	Hypothetical protein unique to L. reuteri	lr0055	EU153352	2.17	0.56
	Hypothetical extracellular protein	lr1055	DQ074875	1.73	0.26
	High-molecular-mass surface protein Lsp, truncated	lr1997	DQ074950	1.12‡	0.17
DOWN-REGULATED GENES:					
Information storage and processing	CopAB ATPases metal-fist type repressor	lr2076	DQ219989	-4.58	0.45
Cellular processes and signalling					
Signal transduction mechanisms	General stress protein, Gls24 family	lr2108	DQ233697	-2.60	1.67
	GTP pyrophosphokinase RelA (EC 2.7.6.5)	lr0021	DQ220000	-1.56	0.25
	Two-component response regulator Rr2	lr0677	DQ219937	-1.65	0.32
Cell wall/membrane biogenesis	Peptidoglycan binding protein, LysM domain	lr1267	DQ074894	-2.34	0.33
	Cell wall-associated glycosyl hydrolase NlpC/P60 superfamily	lr0342	DQ074821	-1.94	0.52
	D-alanine-activating enzyme	lr1652	DQ857795	-1.56	0.18
	Multimodular transpeptidase-transglycosylase	lr1005	DQ074867	-1.53	0.25
	Penicillin-binding protein	lr0889	DQ074857	-1.41	0.11
	D-alanyl carrier protein	lr1650	EU157968	-1.31	0.08
	Phosphoglycerol transferase	lr1145	DQ074881	-1.28	0.46
	Protein DltB	lr1651	EU157969	-1.23‡	0.27
	Phosphoglycerol transferase	lr1734	DQ074927	-1.13‡	0.3
	Protein DltD precursor	lr1649	DQ074918	-1.04‡	0.10

Classification by Cluster of Orthologous Groups (COG)	Description of gene product*	Gene ID	GeneBank accession no.	M value†	SD
Posttranslational modification, protein turnover, chaperones	Glutaredoxin	lr1774	EU038258	-1.56	0.08
Metabolism					
Carbohydrate transport and metabolism	Alcohol dehydrogenase (EC 1.1.1.1)	lr1777	EF421920	-1.87	0.39
	Glycerol uptake facilitator protein	lr1885	DQ233729	-1.78	0.31
	Alcohol dehydrogenase (EC 1.1.1.1)	lr0781	EF421964	-1.60	0.65
Amino acid transport and metabolism	Metalloproteinase (EC 3.4.24.-)	lr1291	AY970991	-1.35	0.38
Lipid transport and metabolism	(3R)-hydroxymyristoyl dehydratase (EC 4.2.1.-)	lr1013	EF421887	-5.08	0.47
	3-oxoacyl reductase (EC 1.1.1.100)	lr1287	EF421909	-4.16	0.34
	Biotin carboxyl carrier protein of acetyl-CoA carboxylase	lr1284	EF421907	-3.66	0.96
	Malonyl-CoA transacylase (EC 2.3.1.39)	lr1009	EF421883	-3.61	0.59
	Acyl carrier protein	lr1010	EF421884	-3.52	0.22
	3-oxoacyl synthase (EC 2.3.1.41)	lr1286	EF421908	-3.23	0.14
	Acetyl-coenzyme A carboxylase carboxyl transferase subunit beta (EC 6.4.1.2)	lr1281	EF421904	-3.13	0.76
	Beta-hydroxyacyl dehydratase FabZ	lr1283	EF421906	-3.05	0.31
	3-oxoacyl synthase III (EC 2.3.1.41)	lr1011	EF421885	-3.03	0.23
	Biotin carboxylase (EC 6.3.4.14)	lr1282	EF421905	-2.97	0.43
	Acetyl-coenzyme A carboxylase carboxyl transferase subunit alpha (EC 6.4.1.2)	lr1280	EF421903	-2.96	0.08
	Enoyl reductase FabL (EC 1.3.1.9)	lr1279	EF421902	-2.5	0.29
Inorganic ion transport and metabolism	Copper-exporting ATPase (EC 3.6.3.4)	lr2077	EU157971	-4.83	0.09
	Copper-transporting ATPase (EC 3.6.1.36)	lr1016	DQ074868	-3.26	0.37
	Copper-translocating P-type ATPase	lr1015	EU157973	-2.89	0.26
Secondary metabolites biosynthesis, transport and catabolism	Propanediol utilization protein PduB	lr1881	DQ233725	-1.65	0.48
	Diol dehydratase gamma subunit (EC 4.2.1.28)	lr1878	DQ233722	-1.56	0.27
	Propanediol utilization protein PduA	lr1882	DQ233726	-1.61	0.11
	Diol dehydratase large subunit (EC 4.2.1.28)	lr1880	DQ233724	-1.22‡	0.10
	Diol dehydratase beta subunit (EC 4.2.1.28)	lr1879	DQ233723	-1.17‡	0.22
Function unknown	Hypothetical protein, RmlC-like cupin family	lr2078	EU157972	-3.80	0.31
	Extracellular transglycosylase-associated protein	lr1351	DQ233687	-3.80	0.44
	General stress protein CsbD-like	lr1350	DQ233686	-3.14	1.19
	Hypothetical protein	lr2050	EU157970	-2.55	0.16
	ABC transporter ATP-binding protein	lr0020	EU157974	-1.63	0.39
	Hypothetical GTP-binding protein	lr0176	EU157966	-1.53	0.20
	Hypothetical protein, streptococcal antigen-like	lr1319	DQ074896	-1.16	0.46

Classification by Cluster of Orthologous Groups (COG)	Description of gene product*	Gene ID	GeneBank accession no.	M value†	SD
	DedA family protein	lr1354	EU157967	-1.07	0.29

* ORFs are designated according to the annotation of the draft genome sequence of *L. reuteri* ATCC 55730 [4] and on the basis of homology to known genes/proteins.

† Genes with M values (mean log2 expression ratios RNA_{dough} / RNA_{CDML}) of ≥+1 (2-fold induction) and ≤-1 (0.5-fold repression) were considered regulated. Significantly regulated genes were detected by applying iterative outlier analysis [8]. Only genes that displayed valid values in at least two of the three biological replicates were considered for analysis.

‡ Regulated genes not detected by iterative outlier analysis but included in the analysis.

Substrate utilization and energy metabolism. Cereal fermentations are mixed substrate fermentations. In rye bran, starch-derived maltose, sucrose, fructose and some hemicelluloses are the main carbohydrates [10]. To facilitate the investigation of gene expression in response to the various carbohydrates in rye bran, the transcriptional response of *L. reuteri* growing in sourdough was compared to that during growth in CDML using glucose as sole carbon source. A number of genes related to carbohydrate metabolism exhibited strong differential expression (Table 3). For example, the induction of alpha-glucosidase/ maltase (lr1335, EC 3.2.1.20), maltose phosphorylase (lr1053, EC 2.4.1.8) and beta-phospho-glucomutase (lr1054, EC 5.4.2.6) indicated the degradation of starch-derived maltose, whereas the elevated expression of sucrose transport proteins (lr0848, lr0128) and sucrose phosphorylase (lr0321, EC 2.4.1.7) denoted the utilization of sucrose present in rye bran. The preferential use of maltose, which is the most abundant fermentable carbohydrate in cereal doughs, is generally regarded as major competitive advantage of dominant sourdough lactobacilli [89]. The gene product of lr1053 was similar to MapA (57% aa identities), a maltose phosphorylase of *L. sanfranciscensis*. MapA is inducible by maltose, in contrast to other maltose utilizing enzymes displaying constitutive expression [22]. Our results suggest a similar regulation in *L. reuteri*.

The transcription of several genes involved in utilization of xylose and arabinose (e.g. D-xylose / D-Arabinose:H(+) symporter lr0053 and L-arabinose isomerase lr0799), alpha-galactosides (lr0910, lr0947, lr0752) and lichenin (lr1095) was heightened in sourdough, indicating that *L. reuteri* also utilizes plant heteropolysaccharides, e.g. the hemicelluloses arabinoxylan or arabinogalactan. Arabinoxylan is the main extractable dietary fiber in rye [39] and undergoes degradation by cereal enzymes during dough resting [44]. Recent studies showed that lactobacilli can make use of non-starch polysaccharide degradation products [29, 33, 35, 85].

The two-component regulatory system (TCS) lr1057/lr1058 showing induction in sourdough relative to *in vitro* conditions could be involved in the regulation of carbohydrate utilization. The product of response regulator gene lr1057 shares high homology (59% amino acid (aa) identities) with SptR of *Streptococcus pyogenes*, a regulator that has recently been shown to be essential for persistence in human saliva and adaptation to the oral habitat [74]. Surprisingly, we noticed a similar set of genes regulated in *L. reuteri* in sourdough and by the SptR response regulator of *S. pyogenes* in saliva. For example, genes related to maltose/ maltodextrin and HePS-derived sugar utilization, pentose phosphate pathway, glycolysis, stringent response (*relA*, GTP binding proteins), cell wall modification and stress response (*gls24*, glutaredoxin) were comparably regulated (see supplementary material of [74]). It is thus tempting to speculate that the lr1057/lr1058 system regulates gene expression of *L. reuteri* in a similar fashion in sourdough. Furthermore, the hypothesis of a metabolic regulative function of the TCS is corroborated by the presence of the GTP pyrophosphokinase (*relA*/*spoT*)-like gene lr1056 upstream of lr1057/lr1058 analogous to *S. pyogenes*, forming a putative tri-cistronic operon. The gene product of lr1056 is putatively involved in degradation or synthesis of the stringent response factor guanosine 3',5'-bisdiphosphate (ppGpp) and hence participating in cellular response changing nutritional conditions (reviewed in [11, 12]). In contrast, the genuine *relA* gene of *L. reuteri* ATCC 55730 (lr0021, EC 2.7.6.5) is significantly repressed in sourdough. The growth of the lr1057 and lr1056 mutant strains (D1057 and D1056) was attenuated in sourdough fermentation (Fig. 4), and preliminary phenotype screening revealed differences in the growth on arabinose as sole sugar for both mutant strains (data not shown). These findings indicate a significance of the TCS operon for

the growth of *L. reuteri* in sourdough, but further experiments should investigate its actual physiological role.

In sourdough, genes encoding enzymes of energy and central intermediary metabolism displayed substantial differences in expression relative to CDML. As an obligate heterofermentative organism, *L. reuteri* ferments both hexoses and pentoses via the pentose phosphate shunt, but the outcome of fermentation is dependent on available sugars and electron acceptors [27, 37]. The enzymes L-ribulose-5-phosphate 4-epimerase (lr0658), L-ribulokinase (lr0657) and phosphopentomutase (EC 5.4.2.7, lr0675) related to the pentose phosphate pathway were significantly induced during sourdough fermentation. This finding and the fact that also the gene coding for the glycolytic enzyme phosphoglycerate mutase (lr1029, EC 5.4.2.1) was induced suggested an increased metabolic activity of *L. reuteri* in sourdough. Furthermore, the gene cluster lr0838 to lr0843 encoding the alpha and beta subunits of tetrameric pyruvate dehydrogenase (PDH), dihydrolipoamide dehydrogenase (EC 1.8.1.4) and acetyltransferase (EC 2.3.1.12) displayed elevated transcription, whereas two alcohol dehydrogenases (EC 1.1.1.1) were repressed. These expression patterns corresponded to previous reports documenting the favored generation of acetate instead of lactate and ethanol by lactobacilli during sourdough fermentation [52, 77], contributing to the shelf-life and especially the sensory quality of bakery products.

The expression of genes lr1875 and lr1878 up to lr1882 encoding diol/glycerol dehydratase subunits and homologues of the *pduAB* genes, both involved in the degradation of 1,2-propanediol/glycerol, was repressed in sourdough. The diol/glycerol dehydratases are cobalamin-dependent isoenzymes (pfam02288.13), displaying two possible modes of action: glycerol dehydratases (EC4.2.1.30) catalyze the conversion of glycerol to 3-hydroxypropion-aldehyde (3-HPA, reuterin) in sugar/glycerol cofermentations [86], whereas diol dehydratases (EC4.2.1.28) convert rhamnose-derived 1,2-propanediol to propionaldehyde as described for *Lactobacillus* species like *L. reuteri*, *Lactobacillus diolivorans* and *Lactobacillus collinoides* [45, 71, 78]. These reactions are part of two different metabolic pathways, but both permit regeneration of NAD^+ resulting in an increased carbon flow through acetyl phosphate and thus greater acetate and ATP yield [17, 86]. Our results document higher transcription of the genes in chemically defined medium than in sourdough, although glycerol and rhamnose/1,2-

propanediol are not exogenously available as such in CDML. Thus, the presence of fructose as electron acceptor in sourdough presumably renders the above-mentioned pathways unnecessary or might lead to a repression of these genes.

The expression of genes involved in lipid metabolism displayed extensive repression in sourdough. Thus, strongly reduced fatty acid biosynthesis of *L. reuteri* in sourdough could be deduced. This was allegeable by the fact that CDML does not contain lipids, forcing the organism to synthesize fatty acids by itself. In contrast to carbon, lipid and energy metabolism, our microarray data proved only minor transcriptional differences of genes involved in peptide and nucleotide metabolism of *L. reuteri* under the investigated conditions. Most notably, a gene encoding a cytosolic dipeptidase (lr2006), similar to PepD of *Lactobacillus helveticus* (49% aa identities) [87], displayed higher transcript ratio RNA_{dough}/ RNA_{CDML}, and a metalloproteinase-encoding gene (lr1291) showed lower ratios.

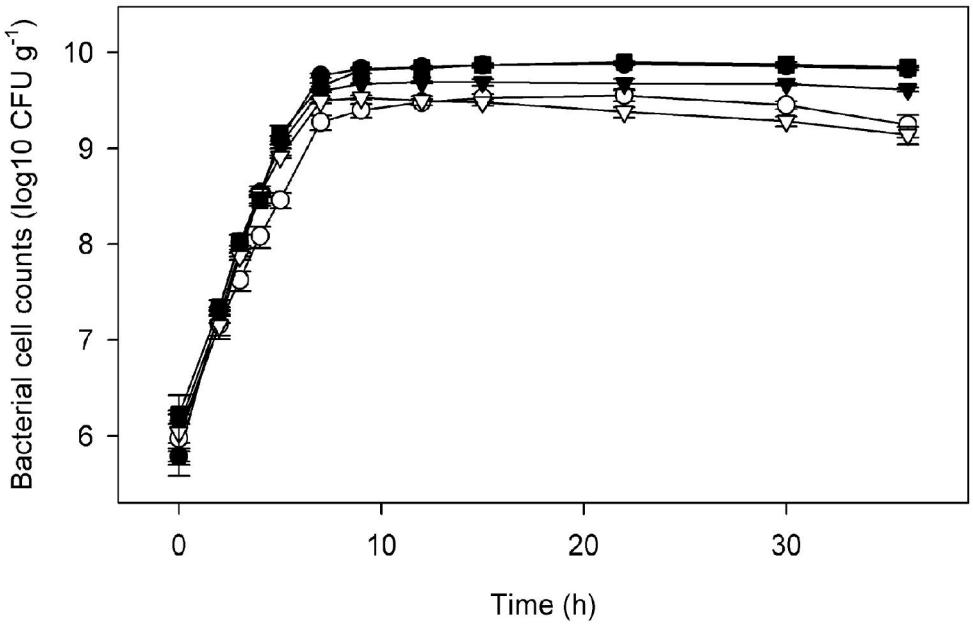

Figure 4. Growth of *L. reuteri* strains in sourdough. Cell counts of wild-type control strain *L. reuteri* D0849 (●) and isogenic mutant strains *L. reuteri* lr0677/rr2 mutant (○), D1056 (▼), D1057 (▽) and D1610 (■). Bacterial cell counts were determined by plating on MRS agar supplemented with erythromycin. Values are means of two independent experiments and one standard deviation is displayed.

Cell envelope, membrane proteins and extracellular polysaccharides. Several genes encoding cell surface proteins and enzymes involved in cell wall biosynthesis were differentially expressed between sourdough fermentation and *in vitro* conditions (Table 3). Genes of the *dltABCD* gene cluster (lr1649-lr1652), responsible for substitution of teichoic acid (TA) with D-alanine (D-ala) in diverse gram-positive bacteria [58], were repressed in sourdough. In lactobacilli, the substitution of D-ala residues of TA impacts acid resistance, autolysis or phage interaction [60, 64, 94]. Recently, a *L. plantarum* Dlt$^-$ mutant characterized by depletion of D-ala was demonstrated to possess enhanced anti-inflammatory capacity in a mouse colitis model [34] and Walter and co-workers found a Dlt$^-$ mutant of *L. reuteri* to be unable to effectively colonize the murine intestinal tract [94]. Taking into account the low pH

of sourdough fermentation, the fact that expression of the *dlt* operon of *L. reuteri* is more repressed in sourdough than in CDML cannot be explained at this point of time.

In addition, the expression of several genes encoding adhesion- and aggregation-related membrane proteins was repressed in sourdough. For example, the gene product of lr1267 displays high homology (59% aa identities) to aggregation promoting factor Apf of *L. gasseri*, a protein involved in cell shape maintenance, autoaggregation and conjugation [41, 66], and lr1897 encodes a putative homologue of EbsC of *Enterococcus faecalis* which regulates mating aggregation formation [5]. In contrast, gene lr1997, encoding a truncated homologue of the high-molecular-mass surface protein Lsp of *L. reuteri* 100-23 involved in effective colonization of the intestinal tract of mice [92], was induced in sourdough. The differential regulation of genes coding for hypothetical proteins containing the LysM peptidoglycan domain (pfam 01476), conferring binding to peptidoglycan [75], the NlpC/P60 domain (pfam 00877.13), characteristic for certain cell wall peptidases [3], a Penicillin-binding protein (lr0889) and two phosphoglycerol transferases (lr1145/lr1734, EC 2.7.8.20) further support the assumption of extensive differences of cell wall properties between both environments. Nevertheless, the growth of *L. reuteri* ATCC 55730 mutant strain D1610 with disrupted lr1610, coding for a cell surface protein containing the NlpC/P60-domain and GW repeats [4], was not negatively affected in sourdough (Fig. 4).

The TCS lr0677/lr0679 showing repression during fermentation could potentially be involved in the observed cell envelope modifications. It is related to the VanRS TCS of diverse gram-positive bacteria including *Lactobacillus sakei*, which are involved in vancomycin resistance and stress resistance [14, 57, 82]. The VanRS system coordinates incorporation of D-alanine-D-lactate (D-Ala-D-Lac) residues to teichoic acids instead of D-alanyl-D-alanine, thus decreasing the affinity for glycopeptide antibiotics. *L. reuteri* ATCC 55730 with inactivated lr0677/rr2 (T. Wall, E. Hüfner, H. Jonsson, C. Hertel and S. Roos, submitted for publication) displayed attenuated growth in sourdough fermentation (Fig. 4), thus demonstrating a contribution of the TCS for the organism's growth in sourdough.

A striking observation was the induction of a putative capsular polysaccharide (CPS) biosynthesis operon (Table 3). Putative functions of gene products and genetic organization

were similar to CPS gene clusters of lactic acid bacteria (LAB) such as *Lactobacillus delbrueckii* subsp. *bulgaricus* [47], *Lactobacillus rhamnosus* [61] and *Streptococcus* spp., and also to more distantly related bacteria like *Bacteroides* spp. and *Clostridium* spp. The first three ORFs of the operon resembled those coding for a LytR-like transcriptional regulator (lr0413), a chain length determination factor (lr0957) and a tyrosine protein kinase (lr0956), which have been shown to possess regulatory functions for exopolysacharide production of various LAB [6, 9, 43, 47]. Genes located downstream encode putative glycosyltransferases, sugar modifying enzymes and transport proteins (data not shown). Based on sequence homology, it is likely that CPS of *L. reuteri* ATCC 55730 is synthesized in a fashion similar to capsule biosynthesis of streptococci and staphylococci, and lipopolysaccharide (O-antigen) bio-synthesis in gram-negative bacteria [9, 42, 59, 95]. In this context the induction of genes involved in the methylerythritol phosphate pathway of isoprenoids synthesis (lr2138, lr1903) in sourdough fermentation suggests the elevated production of C55 UPP, allowing for increased translocation of peptidoglycan precursors as well as for activated nucleotide sugars used for EPS synthesis [67, 96]. The actual physiological function of capsular polysaccharides for the bacterial organism has been for decades the subject of discussion. Protection against adverse environments, phagocytosis or bacteriophages, sequestering of essential cations, biofilm formation, colonization, cellular recognition have been suggested (reviewed in [9, 81], and recently, it has been shown that CPS also elicits immunostimulatory effects for the host organism [13]. Thus we can only speculate about its role for *L. reuteri* in sourdough fermentation. The homology of genes in the CPS cluster of *L. reuteri* to genes of intestinal bacteria suggests genetic exchange by horizontal gene transfer in the intestinal tract. This hypothesis is supported by the fact that sequence analysis revealed numerous transposases and transposon remnants flanking the CPS genes, evidencing transfer events of as proposed for CPS loci of streptococci [7, 42].

Taking into account the repression of the *dlt* operon and induction of CPS genes, we speculate that the cell wall of *L. reuteri* growing in sourdough is characterized by reduced D-ala substitutions of TA and increased amounts of peptidoglycan-linked polysaccharides. This implies changed physico-chemical properties of the cellular envelope, a phenomenon that has been shown to be influenced by environmental changes [58].

Stress response and cobalamin biosynthesis. Interestingly, we observed differential expression of several stress-related genes, for example, the transcription of heat shock response genes *hcrA, grpE, dnaK* and *dnaJ* (lr1123-lr1127), which have been shown to be involved in response to heat, salt and acid stress of lactobacilli [84], was induced in sourdough. In contrast, the expression of two other stress-related genes (lr2108, lr1350) was repressed. The product of lr2108, the general stress protein Gls24, is important for, e.g., virulence and alkaline shock response in gram-positive bacteria [30, 46], and gene lr1350 encodes a CsbD-like stress related protein involved in either a nutritional or an osmotic protection in *Bacillus subtilis* [2, 63]. Since the fermentation parameters pH and temperature of sourdough and CDML were comparable at the time of sampling, regulation of stress-related genes as a response to acid or heat stress was considered unlikely. This assumption was supported by the lack of similarity between gene expression in sourdough and the transcriptional acid shock response of *L. reuteri* ATCC 55730 [90], thus no conclusions can be drawn about the regulation of stress-related gene expression in sourdough.

The ability to synthesize cobalamin de novo is not widely distributed among human intestinal bacteria or bacteria found in food fermentation [68]. *L. reuteri* is not only able to produce vitamin B_{12} [79], a unique property among lactobacilli that are generally characterized by multiple vitamin and amino acid auxotrophies, but also one of the few *Lactobacillus* species commonly associated with both habitats. Therefore, the organism is a potential source of cobalamin for human consumption. Since the transcription of a vast set of genes involved in cobalamin synthesis was induced in sourdough, the in situ production of vitamin B_{12} by *L. reuteri* in sourdough fermentation should be further investigated.

Comparison of IVET and microarray data. Surprisingly, very few regulated genes of *L. reuteri* in sourdough fermentation were detected by both IVET [15] and microarray hybridization, e.g. genes involved in resistance to arsenical compounds (arsenical resistance operon repressor gene lr1999, arsenate reductase family gene *ivi97*), cell envelope synthesis and a gene for a hypothetical extracellular protein (lr1863, *ivi11*). This discrepancy might be explained in several ways. Firstly, IVET and microarray analysis are two very different approaches to investigate specific gene expression with regard to methodology and approach [38, 65]. IVET relies on positive selection of active promoter-containing clones by the use of

reporter genes, in the case of the sourdough IVET a gene conferring lincomycin resistance [15], thus only genes showing elevated expression over long periods of time will be detected. In contrast, microarray hybridizations principally allow for quantifiable expression of all genes present on the array, but conclusions on gene expression can only be drawn for the time point of RNA isolation. Secondly, two different strains of *L. reuteri* were used, a sourdough isolate (IVET) and a human isolate (this study), which might display differences in gene regulation. Thirdly, the *in vitro* reference conditions used to define the borderline of "equally expressed" and "differentially regulated" were different (MRS medium for IVET, CDML for this study), which certainly influenced the results. To summarize it, both techniques should be regarded as complementary to be able to understand the adaptation of *L. reuteri* to the sourdough ecosystem. Our study provides a basis for further investigation of the organism's competitiveness, with possible implementations for the screening for novel starter organisms.

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Chapter VI

Repression of the LEE-encoded regulator gene transcription of *E. coli* O157:H7 by culture supernatants of *Lactobacillus reuteri* is LuxS and strain dependent

Ivan Jelčić[1†], Eric Hüfner[1†], Herbert Schmidt[1] and Christian Hertel[2]

[1] *Institute of Food Science and Biotechnology, Section Food Microbiology, University of Hohenheim, Stuttgart, Germany*

[2] *German Institute of Food Technology (DIL e.V.), Quakenbrück, Germany*

[†] *Both authors contributed equally to this work.*

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Abstract

Culture supernatants of *Lactobacillus reuteri* ATCC 55730 repressed *ler* expression of *Escherichia coli* O157:H7 but neither its isogenic *luxS* mutant nor the *L. reuteri* 100-23C wild-type and its *luxS* mutant elicited a comparable effect. Furthermore, epinephrine-mediated induction of *ler* expression was interfered by secreted substance(s) of *L. reuteri* ATCC 55730.

Bacterial communication by secreted signaling molecules, known as quorum sensing (QS), impacts diverse cellular processes such as virulence gene expression (reviewed in (12, 33)). Enterohemorrhagic *Escherichia coli* (EHEC) strains of serotype O157:H7, the causative agents of bloody diarrhea and hemolytic uremic syndrome in humans (11, 15), harbor QS-regulated virulence genes on a pathogenicity island termed locus of enterocyte effacement (LEE) (23, 27) that are mainly organized in the five polycistronic operons *LEE1* to *LEE5* (6, 11). The first gene in *LEE1*, *ler* (LEE-encoded regulator) encodes the principal transcriptional activator of the LEE genes (5). The expression of *ler* was shown to be controlled by the bacterial QS molecule autoinducer 3 (AI-3) and the eukaryotic catecholamine hormones epinephrine and norepinephrine, thus influencing virulence properties as attachment to host cells by cross-talk of the bacteria and the host organism (24, 25). EHEC senses both AI-3 and catecholamines through the adrenergic two-component systems QseBC and QseEF, which in turn regulate LEE and flagella gene expression via complex and not fully understood signal transduction cascades (3, 20, 26). An active LuxS synthase is the precondition of the formation of AI-3, as well as yet another QS signal, AI-2 (25). LuxS is a metabolic enzyme involved in regeneration of the methyl donor S-adenosyl methionine (SAM) in the activated methyl cycle of methionine metablism (21). While AI-2, a furanosyl borate diester, is the actual product of LuxS activity, the molecular structure and synthesis of AI-3 is unknown (25). Although LuxS does not produce AI-3 directly, a marked reduction of AI-3 activity of an EHEC *luxS* mutant has been observed, which could be restored by expression of amino acid transporters and addition of aspartate (31). LuxS homologues are widespread among both Gram-negative and Gram-positive bacteria, and AI-2/3 are therefore viewed as key mediators of intra- and interkingdom QS (12, 29). Recently, LuxS dependent induction of EHEC

O157:H7 LEE genes by *Lactobacillus reuteri* was demonstrated (28). Stationary phase supernatant of *L. reuteri* 100-23C, a rodent isolate (13), induced *ler* expression, which was abolished in an isogenic *luxS* mutant. Thus, it is very likely that the induction was mediated by AI-3-like molecules. In contrast to these findings, a recent publication showed that secreted molecules of probiotic *Lactobacillus acidophilus* La-5 led to reduced virulence gene expression of EHEC O157:H7, but whether this repression was LuxS-dependent remained undetermined (14).

The aim of the present study was the investigation of LuxS/AI-3-dependent regulation of EHEC O157:H7 virulence genes by secreted substances of *L. reuteri* strains. We constructed an isogenic *luxS* mutant of the probiotic strain *L. reuteri* ATCC 55730 (BioGaia AB, Stockholm, Sweden) and compared the effects of the supernatants of wild-type and *luxS* mutant on EHEC *ler* transcription with the effects of the corresponding strains of *L. reuteri* 100-23C. For this purpose, a novel fluorescence bioassay was developed by transcriptionally fusing the *ler* promoter with a green fluorescent protein (*gfp*) gene. The assay was validated by using supernatants of EHEC O157:H7 (AI-3 producer) and *E. coli* DH5α (natural *luxS* mutant), as well as growth medium with and without epinephrine.

Development and validation of a fluorescence bioassay detecting *ler* promoter activity.

To measure the influence of external signals on *ler* transcription, we fused the *ler* promoter of EHEC O157:H7 EDL933 with the promoterless *gfp*(ASV) located on pJBA89 (1), a plasmid originally constructed to measure acyl homoserine lactone (AHL) QS signals. To do this, the promoter sequence was amplified using primers PlerF (CTGAGAATTCTTAGAGATAC TGGCTTTCAGGAAAC; EcoRI recognition site is underlined) and PlerR (CTGACGCA TGCTTTAATATTTTAAGCTATTAGC; SphI recognition site is underlined). A fragment of pJBA89 containing *luxR* and promoters P*luxR* and P*luxI* was removed and replaced with the 0.9 kb PCR product of the *ler* promoter by digestion with EcoRI and SphI and ligation. Correct insertion was verified by sequencing of the resulting plasmid pIJ01 (5.3 kb) (data not shown). *E. coli* MG1655 (8) was transformed with pIJ01 by electroporation (4), yielding *E. coli* IJ01. The *E. coli* K-12 derivative MG1655 was chosen as host on the basis of the successful utilization of K-12 strains in gene expression studies using reporter fusions of

EHEC genes (10, 18, 22, 23, 25), although the EHEC-specific transcription regulators GrlA/R, EtrA/EivF and Pch that affect LEE gene expression are not present in a K-12 background (32).

The applicability of *E. coli* IJ01 as AI-3/epinephrine responsive reporter organism in a fluorescence bioassay was established using culture supernatants of EHEC O157:H7 strain EDL933 ($LuxS^+$, (16)) and *E. coli* DH5α ($LuxS^-$, Promega) as well as growth medium with (positive control) and without epinephrine (negative control). Supernatants were prepared by taking samples at several time-points from the *E. coli* strains growing in modified LB medium (mLB; with 4 g L^{-1} NaCl) at 37°C, removing cells by centrifugation and sterile-filtering, and adjusting the pH to 7.0 with 1 M NaOH. Prior to start the fluorescence assays, *E. coli* IJ01 was inoculated in mLB medium containing 100 µg ml^{-1} ampicillin and grown aerobically at 30°C to an $OD_{600\ nm}$ of ≤0.2. After two further sub-culturing steps at 30°C, the culture was diluted 1:20 into fresh mLB medium, in mLB containing 50 µM L-epinephrine or in *E. coli* supernatants, all pre-heated to 30°C. Aliquots (200 µl) were transferred to a microtiter plate, which was incubated under shaking at 30°C. Fluorescence (515 nm) and OD_{600} values were measured up to 5 hours using a Cary-Eclipse fluorescence reader (Varian) and a microplate reader 450 (Biorad). Regulation of *ler* was expressed as relative fluorescence by dividing the absolute fluorescence signals by the optical density (OD_{600}) of reporter strain *E. coli* IJ01. For this and all following experiments, comparable growth of strain *E. coli* IJ01 in the different supernatants was checked (OD_{600}) to eliminate cell density-dependent effects. As shown in Fig. 1A, the EDL933 supernatants of early to late stationary phase (OD_{600} 0.8 to 1.2) significantly induced relative fluorescence ($p<0.05$) of the reporter strain IJ01 as compared to mLB medium (negative control) and DH5α supernatants. Similar results have already been demonstrated for EHEC, but maximum AI-3-mediated induction of LEE genes was shown for mid-exponential culture supernatants (32). In addition, mLB with added epinephrine significantly induced relative fluorescence compared to mLB without epinephrine ($p<0.024$) (Fig. 1B). These results confirmed the applicability of the fluorescence assay for detection of *ler* expression.

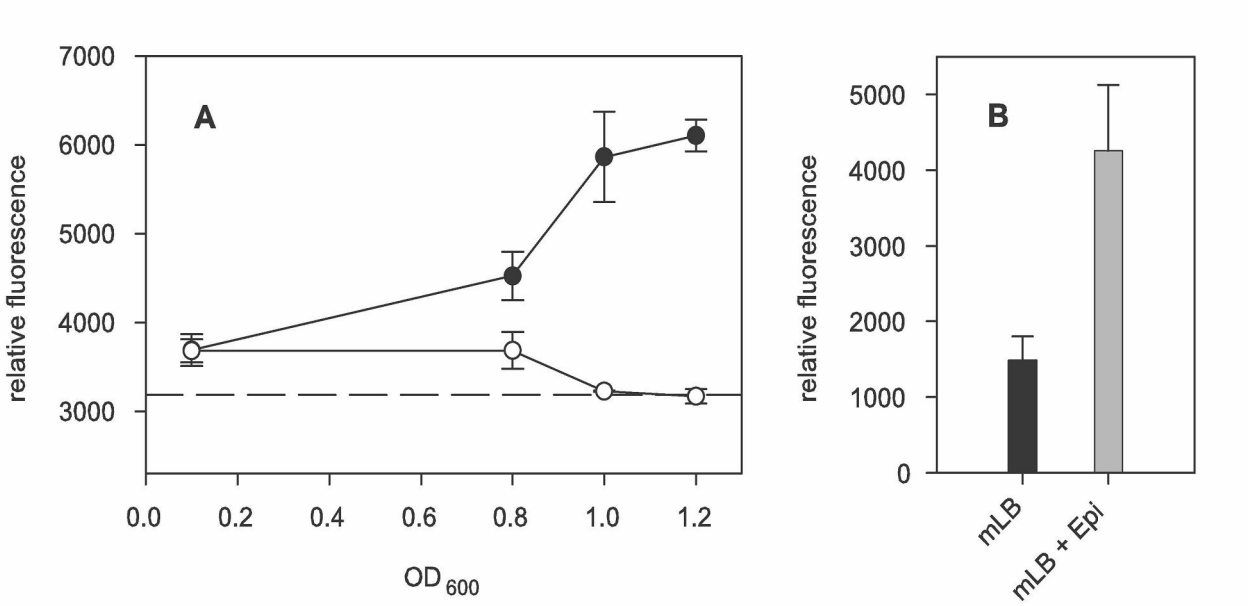

Figure 1. Validation of the *E. coli* IJ01 fluorescence bioassay measuring *ler* promoter regulation. (A) Relative fluorescence induction obtained with culture supernatants of EHEC O157:H7 EDL933 (filled circles) and *E. coli* DH5α (empty circles) of different growth phases and by mLB medium (negative control, dashed line). (B) Relative fluorescence values obtained with mLB medium (black bar) and mLB supplemented with 50 μM L-epinephrine (Epi) (gray bar). Relative fluorescence values were calculated by dividing absolute fluorescence by the corresponding cell density (OD_{600}) values. Error bars denote standard deviations of three independent experiments performed in duplicate.

Construction of the *L. reuteri* ATCC 55730 *luxS* mutant strain *L. reuteri* LTH6560.

An isogenic *luxS* mutant of strain *L. reuteri* ATCC 55730 was constructed by insertional inactivation using the suicide vector pORI28 as described before (30). An internal sequence of the *luxS* gene (bp 46 to 260 of lr0628, GeneBank accession no. DQ233673) was amplified by using primers luxSFor (TGACGAATTCTAAGCACCTTACGTTCGTTTAATTACC, EcoRI recognition site is underlined) and luxSRev (TGACGGATCCGTAATTAAGTGGAA ACCAGTCGG, BamHI recognition site is underlined), cloned into pORI28 using EcoRI and BamHI and inserted in the chromosomal *luxS* ORF by homologous recombination. The

correct localization of pORI28 in the *luxS* ORF and the singular insertion event in the chromosome of the obtained mutant strain *L. reuteri* LTH6560 was verified by PCR using primers LuxCoF (GCACCTTACGTTCGTTTAATTACC) and LuxCoR (TCCCTTCATCAA GAATCTTC) flanking the insertion site and Southern blot hybridization, respectively (data not shown).

Influence of *L. reuteri* supernatants on *ler* expression.

Culture supernatants of *L. reuteri* strains ATCC 55730, LTH6560, 100-23C (13) and 100-23C *luxS* mutant (28) were tested for the ability to influence *ler* expression in the *E. coli* IJ01 bioassay. *L. reuteri* strains were grown anaerobically in modified MRS medium (mMRS) (9) at 37°C. Supernatants were prepared from cultures of different growth phases (OD_{600} of 0.1 to 2.5) by centrifugation, pH adjustment to 7.0, and sterile-filtration. mMRS medium (pH 7.0) was used as AI-3-negative control. Prior to the start of the bioassay, *E. coli* IJ01 was cultured as described above and inoculated 1:20 into *L. reuteri* supernatants and mMRS adjusted to pH 7.0. Aliquots (200 µl) were transferred into a microtiter plate, and incubation and measurements were conducted as described for the testing of *E. coli* supernatants.

The supernatants of the *L. reuteri* strains ATCC 55730 and 100-23C with the respective *luxS* mutants exhibited different effects on *ler* expression (Fig. 2). The *L. reuteri* ATCC 55730 (wild-type) exponential phase supernatants (OD_{600} 0.1) caused lower relative fluorescence than the negative control mMRS and led to a constant decrease in fluorescence with increasing OD_{600} (Fig. 2A). At stationary phase (OD_{600} 2.5), *ler* expression was significantly repressed compared to the *luxS* mutant LTH6560 ($p<0.005$) as well as mMRS medium ($p<0.0001$). Supernatants of strain LTH6560 caused similar *ler* expression as the wild-type at OD_{600} of 0.1 and lower expression at OD_{600} of 1.0, but in stationary phase (OD_{600} of 2.5), the fluorescence exceeded the corresponding wild-type values equalling mMRS medium values. In contrast, *L. reuteri* 100-23C (wild-type) supernatants of exponential and early stationary phase (OD_{600} 0.1 and 1.0) induced *ler* expression compared to its *luxS* mutant and mMRS medium, caused a minimum expression at OD_{600} of 1.5, and induced *ler* expression at OD_{600} 2.5 compared to the *luxS* mutant and mMRS medium (Fig. 2B). The 100-23C *luxS* mutant supernatants did not induce fluorescence above the negative control with mMRS medium.

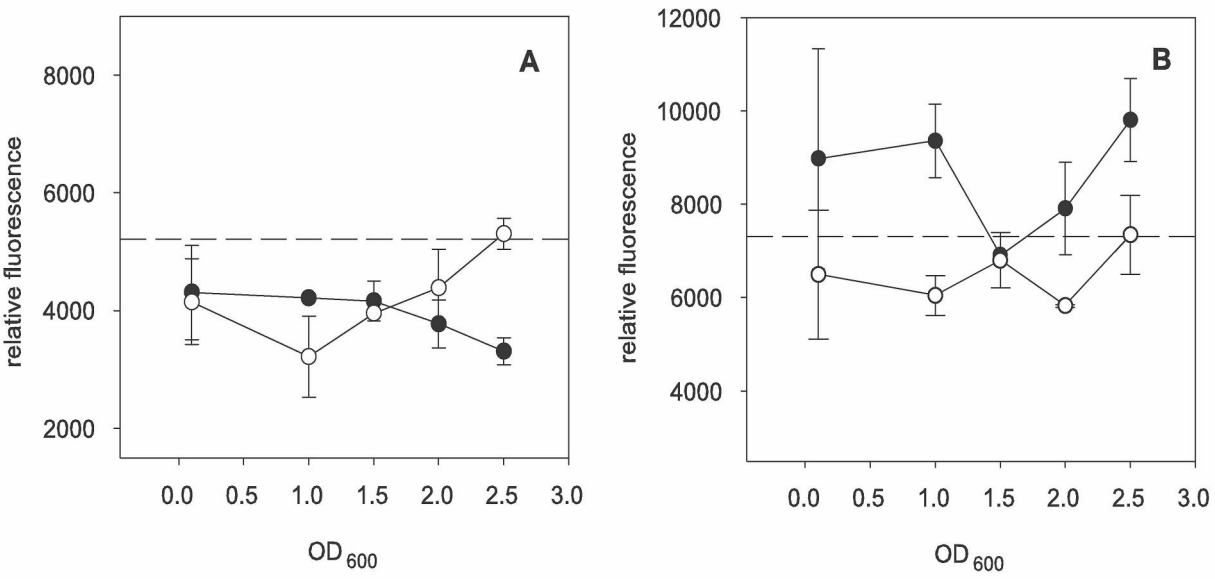

Figure 2. Influence of culture supernatants of *L. reuteri* ATCC 55730 strains (A) and *L. reuteri* 100-23C strains (B) on *ler* expression, detected by applying the *E. coli* IJ01 fluorescence bioassay. Relative fluorescence inductions obtained with supernatants of the wild-type strains (filled circles) and of the corresponding isogenic *luxS* mutants (empty circles). Dashed lines indicate mean relative fluorescence induction of the negative control mLB medium. Error bars denote standard deviations of three independent experiments performed in duplicate.

Surprisingly, the supernatants of *L. reuteri* ATCC 55730 exhibited a negative regulatory effect on *ler* expression compared to the respective *luxS* mutant and growth medium alone, whereas *L. reuteri* 100-23C supernatants induced *ler* transcription as described before (28). These results indicated a LuxS-dependent interference of AI-3-mediated QS. To further investigate the nature of repression, we added epinephrine to supernatants of *L. reuteri* grown to an OD_{600} of 2.0 and 2.5, respectively, and investigated *ler* regulation. mMRS medium (pH 7.0) containing 50 µM L-epinephrine and without epinephrine served as positive and negative control, respectively. The results depicted in Fig. 3 indicated a similar fluorescence induction for spiked 100-23C wild-type and *luxS* mutant supernatants as compared to the positive

control mMRS with epinephrine (Fig. 3B), but reduced fluorescence caused by spiked ATCC 55730 wild-type supernatants (Fig. 3A). Evidently, the full extent of epinephrine-mediated *ler* induction was constrained by secreted substances of *L. reuteri* ATCC 55730, but neither by those of its isogenic *luxS* mutant nor of *L. reuteri* 100-23C wild-type and *luxS* mutant.

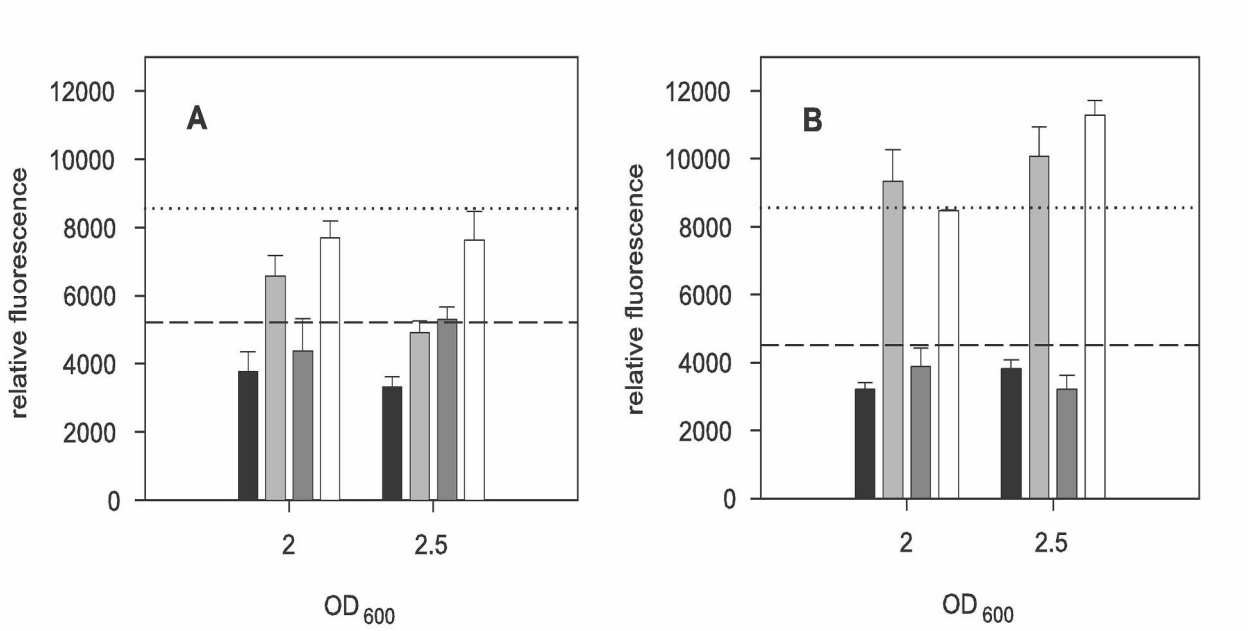

Figure 3. Impact of addition of 50 µM L-epinephrine to stationary phase supernatants of *L. reuteri* on *ler* expression. (A) Relative fluorescence obtained with *L. reuteri* ATCC 55730 (wild-type) supernatants without (black bar) and with added epinephrine (light gray bar), and with *L. reuteri* LTH6560 (*luxS* mutant) supernatants without (dark gray bar) and with added epinephrine (white bar). (B) Relative fluorescence obtained with *L. reuteri* 100-23C (wild-type) supernatants without (black bar) and with added epinephrine (light gray bar), and with *L. reuteri* 100-23C *luxS* mutant supernatants without (dark gray bar) and with added epinephrine (white bar). Dashed lines indicate mean relative fluorescence of the negative control mLB medium, and dotted lines mean relative fluorescence of mLB medium supplemented with epinephrine used as positive control. Error bars denote standard deviations of two independent experiments performed in duplicate.

On the basis of the obtained results, we propose that LuxS of *L. reuteri* ATCC 55730 is responsible, either directly or indirectly, for the production and/or secretion of molecules that negatively regulate *ler* transcription in the stationary phase (OD_{600} 2.5). Since LuxS affects the central metabolism, a mutation consequently leads to pleiotropic effects, thus no conclusion can be made as to the nature of these molecules. Interestingly, the LuxS activity differs among strains of the same species, leading to opposite effects on EHEC virulence gene transcription. A possible explanation of the mode of action would be that the secreted molecules of *L. reuteri* ATCC 55730 share structural homology with AI-3 or epinephrine and bind to the sensor kinases QseE or QseC, blocking the phosphorylation of the cognate response regulators QseF/B. This could abolish the signal transduction cascades analogous to alpha- and beta-adrenergic antagonists (25). However, a negative regulation of *ler* expression via these two-component systems has not been described to date. The facts that *ler* expression is significantly reduced by *L. reuteri* ATCC 55730 compared to medium and *luxS* mutant supernatant, and that the epinephrine stimulus does not result in full induction of *ler* transcription supports the hypothesis of competing antagonistic molecules.

The *in vivo* relevance of EHEC virulence gene repression by *L. reuteri* ATCC 55730 and the underlying principles remain to be investigated. Nevertheless, it seems a promising approach for anti-QS-based therapeutic strategies for the treatment of infectious diseases that recently have gained considerable research interest (2, 7), for example inhibition of *Pseudomonas aeruginosa* or *Staphylococcus aureus* infections by QS-interference (17, 19). Furthermore, the virulence gene repression was shown for the two strains *L. reuteri* ATCC 55730 and *L. acidophilus* La-5 that are proven probiotics, whereas the non-probiotic *L. reuteri* 100-23C does not display this ability. Whether the observed repression is a characteristic of probiotic lactobacilli should be the subject of further investigation.

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Chapter VII

Summary

Lactic acid bacteria (LAB) of the genus *Lactobacillus* represent key microbial agents for the production of fermented foods, which constitute a large part of of the human staple diet (6). Lactobacilli are highly adapted to a great variety of food environments, where the organisms´ metabolic activity is responsible for the biopreservation and the improvement of the sensorical quality of the food produced (1). Selected strains are used as industrial starter organisms, which are characterized by superior ecological competitiveness and the effective production of desired metabolites as organic acids, mainly lactic and acetic acid, and CO_2, and also of antimicrobial and aroma compounds. The ongoing sequencing of *Lactobacillus* genomes provides the basis for the in-depth investigation of the genetic and physiological adaptation of lactobacilli to the food environment with molecular tools, e.g., microarray analysis or bioinformatic comparison of whole genomes. A study of Dal Bello and co-workers was first to investigate bacterial gene expression in a food environment (3). By using *in vivo* expression technology (IVET), 38 genes of *Lactobacillus reuteri* LTH5531 were identified as induced in a type II sourdough fermentation compared to *in vitro* conditions. In Chapter III, the IVET methodology was applied to investigate gene expression of *Lactobacillus sakei* 23K during fermentation in a sausage model system. Fifteen *in carne*-induced genes were identified by screening a genomic library of *L. sakei* established in the IVET vector pEH100. Several genes encoded proteins likely to contribute to stress-related functions. One of the genes was involved in acquisition of ammonia from amino acids (*asnA2*), and the remaining were either part of functionally unrelated pathways or encoded hypothetical proteins. The construction and use of isogenic mutants in the sausage model demonstrated that four genes have an impact on the performance of *L. sakei* during raw sausage fermentation. Inactivation

of the heat shock repressor gene *ctsR* resulted in increased growth, whereas knock-out of the gene *asnA2*, the putative metallo-beta-lactamase gene LSA1065 and the gene LSA1194 encoding a putative membrane protein resulted in attenuated performance when compared to the wild-type strain. This study established a molecular basis which allows the investigation of bacterial properties that contribute to the ecological performance of the organism and to influence the final outcome of sausage fermentation.

On the basis of the insights obtained by using IVET, a strategy to improve sausage fermentation was developed, as described in Chapter IV. The fact that a class III heat shock repressor (*ctsR*) mutant strain displayed accelerated growth led to the assumption that induction of stress response mechanisms might lead to improved sausage fermentation. The class III heat shock system comprises several molecular chaperones and protease complexes involved in the degradation of misfolded or otherwise damaged proteins that occur in the bacterial cell under many stress conditions (4). Sublethal stress treatments of *L. sakei* 23K with cold, salt and heat stress were performend prior to inoculation of the sausage batter in order to investigate the effect on growth and acidification of *L. sakei* 23K. The pH values of sausages fermented with stressed cells attained defined threshold values in distinctly shorter time than those inoculated with unstressed cells. More precisely, the cold-stressed cells reduced the pH to 5.0 within approximately 40 h as compared to approximately 70 h for untreated cells. This enhanced acidification activity of the cold-stressed cells was consistent with an increased growth rate. Additional growth studies in culture medium indicated that the presence of curing salt, the major stressors at the beginning of sausage fermentation, is not alone responsible for increased growth and acidification of stress-treated cells. The results presented in this chapter demonstrate that pre-inoculation stress treatment constitutes a promising way to improve the effectiveness of meat starter lactobacilli.

In Chapter V, the gene expression of *Lactobacillus reuteri* ATCC 55730 during type II sourdough fermentation was investigated. Whole-genome microarrays were utilized to measure relative mRNA levels allowing to compare the transcriptome from *L. reuteri* cells growing in sourdough to that of cells growing in chemically defined medium. Significant differences of gene transcripts were found for 101 genes involved in diverse cellular processes, e.g., carbohydrate and energy metabolism, cell envelope biosynthesis, exopoly-

saccharide production, stress responses, signal transduction and cobalamin biosynthesis. The utilization of starch and non-starch carbohydrates, the remodeling of the cell wall characterized by reduced D-alanylation and increased amounts of cell wall-associated polysaccharides, and regulatory function of two-component systems for cell wall biogenesis and metabolism are suggested by the gene expression data as being important for growth in sourdough. In addition, an impact of several genes of *L. reuteri* for effective growth in sourdough was shown by implementation of mutant strains in sourdough fermentation. This study contributes to the understanding of the molecular fundamentals of *L. reuteri´*s ecological competitiveness, and provides a basis for further exploration of genetic traits involved in adaptation to the food environment. Furthermore, the gene expression data adds to the findings of Dal Bello and co-workers (3), obtained by using IVET to identify induced genes of *L. reuteri* in sourdough fermentation. Due to the radically different approach and methodology of IVET and microarray analysis, the obtained insights are completive for the understanding of the genetic adaptation of *L. reuteri* to the sourdough environment

Certain LAB, including numerous *Lactobacillus* strains, are considered probiotic organisms that exert health benefits for humans and animals (6). In addition to immune-stimulatory effects and activity against immune-mediated intestinal inflammation (2, 7), probiotic organisms have been demonstrated to counteract gastrointestinal infections by pathogenic microorganisms (9). Several mechanisms such as competitive exclusion, the production of antimicrobial substances and the prevention of pathogen attachment to intestinal epithelial cells are responsible for the prevention of an infection. The phenomenon of quorum sensing (QS) has been shown to regulate pathogenesis mechanisms of bacteria like *Escherichia coli* O157:H7 or *Salmonella* spp. via a universal bacterial signal mechanism, the LuxS QS (10). Recently, a repression of virulence gene expression of *E. coli* O157 has been demonstrated for the probiotic strain *Lactobacillus acidophilus* La-5 (8). In Chapter VI, the influence of LuxS-dependent secreted molecules of two *L. reuteri* strains on the expression of *E. coli* O157 virulence regulator gene *ler* was investigated. By using a *ler* promoter fusion with the green fluorescent protein gene *gfp*, a repression of *ler* transcription could be shown for supernatants of the probiotic strain *L. reuteri* ATCC55730, but not for the rodent isolate *L. reuteri* 100-23C. Furthermore, induction of *ler* expression by the catecholamine hormone L-epinephrine, was interfered by secreted substances of *L. reuteri* ATCC 55730. By the use of *L. reuteri*

LuxS mutant strains, it was shown that the observed *ler* repression is LuxS QS as well as strain dependent. The questions about the *in vivo* relevance of virulence gene repression by probiotic lactobacilli and the nature of the active substances are of great interest for the investigation of bacterial QS, and should be adressed by subsequent studies. QS interference represents a promising approach for anti-QS-based therapeutic strategies for the treatment of infectious diseases that recently have gained considerable research interest (5).

The studies of the present thesis provide extensive insights into the ecological adaptation of lactobacilli to food fermentation environments on a whole-genome scale. The exploration of niche-specific global gene expression represents a considerable progress of the state of knowledge of these technologically important organisms, with implications for the enhancement of the selection criteria and improvement of starter organisms.

Zusammenfassung

Milchsäurebakterien des Genus *Lactobacillus* sind wichtige Mikroorganismen für die Herstellung von fermentierten Lebensmitteln, welche einen bedeutenden Teil der menschlichen Ernährung darstellen (6). Laktobazillen sind an eine Vielzahl von Lebensmitteln angepasst und bewirken durch ihre Stoffwechselaktivität die Haltbarmachung und Verbesserung der sensorischen Eigenschaften der Produkte (1). Ausgewählte Stämme, die sich durch effektive Produktion von gewünschten Metabolite wie organischen Säuren, hauptsächlich Milch- und Essigsäure, von antimikrobiell wirksamen Substanzen sowie Aromakomponenten auszeichnen, werden als Starterkulturen für industrielle Fermentationen eingesetzt. Die fortschreitende Sequenzierungen von *Lactobacillus*-Genomen ermöglicht die Erforschung der genetischen und physiologischen Adaptation von Laktobazillen an das Lebensmittelumfeld mit Hilfe molekularbiologischer Methoden, z.B. Microarray-Analysen oder bioinformatische Genomanalysen. Eine Studie von Dal Bello *et al.* untersuchte als erste die Genexpression eines Mikroorganismus im Lebensmittel (3). Mittels *in vivo* expression technology (IVET) wurden 38 Gene von *Lactobacillus reuteri* LTH5531 identifiziert, deren Expression in einer Typ II-Sauerteigfermentation im Vergleich zu *in vitro*-Bedingungen induziert war. In Chapter III wurde die Genexpression von *Lactobacillus sakei* 23K während

der Rohwurstfermentation mit einem neuartigen IVET-System untersucht. Fünfzehn *in carne*-induzierte Gene konnten durch Screening einer *L. sakei*-Genombibliothek in einem Rohwurst-Modell identifiziert werden, die unter Verwendung des IVET-Vektors pEH100 erstellt worden war. Einige Gene codierten für Proteine, die sehr wahrscheinlich eine Rolle bei der bakteriellen Stressantwort spielen. Das Gen *asnA2* war für die Gewinnung von Ammonium aus Aminosäuren verantwortlich, und die restlichen Gene waren entweder Teil weiterer Stoffwechselwege, oder codierten für hypothetische Proteine. Durch die Herstellung isogener Mutantenstämme konnte die Bedeutung von vier Genen für das effiziente Wachstum während der Rohwurstfermentation demonstriert werden. Während die Inaktivierung des Hitzeschock-Regulatorgens *ctsR* in gesteigertem Wachstum resultierte, führten der Knock-out des Gens *asnA2*, des putativen Metallo-beta-Lactamase-Gens LSA1065 und des putativen Membran-protein-Gens LSA1194 zu gehemmtem Wachtum der Mutantenstämme im Vergleich zum Wildtyp-Stamm. Die Erkenntnisse dieser Studie stellen die Basis für eine weitere Erforschung der genetischen Grundlagen der ökologischen Wettbewerbsfähigkeit von *L. sakei* dar.

Aufgrund der Ergebnisse, die durch IVET gewonnen wurden, konnte eine Strategie zur Verbesserung der Rohwurstfermentation mit *L. sakei* entwickelt werden. Die Beobachtung, dass ein Mutantenstamm mit inaktiviertem Klasse-III Hitzeschock-Repressor beschleunigtes Wachstum während der Fermentation zeigte, führte zu der Annahme, dass die Induktion von Stressantwort-Mechanismen zu verbesserter Fermentation führen könnte. Das Klasse-III Hitzeschock-System umfasst mehrere Chaperone und Protease-Komplexe, die für die Degradation fehlgefalteter oder anderweitig beschädigter Proteinen verantwortlich sind, welche in der bakteriellen Zelle bei Stresseinwirkung akkumulieren (4). *L. sakei* 23K wurde vor der Inokulation in die Wurstmasse sublethalen Stress-Behandlungen mit Hitze-, Kälte- und Salz-Stress ausgesetzt. Rohwürste, die mit Kälte-behandelten Zellen fermentiert wurden, erreichten definierte pH-Grenzwerte in wesentlich kürzerer Zeit als solche, die mit ungestressten Zellen inokuliert wurden. Kälte-behandelte Zellen reduzierten den pH-Wert auf 5,0 in ungefähr 40 h, während unbehandelte Zellen ungefähr 70 h zum Erreichen dieser Grenze benötigten. Die gesteigerte Säuerungsleistung der Kälte-behandelten Zellen korrelierte mit einem beschleunigten Wachstum. Durch zusätzliche *in vitro*-Wachstumsversuche in Kulturmedium konnte gezeigt werden, dass das schnellere Wachstum und Ansäuern der Stress-behandelten Zellen nicht alleine durch eine verbesserte Toleranz von Nitrit-Pökelsalz,

dem Haupt-Stressfaktor während der Anfangsphase der Fermentation, begründet ist. Die erzielten Ergebnisse zeigen, dass Prä-Inokulations-Behandlungen mit Stressfaktoren eine vielversprechende Möglichkeit zur Effizienzsteigerung von Rohwurst-Starterorganismen darstellen.

In Chapter V wurde die Genexpression von *Lactobacillus reuteri* ATCC 55730 während einer Typ II-Sauerteigfermentation untersucht. Zur Ermittlung der relativen Transkriptmengen wurden Microarrays verwendet, die nahezu das gesamte Genom des Bakteriums erfassten. Dies ermöglichte einen Vergleich des Transkriptoms von *L. reuteri* während des Wachstums in Sauerteig und in chemisch-definiertem Medium. Für 101 Gene konnten signifikante Änderungen der Transkriptmengen detektiert werden. Die Genprodukte hatten Funktionen im Kohlenhydrat- und Energiestoffwechsel, der Biosynthese der Zellhülle, der Produktion von Exopolysacchariden, der Stress-Antwort, der Signal-Transduktion und der Cobalamin-Biosynthese. Die erhaltenen Erkenntnisse belegen tiefgreifende Unterschiede der bakteriellen Genexpression zwischen dem Wachstum in Sauerteig und *in vitro*-Wachstum, und ermöglichten eine Beschreibung der relevanten Stoffwechselwege und Regulations-mechanismen, die für die Adaptation von *L. reuteri* an das Habitat Sauerteig notwendig sind. Die Relevanz von zellulären Prozessen wie der Verwertung von Stärke und Nicht-Stärke-Polysacchariden, der Umgestaltung der Zelloberfläche durch reduzierte D-Alanyl-Reste und gesteigerter Produktion von Zellwand-assoziierten Polysacchariden, sowie der regulativen Funktion von Zwei-Komponenten-Systemen, die an der Zellwand-Biosynthese beteiligt sind, konnte durch die Transkriptomanalyse belegt werden. Durch die Verwendung von Mutanten-Stämmen wurde ausserdem die Wichtigkeit einzelner Gene für effektives Wachstum in Sauerteig demonstriert. Die Genexpressions-Daten ergänzen des Weiteren die Ergebnisse von Dal Bello *et al*. (3), die durch die Anwendung von IVET erzielt wurden. Aufgrund der völlig unterschiedlichen methodischen Eigenschaften von IVET und Microarray-Analysen ergänzen sich die Ergebnisse beider Studien hinsichtlich des Verständnisses der genetischen Adaptation von *L. reuteri* an das Habitat Sauerteig.

Einige Stämme von Milchsäurebakterien, darunter zahlreiche *Lactobacillus*-Stämme, stellen Probiotika dar, die positive Effekte für die Gesundheit von Menschen und Tieren bewirken können (6). Neben immunstimulatorischen Effekten und Aktivität gegen immun-vermittelte

intestinale Entzündungen (2, 7) weisen probiotische Organismen prophylaktisches und therapeutisches Potential bei gastrointestinalen Infektionen durch pathogene Mikroorganismen auf (9). Mechanismen wie kompetitive Verdrängung, die Produktion von antimikrobiellen Substanzen und Verhinderung der Anheftung von Pathogenen an das Darmepithel sind verantwortlich für die Prävention von Infektionen. Das Phänomen "Quorum Sensing" (QS) reguliert Pathogenitäts-Mechanismen von *Escherichia coli* O157 oder *Salmonella* spp. durch einen universellen bakteriellen Signalmechanismus, das LuxS QS (10). Kürzlich wurde eine Virulenzgen-Repression von *E. coli* O157 durch den probiotischen Stamm *Lactobacillus acidophilus* La-5 nachgewiesen (8). In Chapter VI wurde der Einfluss von LuxS-abhängigen sekretierten Substanzen von zwei *L. reuteri*-Stämmen auf die Expression des Virulenzgen-Regulatorgens *ler* von *E. coli* O157 untersucht. Mittels einer *ler*-Promotorfusion mit dem Green Fluorescent Protein-Gen *gfp* konnte eine Repression der *ler* Transkription durch den Überstand des probiotischen Stammes *L. reuteri* ATCC 55730 nachgewiesen werden, jedoch nicht für den Überstand des Rattenisolats *L. reuteri* 100-23C. Des Weiteren wurde die Induktion der *ler*-Expression durch das Katecholamin-Hormon L-Epinephrin durch sekretierte Substanzen von *L. reuteri* ATCC 55730 gehemmt. Zusätzlich konnte durch den Einsatz von *L. reuteri-luxS*-Mutantenstämmen gezeigt werden, dass die *ler*-Repression sowohl LuxS abhängig als auch stammspezifisch ist. Obwohl die *in vivo*-Relevanz der *ler*-Repression durch Probiotika noch ungeklärt ist, stellt QS-Interferenz einen vielversprechenden Ansatz zur Entwicklung von Anti-QS-basierten Therapieansätzen zur Behandlung und Prophylaxe von Infektionskrankheiten dar (5).

Die Studien der vorliegenden Dissertation liefern tiefgreifende Einsichten in die ökologische Anpassung von Laktobazillen an das Habitat Lebensmittel. Die Erforschung der habitatspezifischen globalen Genexpression stellt eine bedeutende Erweiterung des aktuellen Wissensstandes über diese technologisch bedeutenden Organismen dar, und liefert Grundlageninformationen für die Verbesserung von Starterorganismen beziehungsweise für die Suche nach neuen Selektionskriterien.

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Selbständigkeitserklärung

Hiermit versichere ich, die vorliegende Dissertation eigenständig und ausschließlich unter Verwendung der angegebenen Quellen und Hilfsmittel angefertigt, und wörtlich oder inhaltlich übernommene Stellen als solche gekennzeichnet zu haben. Die vorliegende Arbeit wurde bisher weder im Inland noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde als Dissertation vorgelegt.

Eric Hüfner

Curriculum vitae / Lebenslauf

Name	Eric Hüfner
Adresse	Zur Sieg 8, D-53773 Hennef
Geburtstag	19. April 1976
Geburtsort	Hanau am Main
Familienstand	ledig
1982 – 1986	Johannes-Gutenberg-Grundschule Hainburg
1986 – 1990	Kreuzburgschule Hainburg
1990 – 1995	Einhard-Gymnasium Seligenstadt
1995	Abitur
1996 – 2002	Studium der Ernährungswissenschaften an der Justus-Liebig-Universität Gießen
2001	Diplomarbeit am Institut für Medizinische Mikrobiologie, Universitätsklinikum Gießen
2001	Projektarbeit am Laboratory for Food Microbiology, Wageningen Agricultural University, Niederlande
10/2002	Diplom Ernährungswissenschaften
05/2003	Beginn der Dissertation an der Universität Hohenheim
2007	Projektingenieur am Central Laboratory Unilever Switzerland (CLUS), UNILEVER Schweiz GmbH, Thayngen
seit 05/2008	Wissenschaftlicher Mitarbeiter der Jennewein Biotechnologie GmbH, Rheinbreitbach

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