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Factors contributing to the competitiveness of *Lactobacillus reuteri* in sourdough and rodent gut

by

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Abstract

Lactobacillus reuteri is a common organism in cereal-based foods and a gut symbiont in humans and animals, yet the molecular mechanisms allowing its persistence in various niches are not well understood. *L. reuteri* LTH2584 produces reutericyclin and persists in industrial sourdoughs, where acidic conditions during fermentation cause acid stress to organisms. Another strain, *L. reuteri* 100-23, colonizes the murine forestomach, where this type of lactobacilli contributes to digesta preservation through acid production. *L. reuteri* LTH2584 and 100-23 were studied to gain an understanding of the phylogenetic relationship between these two isolates, as well as the acid resistance mechanisms and the two-component systems that contribute to the persistence of *L. reuteri* in sourdough and the murine gut. Analysis of genomic content revealed a close evolutionary relationship between the *L. reuteri* isolates from sourdough and rodent gut. In addition, a novel double crossover method was developed to generate isogenic deletion mutants for the evaluation of competitiveness, while the acid resistance mechanism was assessed by disruption of glutamate decarboxylase (*gadB*) in *L. reuteri* 100-23. Glutamate decarboxylase enhanced the ability of *L. reuteri* to adapt to both acidic environments (*in vitro*) and sourdough fermentations (*in vivo*) by decarboxylation of glutamate to γ-aminobutyric acid. Biofilm formation may depend on the cross-communication of the *hk430* and *cemAKR* operons. Several osmoregulatory genes that may also be associated with cell envelope architecture and cell morphology were also identified.

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List of Symbols and Abbreviations

A	Absorbance
ABC	ATP-binding cassette
ADI	Arginine deiminase
AIP	Auto-inducing peptide
Amp	Amplicillin
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BAGEL	Bacteriocin genome mining tool
BLASTP	Basic local alignment search tool using a protein query against protein database
bp	Base pair
cDNA	Complementary DNA
CFU	Colony-forming unit
CGH	Comparative genomic hybridization
CRISPR	Clustered regularly interspaced short palindromic repeats
Cy	Cyanine
DNA	Deoxyribonucleic acid
DM	Dried mass
DGGE	Denaturing gradient gel electrophoresis
EDTA	Ethylenediaminetetraacetic acid
Erm	Erythromycin
GABA	γ-aminobutyric acid
g	Gram
glu	Glucose
GG	Double-glycine
HK	Histidine kinase
HCl	Hydrogen chloride

h	Hour
HMDS	Hexamethyldisilazane
HPLC	High performance liquid chromatography
IS	Insertrion sequence
IVET	*In vivo* expression technology system
JGI	Joint Genome Institute
KEGG	Kyoto Encyclopedia of Genes and Genomes
kb	Kilobase
KO	Knockout
L	Liter
LB	Luria-Bertani broth
M	Molar
Mb	Mega bases
mg	Milligram
MIC	Minimal inhibitory concentration
min	Minute
mL	Milliliter
mM	Millimoles per liter
mMRS	Modified deMan-Rogosa-Sharpe medium
Mol	Mole
MRS	deMan-Rogosa-Sharpe medium
N	Nucleotide
NAD	Nicotinamide adenine dinucleotide
OD	Optical density
ORF	Open reading frame
p	Plasmid
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction

qPCR	Quantitative real time polymerase chain reaction
RE	Restriction enzyme
RLF	Reconstituted-lactobacillus-free
RNA	Ribonucleic acid
RR	Response regulator
SD	Standard deviations
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscope
SER	Sourdough extract rye
suc	Sucrose
TCS	Two-component systems
TEM	Transmission electron microscope
UPGMA	The unweighted pair-group method with arithmetic means
UV	Ultraviolet
v	Volume
vol	Volume
w	Weight
WGS	Whole genome shotgun
x g	Times gravity
µL	Microliter

Chapter One: **Introduction**

1.1 Overview of *Lactobacillus reuteri*

Lactobacilli are members of the intestinal microbiota of humans and animals (1). In food production, lactobacilli have been employed in food fermentation since the onset of agriculture (1). Currently, lactobacilli are commonly used as starter cultures in food. Increasing recognition by the scientific community of the relevance of lactobacilli resident and intestinal microbiota has also increased the use of lactobacilli as probiotic cultures to improve host health. There is only a limited overlap between the species of lactobacilli that are used in food fermentation, those that are used as probiotic cultures, and those that occur in the gastrointestinal tracts of humans and animals, but *Lactobacillus reuteri* is one species of lactobacilli that fulfills all these roles. It is present in the intestines of human and animals (2), is used in food production (3), and is used as probiotic (4). *Lactobacillus reuteri* is found in a wide range of cereal fermented foods such as sourdough and fermented porridge (3, 5, 6). Several *L. reuteri* strains isolated from humans modulate host immune response (4, 7). Recently, studies of host-microbiota symbiosis demonstrate a co-evolution between *L. reuteri* and their vertebrate hosts (2, 8). *L. reuteri* is thus well-suited to be a model organism for the comparison of genetic and metabolic traits important for competitiveness in intestines and food ecosystems. Additionally, *L. reuteri* can be used to assess potential probiotic abilities. The availability of molecular tools and genome sequences of *L. reuteri* facilitate elucidation of adaptation to growth and survival in hosts or in cereal-based products by *L. reuteri*. This introduction aims to

provide a brief overview of the ecology of *L. reuteri* in sourdough and in the murine gastrointestinal tract.

1.2 Ecology of *L. reuteri* sourdough isolates

The major nutritional components of dough for microorganisms include starch, sucrose, and gluten proteins (3, 9). Competitiveness of lactobacilli in sourdough is associated with carbohydrate fermentation, regeneration of reduced cofactors, and proteolysis (for reviews, see 3 and 10). Sucrose constitutes approximately 1% of the major sugars in cereals (9). The acidification of dough initiates hydrolysis of polysaccharides and activates endogenous cereal proteases to liberate maltose, peptides, and amino acids that are further used by bacteria. Starch degradation results in maltose becoming the major carbohydrate in sourdough. Because maltose is the preferred carbohydrate for most cereal-associated lactobacilli, the microbial ecology of sourdough is a synergism between maltose-negative yeasts and maltose-fermenting lactobacilli (11).

In artisanal and industrial sourdough fermentations, stable microbiota are established by continuous back-slopping (refreshment) of sourdough type I and II. Type II sourdough has been used as a baking enhancer in industrial processes for forty years (12). Type II sourdough is semi-liquid dough fermented for several two- to five-day periods at temperatures above 37ºC (13), which accelerates bacterial growth and elevates acid production by *L. reuteri*. Studies in microbial ecology have monitored bacterial population dynamics in sourdough fermentation using PCR-denaturing gradient gel electrophoresis (14). These studies of *L. reuteri* in type II sourdough fermentation have established the ecological factors

that affect fermentation, such as temperature, dough yield, dough refreshment, ionic strength, and microbial products. Because of the acidic environment in the late stage of sourdough fermentation, *L. reuteri* sourdough isolates LTH2584, LTH5331, LTH5448, and TMW1.106 have developed several mechanisms to adapt to highly acidic ecosystems (15, 16, 17, 18).

L. reuteri LTH2584 was isolated from type II sourdough, a German Sourdough Extract Rye (SER) sourdough in 1988 (12), and found to produce reutericyclin, which is an antimicrobial tetramic acid-derivative (16). Its antimicrobial activity targets Gram-positive bacteria, including both spoilage organisms and pathogens such as *Staphylococcus aureus*, *Bacillus cereus*, and *Listeria monocytogenes*. Reutericyclin is produced in sourdough and believed to allow the persistence of *L. reuteri* throughout the continuous production of industrial sourdough (17).

L. reuteri generally uses sucrose as a substrate for the production of exopolysaccharides such as glucan (glucose) and fructan (fructose) to cope with acid stress (19, 20). The formation of exopolysaccharides in *L. reuteri* TMW1.106 and LTH5448 is mediated by glucansucrase (*gtfA*) and fructansucrase (*ftfA* or *inu*) (Table 1-1, 18). *L. reuteri* TMW1.106 synthesizes large amounts of glucan and gluco-oligosaccharides through the expression of glucansucrase (*gtfA*), and small amounts of fructan and fructan-oligosaccharides through the expression of inulosucrase (*inu*). Strain LTH5448 only forms fructan and fructan-oligosaccharides from sucrose through the expression of levansucrase (*ftfA*). Fructan and fructan-oligosaccharides can protect bacterial membranes from

Table 1-1 Genes of *Lactobacillus reuteri* that have been known or suggested to contribute to the competitiveness in sourdough or the gastrointestinal tract

Gene	Protein encoded	Strain	Origin	Physiological features	Ref.
gtfA	Glucansucrase	TMW1.106	Sourdough	Production of EPS and bacterial biofilm formation	31
inu	Fructansucrase	TMW1.106	Sourdough	Production of EPS and bacterial biofilm formation	31
ftfA	Fructansucrase	LTH5448	Sourdough	Production of EPS	31
ftf	Fructansucrase	100-23	Rat	Production of EPS, biofilm formation and immunomodulation	32
lsp	Large surface protein	100-23	Rat	Adherence to the murine forestomach epithelium	30
msrB	Methionine sulfoxide reductase B	100-23	Rat	Reduction of oxidized methionine residues, resistance to nitric oxide produced by epithelial cells	30
met	Methionine synthase II	100-23	Rat	No effect on ecological performance	30
xylA	Xylose isomerases A	100-23	Rat	No effect on ecological performance	30
dltA	D-Alanine-D-alanyl carrier protein ligase (Dcl)	100-23	Rat	Acid resistance and resistance to defensins, biofilm formation, and adhesion	33
luxS	Quorum sensing AI-2	100-23	Rat	Formation of biofilms by Gram-positive bacteria	34
mub	Mucus-binding protein	ATCC 53608	Pig	Adhesion to mucus	35
cyuC (also referred to as *bspA*; *mapA*; *cnBP*; *p29Cnb*)	Cystine transporter, ATP-binding cassette transporter, collagen binding protein, and mucus adhesion-promoting binding protein	JCM1081, 104R, BR11	Chicken, pig, and guinea pig	Cystine-mediated oxidative defense; adhesion to human HT-29 cell lines; adhesion to mucus; inhibition of the adhesion of *Staphylococcus aureus* to surfaces	36, 37, 38, 39, 40, 43
cgl	Cystathionine gamma-lyase	BR11	Guinea pig	A factor required for cystine-mediated oxidative defense	44
pdu/*cbi*/*cob*/*hem* cluster		CRL1098, KCTC 3594, and JCM 1112^{T}	Sourdough and human isolates	Biosynthesis of reuterin and cobalamin (vitamin B_{12}); glycerol utilization; propanediol fermentation	45, 46, 47, 48

stresses induced by antibiotics, membrane permeable compounds, and high temperature (21). Exopolysaccharides not only protect *L. reuteri* from acid stress in sourdough, but also reduce agglutination of enterotoxigenic *Escherichia coli* to porcine erythrocytes (22). Moreover, the fructan and fructan-oligosaccharides are not digestible but can be utilized by *Bifidobacterium* (23), which enhances the functionality of exopolysaccharide as a prebiotic.

Transcriptional analysis of the type II sourdough isolate L. reuteri LTH5531 identified 29 genes that were highly enriched during sourdough fermentation using *in vivo* expression technology (IVET) (15). These genes are related to metabolism of amino acids and nucleotides; transportation of protein and peptides; transcription and translation; and cell envelope synthesis. Interestingly, an arginine/ornithine antiporter and two glutamine converting proteins are induced during sourdough fermentation, implying that *L. reuteri* has adapted to harsh acid stress by expressing genes coding for an arginine deiminase pathway and glutamine metabolism (24).

1.3 Ecology of *L. reuteri* rodent isolates

The rodent stomach is comprised of two sections: the forestomach, which is lined with cornified non-secretory squamous epithelium, and the glandular portion, which is filled with secretory epithelium that secretes gastric acid. Following food consumption, the feed can be stored in the rodent forestomach for more than 20 hours. During this time, the pH of digesta is 4 to 5 (25), which is optimal for starch hydrolysis that results in the production of maltose and oligosaccharides. *L. reuteri* 100-23, which was isolated from a rat, can colonize

the forestomach of the reconstituted-lactobacillus-free (RLF) mice (26). The abundant supplies of di- and oligo-saccharides therefore provide a suitable substrate for *L. reuteri*.

The RLF mouse model was generated by Tannock *et al.* (27) to assess the effects of lactobacilli on the murine intestine. Different from conventional mice, the RLF mice possess a functional complex intestinal microflora with the exclusion of lactobacilli. Lactobacilli colonize the gastrointestinal tract of mice immediately following birth, and become persistent resident microflora of the gastrointestinal tract. Reconstitution of the RLF mice with defined strains of lactobacilli has allowed for evaluation of the influence of lactobacilli on host physiology.

Moreover, strain-specific traits of lactobacilli necessary for successful colonization of mice can be determined. *L. reuteri* 100-23 adheres to the forestomach but not other secretory epithelia in the gastrointestinal tract of mice. The population of *L. reuteri* 100-23 found in the jejunum and cecum is suggested to originate from the population in the forestomach (30).

The IVET system was initially developed for the identification of the virulence factors of *Salmonella enterica* serovar Typhimurium in mice (28). This promoter-based IVET system was adapted to identify *in vivo* expressed genes in complex environments. Three induced genes of *L. reuteri* 100-23 in the RLF-mice were identified and characterized using the IVET system (29) (Table 1-1). A large surface protein (Lsp) and methionine-sulfoxide reductase B (MsrB) are involved in the adhesion and biofilm formation processes, although the *mrsB*-deficient

mutant does not lose its ecological competitiveness completely (30). Mutation of the other *in vivo* over-expressed genes, xylose isomerases A (*xylA*) and methionine synthase II (*met*), did not decrease the competitiveness of the mutants in competition with the wild-type strains (30). Interestingly, the insertional disruptions of either *gtfA* or *inu* in *L. reuteri* have different impacts on the strain's ecological performance. GtfA appears to be a relevant factor for the glucan synthesis that provides the extracellular biofilm matrix. Inu, in contrast, plays a role as a glucan-binding protein. When the *gtfA*-deficient mutant is cultured with the glucan-producing strain, it maintains the ability to form a biofilm on the surface of the forestomach, while the *inu*-deficient mutant loses its ability to interact with glucan and fails to colonize in the gut of mice (Table 1-1, 31). The role of fructansucrase in colonization was confirmed with the rodent isolate *L. reuteri* 100-23 and a cognate *ftf*-deficient mutant (32). Moreover, *L. reuteri* 100-23 was shown to increase the proportions of regulatory T cells (Foxp3+) in the spleen of mice, whereas the *ftf* mutant did not (32). Other characterized genes related to biofilm formation include *dltA*, which encodes D-Alanine-D-alanyl carrier protein ligase (involved in the development of cell envelope structure) (33); *luxS*, which encodes AI-2 (involed in quorum sensing) (34); *mub*, which encodes a mucus-binding protein (involved in adhesion) (35), and *mapA*, which encodes a mucus adhesion-promoting protein (involved in adhesion) (36). The adhesive activity of Mub is variable among different isolates of *L. reuteri* (35). However, MapA, a cystine transporter, is the same protein as BspA/CyuC, p29CnB, and CnBP, and is required for cystine-mediated oxidative defense,

antimicrobial activity, and collagen binding (37, 38, 39, 40). Although the contribution of MapA to initiate biofilm formation in *L. reuteri* 100-23 has not been established, it is presumed to contribute to adhesion.

Most *L. reuteri* strains isolated from humans produce reuterin (3-hydroxy propionaldehyde) by metabolizing glycerol (for review, see 41). Reuterin inhibits the growth of Gram-positive and Gram-negative bacteria, yeast, molds, and protozoa (42). In contrast, strains of *L. reuteri* isolated from either rodents or sourdough usually do not synthesize reuterin (2, 16).

1.4 Research hypothesis and objectives

The low pH (4-5) of the murine forestomach readily allows the degradation of dietary starch. In sourdough, starch degradation begins when flour is hydrated. During fermentaion, lactobacilli consume carbohydrates, producing energy and lactate/acetate. According to the co-evolutionary theory proposed by Oh *et al.* (8), *L. reuteri* is a symbiont that has evolved along with specific hosts. In other words, isolates of *L. reuteri* originating from different hosts generally continue to colonize the gastrointestinal tract of these respective hosts.

This thesis aimed to test the hypothesis that the isolates of *L. reuteri* originating from rodents and sourdough have genetic and biochemical traits that differentiate them from other strains in the species.

The objectives of this study are as follows:

1. Develop a double crossover method to generate multiple-deletion and complemented mutants.

2. Determine the contribution of glutamate decarboxylase in *L. reuteri* to acid resistance and persistence in sourdough fermentation.
3. Elucidate the phylogenetic relationship of the *L. reuteri* sourdough strain by comparative genomic hybridization.
4. Characterize the novel two-component regulatory systems of *L. reuteri* LTH2584 and 100-23.

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Chapter Two: **Contribution of glutamate decarboxylase in *Lactobacillus reuteri* to acid resistance and persistence in sourdough fermentation**

2.1 Introduction

Sourdough is used in bread production as a leavening agent, or as an agent to improve the texture, flavour, and shelf-life of bread (1, 2). Sourdoughs used as the sole leavening agent (type I sourdoughs) are maintained by frequent back-slopping. These conditions select for fast-growing microorganisms, and, as a result, the most common type I sourdough microbiota is *Lactobacillus sanfranciscensis* (3, 4, 5). Industrial sourdoughs used for the production of baking enhancers (type II sourdoughs) are fermented at 35 – 40°C for extended fermentation times (2 – 5 days) to achieve high levels of total titrable acidity (1, 3). The most common sourdough strains in this category are thermophilic, acid-tolerant lactobacilli, including *Lactobacillus pontis* and *Lactobacillus panis* (3, 4, 5). *Lactobacillus reuteri* is generally considered an intestinal organism (6) but also occurs in type II sourdoughs (3, 4, 5). *Lactobacillus reuteri* was shown to persist in an industrial sourdough fermentation over a period of more than a decade, corresponding to about 210 000 generations of bacterial growth (7). As well, model sourdough fermentations confirm that long fermentation times at a high temperature (42°C) select for *L. reuteri* (8, 9).

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These long fermentation times cause acid stress to impact the persistence of lactobacilli in type II sourdough fermentation. Mechanisms of acid tolerance in Gram-positive bacteria generally include the expression of stress proteins, overexpression of proton pumps, and modification of metabolic pathways to consume protons (10, 11). Remarkably, metabolic pathways contributing to acid resistance of *L. reuteri* also improve bread quality. Exopolysaccharide formation including dextran, reutern, and levan by lactic acid bacteria contributes to acid resistance (12, 13) and improves bread texture and volume (14), while conversion of arginine to ornithine improves the acid resistance of *L. reuteri* (10, 15, 16). Ornithine is also a precursor of the characteristic flavor compound of wheat bread crust, 2-acetyl-1-pyrroline (17). In addition, deamidation of glutamine to glutamate by sourdough lactobacilli generates umami taste in sourdough bread and improves growth of *L. reuteri* at low pH levels (18).

Glutamate decarboxylation to γ-aminobutyric acid (GABA) contributes to acid resistance of *Escherichia coli*, *Listeria monocytogenes,* and *Lactococcus lactis* (19, 20, 21). The antiport of glutamate and GABA generates a ΔpH and $\Delta\Psi$ contributing to a proton motive force for ATP synthesis (22). However, although glutamate decarboxylases were biochemically characterized in *L. brevis* and *L. paracasei* (23, 24), the physiological function of glutamate decarboxylase in aciduric *L. reuteri* is unclear. Moreover, experimental evidence is lacking for the contribution of any acid resistance mechanism to the competitiveness of lactobacilli in sourdough. Thus, the aim of this study was to investigate the ecological role of glutamate decarboxylase in cereal-associated *L. reuteri*.

Experiments were conducted with *L. reuteri* 100-23 (25), which has a known genome sequence, harbors a glutamate decarboxylase, and decarboxylates glutamate during sourdough fermentation (26). An isogenic deletion mutant, *L. reuteri* Δ*gadB*, was generated by a double crossover method to elucidate the importance of GadB in acid resistance and *L. reuteri*'s competitiveness in type II sourdoughs.

2.2 Materials and methods

2.2.1 Strains, plasmids and media

Bacterial strains and plasmids used in this study are shown in Table 2-1. *Escherichia coli* JM109 (Promega, Nepean, Canada) was cultured in Luria-Bertani (LB) broth at 37°C or 30°C for the maintenance of pJRS233, a temperature-sensitive plasmid, and its derived plasmids. *L. reuteri* was cultured at 37°C in deMan-Rogosa-Sharpe broth (MRS) (27) (Difco, Becton Dickinson, Mississauga, Canada) or modified MRS medium (7) under micro-aerobic conditions (1% O_2, 5% CO_2, and 94% N_2). Ampicillin (100 mg L^{-1}) or erythromycin (500 mg L^{-1}) was added to LB for selecting antibiotic-resistant *E. coli*. Erythromycin (10 mg L^{-1}) was added to the MRS medium to select erythromycin-resistant *L. reuteri.*

2.2.2 DNA manipulation

DNA was isolated from overnight grown cultures using the Blood & Tissue Kit (Qiagen, Mississauga, Canada) according to manufacturer instructions. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville,

IA), restriction enzymes were purchased from New England Biolabs (Pickering, Canada), T4 DNA ligase were purchased from Epicentre (Markham, Canada) and Taq DNA polymerase were purchased from Invitrogen (Burlington, Canada). DNA sequencing was performed by MacrogenUSA (Rockville, MD).

Table 2-1 Bacterial strains and plasmids used in this study

Strain or plasmid	**Genotype**	**Source or reference**
Strains		
Escherichia coli JM109	Cloning host for pGEMTeasy- and pJRS233-derivative plasmids	Promega
Lactobacillus reuteri 100-23	Rodent isolate; wild type strain	(25)
Lactobacillus reuteri Δ*gadB*	Wild-type strain derivative with a deletion in *gadB*	This study
Plasmids		
pGEMTeasy	Cloning vector used in *E. coli*; 3.0 kb; Amp^r	Promega
pGadB-A	pGEMTeasy containing 1 kb of the DNA sequence upstream of *gadB*; 4.0 kb; Amp^r	This study
pGadB-B	pGEMTeasy containing 0.9 kb of the DNA sequence downstream of *gadB*; 3.9 kb; Amp^r	This study
pGadB-AB	pGEMTeasy containing the upstream and downstream sequences of *gadB*; 4.9 kb; Amp^r	This study
pJRS233	Shuttle vector used in the hosts *E. coli* and *L. reuteri* 100-23; 6.0 kb; Erm^r	(28)
pKO-*gadB*-AB	pJRS233 containing 1.9 kb of the flanking sequences of *gadB*; 7.9 kb; Erm^r	This study

Amp^r: amplicillin-resistance gene; Erm^r: erythromycin-resistance gene

2.2.3 Amino acid comparison of **gadB** *genes and the genetic loci in lactic acid bacteria*

The glutamate decarboxylase (GadB) protein sequence of *L. paracasei* (24) (GI:169264609) was used to identify a homologous gene encoding *gadB* in the genome of *L. reuteri* 100-23 (http://www.jgi.doe.gov/) (29). The genetic loci of putative *gadB* genes and flanking nucleotide sequences in *Lactococcus lactis* subsp. *cremoris* MG1363 (21), *L. reuteri* 100-23, and *L. plantarum* WCFS1 (30) were analyzed with the BLASTx program against the National Center for Biotechnology Information databases (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The protein sequences of GadB from various species were retrieved from Uniprot database and aligned to calculate the similarity score using Geneious alignment (Geneious version 5.1.6, www.geneious.com).

2.2.4 Generation and verification of the L. reuteri ΔgadB *mutant*

The gene coding for GadB in *L. reuteri* 100-23 was truncated using the temperature-sensitive shuttle vector pJRS233 (28) according to a deletion strategy described previously (31). A 5' 1016-bp fragment of *gadB* was amplified from genomic DNA of *L. reuteri* 100-23 using primers *gadB*-KO1-*Pst*I (5'-AACTGCAGGTTCAATTTTCAGCACATG-3') and *gadB*-KO2-*Xba*I (5'-GCTCTAGATATCCTGCCATAGATAAAACCTC-3'). The amplicon was ligated into pGEMTeasy vector (Promega) to generate pGadB-A. The plasmid pGadB-B containing the 3' flanking fragment of *gadB* was created using *gadB*-KO3-*Xba*I (5'-GCTCTAGATTCACTCATTAAACCTTAGAA-3') and *gadB*-KO4-*Bam*HI (5'-CGGGATCCAATGGCTGCAGGGATA-3'). The flanking fragments of *gadB* from these plasmids were digested with the respective restriction enzymes, purified, and ligated to create pGadB-AB. By ligating these

restriction enzyme-cut flanking sequences of the *gadB* gene in pGadB-AB, the truncated form of *gadB* was created. The DNA fragment in pGadB-AB was cut with *Pst*I and *Bam*HI and ligated into the *Pst*I-*Bam*HI sites in pJRS233. The resulting plasmid pKO-*gadB*-AB was transformed by electroporation into competent *L. reuteri* 100-23 cells suspended in water with 30% (w/v) polyethylene glycol (MW 3350; J.T. Baker Chemical, Phillipsburg, NJ). Since pJRS233 carries a temperature-sensitive replication origin pSC101 and an erythromycin-resistance gene, the increase of growth temperature and the addition of erythromycin promote the integration of pKO-*gadB*-AB into the chromosome of erythromycin-sensitive *L. reuteri* 100-23 by homologous recombination.

Transformants of *L. reuteri* were grown in mMRS-erythromycin broth at 42 to 44°C for 80 generations to select for single crossover mutants. *L. reuteri* with pKO-*gadB*-AB integrated into the chromosome was cured by cultivation in mMRS broth without the addition of erythromycin at 37°C for 100 generations. During the inoculation of the plasmid-curing test, the second homologous recombination occurred. The culture was plated on mMRS agar and erythromycin-sensitive double crossover mutants were identified by replica-plating onto mMRS and mMRS-erythromycin agar. The truncation of *gadB* in *L. reuteri* 100-23Δ*gadB* was confirmed by PCR with the primers *gadB*-KO1-*Pst*I and *gadB*-KO4-*Bam*HI. An amplicon with an expected size of 3000 bp was obtained with DNA from the *L. reuteri* 100-23 as a template. PCR with DNA from *L. reuteri* 100-23Δ*gadB* yielded a 2000 bp amplicon. A second PCR with primers *gadB*-5F (5'-GGTCTTATTACCGTTCCTAAT-3') and *gadB*-6R (5'-

ACATTTCTTATGGGATTGCAT-3') yielded a 1700 bp and a 500 bp amplicon from the *L. reuteri* 100-23 and *L. reuteri* Δ*gadB*, respectively. DNA sequencing was conducted to verify the deletion region using primers *gadB*-5F and *gadB*-6R.

2.2.5 Growth in mMRS and survival at pH 2.5

Growth of *L. reuteri* 100-23 and *L. reuteri* Δ*gadB* was assessed in mMRS (pH 6.7) and mMRS acidified to pH values of 4.7 and 3.8. Media were inoculated with cultures grown overnight and incubated at 37°C. Growth was monitored by optical density (OD) measurement at 600 nm. To evaluate acid resistance, cultures grown overnight were harvested by centrifugation, washed in 50 mM Na_2HPO_4 buffer (pH 7), and resuspended in 50 mM potassium phosphate buffer (pH 2.5) to an OD_{600nm} of 1.0. To assess the contribution of amino acid metabolism to acid resistance, parallel experiments were conducted in potassium phosphate buffer (pH 2.5) supplemented with 10 mM glutamate or arginine. A pH of 2.5 adjusted with HCl was chosen to match conditions previously used to determine the effect of glutamate decarboxylase on acid resistance in *E. coli* and *L. monocytogenes* (10, 19, 20). Samples were taken after 0, 1, 3, 5, 8 and 24 h of incubation at pH 2.5 for quantification of amino acids (see below), and to monitor bacterial survival. For determination of bacterial survival, samples were immediately mixed with phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 8.0) and diluted in PBS buffer prior to plating on MRS agar. For the samples of 24 h acid treatment, 10 mL of culture was centrifuged, resuspended in PBS buffer and spread on MRS agar

plates to determine the bacterial survival. Growth curves and the rate of survival at pH 2.5 were determined in three independent experiments.

2.2.6 Sourdough fermentations and sampling

Sourdough fermentations were performed with *L. reuteri* 100-23 and *L. reuteri* Δ*gadB* to examine the effect of glutamate decarboxylase on growth, acidification, pH, and amino acid concentrations. Doughs were prepared from 40 g whole wheat flour (Rogers Food Ltd, Armstrong, Canada) and sterile tap water to achieve a dough yield of 200 [(dough mass/flour mass) × 100], and inoculated at 37°C with an initial cell count of $1 \pm 0.5 \times 10^7$ CFU g^{-1}. Initially, sourdoughs were inoculated with *L. reuteri* 100-23 or *L. reuteri* Δ*gadB* separately, and samples were collected after 0, 6, 12, 24, 48, 72, and 96 h of fermentation. In a second experiment, *L. reuteri* 100-23 and Δ*gadB* were co-cultured in the same sourdough. Doughs were fermented for 96 h (trial I), or for 240 h by refreshing (also referred to as backslopping) every 48 h (trial II) to match conditions known to select for *L. reuteri* (8). At each refreshment step, 5% (w/v) of the ripe sourdough was used as an inoculum for the subsequent fermentation. Samples were taken to measure pH and viable cell counts. The dough samples were stored at -20°C for subsequent DNA extraction and amino acid analysis (see below). Sourdough fermentations were carried out in two independent experiments, and each sample was analyzed in duplicate.

2.2.7 Quantification of amino acids by high performance liquid chromatography (HPLC)

Cells from phosphate buffer (pH 2.5) were removed by centrifugation. The supernatant (1 vol) was mixed with deionized water (4 vol), saturated potassium borate (4 vol), and β-aminobutyric acid, which was used as an internal standard (1 vol). Sourdough samples were lyophilized, extracted with water at an extraction ratio of 1:6 (w/v) and diluted with water, potassium borate, and the internal standard described above. Amino acids were quantified by HPLC after derivatisation with *o*-phtaldialdehyde (32).

2.2.8 Extraction of DNA from sourdough

Two grams of sourdough were washed with 4 mL of PBS buffer (pH 7.8) and resuspended in PBS. Flour solids were removed by centrifugation at 1500 × *g* for 10 min, while cells were harvested by centrifugation at 13,000 × *g*. Cells were resuspended in 660 µL lysis buffer (10 mM Tris-Cl, 0.5 mM EDTA, 10% (w/v) SDS, pH 7.5), and incubated at 65°C for 10 min. Protein was precipitated by addition of 340 µL of 5 mol L^{-1} potassium acetate and incubation at -20°C for 10 min. After centrifugation, 900 µL of the supernatant was mixed with an equal volume of isopropanol, DNA was collected by centrifugation, and the pellet was washed with 95% ice-cold ethanol. DNA was resuspended in 20 µL of 10 mM Tris-Cl (pH 8.5), and the DNA concentration was determined by UV spectrometry using a NanoDrop spectrophotometer ND-1000 (Thermo Fisher Scientific Inc., Wilmington, DE).

2.2.9 Quantification of bacterial population in sourdough using quantitative PCR (qPCR)

The cell counts of *L. reuteri* 100-23 relative to the *L. reuteri* Δ*gadB* were quantified using a probe-based qPCR assay targeting the native and disrupted regions of *gadB*, respectively. Two micrograms of chromosomal DNA from *L. reuteri* 100-23 and *L. reuteri* Δ*gadB* were digested separately with *Not*I, purified, and diluted to a known copy number of DNA for use as standards. The copy numbers of the genomic DNA of *L. reuteri* strains 100-23 and Δ*gadB* range from 10^2 – 10^6 and are calculated based on the reference provided by Applied Biosystems (Streetsville, Canada) (http://www.appliedbiosystems.com/support/tutorials/pdf/quant_pcr.pdf). DNAs extracted from sourdough were also digested with *Not*I and purified using the QIAquick PCR Purification kit (Qiagen). Strain-specific primers and probes were designed using Primer Express Software 3.0 (Applied Biosystems). Primers and probes specific for *L. reuteri* 100-23 (WT-qPCR-F, 5'-TGGGATTTCCAACTAAAGAATGTTG-3'; WT-qPCR-R, 5'-CAACCAATACCAGGATAAACTAAACCA-3'; and WT-qPCR-probe, 5'-Hex/TCCATTAACGCTTCCGGCCACAAGT/IABkFQ-3') were used to target the DNA region of gene *gadB*, which has been deleted in the *L. reuteri* Δ*gadB* strain. Primers and probes specific for *L. reuteri* Δ*gadB* (Δ*gadB*-qPCR-F, 5'-CATATCATTGCAAATTCAGACGAAA-3'; Δ*gadB*-qPCR-R, 5'-ATCTAAGCAAGTTGTTATGCTTGTTTAGATC-3'; and Δ*gadB*-qPCR-probe 5'-TET/ CCTAGGAGGTTTTATCTATGGCAGGATAATCTAGATTCAC/ IABkFQ-3') target the joint site of the deleted *gadB* sequence, which is absent in the wild type strain. PCR was carried out with Rox Brilliant II Master Mix

(Stratagene, Mississauga, Canada) in a 7500 Fast Real-time PCR instrument (Applied Biosystems). The amplification program was 95°C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Data was collected at 60°C.

2.2.10 Nucleotide accession numbers

DNA sequences of *L. reuteri* 100-23 were obtained from the National Center for Biotechnology Information databases (GenBank: AAPZ02000002.1). The gene regions of predicted glutamate/γ-aminobutyrate antiporter (*gadC2*), glutaminase (*gls3*), glutamate/γ-aminobutyrate antiporter (*gadC1*), glutamate decarboxylase (*gadB*), and xanthine/uracil/vitamin C permease are 549316..550869, complement (548219..549139), complement (546271..547809), complement (544847..546253), and 543444..544787, respectively. The sequence of the truncated *gadB* in *L. reuteri* Δ*gadB* was deposited with accession number JF339969.

2.3 Results

2.3.1 Genetic locus coding for glutamate metabolism in L. reuteri

Genome analysis of *L. reuteri* 100-23 revealed a gene cluster encoding proteins homologous to glutamate decarboxylase (*gadB*), glutamate: γ-aminobutyric acid (GABA) antiporters (*gadC1* and *gadC2*), a glutaminase (*gls3*), and a xanthine/uracil/vitamin C permease (Fig. 2-1). The GadB sequence is 69%, 64.4%, 63.7%, and 51.1% similar to that of *L. lactis* MG1363 (21), *L. plantarum* WCFS1 (30), *L. brevis* OPK-3 (33), and *L. paracasei* (24), respectively, suggesting that *gadB* of *L. reuteri* 100-23 encodes a glutamate decarboxylase.

However, the organisation of the *gadB* gene locus in *L. reuteri* differs from related lactic acid bacteria as it also contains one of three glutaminases encoded in the genome of *L. reuteri* 100-23. In *L. lactis* subsp. *cremoris* MG1363, *gadB* is accompanied by *gadC* and *gadR* (Fig. 2-1). GadR is a positive regulator of *gadBC*, and is adjacent to the glutamate synthase domains *gltB* and *gltC* (21). In *L. plantarum* WCFS1, *gadB* is not located adjacent to other genes related to glutamine or glutamate metabolism.

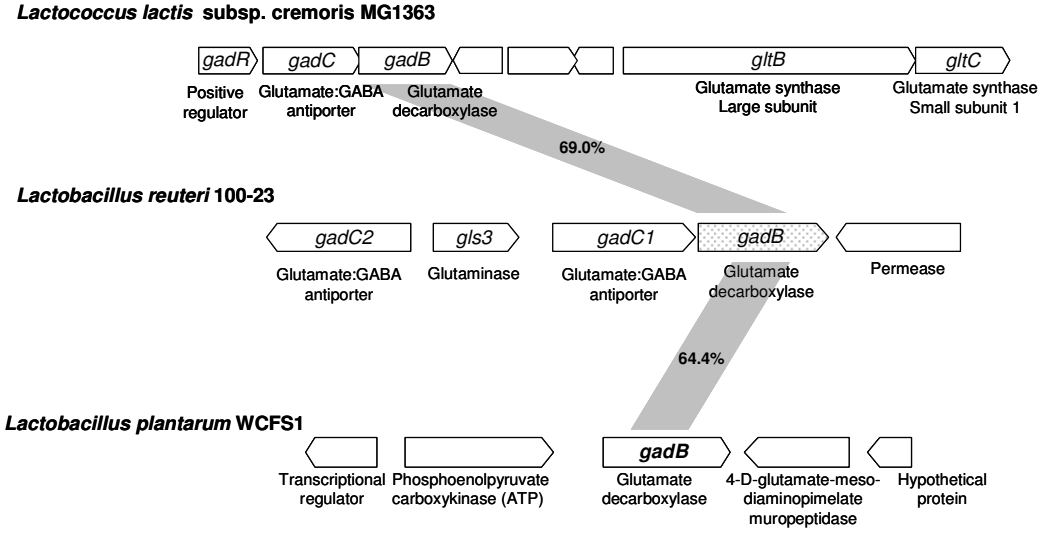

Figure 2-1 Representation of genetic loci encoding glutamate decarboxylases in *Lactococcus lactis* MG1363, *Lactobacillus reuteri* 100-23, and *Lactobacillus plantarum* WCFS1. Numbers indicate protein similarity.

2.3.2 Creation of a gadB *deletion mutation in* L. reuteri

A deletion in *gadB* of *L. reuteri* 100-23 was generated by a double crossover mutagenesis method. The deletion in the resulting strain *L. reuteri* 100-23 Δ*gadB* was confirmed by DNA sequencing. The impact of *gadB* mutation on bacterial growth was investigated in mMRS and acidified mMRS (Fig. 2-2).

Wild-type *L. reuteri* and the Δ*gadB* mutant grew similarly in mMRS at all pH values tested, indicating that the growth of *L. reuteri* Δ*gadB* in complex media was not affected.

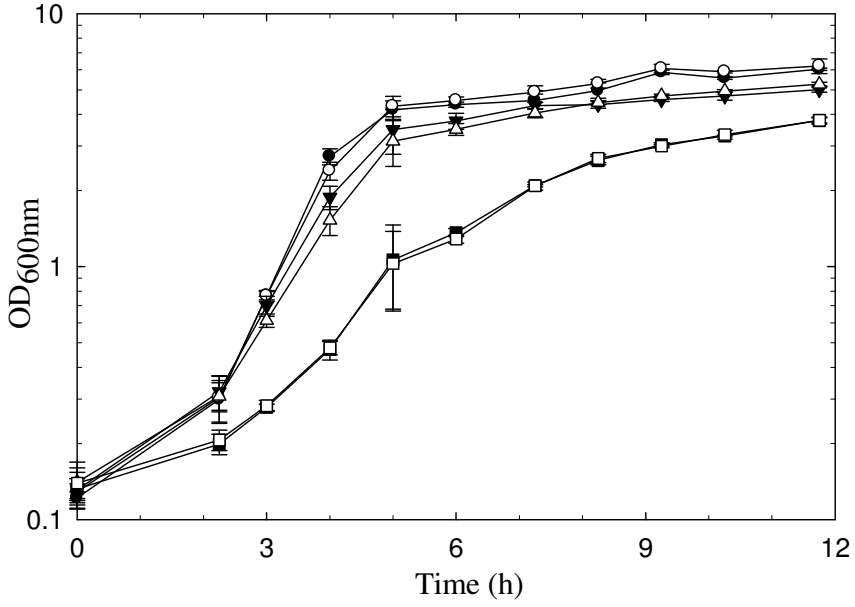

Figure 2-2 Bacterial growth curve of *L. reuteri* 100-23 (black symbols) and Δ*gadB* (open symbols) strains in mMRS (pH 6.7, ●, ○) and HCl-acidified mMRS (pH 4.7, ▼, △; pH 3.8, ■, □). Results are shown as means ± standard deviations of three independent experiments.

2.3.3 Survival of L. reuteri ΔgadB *at pH 2.5*

To confirm that *gadB* of *L. reuteri* encodes glutamate decarboxylase, this study compared the wild-type strain and the Δ*gadB* mutant strain. It compared acid resistance, amino acid metabolism, and the rate of survival at pH 2.5 between these two strains. Disruption of *gadB* did not influence the survival of *L. reuteri* at pH 2.5 in the absence of glutamate (Fig. 2-3). However, the addition of glutamate

increased the survival of *L. reuteri* 100-23 by 3 log CFU mL^{-1} at 8 h of incubation and more than 2 log CFU mL^{-1} at 24 h of incubation. A transient effect on the survival of *L. reuteri* Δ*gadB* was observed at 8 h of incubation, but not at 24 h of incubation (Fig. 2-3). Amino acid analysis confirmed that *L. reuteri* 100-23 converted glutamate to GABA, but *L. reuteri* Δ*gadB* did not. After *L. reuteri* 100-23 was incubated for 24 h in phosphate buffer in either the presence or absence of glutamate, GABA concentration was 2.5 ± 0.1 and 0.4 ± 0.03 mM, respectively. In contrast, GABA was not detected in the supernatant after incubation of *L. reuteri* Δ*gadB* under the same conditions. Together, these results demonstrate that

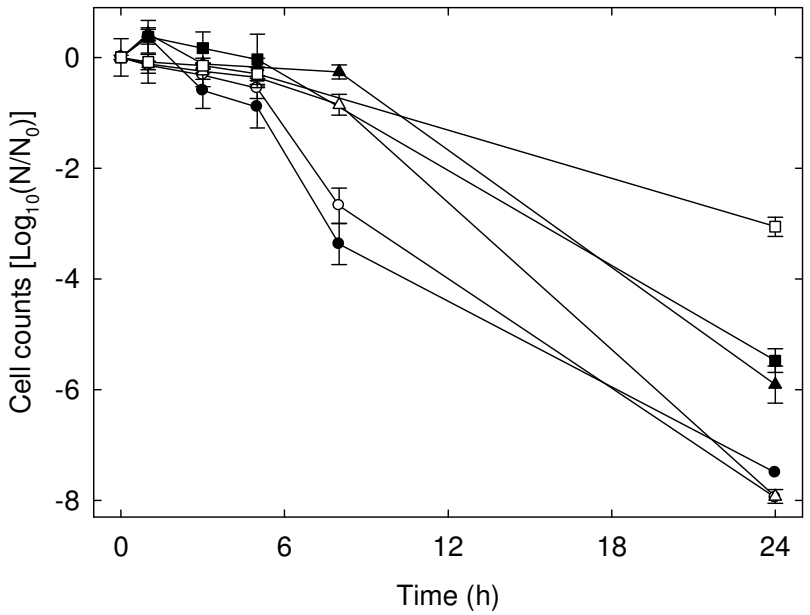

Figure 2-3 Acid resistance of *L. reuteri* 100-23 (black symbols) and *L. reuteri* Δ*gadB* (open symbols) in phosphate buffer (pH 2.5) (●, ○), in phosphate buffer with 10 mM glutamate (▲, △), or in phosphate buffer with 10 mM arginine (■, □). Cell counts are plotted as $\log_{10}$ (N/N_0). Data are the mean of three independent experiments, and the standard deviations are indicated as error bars.

gadB in *L. reuteri* encodes a glutamate decarboxylase which contributes to acid resistance at pH 2.5.

The effect of glutamate on acid resistance of *L. reuteri* 100-23 and *L. reuteri* Δ*gadB* was compared to the effect of arginine. The effect of arginine on the survival of *L. reuteri* 100-23 was comparable to the effect of glutamate (Fig. 2-3). Notably, *L. reuteri* Δ*gadB* was more resistant in arginine-containing medium than the wild-type strain after 24 h of incubation, indicating that over-expression of alternative pathways to achieve pH homeostasis may compensate for the loss of the acid resistance function.

2.3.4 Glutamine metabolism of L. reuteri ΔgadB *in sourdough*

Because *in vitro* acid challenge demonstrated that GadB contributed to bacterial survival under acidic conditions after 24 h of incubation, stationary phase survival of *L. reuteri* Δ*gadB* in sourdough was compared to that of the wild type strain (Fig. 2-4A). Within 12 h of fermentation, the pH decreased from 6.6 to 3.8, and growth of the wild-type and Δ*gadB* strains in sourdough was virtually identical, with maximum cell counts of 10.7 ± 0.9 and 10.5 ± 0.6 log CFU g^{-1}, respectively. At 96 h after fermentation, the pH remained at 3.8, while the cell counts decreased to 6.5 - 7.7 log CFU g^{-1}. Cell counts of *L. reuteri* Δ*gadB* in sourdough consistently decreased faster than cell counts of the wild type strain. A comparable trend towards improved survival was observed when a GadB-positive wild-type strain was compared to a GadB-negative wild-type strain (26). However, in both cases, the difference was not significant (Fig. 2-4A, and 26).

The evaluation of glutamine and glutamate metabolism by *L. reuteri* 100-23 and *L. reuteri* Δ*gadB* in sourdough revealed that disruption of *gadB* had no

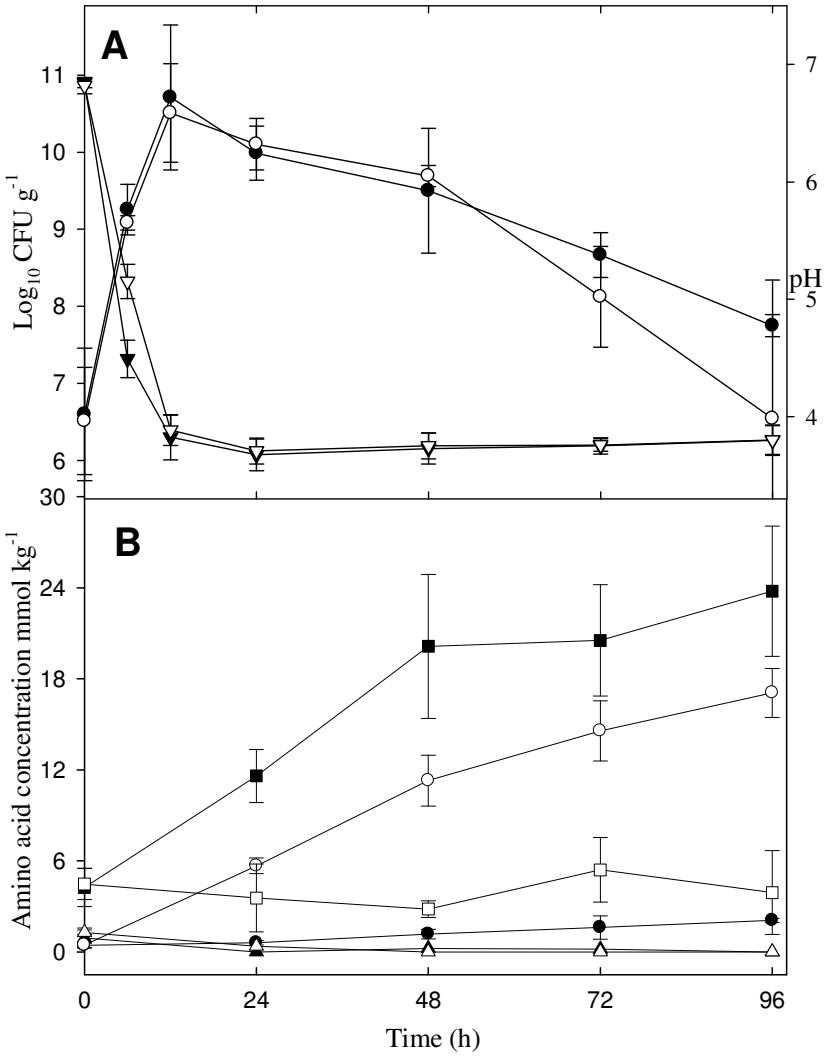

Figure 2-4 (A) Cell counts (●, ○) and pH (▼, ▽) of sourdough fermented with *L. reuteri* 100-23 (black symbols) or *L. reuteri* Δ*gadB* (open symbols) over 96 h. (B) Concentrations of glutamine (▲, △), glutamate (●, ○) and γ-aminobutyric acid (■, □) during sourdough fermentation over 96 h in *L. reuteri* 100-23 (black symbols) and *L. reuteri* Δ*gadB* (open symbols). Symbols indicate means ± standard deviation from quadruplicate determinations.

influence on the concentration of total amino acids or the total combined concentration of glutamine, glutamate, and GABA. After 96 h of incubation, the concentration of the total amino acids for the wild type and Δ*gadB* strains were 141 ± 7 and 129 ± 8 mmol kg^{-1} DM, respectively. The total combined concentrations of glutamine, glutamate, and GABA, for the wild type and Δ*gadB* strains were 26 ± 4 and 21 ± 4 mmol kg^{-1} DM, respectively. In sourdough fermentation, the level of glutamine remained low throughout the 96 h of incubation, indicating a conversion of glutamine to glutamate in the Δ*gadB* mutant, and a coversion of glutamine to GABA in the wild-type strain (Fig. 2-4B). *L. reuteri* 100-23 converted glutamine to GABA, which accumulated to 25 mmol kg^{-1} DM after 96 h of fermentation (Fig. 2-4B). In contrast, *L. reuteri* Δ*gadB* only accumulated glutamate. Low levels of GABA at the beginning of the fermentation and in the sourdoughs fermented with *L. reuteri* Δ*gadB* were attributable to the interference by tyrosine, whose retention time was close to that of GABA.

2.3.5 Role of **gadB** *in long-term sourdough fermentation*

The disruption of *gadB* did not significantly influence survival of *L. reuteri* over 96 h of fermentation. However, in sourdoughs maintained by continuous backslopping, relatively small differences in a single batch will accumulate over subsequent fermentation cycles. Therefore, this study investigated whether *gadB* disruption affects the ecological fitness of *L. reuteri* Δ*gadB* in competition with the wild type strain in a sourdough that was

backslopped every 48 h. A sourdough fermented over 96 h without backslopping was used as a control. In sourdough without backslopping, the growth of the co-culture was similar to that of single strains (Fig. 2-4A and Fig. 2-5).

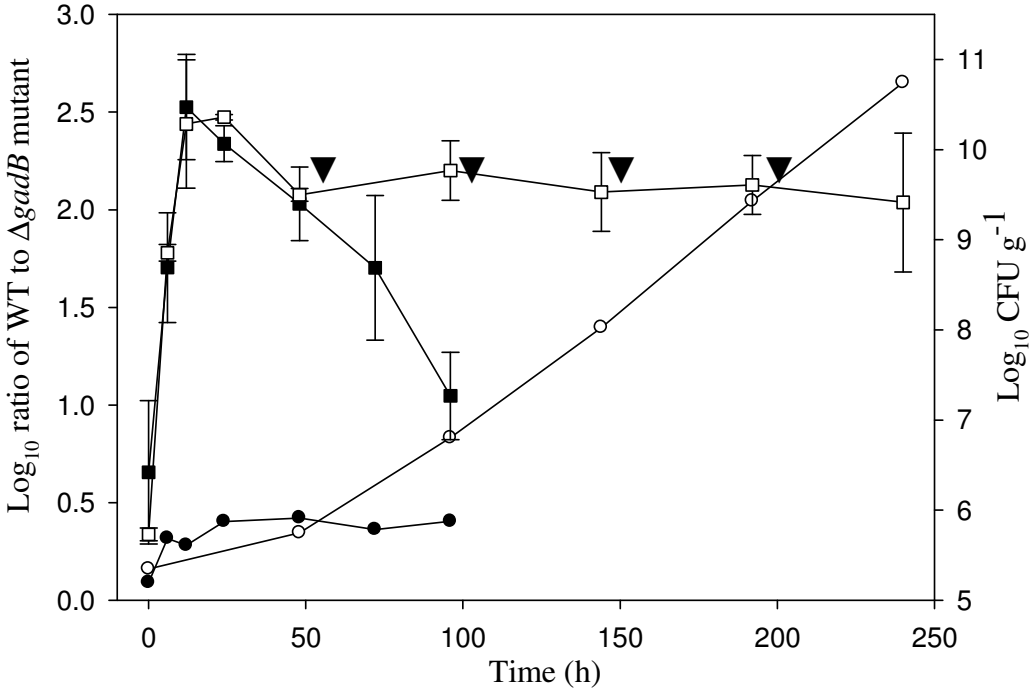

Figure 2-5 Kinetics of bacterial population in type II sourdough fermentations. Sourdoughs were fermented in a single batch over 96 h (trial I; filled symbols), or over 240 h with refreshment (backslopping with 5% inoculum) every 48 h (trial II; open symbols). Total bacterial cell counts (■, □) were enumerated by plating on mMRS agar. The relative quantification of wild type and mutant strains was achieved by qPCR (●, ○). The time points of refreshment are indicated (▼). Symbols indicate means ± standard deviation from duplicate independent experiments analysed in duplicate. The standard deviations of cell copy number ratio were less than 0.2 log.

The relative cell counts of *L. reuteri* 100-23 and *L. reuteri* Δ*gadB* were determined by qPCR using strain-specific primers and probes. Cell counts of *L. reuteri* 100-23 relative to *L. reuteri* Δ*gadB* increased slightly from 1.3 to 2.7 log

during 96 h of incubation, suggesting that disruption of *gadB* compromised stationary phase survival slightly. In the backslopped sourdoughs, the relative cell counts of *L. reuteri* 100-23 and *L. reuteri* Δ*gadB* also increased from about 1.5 to 2 log during the first fermentation cycle. With every subsequent fermentation cycle, the log ratio (the wild-type/Δ*gadB*) increased by about 0.5 (Fig. 2-5). At the end of the fifth fermentation cycle, cell counts of *L. reuteri* 100-23 relative to *L. reuteri* Δ*gadB* were 460:1. This demonstrates that *gadB* in *L. reuteri* is essential for its competitiveness in type II sourdoughs maintained by continuous propagation**.**

2.4 Discussion

This study investigated the role of *gadB*, which codes for glutamate decarboxylase, in the acid resistance of *L. reuteri*, and demonstrated that glutamate decarboxylation contributes to the competitiveness of *L. reuteri* in type II sourdoughs that are propagated by continuous backslopping. In *L. reuteri*, the contribution of metabolic traits to competitiveness in the gastrointestinal tract was previously evaluated with insertional mutations generated by single-crossover mutagenesis (6, 34). In contrast, the current study employed a double crossover method to generate an in-frame deletion of *gadB* in *L. reuteri* 100-23, which avoids interference by antibiotic-resistance genes or other plasmid-borne foreign genes that remain on the chromosome of mutant strains generated by single-crossover mutagenesis.

Lactic acid bacteria in sourdoughs were previously identified using species-specific, semi-quantitative denaturing gradient gel electrophoresis (DGGE) (8, 9, 35, 36) or culture-dependent enumeration, followed by strain identification through random amplified polymorphic DNA (35, 37). However, culture-dependent enumeration and DGGE both fail to account for species or strains that contribute less than 1% to the total cell counts. The qPCR method used in this study achieved a relative precise quantification of *L. reuteri* 100-23 and *L. reuteri* Δ*gadB* in sourdoughs in which *L. reuteri* Δ*gadB* contributed less than 0.5% of the total population.

Type II sourdough fermentations are characterized by a short period of growth, followed by an extended period of fermentation at pH 3.2 – 3.6 (3, 4). In long-term sourdough fermentations, endogenous cereal proteases provide a continuous amino acid supply from cereal proteins, and thus support bacterial acid resistance through amino acid metabolism (17, 18). Arginine and glutamate often contribute to acid resistance of Gram-negative and Gram-positive bacteria (Fig. 2-6) (10, 15, 38).

The arginine deiminase (ADI) pathway generates ATP, consumes intracellular protons, and causes the alkalization of the fermentation substrate (Fig. 2-6; 10, 11, 15). In the current study, arginine improved the survival of *L. reuteri* at pH 2.5 about 10-100 fold, which is consistent with the findings of previous studies (16). Glutamate decarboxylase was characterized in *L. brevis* and *L. paracasei* (23, 24), and GadB-positive lactobacilli were employed to produce GABA as a functional food ingredient in food fermentations (26, 39). The strain-

dependent expression of glutamate decarboxylase in lactobacilli, particularly *L. reuteri*, indicates that GadB is not essential for growth (26). However, the previous work did not determine the contribution of glutamate decarboxylation to acid resistance. Although glutamate decarboxylation generally contributes to bacterial resistance to acid (10, 15), its contribution to acid resistance in acid-tolerant lactobacilli has not been demonstrated experimentally (26). Glutamate decarboxylation is particularly effective at pH values below 4.23, the pK_A of GABA, as protonation of GABA below the pK_A results in alkalization of the external fermentation substrate (Fig. 2-6). Accordingly, this study demonstrated a protective effect of glutamate decarboxylation at pH 2.5 in buffer, and at pH 3.6 in sourdough. Sourdough is acidified by lactic and acetic acids rather than HCl, but the growth and survival of lactobacilli in sourdough is determined by the pH and not by the concentration of undissociated organic acids (40). The protective effect of glutamate on the survival of *L. reuteri* at pH 2.5, as demonstrated by a 100-fold increase of cell counts after acid challenge, is comparable to the effect in *E. coli* (19, 38). In *L. monocytogenes*, the addition of glutamate improved the survival of a wild type strain after exposure to pH 2.5 more than 6 log CFU mL^{-1} compared to a *gadAB* deficient mutant (20).

The contribution of glutamine deamidation to bacterial acid resistance has not been demonstrated (10, 15, 41) but glutamine improved the growth of *L. reuteri* and *L. sanfranciscensis* at low pH levels (18). Glutamine accounts for about 30% of the amino acids in wheat proteins, but because the amount of glutamate is low (42), proteolysis in wheat dough liberates glutamine rather than

glutamate (18, 26). Glutamate-mediated acid resistance in sourdough thus depends on the conversion of glutamine to glutamate. The amount of the conversion of glutamine to glutamate in *L. reuteri gadB*–negative strain TMW 1.106 is comparable to the amount of the conversion of glutamine/glutamate to GABA in the *L. reuteri gadB*–positive strain LTH5448 in sourdough fermentation (26). In this study the amino acid analysis of sourdough fermentation using the *L.*

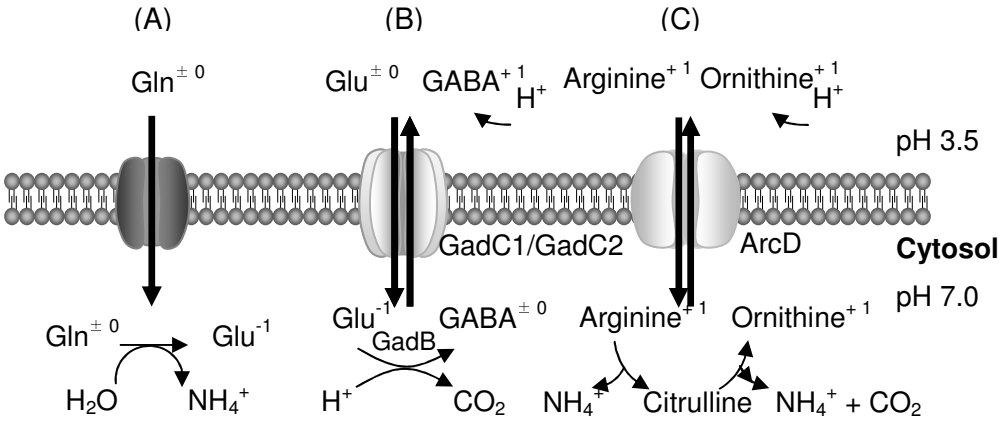

Figure 2-6 Amino-acid based acid resistance mechanisms in *L. reuteri*. (A) Following uptake of glutamine by unknown mechanisms, glutaminase catalyzes deamidation to glutamate and ammonia. (B) Glutamate is taken up by the electrogenic glutamate/γ-aminobutyric acid (GABA) antiport system. Decarboxylation of glutamate consumes an intracellular proton and contributes to generation of $\Delta\Psi$ and ΔpH. Extracellular protonation of GABA consumes additional protons. (C) Arginine is brought into cells by electroneutral arginine/ornithine exchange. Ammonia generated from arginine contributes to intracellular pH homeostasis; intra- or extracellular protonation of ornithine consumes additional protons. GadB: glutamate decarboxylase; GadC: putative glutamate:GABA antiporter; ArcD: putative arginine:ornithine antiporter. Relevant pK_A values of amino acid side chains and GABA are: glutamate, 4.25; GABA, 4.23; arginine, 12.5; ornithine, 8.69.

reuteri 100-23 wild-type and Δ*gadB* mutant strains confirmed the previous finding (Fig. 2-4). *L. reuteri* 100-23 harbors three putative glutaminase genes (*gls*), and *gls3* is located adjacent to *gadB*. The substrate availability in cereal fermentations and the arrangement of gene loci indicates that the glutamate-based acid resistance of *L. reuteri* 100-23 uses glutamine as a substrate (Fig. 2-6). In contrast, sourdough isolate *L. lactis* MG1363 has genes coding for glutamine synthase downstream of the *gadB*C operon. In human isolate *L. plantarum* WCFS1 no genes related to glutamine metabolism are located close to *gadB* (Fig. 2-1). The exceptional arrangement of glutamine/glutamate catabolic genes in *L. reuteri* may reflect the adaptation to cereal substrates. The encoding of Glutamine:GABA antiport by *gadC* or analogues has not been shown experimentally (22). However, ATP-dependent glutamate transport enzymes in *L. lactis* and *L. delbrueckii* also have a high affinity to glutamine (43, 44).

The disruption of *gadB* did not influence the survival of *L. reuteri* during long-term sourdough fermentation unless the sourdough was backslopped. The loss of one metabolic pathway for pH homeostasis in *L. reuteri* is apparently partially compensated for by alternative pathways (Fig. 2-6). However, monitoring of the microbiota in sourdoughs maintained by continuous backslopping is considered the most appropriate tool to identify competitive strains (8, 35, 36, 37). The observation that the wild-type strain displaced *L. reuteri* Δ*gadB* after only a few refreshments demonstrates that acid resistance in general, and glutamate decarboxylase in particular, contribute to the competitiveness of *L. reuteri* in type II sourdoughs.

In addition to its occurrence in sourdough fermentations, *L. reuteri* is recognized as a member of the intestinal microbiota of humans and animals, and it has evolved to colonize the gastrointestinal tract of its specific hosts (6). In pigs, poultry, and rodents, *L. reuteri* colonizes the pars oesophagus, the crop, and the forestomach, respectively (45). It is interesting to note that *L. reuteri* sourdough isolates, but not human or poultry isolates, are able to colonize rodents (46, 47). In animals, *L. reuteri*'s colonization and biofilm formation occur on the stratified squamous epithelium lining of the proximal gastrointestinal tract upstream of the stomach (45, 46). Therefore, colonized *L. reuteri* are exposed to stomach acidity. The conversion of glutamate to GABA in *L. reuteri* is strain-specific but was reported in human, rodent, and sourdough isolates (this study, 26). Mechanisms of acid resistance that are relevant in sourdough thus likely contribute to the competitiveness of *L. reuteri* in intestinal ecosystems, and may improve gastro-intestinal survival of *L. reuteri* commercially used as probiotics.

This study demonstrated that the conversion of glutamate to GABA by *L. reuteri* 100-23 contributes to acid resistance and to competitiveness in type II sourdough fermentations. The organization of the gene cluster for glutamate conversion and the availability of amino acids in cereals imply that glutamine rather than glutamate functions as the substrate for GABA formation. The exceptional coupling of glutamine deamidation to glutamate decarboxylation in *L. reuteri* may reflect ecological adaptation to cereals, or to the proximal intestinal tracts of animals that predominantly feed on cereal grains.

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Chapter Three: **Elucidation of phylogenetic relationship of *Lactobacillus reuteri* sourdough strain by comparative genomic hybridization**

3.1 Introduction

Lactobacillus reuteri has been used as a model species to investigate the evolutionary and ecological adaptation of symbiotic organisms that are associated with vertebrate hosts (1, 2). *L. reuteri* forms biofilms on non-secretory stratified squamous epithelium in the upper intestinal tract of animals, including the pars oesophagus of pigs, the crop of poultry, and the forestomach of rats and mice (for review, see 3). Moreover, *L. reuteri* is one of the dominant species found in industrial sourdoughs (4), where they are used to acidify dough, improve flavor and texture, and extend the shelf life of bread by producing of organic acids and antimicrobial compounds (5, 6, 7). *L. reuteri* LTH2584 was isolated from industrial rye sourdough in 1988 (4), and was found to produce reutericyclin, an antimicrobial compound that contributes to its persistence in sourdough (8, 9). This strain was also found to colonize the murine forestomach, as the rodent isolates of *L. reuteri* do (10). In general, *L. reuteri* human, poultry, and swine isolates do not colonize in RLF mice (11, 12). In addition to their shared ability to colonize in mice, *L. reuteri* LTH2584 and rodent isolates differ from human isolates of *L. reuteri* in their lack of the production of the antimicrobial compound reuterin (2, 8). This increases the attractiveness of *L. reuteri* LTH2584 as a

candidate for the study of sourdough microorganisms and *L. reuturi* sourdough isolates' relationship to gut ecosystems.

The accessibity of the genome sequences of *L. reuteri* allows the development of whole-genome microarrays to investigate gene expression and perform phylogenetic studies (12, 13, 14). The genome size of *L. reuteri* is relatively small (1.9 – 2.3 Mb), compared to that of commensal *Escherichia coli* (4.3 – 6.2 Mb), which reflects *L. reuteri*'s particular adaptation to ecological niches through the reduction of its genome size (15). The genome sequences are available for *L. reuteri* DSM 20016^T and 100-23, which were isolated from humans and rats, respectively (12, 16, 17). Nevertheless, the genome information provides good criteria for strain selection and for the characterization of differential gene features between *L. reuteri* strains isolated from sourdough and animals (18, 19).

L. reuteri LTH2584 is not only a persistent strain in type II sourdough fermentation but also a strain that colonizes in the forestomach of mice (10). Moreover, the global gene expression of *L. reuteri* in type II sourdough fermentation reveals that two-component regulatory systems are induced by acid stress (13, 20). However, the phylogeny of *L. reuteri* sourdough isolates has not been investigated yet, and the contribution of two-component regulatory systems to the adaptation of *L. reuteri* isolated from different niches is not well established. Therefore, the aims of this study were to analyze the genome content of *L. reuteri* LTH2584 by comparing it with that of the rodent strains 100-23 and

human strain DSM 20016^T using DNA microarrays, and to analyze the genes encoding two-component regulatory systems in *L. reuteri* LTH2584 and 100-23.

3.2 Materials and methods

3.2.1 Strains, media and growth conditions

Table 3-1 lists the bacterial strains used in this study. *L. reuteri* was cultured at 37°C in deMan-Rogosa-Sharpe broth (MRS) (Difco, Becton Dickinson, Mississauga, Canada) or in modified MRS medium without agitation (8, 21) under micro-aerobic conditions (1% O_2, 5% CO_2, and 94% N_2).

Table 3-1 Bacterial strains used in this study

Strain	Genotype	Source
L. reuteri LTH2584	Sourdough isolate; reutericyclin-producing strain	4, 8
L. reuteri 100-23	Rodent isolate; MSLA lineage III	1, 12, 17
L. reuteri DSM 20016^T	Human isolate F275; MLSA lineage II; reuterin-producing strain	12, 18
L. reuteri ATCC 55730	Human isolate; MLSA lineage VI; reuterin-producing strain	1, 12
L. reuteri CSF8	Chicken isolate; MLSA lineage VI	1, 12
L. reuteri JW2015	Swine isolate; MLSA lineage IV	1, 12

3.2.2 DNA manipulation

Two milliliters of overnight grown cultures were harvested and washed with lysis buffer (20 mM Tris·Cl, pH 9.0; 2 mM sodium EDTA; 1.2% Triton X-100 (w/v)), and chromosomal DNA was extracted using the Blood & Tissue Kit (Qiagen, Mississauga, Canada) according to the manufacturer's instructions. DNA was resuspended in 20 µL of 10 mmol L^{-1} Tris-Cl (pH 8.5), and the DNA

concentration was determined by UV spectrometry using a NanoDrop spectrophotometer ND-1000 (Thermo Fisher Scientific Inc., Wilmington, DE). Oligonucleotides listed in Table 3-2 were purchased from Invitrogen (Burlington, Canada) and Integrated DNA Technologies (San Diego, CA). Taq DNA polymerase was purchased from Invitrogen, and DNA sequencing was performed by the Molecular Biology Services Unit (Department of Biological Sciences, University of Alberta).

3.2.3 Bioinformatic prediction of protein function

The genome sequences of *L. reuteri* DSM 20016^T and 100-23 were retrieved from the DOE Joint Genome Institute (www.jgi.doe.gov, 19). BLASTP analysis was performed to retrieve homologous proteins. The prediction of histidine kinase and response regulator has been previously reported (22, 23). The CheY-like receiver domain and the LytTr DNA binding domain were identified using the Pfam database (pfam00072 and pfam04397; pfam.sanger.ac.uk). The gene loci of *aip*/*tcsKR* and *cemAKR* were predicted using a web-based bacteriocin genome mining tool (BAGEL) (24) and GeneMark.hmm for Prokaryotes (Version 2.4) (exon.biology.gatech.edu, 25).

3.2.4 Comparative genomic hybridization (CGH) using custom microarrays

Custom microarrays were purchased and designed by the Food Processing Center at the University of Nebraska-Lincoln, and the probes were designed to contain 2170 predicted open reading frames (ORFs) of strain 100-23 and 320 distinctive ORFs of strain DSM 20016^T (12). The detailed method has already

been described (12). In brief, a two-color labeling protocol was used to prepare synthetic amplicons for hybridization. Genomic DNAs of strains 100-23 and DSM 20016^T were equally mixed, and two micrograms of the mixed DNA were sheared. This was followed by amplification using the BioPrime DNA labeling kit (Life Technologies, Rockville, MD) along with random primers and Cy5 dye-labeled nucleotides to generate reference DNA. Sample DNA of strain LTH2584 was prepared using the same method as the reference DNA, but Cy-3 dye labeled nucleotides were used instead. In the dye-swap experiment, reference DNA of strains 100-23 and DSM 20016^T, and sample DNA of strain LTH2584 were labeled with Cy-3 and Cy-5 dyes, respectively. Cy-5 and Cy-3 labeled amplicons were co-purified and hybridized in formamide-containing buffer (Array Hyb Low Temp; Sigma, St. Louis, MO) for 20 h at 43°C. After hybridization, slides were washed once in a 1× SSC (0.15 M NaCl; 0.015 M sodium citrate) solution containing 0.03% sodium dodecyl sulfate, 0.2× SSC, and 0.05× SSC. The fluorescence intensity of each spot was determined using a GenePix4000 multicolor microarray scanner and GenePix software (Axon Instruments, Union City, CA).

3.2.5 Genome content comparison

The MARKFIND program was used for the conversion of CGH data as described previously (12, 26). The principle of MARKFIND is based on the calculation of the unweighted pair-group method with arithmetic means (UPGMA) to represent genome polymorphisms for cluster analysis by sorting polymorphic characters in the binary strings relative to user-specified groups. Data from all

arrays are composited and transformed into binary elements using a Perl-based program FormatALL (27).

3.2.6 Polymerase chain reaction (PCR) analysis of genes coding for two-component regulatory systems

Some of convergent genes identifed in the sourdough strain LTH2584 and the rodent strain 100-23 were verified by PCR analysis using Taq DNA polymerase (Invitrogen) according to the manufacturer's instructions. The cystine transporter protein (*cyuC*) and cystathionine gamma-lyase (*cgl*) of the cystine-mediated oxidative defense contribute to the survival of *L. reuteri* under aerobic conditions (28, 29). Isoleucyl-tRNA synthetase (*ileS*), a chaperon protein (*dnaK*), and a recombinase (*recA*) were found to be endogenous genes in lactobacilli (30). The two-component systems in *L. reuteri* LTH2584 (*tcsK*/*tcsR*) and in *L. reuteri* 100-23 (*cemK*/*cemR* and *hk430*/*rr431*/*abc432*) were also included. Specific primers for the above genes are listed in Table 3-2.

3.2.7 Genome annotation and nucleotide accession numbers

The genome records of *L. reuteri* 100-23 and DSM 20016^{T} were retrieved from the Joint Genome Institute (JGI) and annotated using the JGI annotation pipeline (Walnut Creek, CA). DNA sequences of *L. reuteri* 100-23 and DSM 20016^{T} were obtained from the National Center for Biotechnology Information's databases (GenBank: AAPZ02000002.1 and NC_009513, respectively).

Table 3-2 Primers used in this study

Primer name	Sequence (5' - 3')	Target gene
iresregV	CAACGCATGAAGAACTAG	Forward primer for sequencing the full-length of *tcsR* in strain LTH2584
dsResReg-R	AGATAATCATCATTGTTGGA	Reverse primer for sequencing the downstream of *tcsR* in strain LTH2584
ABC-R	AGGGAGTGAGAGGTTGTC	Reverse primer for sequencing the downstream of *tcsR* in strain LTH2584
CyuC-qPCR-F2	ACTCCCGCGAAGCATGGTACG	Forward primer for cystine transporter in strains 100-23 and LTH2584
CyuC-qPCR-R2	AATTTTGGCTGGTTTTTGTTCACTAGAAACA	Reverse primer for *cyuC* cystine transporter in strains 100-23 and LTH2584
Cgl-qPCR-F2	AATACCTTTGCTACTCCTTATAACCAACAAC CA	Forward primer for cystathionine gamma-lyase in strains 100-23 and LTH2584
Cgl-qPCR-R2	CGACAACATCACTATGACCGCCTAAA	Reverse primer for cystathionine gamma-lyase in strains 100-23 and LTH2584
Cgl-qPCR-F3	GATGTTGTCGCCGGATTAGCAGTTAC	Forward primer for cystathionine gamma-lyase in strains 100-23 and LTH2584
Cgl-qPCR-R3	GCCAACTATCATCAGGACCAAGCG	Reverse primer for cystathionine gamma-lyase in strains 100-23 and LTH2584
IleS-qPCR-F2	CGGCGGTCAATGCAAACTGTCT	Forward primer for isoleucyl-tRNA synthetase in strains 100-23 and LTH2584
IleS-qPCR-R2	CTTCTTCTGTAGTGTGAGGAAGGATTGGTG	Reverse primer for isoleucyl-tRNA synthetase in strains 100-23 and LTH2584
DnaK-qPCR-F2	AACACAACTATTCCTACTTCAAAGAGCCAA ATC	Forward primer for *dnaK* in strains 100-23 and LTH2584
DnaK-qPCR-R2	CTGCTGCCATTGGACGTTCACC	Reverse primer for *dnaK* in strains 100-23 and LTH2584
RecA-qPCR-F2	CAACTATCCGGATGGAAATTCGTCG	Forward primer for *recA* in strains 100-23 and LTH2584
RecA-qPCR-R2	TGTCAACTTCACAACGTTTGAATGGC	Reverse primer for *recA* in strains 100-23 and LTH2584

Table 3-2 –continued- Primers used in this study

Primer name	Sequence (5' - 3')	Target gene
HK2584-qPCR-F2	CGATAACGCCCTCGAAGAATGCA	Forward primer for *cemK* in strain 100-23 and *tcsK* in strain LTH2584
HK2584-qPCR-R2	TTAGGAGCGTCTTGATATAGCATTGAATTGA	Reverse primer for *cemK* in strain 100-23 and *tcsK* in strain LTH2584
RR2584-qPCR-F2	TGTCTGCGATGATGACAGGACTCAAG	Forward primer for *cemK* in strain 100-23 and *tcsK* in strain LTH2584
RR2584-qPCR-R2	TGCAATCAAATCAATGGTCGGATGA	Reverse primer for *cemK* in strain 100-23 and *tcsK* in strain LTH2584
HK1745-qPCR-F2	TGCTGTATCAAAGTGCCCCTAACAAGTTAG	Forward primer for *hk430* in strains 100-23 and LTH2584
HK1745-qPCR-R2	GCTAATCCAAGTCCCTCATGGTTAGTCTTATTT	Reverse primer for *hk430* in strains 100-23 and LTH2584
RR1746-qPCR-F2	CTCAGCAAATTCAAAAAAAGCACCGT	Forward primer for *rr431* in strains 100-23 and LTH2584
RR1746-qPCR-R2	ATCGCCGTTGCAATTTTCGTTG	Reverse primer for *rr431* in strains 100-23 and LTH2584
ABC1747-qPCR-F3	ACTAAAGCCTGCAAAGTTGCGATGAT	Forward primer for *abc432* in strains 100-23 and LTH2584
ABC1747-qPCR-R3	TTGTCCACCTGAAAGGGTAGTAGCATTTTC	Reverse primer for *abc432* in strains 100-23 and LTH2584
RR431-seq-F2	CAAGGRGAATTCTCMGG	Forward primer for *rr431* in strains 100-23 and LTH2584
RR431-seq-R2	CCKGAGAATTCYCCTTG	Reverse primer for *rr431* in strains 100-23 and LTH2584
ABC-seq-F2	AASARATGGAAAGTAATGCT	Forward primer for *abc432* in strains 100-23 and LTH2584
ABC-seq-R2	AGCATTACTTTCCATYTSTT	Reverse primer for *abc432* in strains 100-23 and LTH2584
ABC-seq-F3	TGAGYGGTTCWGGWAA	Forward primer for *abc432* in strains 100-23 and LTH2584
ABC-seq-R3	TTWCCWGAACCRCTCA	Reverse primer for *abc432* in strains 100-23 and LTH2584
IS4-Kath-F1	GCTGGTA TAGTTCTCCAC	Forward primer for IS*4* in strains 100-23 and LTH2584
IS4-Kath-R1	CGTAAGTATTGTTTAGCTGTT	Reverse primer for IS*4* in strains 100-23 and LTH2584
IS4-Kath-F2	CACTAAGCTAA CACCACA	Forward primer for IS*4* in strains 100-23 and LTH2584
IS4-Kath-R2	AGAGCATTATCATAAGCGAT	Reverse primer for IS*4* in strains 100-23 and LTH2584
HK430-Kath-F1	ATCGCTTATGATAATGCTCT	Forward primer for *hk430* in strain 100-23 and LTH2584

Table 3-2 –continued- Primers used in this study

Primer name	**Sequence (5' - 3')**	**Target gene**
HK430-Kath-R1	CTGCATCAGTGGCAAT	Reverse primer for *hk430* in strain 100-23 and LTH2584
RR431-Kath-F1	CAAGCAGGTTTAGCATTAG	Forward primer for *rr431* in strains 100-23 and LTH2584
RR431-Kath-R1	CGCCATTGATAAACGTTAC	Reverse primer for *rr431* in strains 100-23 and LTH2584
ABC432-Kath-F1	GTTGACGAAGCAGATTGT	Forward primer for *abc432* in strains 100-23 and LTH2584
ABC432-Kath-R1	GTTTTCGATAGTCTTTGACC	Reverse primer for *abc432* in strains 100-23 and LTH2584
ABC432-Kath-R2	ATTGATGCACTTACAGTAGC	Reverse primer for *abc432* in strains 100-23 and LTH2584

3.3 Results

3.3.1 Genome comparison of L. reuteri *LTH2584, 100-23 and DSM 20016^T using custom microarrays*

Since the ecological niches of *L. reuteri* 100-23 and DSM 20016^T are different, the representative genes of *L. reuteri* rodent and human isolates that were used in the DNA microarray were chosen for their uniqueness. This DNA microarray showed the variation of genome contents among the different isolates of *L. reuteri* (12). The same approach was taken to study the phylogenetic relationship between *L. reuteri* sourdough strain LTH2584 and other *L. reuteri* strains in this study. The results of comparative genomic hybridization were analyzed and converted to a phylogenetic tree using the MARKFIND program (Fig. 3-1). The analysis of gene contents showed that strain 100-23 has a higher correlation to strain LTH2584 than it has to the human strains DSM 20016^T and ATCC 55730, the chicken strain CSF8, or the swine strain JW2015 (Fig. 3-1).

In addition to gene polymorphism, the genes whose signals were below the threshold value calculated using the FormatALL method were extracted. These genes indicated that the DNA sequences of strain LTH2584 showed hybridization signals that were either much stronger or weaker than that of reference DNA on the arrays, implying the divergence of these genes in sourdough strain LTH2584. Fifty-three unique genes of strain 100-23 were identified and differentiated from those of strain LTH2584 in functional groups (Table 3-3). Of these, 31 were genes with unknown functions and 8 were genes of

integrase/transposase/phage-related proteins. The majority of divergent genes found in the human strain DSM 20016^T encoded hypothetical proteins; encoded

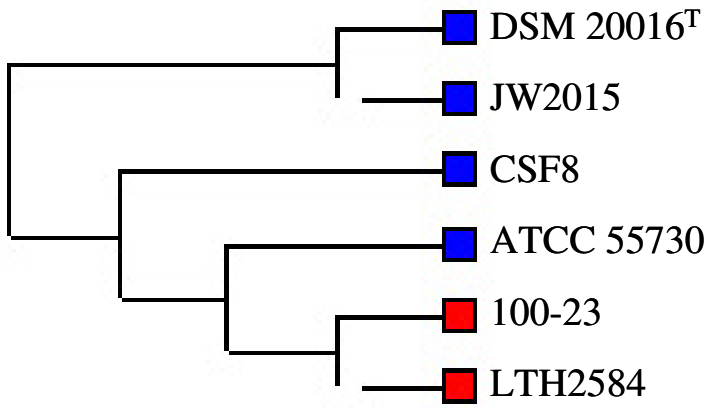

Figure 3-1 Genome content comparison by microarray analysis. The genes of sourdough strain LTH2584 were identified and compared to the reference strain 100-23 and other animal isolates of *L. reuteri* using the MARKFIND program. *L. reuteri* DSM 20016^T, JW2015, CSF8, ATCC 55730, 100-23, and LTH2584 were isolated from humans, swine, chickens, humans, rats, and sourdough, respectively. The CGH data on *L. reuteri* human and animal isolates was provided by Dr. Jens Walter (12).

integrase, transposase, or phage-related proteins; or encoded coenzyme, vitamin, amino acid, or protein metabolism. A specific gene cluster *pdu*/*cbi*/*cob*/*hem* encoding reuterin production and cobalamin synthesis was identified in the human strain DSM 20016^T. Some of the unique genes of the human and rodent isolates (Tables 3-4 and 3-5), which were divergent in the sourdough strain, may be the result of an evolutionary process resulting from their adaptation to different ecological niches. The results of the CGH analysis showed

that 2117 out of 2170 genes of strain 100-23 and 180 out of 320 genes of strain DSM 20016^T were identified as convergent with genes in strain LTH2584 (data not shown).

Table 3-3 Gene groups that are divergent in *L. reuteri* LTH2584 compared to rodent strain 100-23 and human strain DSM 20016^T

Gene group	100-23	DSM 20016^T
Integrases, transposases and phage-related proteins	8	33
DNA binding, restriction endonucleases and recombination/repair	2	9
Transcription and translation	3	5
Coenzyme/ vitamin/ amino acid/ protein metabolism	2	37
Transport proteins (ion, peptide and nucleotide)	2	2
Cell cycle control and cell division	0	1
Signal transduction and regulatory proteins	2	3
Cell wall/ membrane/ envelope biogenesis	3	4
Glycosyl transferases and sugar metabolism	0	6
Hypothetical and unknown proteins	31	40
Total	53	140

3.3.2 Analysis of selected genes in L. reuteri *100-23 and LTH2584*

The CGH analysis using the MARKFIND program revealed that sourdough strain LTH2584 has a closer phylogenetic relationship to the rodent strain 100-23 than to the isolates of other hosts. Some of the convergent genes identified in *L. reuteri* LTH2584 and 100-23 were chosen for PCR analysis because their functions have been characterized or discovered in recent studies of *L. reuteri*. Genes involved in the cystine-mediated oxidative defense (*cyuC* and *cgl*) were selected. Moreover, two-component systems of *L. reuteri* LTH2584 (*tcsK*/*tcsR*) and *L. reuteri* 100-23 (*cemK*/*cemR* and *hk430*/*rr431*/*abc432*) were chosen since response regulators are expressed during type-II sourdough

Table 3-4 Unique genes of *L. reuteri* human isolate DSM 20016^T that were divergent in the sourdough isolate LTH2584 and identified by FormatALL. Genes specific to the human MLSA lineage II are shown in bold while the conserved genes of the human MLSA lineage II are shown in bold and underlined according to the findings of Frese *et al*. (12)

Gene ID[a]	Annotation
Integrases, transposases and phage-related proteins	
640589868	Transposase, IS605 OrfB family
640589947	Transposase IS66
640590041	Integrase, catalytic region
640590498	Phage/plasmid primase, P4 family
640590499	**Phage head-tail adaptor, putative**
640590503	**Phage Terminase**
640590505	**Phage major capsid protein, HK97 family**
640590506	**Phage transcriptional regulator, RinA family**
640590635	Prophage antirepressor
640590645	Primosome, DnaD subunit
640590667	**Phage putative head morphogenesis protein, SPP1 gp7 family**
640590669	Phage putative head morphogenesis protein, SPP1 gp7 family
640590678	**Lytic transglycosylase, catalytic**
640590704	Phage integrase family protein
640590909	Integrase, catalytic region
640590948	Phage major tail protein
640590950	Phage protein, HK97 gp10 family
640590952	Uncharacterized phage protein (possible DNA packaging)
640590959	Phage terminase, small subunit, putative, P27 family
640590960	HNH endonuclease Phage-associated homing endonuclease
640590976	Primosome, DnaD subunit
640590981	**Phage antirepressor protein**
640590983	**Helix-turn-helix domain protein**
640590987	Phage integrase family protein
640591192	Integrase, catalytic region
640591194	Transposase IS3/IS911 family protein
640591254	Transposase, IS605 OrfB family
640591280	**Phage integrase family protein**
640591431	Integrase, catalytic region
640591453	**Phage integrase family protein**
640591528	Transposase, IS605 OrfB family
640591618	Transposase and inactivated derivatives IS30 family-like protein

[a] This gene ID system is the same as that used in the DOE Joint Genome Institute databases

Table 3-4 –continued- Unique genes of *L. reuteri* human isolate DSM 20016^T that were divergent in the sourdough isolate LTH2584 and identified by FormatALL. Genes specific to the human MLSA lineage II are shown in bold while the conserved genes of the human MLSA lineage II are shown in bold and underlined according to the findings of Frese *et al*. (12)

Gene ID[a]	Annotation
Integrases, transposases and phage-related proteins	
640591619	Transposase and inactivated derivatives IS30 family-like protein
DNA binding, restriction endonucleases and recombination/repair	
640590702	Restriction modification system DNA specificity domain
640590703	Restriction modification system DNA specificity domain
640590705	Restriction modification system DNA specificity domain
640590706	Type I site-specific deoxyribonuclease, HsdR family
640590628	Recombinase
640590663	**ParB domain protein nuclease**
640590890	**Superfamily I DNA and RNA helicase-like protein**
640591279	**Restriction modification system DNA specificity domain**
640591281	**Restriction modification system DNA specificity domain**
Transcription and translation	
640590634	Transcriptional regulator, XRE family
640590639	Transcriptional regulator, XRE family
640590642	**Putative transcriptional regulator, XRE family**
640590881	Transcriptional regulator, XRE family
640591675	**Histidyl-tRNA synthetase**
Coenzyme/ vitamin/ amino acid/ protein metabolism	
640590661	Phosphoadenosine phosphosulfate reductase
640591540	Nicotinate-nucleotide--dimethylbenzimidazole phosphoribosyltransferase
640591544	Adenosylcobinamide kinase
640591545	Glutamate-1-semialdehyde 2,1-aminomutase
640591547	Hydroxymethylbilane synthase
640591548	Glutamyl-tRNA reductase
640591549	Siroheme synthase (precorrin-2 oxidase/ferrochelatase domain)-like protein
640591550	Adenosylcobyric acid synthase (glutamine-hydrolysing)
640591556	Anaerobic cobaltochelatase
640591558	Precorrin-6A reductase

[a] This gene ID system is the same as that used in the DOE Joint Genome Institute databases

Table 3-4 –continued- Unique genes of *L. reuteri* human isolate DSM 20016^T that were divergent in the sourdough isolate LTH2584 and identified by FormatALL. Genes specific to the human MLSA lineage II are shown in bold while the conserved genes of the human MLSA lineage II are shown in bold and underlined according to the findings of Frese *et al*. (12)

Gene ID[a]	Annotation
Coenzyme/ vitamin/ amino acid/ protein metabolism	
640591561	Precorrin-4 C11-methyltransferase
640591562	Precorrin-6Y C5, 15-methyltransferase (decarboxylating), CbiT subunit
640591564	Cobalamin biosynthesis protein CbiD
640591566	Adenosylcobinamide-phosphate synthase
640589953	ADP-ribosylation/Crystallin J1
640590711	Riboflavin synthase, alpha subunit
640590712	GTP cyclohydrolase II riboflavin metabolism
640590920	Methionine synthase, vitamin-B12 independent
640590940	**Lipolytic protein, G-D-S-L family; GDSL-like lipase/ acylhydrolase**
640590954	Peptidase S14, ClpP
640591567	Cobyrinate a,c-diamide synthase (EC 6.3.5.-) / hydrogenobyrinic acid a,c-diamide synthase (glutamine-hydrolysing)
640591568	L-threonine O-3-phosphate decarboxylase
640591571	NADPH-dependent FMN reductase
640591577	Microcompartments protein
640591585	Propanediol utilization protein
640591587	Microcompartments protein
640591590	Dehydratase, small subunit
640591591	Dehydratase, medium subunit
640591593	Microcompartments protein
640591596	Ethanolamine utilization protein EutJ family protein
640591677	**Histidine decarboxylase, pyruvoyl type**
640591738	Putative NADH-flavin reductase-like protein
640591747	Hydratase/decarboxylase
640591216	Acyltransferase 3
640591426	Alcohol dehydrogenase GroES domain protein
640591582	ATP--cobalamin adenosyltransferase
640591761	Antibiotic biosynthesis monooxygenase

[a] This gene ID system is the same as that used in the DOE Joint Genome Institute databases

Table 3-4 –continued- Unique genes of *L. reuteri* human isolate DSM 20016^T that were divergent in the sourdough isolate LTH2584 and identified by FormatALL. Genes specific to the human MLSA lineage II are shown in bold while the conserved genes of the human MLSA lineage II are shown in bold and underlined according to the findings of Frese *et al*. (12)

Gene ID[a]	Annotation
Transport proteins (ion, peptide and nucleotide)	
640589954	Permease for cytosine/purines, uracil, thiamine, allantoin
640591552	Cobalt ABC transporter, inner membrane subunit CbiQ
Cell cycle control and cell division	
640590943	Chromosome segregation ATPase-like protein
Signal transduction and regulatory proteins	
640591099	YSIRK Gram-positive signal peptide
640591576	Protein tyrosine phosphatase
640591730	Diguanylate phosphodiesterase
Cell wall/ membrane/ envelope biogenesis	
640590251	Beta-lactamase
640590691	N-acetylmuramoyl-L-alanine amidase, family 2
640591191	Putative cell wall binding repeat-containing protein
640591212	NLP/P60 protein Cell wall-associated hydrolases (invasion-associated proteins)
Glycosyl transferases and sugar metabolism	
640591219	Polysaccharide biosynthesis protein
640591733	Glycosyl transferase, family 2
640591190	Glycosyl hydrolase 53 domain protein
640591196	Dextransucrase
640591215	Mannosyl-glycoprotein endo-beta-N-acetylglucosamidase
Hypothetical and unknown proteins	
640590227	Hypothetical protein
640590249	Hypothetical protein
640590364	Hypothetical protein
640590496	**hypothetical protein**
640590497	**hypothetical protein**
640590629	Hypothetical protein

[a] This gene ID system is the same as that used in the DOE Joint Genome Institute databases

Table 3-4 –continued- Unique genes of *L. reuteri* human isolate DSM 20016^T that were divergent in the sourdough isolate LTH2584 and identified by FormatALL. Genes specific to the human MLSA lineage II are shown in bold while the conserved genes of the human MLSA lineage II are shown in bold and underlined according to the findings of Frese *et al*. (12)

Gene ID[a]	Annotation
Hypothetical and unknown proteins	
640590637	Hypothetical protein
640590638	Hypothetical protein
640590644	Hypothetical protein
640590648	**Hypothetical protein**
640590653	**Hypothetical protein**
640590656	**Hypothetical protein**
640590664	Hypothetical protein
640590673	Hypothetical protein
640590675	Hypothetical protein
640590676	Hypothetical protein
640590685	**Hypothetical protein**
640590880	Hypothetical protein
640590885	**Hypothetical protein**
640590887	**Hypothetical protein**
640590908	Hypothetical protein
640590937	**Hypothetical protein**
640590938	**Hypothetical protein**
640590945	**Hypothetical protein**
640590947	Hypothetical protein
640590949	Hypothetical protein
640590966	Hypothetical protein
640590969	Hypothetical protein
640590977	**Hypothetical protein**
640590980	Hypothetical protein
640591189	Hypothetical protein
640591193	Hypothetical protein
640591207	Hypothetical protein
640591221	**Hypothetical protein**
640591264	Hypothetical protein
640591283	**Hypothetical protein**
640591538	Hypothetical protein
640591574	Hypothetical protein

[a] This gene ID system is the same as that used in the DOE Joint Genome Institute databases

Table 3-4 –continued- Unique genes of *L. reuteri* human isolate DSM 20016^T that were divergent in the sourdough isolate LTH2584 and identified by FormatALL. Genes specific to the human MLSA lineage II are shown in bold while the conserved genes of the human MLSA lineage II are shown in bold and underlined according to the findings of Frese *et al*. (12)

Gene ID[a]	Annotation
Hypothetical and unknown proteins	
640591676	**Hypothetical protein**
640591712	Hypothetical protein

[a] This gene ID system is the same as that used in the DOE Joint Genome Institute databases

Table 3-5 Unique genes of *L. reuteri* rodent isolate 100-23 that were divergent in the sourdough isolate LTH2584 and identified by FormatALL. Genes specific to the rodent-lineage are shown in bold while conserved genes among rodent strains (Type I genes) are shown in bold and underlined according to the findings of Frese *et al*. (12)

Gene ID [a]	Annotation
Integrases, transposases and phage-related proteins	
2500069124	**Phage transcriptional activator, RinA family**
2500069164	Phage terminase-like protein, large subunit
2500069509	**Death-on-curing family protein**
2500069747	**Tape measure domain; phage-related protein**
2500069750	**Phage major tail protein, TP901-1 family**
2500069759	Phage portal protein, SPP1 family
2500070705	**Phage integrase family**
2500070723	**Phage integrase family**
DNA binding, restriction endonucleases and recombination/repair	
2500069119	**Deoxynucleoside kinases**
2500069792	**Site-specific recombinase XerD**
Transcription and translation	
2500069083	Helix-turn-helix
2500069125	tRNA-Val
2500069165	**Group I intron endonuclease**
Coenzyme/ vitamin/ amino acid/ protein metabolism	
2500069899	Coenzyme F420-dependent N5,N10-methylene tetrahydromethanopterin reductase and related flavin-dependent oxidoreductases
2500071339	Acetyltransferase (isoleucine patch superfamily)
Transport proteins (ion, peptide and nucleotide)	
2500069907	Ammonium transporter
2500069910	ABC-type multidrug transport system, ATPase component
Signal transduction and regulatory proteins	
2500069919	cAMP-binding proteins - catabolite gene activator and regulatory subunit of cAMP-dependent protein kinases
2500071110	SOS regulatory protein LexA

[a] This gene ID system is the same as that used in the DOE Joint Genome Institute databases

Table 3-5 -continued- Unique genes of *L. reuteri* rodent isolate 100-23 that were divergent in the sourdough isolate LTH2584 and identified by FormatALL. Genes specific to the rodent-lineage are shown in bold while conserved genes among rodent strains (Type I genes) are shown in bold and underlined according to the findings of Frese *et al*. (12)

Gene ID [a]	Annotation
Cell wall/ membrane/ envelope biogenesis	
2500069518	Cell wall-associated hydrolases
2500070697	**Large surface protein with LPXTG-motif cell wall anchor domain**
2500070943	Surface protein with LPXTG-motif cell wall anchor domain
Hypothetical and unknown proteins	
2500069101	**Hypothetical protein**
2500069104	**Hypothetical protein**
2500069118	Hypothetical protein
2500069128	**Uncharacterized conserved protein**
2500069137	**Hypothetical protein**
2500069145	**Hypothetical protein**
2500069146	**Hypothetical protein**
2500069150	**Hypothetical protein**
2500069154	Hypothetical protein
2500069161	Hypothetical protein
2500069174	**Hypothetical protein**
2500069179	Hypothetical protein
2500069182	Hypothetical protein
2500069184	Hypothetical protein
2500069237	Hypothetical protein
2500069514	Hypothetical protein
2500069524	Hypothetical protein
2500069525	Hypothetical protein
2500069538	Hypothetical protein
2500069545	Hypothetical protein
2500069548	**Hypothetical protein**
2500069549	**Hypothetical protein**
2500069560	**Hypothetical protein**
2500069782	**Hypothetical protein**
2500069847	Hypothetical protein
2500069851	**Hypothetical protein**

[a] This gene ID system is the same as that used in the DOE Joint Genome Institute databases

Table 3-5 -continued- Unique genes of *L. reuteri* rodent isolate 100-23 that were divergent in the sourdough isolate LTH2584 and identified by FormatALL. Genes specific to the rodent-lineage are shown in bold while conserved genes among rodent strains (Type I genes) are shown in bold and underlined according to the findings of Frese *et al*. (12)

Gene ID [a]	**Annotation**
Hypothetical and unknown proteins	
2500069913	Hypothetical protein
2500070717	Hypothetical protein
2500070951	Hypothetical protein
2500071113	Hypothetical protein
2500071114	**Hypothetical protein**

[a] This gene ID system is the same as that used in the DOE Joint Genome Institute databases

fermentation (13, 20). Also, the study of *hk430* in strain 100-23 shows that the conservation of *hk430* is only found in rodent isolates of *L. reuteri* and HK430 has ecological importance to the colonization of the murine forestomach (12). Endogenous genes such as isoleucyl-tRNA synthetase (*ileS*), a heat-shock/chaperon protein (*dnaK*), and a recombinase (*recA*) were also included. The PCR amplifications of *cyuC*, *cgl*, *ileS*, *dnaK*, *recA*, *tcsK*/*tcsR*, and *cemK*/*cemR* were carried out in both *L. reuteri* strains LTH2584 and 100-23 (Table 3-6). However, the two-component system *hk430*/*rr431*/*abc432* was only found in the *L. reuteri* rodent strain 100-23, and not in the sourdough strain LTH2584, which suggests a sequence divergence of the two-component system *hk430*/*rr431*/*abc432* in the sourdough strain LTH2584.

Table 3-6 PCR analysis of genes that were convergent in *L. reuteri* strains 100-23 and LTH2584

Gene	100-23	LTH2584
cyuC	Present	Present
cgl	Present	Present
ileS	Present	Present
dnaK	Present	Present
recA	Present	Present
cemK or *tcsK*	Present	Present
cemR or *tcsR*	Present	Present
hk430	Present	Absent
rr431	Present	Absent
abc432	Present	Absent
IS*4*	Present	Absent

To clarify the results of the PCR analysis in the sourdough strain, degenerate primers were designed based on the conserved domains of HK430, RR431, and ABC432, and encompassing the region of IS*4* and *abc432*. The

negative results of the PCR analysis implied that the genetic loci of IS*4*, *hk430*, *rr431*, and *abc432* found in *L. reuteri* 100-23 is either not present or very divergent in the genome of *L. reuteri* LTH2584.

3.3.3 In silico prediction of two-component systems

Because of the evolutionary and ecological importance of *hk430* in the rodent strain 100-23, the two-component systems identified in the sourdough strain LTH2584 and the rodent strain 100-23 were analyzed using bioinformatic programs. The prediction of *tcsK*/*tcsR* in *L. reuteri* LTH2584 using GeneMark.hmm and BAGEL showed the genetic loci that encode an auto-inducing peptide, a histidine kinase, and a response regulator, suggesting a putative peptide-based quorum sensing two-component regulatory system (Fig. 3-2). A short DNA fragment upstream of *tcsK*, named *aip*, was predicted to encode a putative auto-inducing peptide for the regulation of bacteriocin synthesis. The putative auto-inducing peptide has a typical double-glycine-type leader sequence (31), followed by the heptapeptide IYSLLSL. The histidine kinase TcsK belongs to the HPK_{10} subfamily, whose members have five to seven transmembrane segments at the amino-terminus as well as homology boxes on the ATP-binding domain at the carboxyl-terminus (22). The predicted response regulator TcsR has two domains, the CheY-like receiver domain with conserved aspartate and lysine residues, and the LytTr DNA-binding domain that is common to proteins in the LytR/AlgR family (23). Likewise, the prediction of *cemK*/*cemR* in *L. reuteri* 100-23 revealed a similar genetic organization, and the name of the genetic loci *cemAKR* stands for an auto-inducing peptide (*cemA*), a histidine kinase (*cemK*),

and a response regulator (*cemR*). The results of the BLASTP analysis showed the orthologous genes of *tcsK* and *tcsR* in *L. reuteri* rodent isolate 100-23 but not in other human isolates (DSM 20016T, MM4-1A, JCM1112, MM2-3, CF48-3A, and ATCC 55730). These orthologous operons of *tcsKR* in *L. reuteri* 100-23 were named as *hk430*/*rr431*/*abc432* and *cemAKR* (Fig. 3-3). The *hk430* operon consists

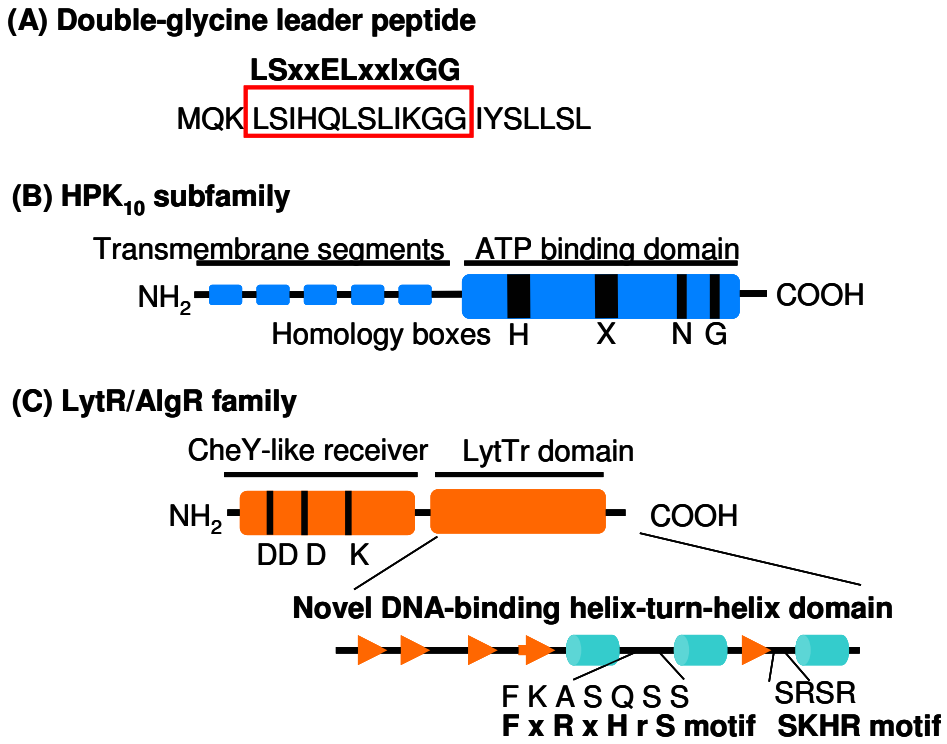

Figure 3-2 Prediction of genetic loci coding for a peptide-based quorum sensing two-component regulatory system in *L. reuteri* LTH2584. (A) The bacteriocin-like autoinducing peptide contains a conserved double-glycine (GG) motif and its potential heptapeptide IYSLLSL works as a signal peptide or peptide lactone. (B) The deduced protein sequence of the *tcsK* gene has specific homology to the HPK_{10} subfamily. (C) The TcsR contains an N-terminal CheY-like receiver domain and a C-terminal DNA-binding domain (LytTr domain).

of a histidine kinase (*hk430*), a response regulator (*rr431*), and an ATP-binding cassette transporter (*abc432*). The *tcsKR* and *cemKR* operons are highly homologous, being more than 90% similar in both DNA and protein levels. In strain 100-23, a homologue of *aip* was found and named as *cemA*. In addition, the DNA sequences of *hk430* and *tcsK* are 82% similar and the protein sequences are 80% similar. The DNA sequences of *rr431* and *tcsR* are 73% similar, and the protein sequences are 86% similar (Fig. 3-3).

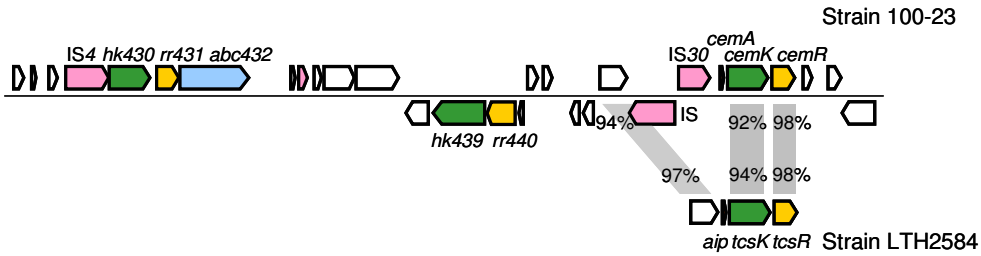

Figure 3-3 Comparison of novel two-component regulatory systems in *L. reuteri* rodent and sourdough isolates. Sequencing of a two-component regulatory system of *L. reuteri* LTH2584 revealed an operon of a membrane-bound histidine kinase (*tcsK*), its cognate cytosolic response regulator (*tcsR*), and one bacteriocin-like autoinducing peptide (*aip*). The comparison between operon *tcsKR* of strain LTH2584 and operon *cemAKR* of strain 100-23 in nucleotide and deduced protein sequences shows a similarity from 92 – 98%.

3.4 Discussion

This study aimed to discover the genes of *L. reuteri* sourdough strain LTH2584 associated with adaptation by comparing their genome content to that of *L. reuteri* isolates from humans and rats, and by analyzing the genes coding for two-component systems in the sourdough strain LTH2584 and rodent strain 100-23. The characterization of the population and phylogeny of lactic acid bacteria

isolated from different niches was previously performed using amplified-fragment length polymorphism, multi-locus sequence analysis, and quantitative PCR (1, 32; Chapter 2, this thesis). Such approaches are used to clarify the evolutionary history rather than to provide the genomic information of gene variation and conservation. The microarray analysis has advantages when used to analyze gene polymorphisms and genome content (12, 14). Although the custom microarrays of *L. reuteri* only consist of the genes of rodent and human isolates, these arrays were verified as having relatively reliable gene polymorphism information, and this information serves as a powerful tool for the study of phylogeny. It is not, however, useful for obtaining accurate information on the presence or absence of genes (12).

The gene polymorphisms of the CGH analysis in this study showed a highly phylogenetic correlation between *L. reuteri* sourdough strain LTH2584 and rodent strain 100-23, suggesting that the sourdough strain shared a more recent common ancestor with the rodent strain than with other vertebrate strains. Moreover, sourdough isolates are capable of producing exopolysaccharides to colonize the murine forestomach where they can compete with the rodent strains (10, 33, 34). In contrast, most of the *L. reuteri* isolates from humans and chickens cannot colonize or form biofilms on the epithelium of the murine forestomach, reflecting evolutionary adaptations to their hosts (1, 12, 35). The gene polymorphism analysis of *L. reuteri* LTH2584 showed that strain LTH2584 shares a great number of convergent genes with strain 100-23, many of which are conserved in other rodent-lineage strains of *L. reuteri*. However, strain LTH2584

did not share most of the human-conserved genes that are found in the human MLSA lineage II of *L. reuteri* (this thesis, 12).

The CGH analysis revealed the majority of rodent-unique and human-unique genes are phage-related proteins and hypothetical proteins. Phages have an important role in the lateral gene transfer for bacterial evolution (36). Bacterial phage resistance is mediated by Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) elements (37, 38). However, none of the CRISPR-associated proteins, Cas1, Cas2, or Cas3, were found in *L. reuteri* 100-23. Most genes of the *pdu-cbi-cob-hem* gene cluster responsible for reuterin and cobalamin production (18) in the human strains of *L. reuteri* were not shown to be convergent with the genes of sourdough strain LTH2584 (Table 3-3). This was expected based on the inability of strain LTH2584 to synthesize reuterin or cobalamin, which resembles the physiology of *L. reuteri* 100-23. Moreover, the production of fructan and fructoligosaccharides through the use of fructansucrases contributes to the competitiveness of *L. reuteri* in sourdough and the murine forestomach (33). Taken together, the genome polymorphism of the sourdough strain LTH2584 and its colonization in the murine guts imply the source of the original *L. reuteri* sourdough strains may have come from rodent feces.

Two-component systems are critical regulatory mechanisms for microorganisms' adaptation in various environments (22, 23). The two-component system *hk430*/*rr431*/*abc432* was found by the BLASTP analysis in *L. reuteri* strain 100-23, but not in the sourdough strain LTH2584 or in other animal isolates of *L. reuteri* (data not shown). The disruption of *hk430* reduces the ability

of *L. reuteri* 100-23 to colonize in the murine gut (12). It is notable that in strain 100-23, IS elements are located adjacent to *hk430* and *cemK*, and the orthologs of *hk430/rr431/abc432* are absent in the sourdough strain LTH2584 (Fig. 3-3). This study demonstrated the close evolutionary relationship between the *L. reuteri* rodent strain 100-23 and sourdough strain LTH2584. The few genetic differences between these two strains may reflect their adaptations to different ecological niches.

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Chapter Four: **Characterization of the novel two-component regulatory systems of *Lactobacillus reuteri* strains LTH2584 and 100-23**

4.1 Introduction

Lactobacillus reuteri is heterofermentative, thermophilic lactic acid bacterium occurring in food fermentations and vertebrate intestinal habitats (1, 2). Extraintestinal habitats include industrial sourdough fermentations and cereal fermentations in tropical climates (1, 3). In humans, the species is present in the gastrointestinal tract and the female urogenital tract (2). *L. reuteri* forms biofilms on non-secretory stratified squamous epithelia in the upper intestinal tract of animals (2, 4), including the pars esophagus of pigs, the crop of poultry, and the forestomachs of horses, rats, and mice (for reviews, see 2, 5). Because of its stable association with diverse mammalian hosts, *L. reuteri* has been used as a model organism to study host-microbe interaction and host-specific adaptation (2). The cell wall-associated proteins BspA/CnBP/MapA/CyuC, Mub, and Lsp mediate adhesion to mucus or intestinal surfaces (6, 7, 8, 9, 10). The biofilm formation by *L. reuteri* TMW1.106 and its competitiveness in the rodent forestomach depend on the expression of a reuteransucrase to produce an extracellular polysaccharide matrix (4). It has been suggested that fructansucrases in *L. reuteri* act as matrix-binding proteins and protect against environmental insults (4, 11). Expression of GtfA coding for reuteransucrase in *L. reuteri* TMW 1.106 is constitutive (12). The fructansucrases in *L. reuteri* were shown to be up-regulated in response to

membrane stress, whereas sucrose-dependent expression was observed only in some strains (12, 13).

The ecotype of *L. reuteri* is remarkably analogous to *Streptococcus mutans*, a phylogenetically related bacterium. *S. mutans* forms biofilms on tooth enamel, and causes cariogenic lesions by acid production from glucose. Biofilm formation in oral streptococci is mediated by glucansucrases and fructansucrases that act as key virulence factors, and are dependent on signal transduction using several regulators and two-component systems (14, 15, 16). A typical two-component system consists of a histidine kinase and a response regulator. The histidine kinase autophosphorylates in response to environmental stimuli, and relays a phosphoryl group to activate its cognate response regulator, which then binds to DNA and alters gene expression (17). The *L. reuteri* LTH2584 genome contains genes encoding a novel two-component system *tcsKR* (Chapter 3, this thesis). In addition, the *L. reuteri* 100-23 genome contains 17 genes that encode response regulators (18). However, it remains unclear whether two-component systems known to control biofilm formation in oral streptococci (14) are also involved in the regulation of biofilm formation in *L. reuteri*.

Gene *lr70430*, which codes for a histidine kinase, is unique to the rodent isolates of *L. reuteri* and contributes to the colonization of *L. reuteri* rodent strain 100-23 in the murine forestomach (19). Gene *lr70430* and its cognate response regulator were identified as convergent genes of strain LTH2584 by CGH analysis (Chapter 3, this thesis), and the genetic loci were named the "*hk430* operon." This operon comprised *hk430*, *rr431*, and *abc432*, coding for histidine

kinase, a response regulator, and an ABC transporter, respectively. Paralogous genes of *hk430* and *rr431* were also found in strain 100-23 and this gene cluster was named the "*cemAKR* operon." This operon encoded a putative autoinducing peptide, a histidine kinase, and a response regulator. The genetic loci of *aip*/*tcsKR* found in the sourdough strain LTH2584 was orthologous to the *cemAKR* operon of *L. reuteri* 100-23. However, the role of the *hk430* and *cemAKR* operons in *L. reuteri* 100-23 (20) and the *aip*/*tcsKR* operon in strain LTH2584 (21) related to the adaptation of *L. reuteri* in different niches has not yet been deduced. It was therefore the aim of this study to characterize the role of two-component systems in *L. reuteri* rodent isolate 100-23 and sourdough isolate LTH2584. A novel method for the multi-deletion mutagenesis in *L. reuteri* was employed. The genetic loci of *aip*/*tcsKR* in strain LTH2584 and the genetic loci of the *hk430* and *cemAKR* operons in strain 100-23 were disrupted by homologous recombination. The phenotype of mutant strains, including their ability to adhere in the presence of glucose or sucrose, was characterized.

4.2 Materials and methods

4.2.1 Bacterial growth

Bacterial strains and plasmids used in this study are listed in Table 4-1. *Escherichia coli* JM109 (Promega, Nepean, Canada) was cultured in Luria-Bertani (LB) broth with agitation at 37°C in most cases. When *E. coli* contains pJRS233-derived plasmids, it was cultured at 30°C to maintain the plasmids. *L. reuteri* was usually cultured at 37°C under micro-aerobic conditions (1% O_2, 5%

Table 4-1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
Strains		
Escherichia coli JM109	Cloning host for pGEMTeasy- and pJRS233-derived plasmids	Promega
Lactobacillus reuteri		
LTH2584	Sourdough isolate; reutericyclin-producing strain; wild type strain	(21)
Δ*tcsK*	A deletion mutant of *L. reuteri* LTH2584 on *tcsK* homologous to *cemK* of strain 100-23	This study
Δ*tcsR*	A deletion mutant of *L. reuteri* LTH2584 on *tcsR* homologous to *cemR* of strain 100-23	This study
100-23	Rodent isolate; wild type strain	(20)
Δ*hk430*	A deletion mutant of *L. reuteri* 100-23 on *hk430*	This study
Δ*rr431*	A deletion mutant of *L. reuteri* 100-23 on *rr431*	This study
Δ*hk430*Δ*rr431*	A deletion mutant of *L. reuteri* 100-23 on *hk430* and *rr431*	This study
Δ*cemA*	A deletion mutant of *L. reuteri* 100-23 on DNA region encoding putative autoinducing peptide upstream of *cemK*	This study
Δ*cemK*	A deletion mutant of *L. reuteri* 100-23 on *cemK*	This study
Δ*cemK*Δ*cemR*	A deletion mutant of *L. reuteri* 100-23 on *cemK* and *cemR*	This study
Δ*hk430*Δ*cemK*	A deletion mutant of *L. reuteri* 100-23 on *hk430* and *cemK*	This study
Δ*rr431*Δ*cemK*	A deletion mutant of *L. reuteri* 100-23 on *rr431* and *cemK*	This study
Plasmids		
pUC19	Cloning vector used in *E. coli*; 2.7 kb; Amp^r	New England Biolabs
pGEMTeasy	Cloning vector used in *E. coli*; 3.0 kb; Amp^r	Promega
pHK430-A	pGEMTeasy containing 1.1 kb of the DNA sequence upstream of *hk430*; 4.1 kb; Amp^r	This study
pHK430-B	pGEMTeasy containing 1.0 kb of the DNA sequence downstream of *hk430*; 4.0 kb; Amp^r	This study

Amp^r, amplicillin resistance gene; Erm^r, erythromycin resistance gene

Table 4-1 -continued- Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
Plasmids		
pRR431-A	pGEMTeasy containing 0.7 kb of the DNA sequence upstream of *rr431*; 3.7 kb; Amp^r	This study
pRR431-B	pGEMTeasy containing 1.0 kb of the DNA sequence downstream of *rr431*; 4.0 kb; Amp^r	This study
pCemA-A	pGEMTeasy containing 1.0 kb of the DNA sequence upstream of *cemA*; 4.0 kb; Amp^r	This study
pCemA-B	pGEMTeasy containing 1.0 kb of the DNA sequence downstream of *cemA*; 4.0 kb; Amp^r	This study
pCemK-A	pGEMTeasy containing 1.2 kb of the DNA sequence upstream of *cemK*; 4.2 kb; Amp^r	This study
pCemK-B	pGEMTeasy containing 1.0 kb of the DNA sequence downstream of *cemK*; 4.0 kb; Amp^r	This study
pCemR-B	pGEMTeasy containing 909 bp of the DNA sequence downstream of *cemR*; 3.9 kb; Amp^r	This study
pJRS233	Shuttle vector used in the hosts *E. coli* and *L. reuteri* strains LTH2584 and 100-23; 6.0 kb; Erm^r	(23)
pKO-*tcsK*-AB	pJRS233 containing 1.9 kb of the *tcsK* flanking DNA sequences by ligating *Pst*I/*Xho*I-cut 830 bp of amplicon TcsK-A with *Xho*I/*Hind*III-cut 1066 bp of amplicon TcsK-B; 7.9 kb; Erm^r	This study
pKO-*tcsR*-AB	pJRS233 containing 1.1 kb of the *tcsR* flanking DNA sequences by ligating *Pst*I/*Xho*I-cut 535 bp of amplicon TcsR-A with *Xho*I/*Hind*III-cut 536 bp of amplicon TcsR-B; 7.1 kb; Erm^r	This study
pKO-*hk430*-AB	pJRS233 containing 2.1 kb of the *hk430* flanking DNA sequences by ligating *Bam*HI/*Sal*I-cut 1.1 kb of pHK430-A with *Sal*I/*Pst*I-cut 1.0 kb of pHK430-B; 8.1 kb; Erm^r	This study
pKO-*rr431*-AB	pJRS233 containing 1.7 kb of the *rr431* flanking DNA sequences by ligating *Bam*HI/*Sal*I-cut 0.7 kb of pRR431-A with *Sal*I/*Pst*I-cut 1.0 kb of pRR431-B; 7.7 kb; Erm^r	This study
pKO-*hk430rr431*-AB	pJRS233 containing 2.1 kb of the *hk430* and *rr431* flanking sequences by ligating *Bam*HI/*Sal*I-cut 1.1 kb of pHK430-A with *Sal*I/*Pst*I-cut 1.0 kb of pRR431-B; 8.1 kb; Erm^r	This study

Amp^r, amplicillin resistance gene; Erm^r, erythromycin resistance gene

Table 4-1 -continued- Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
Plasmids		
pKO-*cemA*-AB	pJRS233 containing 1.9 kb of the *cemA* flanking DNA sequences by ligating *Pst*I/*Sal*I-cut 0.9 kb of pCemA-A with *Sal*I/*Bam*HI-cut 1.0 kb of pCemA-B; 7.9 kb; Erm^r	This study
pKO-*cemK*-AB	pJRS233 containing 2.1 kb of the *cemK* flanking DNA sequences by ligating *Pst*I/*Sal*I-cut 1.1 kb of pCemK-A with *Sal*I/*Bam*HI-cut 1.0 kb of pCemK-B; 8.1 kb; Erm^r	This study
pKO-*cemKcemR*-AB	pJRS233 containing 2.0 kb of the *cemK* and *cemR* flanking DNA sequences by ligating *Pst*I/*Sal*I-cut 1.1 kb of pCemK-A with *Sal*I/*Bam*HI-cut 0.9 kb of pCemR-B; 8.0 kb; Erm^r	This study

Amp^r, amplicillin resistance gene; Erm^r, erythromycin resistance gene

Table 4-2 Primers used in this study

Primer name	Sequence (5' - 3')	Features
tcsK-KO-1-*Pst*I	AACTGCAGATGGCGAAGATTAGT	Forward primer for 5'-flanking sequence of *tcsK*
tcsK-KO-2-*Xho*I	CCGCTCGAGCTAAAAGAATGTGAAAAGTT	Reverse primer for 5'-flanking sequence of *tcsK*
tcsK-KO-3-*Xho*I	CCGCTCGAGACATTGCTTACTCCTCT	Forward primer for 3'-flanking sequence of *tcsK*
		Sequencing primer for mutant Δ*tcsR*
tcsR-KO-1-*Pst*I	AACTGCAGTCTCGTGATAATCTTGG	Forward primer for 5'-flanking sequence of *tcsR*
tcsR-KO-2-*Xho*I	CCGCTCGAGAGTCAATGGTAGTCCATA	Reverse primer for 5'-flanking sequence of *tcsR*
		Sequencing primer for mutant Δ*tcsK*
tcsR-KO-3-*Xho*I	CCGCTCGAGTAAAGTTGGTACAAGGAT	Forward primer for 3'-flanking sequence of *tcsR*
tcsR-KO4-*Hind*III	CCCAAGCTTGAGTGAGAGGTTGTC	Reverse primer for 3'-flanking sequence of *tcsK* and *tcsR*
hk430-KO-1a-*Bam*HI	CGGGATCCTTCATTACTTCGTCCATT	Forward primer for 5'-flanking sequence of *hk430*
		Sequencing primer for mutants Δ*hk430* and Δ*hk430*Δ*rr431*
hk430-KO-2-*Sal*I	ACGCGTCGACCTAAAGGACCGGAGC	Reverse primer for 5'-flanking sequence of *hk430*
hk430-KO-3-*Sal*I	ACGCGTCGACCCTCCGATAATGGTTACT	Forward primer for 3'-flanking sequence of *hk430*
hk430-KO-4-*Pst*I	AACTGCAGAGCGGCTTCGACAATACCT	Reverse primer for 3'-flanking sequence of *hk430*
		Sequencing primer for mutants Δ*rr431* and Δ*hk430*Δ*rr431*
rr431-KO-1a-*Bam*HI	CGGGATCCAATCGAAGTTAAGACGA	Forward primer for 5'-flanking sequence of *rr431*
rr431-KO-2-*Sal*I	ACGCGTCGACTTACAGAATAATTGACGAC	Reverse primer for 5'-flanking sequence of *rr431*
		Sequencing primer for mutant Δ*hk430*
rr431-KO-3-*Sal*I	ACGCGTCGACCGGAGTCGAACAAAAG	Forward primer for 3'-flanking sequence of *rr431*
rr431-KO-4-*Pst*I	AACTGCAGCAAGGTATACGGGAAT	Reverse primer for 3'-flanking sequence of *rr431*
abc432-KO-1-*Bam*HI	CGGGATCCTAAGACTAACCATGAGG	Sequencing primer for mutant Δ*rr431*
cemA-KO-1-*Not*I	ATAAGAATGCGGCCGCCCAATATCTTCAGAC TTATCAG	Forward primer for 5'-flanking sequence of *cemA* or *cemK*
cemA-KO-2-*Sal*I	ACGCGTCGACTTACATAAATATATCATGTCT CCTA	Reverse primer for 5'-flanking sequence of *cemA*

Table 4-2 -continued- Primers used in this study

Primer name	Sequence (5' - 3')	Features
cemA-KO-3-*Sal*I	ACGCGTCGACTAAATAATAATACATATACCTACACTAA	Forward primer for 3'-flanking sequence of *cemA*
		Sequencing primer for mutants Δ*cemK* and Δ*cemK*Δ*cemR*
cemA-KO-4-*Bam*HI	CGGGATCCAAGCGATTCCAATTATTCG	Reverse primer for 3'-flanking sequence of *cemA*
cemA-5-F	GACATTTCAGGTTAAGAAAGAT	Sequencing primer for mutant Δ*cemA*
cemA-6-R	GCTCCTGTATAACCTAAGC	Sequencing primer for mutant Δ*cemA*
cemK-KO-2-*Sal*I	ACGCGTCGACTCATTGAATTACTGAAATAATTTG	Reverse primer for 5'-flanking sequence of *cemK*
		Sequencing primer for mutant Δ*cemA*
cemK-KO-3-*Sal*I	ACGCGTCGACGAATAGAAAGGAATACTTACG	Forward primer for 3'-flanking sequence of *cemK*
cemK-KO-4-*Bam*HI	CGGGATCCGAGTGAGAGGTTGTCTT	Reverse primer for 3'-flanking sequence of *cemK*
		Sequencing primer for mutant Δ*cemK*Δ*cemR*
cemR-KO-1-*Pst*I	AACTGCAGCTCTACTATTACCATCCTCT	Forward primer for 5'-flanking sequence of *cemR*
cemR-KO-2-*Sal*I	ACGCGTCGACTCACATTTTAAGTAGTGTAGC	Reverse primer for 5'-flanking sequence of *cemR*
		Sequencing primer for mutant Δ*cemK*
cemR-KO-3-*Sal*I	ACGCGTCGACTAAGACTGATGTAGATTAGCA	Forward primer for 3'-flanking sequence of *cemR*
cemR-KO-4-*Bam*HI	CGGGATCCCTGAAGGTGGCTCTG	Reverse primer for 3'-flanking sequence of *cemR*
RecA-qPCR-F2	CAACTATCCGGATGGAAATTCGTCG	qPCR forward primer for endogenous gene *recA*
RecA-qPCR-R2	TGTCAACTTCACAACGTTTGAATGGC	qPCR reverse primer for endogenous gene *recA*
pHK1745-qPCR-F1	CGGACTAGGCTATATTGGATCGTATT	qPCR forward primer for *hk430*
pHK1745-qPCR-R1	GTTGGATGCCCTTCGTTTGTA	qPCR reverse primer for *hk430*
RR1746-qPCR-F2	CTCAGCAAATTCAAAAAAAGCACCGT	qPCR forward primer for *rr431*
RR1746-qPCR-R2	ATCGCCGTTGCAATTTTCGTTG	qPCR reverse primer for *rr431*
ABC1747-qPCR-F3	ACTAAAGCCTGCAAAGTTGCGATGAT	qPCR forward primer for *abc432*
ABC1747-qPCR-R3	TTGTCCACCTGAAAGGGTAGTAGCATTTTC	qPCR reverse primer for *abc432*
70449-qPCR-F1	AGGACTTACTTTTGAACTTTTCACATTCTT	qPCR forward primer for *cemK*

Table 4-2 -continued- Primers used in this study

Primer name	Sequence (5' - 3')	Features
70449-qPCR-R1	CATATTCCTTATGATTGGCTTAGGTTATAC	qPCR reverse primer for *cemK*
70450-qPCR-F2	CAGTCTAGCTTAATTAACTTACAAAATGTTGA	qPCR forward primer for *cemR*
70450-qPCR-R2	CGGCTTATTAAGTTTTCCAACAATG	qPCR reverse primer for *cemR*
AIP-qPCR-F2	TGATATATTTATGCAAAAACTATCAATTCATC	qPCR forward primer for *cemA*
AIP-qPCR-R2	TTATTTACAGACTTAAAAGTGAGTATATACCACCC	qPCR reverse primer for *cemA*

CO_2, and 94% N_2) without agitation in deMan-Rogosa-Sharpe broth (MRS) (Difco, Becton Dickinson, Mississauga, Canada) (22). In addition to the unmodified MRS, three other types of modified MRS media were also used in this study. The first type of modified MRS medium was referred to as mMRS, and contained 1% maltose, 0.5% glucose, and 0.5% fructose (21). The second type was referred to as gluMRS, and contained 2% glucose. The third type was referred to as sucMRS, and contained 2% sucrose. Ampicillin (100 mg L^{-1}) and erythromycin (500 mg L^{-1}) were added to the LB medium for plasmid maintenance in *E. coli*. 10 mg L^{-1} of erythromycin was added to the MRS medium for the growth of erythromycin-resistant *L. reuteri.*

4.2.2 DNA isolation and manipulation

Genomic DNA was isolated using the Blood & Tissue Kit (Qiagen, Mississauga, Canada) according to the protocol provided by the manufacturer. Oligonucleotides (Table 4-2) were purchased from Integrated DNA Technologies (San Diego, CA). Restriction enzymes (New England Biolabs, Pickering, Canada), T4 DNA ligase (Epicentre, Markham, Canada), and Taq DNA polymerase (Invitrogen, Burlington, Canada) were used for cloning. DNA sequencing was performed after polymerase chain reaction (PCR) cloning (TA Vector, Invitrogen) (Macrogen, Rockville, MD).

4.2.3 Bioinformatic prediction of promoter regions

The genome sequence of *L. reuteri* 100-23 was retrieved from the Whole Genome Shotgun (WGS) project at the National Center for Biotechnology

Information databases (accession number: AAPZ00000000) (http://www.ncbi.nlm.nih.gov/Traces/wgs/?val=AAPZ02). BLASTP analysis was performed to retrieve homologous proteins, which were further analyzed at the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. The protein sequence of histidine kinases and response regulators from *L. reuteri* LTH2584 and 100-23 were aligned to calculate the similarity scores using Geneious Alignment (Geneious version 5.1.6, www.geneious.com). The ClustaW2 program was used to align the promoter regions of *rr431* and *cemR* (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The pattern search of the promoter regions was executed using the Correlator program. The identification of genes near promoters with the direct repeats was executed using the Crosscorrelator program (24, Appendix A).

4.2.4 Generation of the **L. reuteri** *single- and double-gene knockout mutants*

The double crossover method by homologous recombination has been described (Chapter 2, this thesis). In brief, the 5'- and 3'-flanking sequences of the target gene were amplified by PCR, and were thereafter referred to as amplicon-A and amplicon-B, respectively. A knockout plasmid, pKO-*Gene*-AB, was generated using three different methods, A1, A2, and A3 (Fig. 4-1). In A1, PCR products A and B were inserted separately into pGEMTeasy vectors to produce pGene-A and pGene-B. Next, the restriction enzymes (RE) were used to cut out two amplicons from pGene-A and pGene-B. These two amplicons were then ligated into a pGEMTeasy vector using T4 DNA ligase, which produced pGene-AB. The ligated DNA fragment AB was cut out of pGene-AB with

suitable RE and inserted into the integration shuttle vector pJRS233 (23). In A2, amplicons A and B were cut using RE and ligated into pJRS233. In A3, amplicons A and B were ligated into pGEMTeasy vectors, and became pGene-A and pGene-B. Then two amplicons were cut from pGene-A and pGene-B using RE, and then were inserted into the integration shuttle vector pJRS233.

A single-gene knockout mutant was generated by temperature-impulse integration and a plasmid-curing test (Fig. 4-1B to 4-1E) as described in Chapter 2 (this thesis). The same strategy was used to generate a double-gene knockout mutant by introducing a pJRS-derived plasmid into a single-gene knockout *L. reuteri* mutant to disrupt the second target gene. The mutants of *L. reuteri* generated in this study were all verified by PCR analysis and DNA sequencing.

4.2.5 Light microscope (LM) analysis

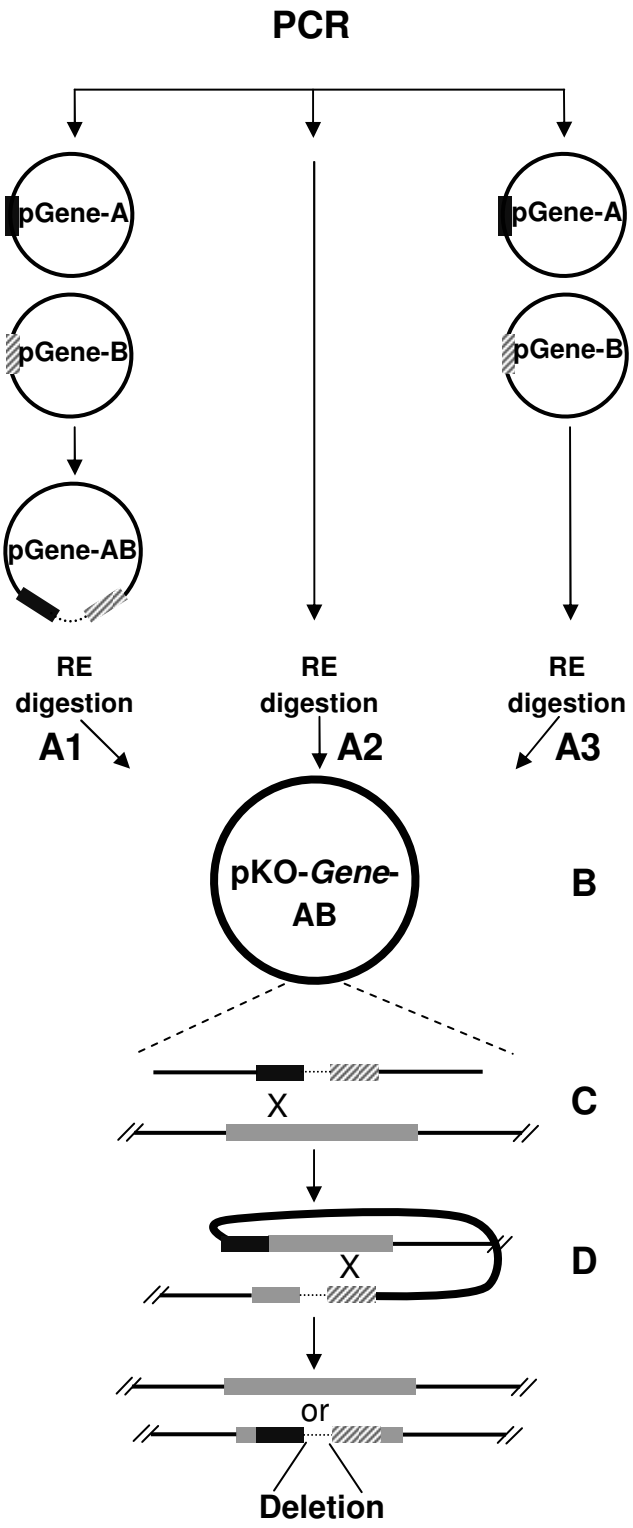
Colony morphology of cells grown on mMRS agar containing 2% glucose after 24 h of inoculation at 37°C under micro-aerobic conditions (1% O_2, 5% CO_2, and 94% N_2) was examined using a light microscope NCCD (Nikon, Mississauga, Canada).

4.2.6 Scanning electron microscope (SEM) analysis: Preparation of cells from mMRS agar

Cells from a stationary culture were harvested from mMRS agar containing 2% glucose after 24 h of inoculation and fixed overnight with 2% glutaraldehyde in 10 mM of phosphate buffered saline (PBS) buffer (pH 7.4) at 4°C. Fixed cells were washed twice in PBS buffer, centrifuged at 3000 × *g* for

four minutes, and dehydrated by adding one mL of ethanol in water in increasing concentrations of (70, 85, 95, 100, and 100%, v/v) at room temperature. Cells were incubated in each ethanol concentration for 10 min. Hexamethyldisilazane (HMDS) (Sigma-Aldrich, Munich, Germany) was introduced into the cells by gradually increasing the concentration of HMDS in ethanol, with the following series: 75% ethanol/25% HMDS, 50% ethanol/50% HMDS, 25% ethanol/75% HMDS, and three volumes of 100% HMDS. Samples were air-dried overnight, mounted on SEM stubs, and then immediately coated with Au/Pd on a sputter coater (Hummer 6.2) (Anatech, Union City, CA). The examination was performed by a scanning electron microscope XL30 (FEI, Hillsboro, OR) at an acceleration voltage of 20 kV.

Figure 4-1 The double crossover mutagenesis strategy developed in this study. (A) The flanking sequences of the target gene were amplified by PCR, and became amplicons A and B. A knockout plasmid, pKO-*Gene*-AB, was generated using three different methods, A1, A2, and A3 (for a detail description, see method section ***4.2.4***). The most direct method, A2, was performed first. A1 or A3 were employed in cases where A2 was not successful. (B) *L. reuteri* was transformed with the placement of the pKO-*Gene*-AB into its cytoplasm. (C) Erythromycin-resistant transformants were inoculated in mMRS-Erm at 42 - 44°C for 80 generations. (D) Erythromycin-resistant transformants were subsequently transferred to mMRS at 37°C and inoculated for 100 generations. (E) Erythromycin-sensitive strains were identified by replica plating on mMRS-Erm and mMRS agar. The gene replacement was verified by PCR analysis and DNA sequencing.


PCR
pGene-A
pGene-B
pGene-AB
pGene-A
pGene-B
RE digestion
A1
RE digestion
A2
RE digestion
A3
pKO-*Gene*-AB
B
X
C
X
D
or
Deletion

4.2.7 Autoaggregation

Autoaggregation was determined as described by Walter *et al.* (4). Cells of *L. reuteri* were inoculated in mMRS or sucMRS broth for 16 h. Cells were then harvested by centrifugation, washed, and suspended in a 0.7 volume of a corresponding buffer: mMRS (pH 6.4), 25 mM NaH_2PO_4 buffer (pH 7), or 25 mM lactate buffer (pH 4). Cells were incubated at room temperature and autoaggregation was observed visually.

4.2.8 Membrane fluidity assay

The fluorescence probe Laurdan (6-dodecanoyl-2-di-methyl-amino-naphtalene, Invitrogen) was used to determine the membrane fluidity as described previously (25). In brief, cells grown overnight were washed twice in phosphate buffer, adjusted to an OD_{600} of 3, and stained with Laurdan by incubation for 30 min in the dark at 37°C. Stained cells were then washed, and the Laurdan emission spectrum was measured with a spectrofluorometer FP-6300 (Jasco, Easton, MD). The generalized polarization of Laurdan was calculated as described by Ulmer *et al.* (25).

4.2.9 Growth curves

L. reuteri was grown in the following conditions: in mMRS broth at 37°C and 42°C; in sucMRS broth at 37°C; in mMRS broth with 4% (w/v) NaCl; in mMRS medium without 0.05% (w/v) cysteine or without 0.01% (w/v) Mn^{2+} and 0.001% (w/v) Mg^{2+}; in mMRS medium with 0.018% (v/v) isohumulone; and in mMRS broth at 25°C for 20 h then 37°C for 24 h. All cultures were incubated

under micro-aerobic conditions (1% O_2, 5% CO_2, and 94% N_2), and the OD_{630nm} was measured hourly.

4.2.10 Stress assays

L. reuteri grown overnight were washed, resuspended in media to an initial cell concentration of 10^8 CFU mL^{-1}, transferred to microtiter plates, and covered with 50 microliters of paraffin oil unless specified conditions were mentioned. Plates were incubated at 37°C unless otherwise specified and the OD_{630} was determined after 18 h of incubation. The media composition and incubation conditions were modified as follows: growth temperature of 25°C and 45°C; omission of paraffin oil to generate aerobic conditions; adjustment of the initial pH of the medium to 3, 4, 6, 8, or 10; adjustment of the NaCl concentration to a range of 3 to 16% (w/v); adjustment of the glucose concentration to a range of 1.6 to 8% (w/v); adjustment of the sucrose concentration to a range of 10 to 40% (w/v); and adjustment of the glycerol concentration to a range of 2 to 10% (v/v).

4.2.11 Minimum inhibitory concentration (MIC) measurements

The MICs of reutericyclin, isohumulone and phenylethanol towards *L. reuteri* were determined as described previously (21). Sensitivity to reutericyclin was tested with a culture supernatant of *L. reuteri* LTH2584 containing approximately 1 mg L^{-1} reutericyclin (21). Isomerized hop extract contained 30% iso-α-humulone (Yakima Chief Inc., Sunnyside, WA). Reutericyclin and

isohumulone act as proton-ionophores (12), while phenylethanol (Sigma-Aldrich) influences the fluidity of the cytoplasmic membrane (26).

4.2.12 Autolysis

To measure the autolysis ability of *L. reuteri* 100-23, a previous method was modified (27). Cells grown overnight were harvested and suspended in 50 mM NaH_2PO_4 containing 0.05% (v/v) Triton X-100 (pH 6) and incubated at 37°C with agitation. Autolysis was determined as A/A_0 where A is OD_{600} read after 5 h incubation time and A_0 is OD_{600nm} read at time zero. The data represent five independent experiments.

4.2.13 Analysis of organic acid production and carbohydrate utilization in MRS broth

L. reuteri strains LTH2584, Δ*tcsK*, and Δ*tcsR*, grown at 37°C in mMRS or sucMRS broth, were centrifuged at 8000 × *g* for 10 min to collect the supernatants, which were then precipitated by being mixed with an equal volume of 7% perchloric acid at 4°C overnight. After centrifugation at 10 000 × *g* for 10 min, the organic acids in the supernatants were identified by high-performance liquid chromatography (HPLC) according to the method used by Galle *et al.* (28). For the analysis of carbohydrate utilization, culture supernatant, after 18 h of inoculation at 37°C in mMRS broth or sucMRS broth, was directly examined by the HPLC analysis according to the method described previously (28).

4.2.14 RNA extraction

The MasterPure™ RNA Purification protocol (Epicentre Technologies, Markham, Canada) was used with slight modifications. A culture grown overnight was diluted fifty-fold in gluMRS broth and incubated at 37°C until an OD_{600nm} of 0.4 was reached. Cells were harvested from 10 mL of culture, and RNA synthesis was halted by adding 1.25 mL of ice-cold ethanol/phenol Stop Solution (5% acidic phenol in ethanol, pH < 7). Three milliliters of culture were centrifuged at 10 000 × *g* for one min, and the cells were lysed with 300 µL of Tissue and Cell Lysis Solution containing 5.5 micrograms of Proteinase K. The lysis was performed at 65°C for 15 min with vortexing at five min intervals. After incubating on ice for five min, 175 µL of MPC Protein Precipitation Reagent was added to denature proteins, followed by centrifugation at 10 000 × *g* for 10 min. The supernatant containing nucleic acids was mixed with 500 µL of isopropanol, and collected by centrifugation. The resulting pellets were rinsed with a 75% ethanol solution and suspended in 30 µL of nuclease-free water (Ambion) for RNase-free DNase I treatment (Ambion, Streetsville, Canada). The DNase I digestion was performed at 37°C for 2 h, after which 5 µL of 50 mM EDTA was added to stop the reaction. Five units of SUPERase·In (RNase inhibitor, Ambion) were added to the resulting RNA and stored at 4°C.

4.2.15 Complementary DNA synthesis

Two micrograms of total RNA was used as a template for cDNA synthesis using Random Primers (Invitrogen), dNTPs (Invitrogen), and nuclease-free water (Ambion) for primer annealing. The RNA/Primer mixture was incubated at 70°C for 10 min, then 25°C for 10 min, and finally chilled to 4°C. The reaction mix was

prepared with the RNA/Primer mixture, 5X 1st Strand Buffer, 100 mM DTT, SUPERase·In, SuperScript III (reverse transcriptase, Invitrogen), and nuclease-free water. This reaction mixture was then incubated at 25°C for 10 min, 37°C for 1 h, 42°C for 1 h, and then 70°C for 10 min to inactivate SuperScript III. The cDNAs were stored at 4°C. Amplifications were carried out in a GeneAmp PCR System 9700 (Applied Biosystems, Streetsville, Canada).

4.2.16 Relative quantification of gene expression using quantitative PCR (qPCR)

Gene expression was quantified with cDNA using a SYBR Green-based assay. Gene-specific primers were designed to have amplicons of 90-150 bp in size using Primer Express Software 3.0 (Applied Biosystems). PCR was carried out with custom SYBR Green Master Mix (the MBSU facility, University of Alberta) in a 7500 Fast Real-time PCR instrument (Applied Biosystems). The calculation of the relative gene expression was carried out according to the ΔΔCt methods (29). Exponentially growing cells of *L. reuteri* 100-23 were used as a reference condition, and *recA* was used as endogenous gene control. The PCR-efficiencies of the primers were experimentally determined with serial dilutions of the cDNA of *L. reuteri* 100-23 and calculated as described (29) with ABI software. The amplification program was 95 °C for 2 min; 40 cycles of 95 °C for 15 s, and 60 °C for 1 min. Data was collected at 60°C followed by a dissociation curve. Analysis was performed in triplicate with three independent experiments. The DNase I-treated RNA and genomic DNA were used as PCR negative and positive controls, respectively.

4.2.17 Adherence assay

The adherence assay was based on the method of Loo *et al*. with modifications (30). Cells from cultures grown overnight were washed, sub-cultured in two milliliters of gluMRS or sucMRS media, and incubated in 35 mm x 10 mm polystyrene petri dishes. After 24 h of inoculation at 37°C under micro-aerobic conditions (1% O_2, 5% CO_2, and 94% N_2), the supernatants were discarded, and cells were washed twice with 50 mM NaH_2PO_4 (pH 6) buffer. Cells adhering to the plate were scraped using plastic tips, and then resuspended in one milliliter of phosphate buffer. The cell density was determined by measuring the OD_{600nm}. Analysis was performed in triplicate with three independent experiments under different growth conditions.

4.2.18 Statistical analysis

All experiments were performed at least in duplicate. Statistical analysis was performed using the *t*-test (SigmaPlot, version 11.0; Chicago, IL).

4.2.19 Sequences and accession numbers

The nucleotide sequence of the *tcsKR* operon is shown in Appendix B. The nucleotide sequence of the *hk430* operon and *cemAKR* were retrieved from the GenBank database (accession number: AAPZ02000001; locus tag: Lreu23DRAFT_4807 for *hk430*; Lreu23DRAFT_4808 for *rr431*; Lreu23DRAFT_4809 for *abc432*; Lreu23DRAFT_4825 for *cemK*; and Lreu23DRAFT_4826 for *cemR*). The nucleotide sequence of *cemA* is 5'-ATGCAAAAACTATCAATTCATCAACTATCTTTAATTAAGGGTGGTATA

TACTCACTTTTAAGTCTGTAAA-3', and the predicted protein sequence is MQKLSIHQLSLIKGGIYSLLSL. The nucleotide sequence of *L. reuteri* 100-23 Δ*cemA* is deposited with GenBank accession number JF339968. Sequencing results confirmed the deletion of *L. reuteri* LTH2584 Δ*tcsK* and Δ*tcsR*, and *L. reuteri* 100-23 Δ*hk430*, Δ*rr431*, Δ*hk430*Δ*rr431*, Δ*cemK*, Δ*cemK*Δ*cemR*, Δ*hk430*Δ*cemR*, and Δ*rr431*Δ*cemR* (Appendix C).

4.3 Results

4.3.1 Disruption of genes encoding a two-component system and phenotypic analysis in L. reuteri *LTH2584*

A novel two-component system *tcsKR* and *aip* coding for an auto-inducing peptide were identified in *L. reuteri* sourdough isolate LTH2584 and named the "*tcsKR* operon." Prediction of the genetic loci using the BAGEL program suggested that regulation may be controlled by cell density quorum-sensing molecules (Fig. 4-2A). To identify functions of the genetic loci *tcsKR*, deletions in *tcsK* and *tcsR* were generated by double crossover (Fig. 4-1). Sequencing of the PCR fragments of *L. reuteri* LTH2584 Δ*tcsK* and Δ*tcsR* was used to confirm the deletions downstream of the translational initiation codon were made. The colony morphologies of *L. reuteri* LTH2584 Δ*tcsK* and Δ*tcsR* were not different from that of *L. reuteri* LTH2584 when cultured in mMRS agar at 37°C for 24 h (Fig. 4-2B). In addition, a series of phenotypic assays on *L. reuteri* strains LTH2584, Δ*tcsK,* and Δ*tcsR* were performed (Table 4-3). The

mutation of *tcsK* or *tcsR* did not affect the growth rate of *L. reuteri* at 37°C or 42°C. Because *L. reuteri* LTH2584 produces reutericyclin, the sensitivity of both

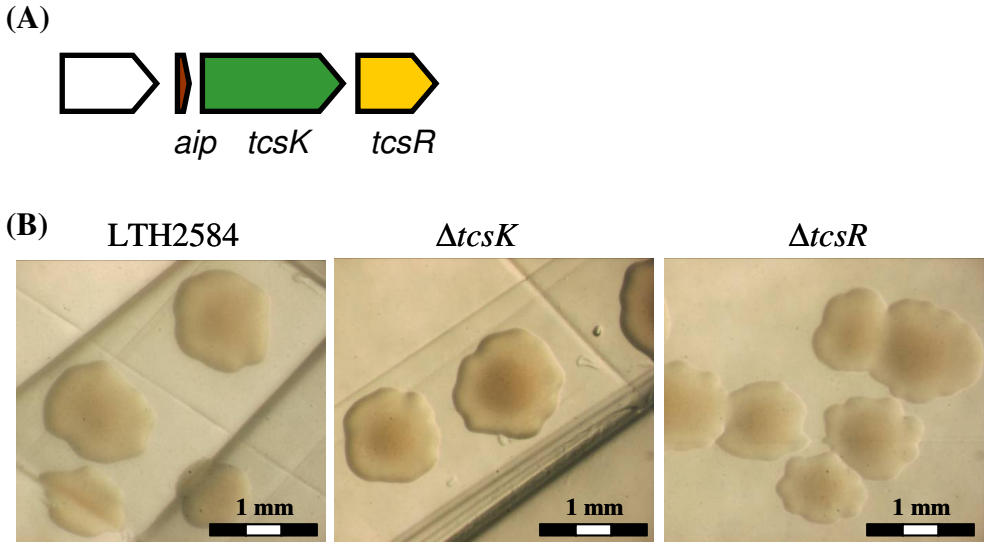


Figure 4-2 (A) Schematic representation of genes coding for a novel two-component regulatory system in *L. reuteri* LTH2584. *aip*: autoinducing peptide; *tcsK*: histidine kinase; *tcsR*: response regulator. (B) Colony morphology of *L. reuteri* LTH2584 and its isogenic mutant strains Δ*tcsK* and Δ*tcsR* grown on mMRS agar containing 2% glucose. Cell morphology was observed by light micrographs, which were taken at 3 different fields in 2 independent experiments.

mutant strains to reutericyclin was assessed by measuring the minimal inhibitory concerntration (MIC), and these measurements showed that reutericylin sensitivity remained unchanged. Production of exopolysaccharide, acetate, lactate, and ethanol in *L. reuteri* LTH2584 Δ*tcsK* and Δ*tcsR* were comparable to that of the wild-type strain LTH2584. In short, the deletion of *tcsK* and *tcsR* did not change the major phenotypic characteristics of *L. reuteri* LTH2584.

4.3.2 In silico prediction of operons hk430 *and* cemAKR *in* L. reuteri *100-23*

Since *L. reuteri* sourdough strain LTH2584 has a close phylogenetic relationship with rodent strain 100-23 (Chapter 3, this thesis), the orthologous genetic loci of *tcsKR*, the *cemAKR* operon, were investigated in the strain 100-23 of *L. reuteri* for which the genome sequence is available. Another two-component system *hk430*/*rr431*/*abc432* (the *hk430* operon) absent in the sourdough strain LTH2584 was also investigated (Fig. 4-3A). The colonization-related histidine kinase *lr70430* is referred to as *hk430* in this study. The *hk430* operon consists of genes coding for a histidine kinase, a response regulator of the LytR/AlgR family, and an ATP-binding cassette-type transporter, whereas the *cemAKR* operon is composed of genes coding for an auto-inducing peptide, a histidine kinase and a response regulator of the LytR/AlgR family. Analysis using the BAGEL program identified identical DNA regions encoding the putative signal transduction peptide IYSLLSL as *cemA* in *L. reuteri* 100-23 and as *aip* in *L. reuteri* LTH2584. In addition, the deduced amino acid sequences of HK430 and CemK are highly similar (77%), as are RR431 and CemR (76%) (Fig. 4-3A). The pathway prediction also demonstrated the similarity of *hk430*/*rr431*and *cemKR* to the bacteriocin-related two-component system *abpKR*, and also demonstrated the similarity of *abc432* to *abpT*, a bacteriocin export accessory protein in *Lactobacillus salivarius* UCC118 (KEGG database). These findings of genetic organization implied a co-operation of the *hk430* and *cemAKR* operons regulated by the response regulators RR431 and CemR. Therefore, the promoter regions of *rr431* and *cemR* were examined. When aligned using ClustalW2, the promoters of *rr431* and *cemR* shared a high homology (identity scores of 81). The pattern

Table 4-3 Phenotypic analysis of *L. reuteri* mutant strains compared to the wild type strains

	LTH2584		100-23							
	Δ*tcsK*	Δ*tcsR*	Δ*hk430*	Δ*rr431*	Δ*hk430* Δ*rr431*	Δ*cemA*	Δ*cemK*	Δ*cemK* Δ*cemR*	Δ*hk430* Δ*cemK*	Δ*rr431* Δ*cemK*
Autoaggregation	n.d.	n.d.	-	-	-	-	-	-	-	-
Membrane fluidity (Laurdan GP)	n.d.	n.d.	-	-	-	-	-	-	-	-
Growth curves										
mMRS (37°C)	-	-	-	-	-	-	-	-	-	-
mMRS (42°C)	-	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
sucMRS (37°C)	n.d.	n.d.	-	-	-	-	-	-	-	-
mMRS (4% NaCl): longer lag phase	n.d.	n.d.	-	-	-	-	-	-	-	+
mMRS without cysteine	n.d.	n.d.	-	-	-	-	-	-	-	-
mMRS without Mn^{2+} and Mg^{2+}	n.d.	n.d.	-	-	-	-	-	-	-	-
mMRS (0.018% isohumulone)	n.d.	n.d.	-	-	-	-	-	-	-	-
mMRS (25°C for 20 h and 37°C for 24 h)	n.d.	n.d.	-	-	-	-	-	-	-	-
Stress assays (OD_{630} after 18 h)										
mMRS (25°C)	n.d.	n.d.	-	-	-	-	-	-	-	-
mMRS (45°C)	n.d.	n.d.	-	-	-	-	-	-	-	-
Oxygen tolerance	n.d.	n.d.	-	-	-	-	-	-	-	-
mMRS pH 3, 4, 6, 8, 10	n.d.	n.d.	-	-	-	-	-	-	-	-
mMRS (4% NaCl)	n.d.	n.d.	-	-	-	-	-	-	-	-
mMRS (8% glucose)	n.d.	n.d.	-	-	-	-	-	-	-	-
mMRS (10, 15 and 20% sucrose)	n.d.	n.d.	-	-	-	-	-	-	-	-
mMRS (2- 10% glycerol)	n.d.	n.d.	-	-	-	-	-	-	-	-
MIC										
mMRS (reutericyclin)	-	-	-	-	-	-	-	-	-	-
mMRS (isohumulone)	n.d.	n.d.	-	-	-	-	-	-	-	-
sucMRS (phenylethanol)	n.d.	n.d.	-	-	-	-	-	-	-	-
Autolysis	n.d.	n.d.	-	-	-	-	-	-	-	-
Carbohydrate utilization	-	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Organic acids production	-	-	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d. not determined; – indicates no significant change detected in a mutant strain

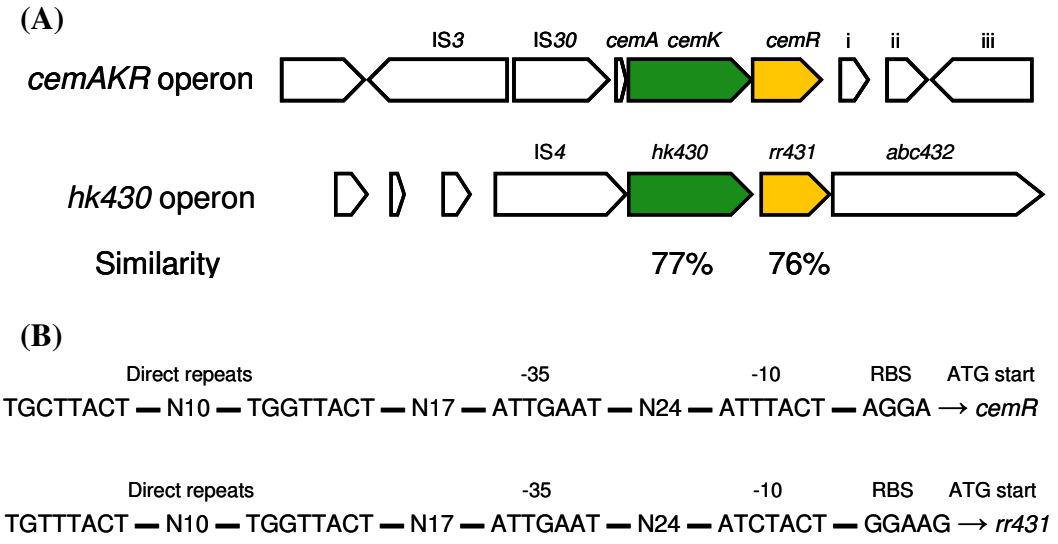


Figure 4-3 Comparison of novel two-component regulatory systems in *L. reuteri* 100-23. (A) The genetic loci of *cemAKR* were identified with a bacteriocin genome mining tool and predicted to be a putative autoinducing peptide, histidine kinase, and response regulator, respectively. The neighbour genes were predicted from the results of BLASTP analysis: i, YbaK/prolyl-tRNA synthetase associated protein; ii, protein tyrosine phosphatase; and iii, beta-lactamase. The genetic loci of *hk430/rr431/abc432* were annotated as histidine kinase, response regulator, and ATP-binding cassette transporter, respectively. The similarity of protein histidine kinases and response regulators are 77% and 76%, respectively. (B) Direct repeats in the promoter of *cemR* and *rr431* are similar to those in the promoter region of *agrA* family. -35 and -10 indicate the hypothetical -35 and -10 boxes in the *cemR* promoter; RBS indicates the ribosome binding site; N indicates nucleotides.

search of the promoter regions of *rr431* and *cemR* by the Correlator program identified the direct repeat (Fig. 4-3B), 5'-TGCTTACT. This direct repeat resembles the characterized direct repeats in the promoter region of *agrA*. AgrA is

a response regulator in the LytR/AlgR family that is regulated by auto-inducing peptides and is responsible for the virulence in *Staphylococcus aureus* (31).

4.3.3 Bioinformatic prediction of the regulon of RR431 and CemR

The identity of the regulon of RR431 and CemR was predicted using the Crosscorrelator program, which searches for the direct repeat patterns in the promoter regions of genes across the *L. reuteri* 100-23 genome (Table 4-4). In total, the regulon was found to have 24 genes: five genes were related to sugar metabolism and transport; five genes involved the two-component regulatory systems; three genes were amino acid transporters; three genes contributed to vitamin synthesis and cofactor (NAD) regeneration; and three genes were associated with cell division and cell envelope biosynthesis. The Crosscorrelator program also found a hypothetical membrane protein, a DNA polymerase III catalytic subunit, and an arginyl-tRNA synthetase. Specifically, gene 2500070172, which codes for putative glutamate-cysteine ligase (a protein that is also called gamma-glutamylcysteine synthetase), works as the first enzyme in the glutathione biosynthesis pathway. Gene 2500071440 encodes the chloride channel protein EriC, which is linked to acid resistance in *Escherichia coli* (32). Of note, the genes related to two-component systems (*rr431* and *cemR*) were identified, confirming the accuracy of the Crosscorrelator program.

Table 4-4 Genes of regulon that are controlled by response regulators RR431 and CemR predicted to be present by the Crosscorrelator program. This gene ID system is the same as that used in the DOE Joint Genome Institute databases.

Gene ID	Annotation	Score of identity (%)
Replication and translation		
2500069069	DNA polymerase III catalytic subunit, DnaE type	79.184
2500069669	Arginyl-tRNA synthetase (EC 6.1.1.19)	76.314
Carbohydrate metabolism		
2500069228	Gluconate transporter	75.537
2500071258	Acetate kinase (EC 2.7.2.1)	75.842
2500071405	Sugar (Glycoside-Pentoside-Hexuronide) transporter	77.855
2500071445	Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12)	75.175
2500071427	Ribokinase	75.018
Signal transduction		
2500069227	Transcriptional regulator, LacI family	75.537
2500070431	Response regulator of the LytR/AlgR family; *rr431*	100
2500070450	Response regulator of the LytR/AlgR family; *cemR*	91.489
2500070531	Bacteriocin-type signal sequence	75.175
2500070532	ABC-type bacteriocin transporter	75.175
Amino acid metabolism		
2500070018	Amino acid transporters	75.018
2500070172	Putative glutamate-cysteine ligase/putative amino acid ligase	76.433
2500070205	Amino acid transporters	76.314
2500070246	Amino acid transporters	76.314
Vitamin and cofactor synthesis		
2500071136	1-deoxy-D-xylulose-5-phosphate synthase (EC 2.2.1.7)	81.624
2500071165	Short-chain dehydrogenases of various substrate specificities	75.911
2500071311	Folylpolyglutamate synthase/dihydrofolate synthase	80.454
Membrane protein and transporter		
2500070342	Predicted membrane protein	75.532
2500071440	Chloride channel protein EriC	75.018
Cell envelope and division		
2500071188	Bacterial cell division membrane protein FtsW	78.246
2500071234	UDP-N-acetylglucosamine—N-acetylmuramyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase (EC 2.4.1.227)	76.314
2500071320	Negative regulator of Septation ring formation EzrA	75.2

4.3.4 Generation of L. reuteri *100-23 deletion mutants and characterization of the* hk430 *and* cemAKR *operons*

To test the hypothesis that cooperative regulation between *hk430* and *cemAKR* operons, the single-gene knockout mutants Δ*hk430*, Δ*rr431*, Δ*cemA*, and Δ*cemK* as well as the double-gene knockout mutants Δ*hk430*Δ*rr431*, Δ*cemK*Δ*cemR*, Δ*hk430*Δ*cemK*, and Δ*rr431*Δ*cemK*, were generated using deletion mutagenesis and validated by DNA sequencing (Fig. 4-1, Table 4-1).

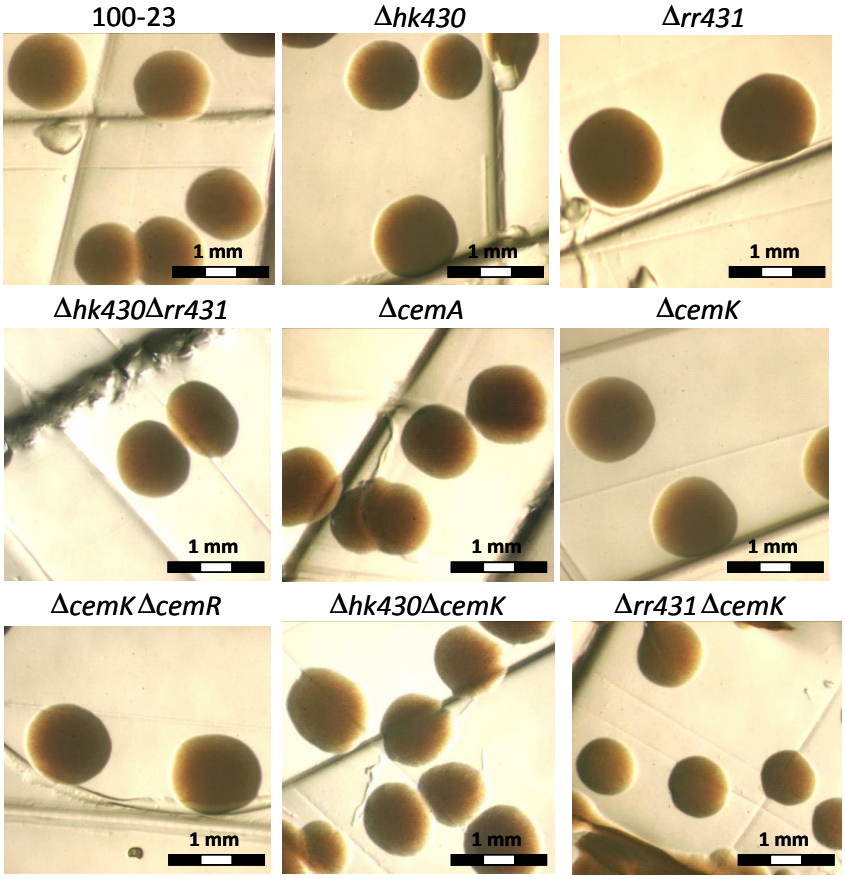


Figure 4-4 Colony morphology of *L. reuteri* 100-23 and its isogenic mutant strains grown on mMRS agar containing 2% glucose. Colony morphology was observed with light micrographs, which were taken at 6 fields in 2 independent experiments.

None of the colony morphologies of all *L. reuteri* mutants differed from that of the wild type strain 100-23 (Fig. 4-4). Likewise, the deletions of genes in the *hk430* operon and the *cemAKR* operon had no observable significant effect on cell properties when observed using scanning electron microscopy (Fig. 4-5). From the results of detailed biochemical assays (Table 4-3), *L. reuteri* 100-23 and its isogenic mutants were found to aggregate at comparable incubation times in buffers with pH values of 4, 6.4, and 7. Membrane fluidity as assessed by the

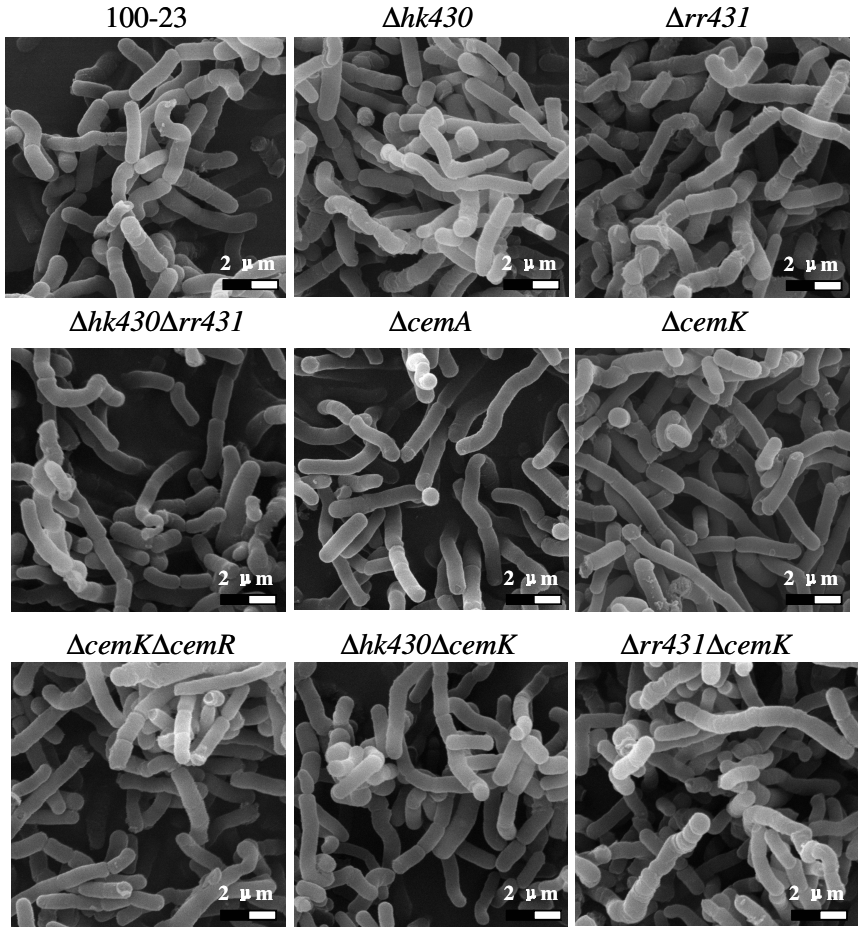


Figure 4-5 Cell morphology of *L. reuteri* 100-23 strains grown on mMRS agar containing 2% glucose observed with scanning electron micrographs, which were taken at 6 fields in 2 independent experiments.

Laurdan generalized polarization did not change after the disruption of the *hk430* and *cemAKR* operons. The mutation of *hk430*, *rr431*, or *cemAKR* did not affect the growth curve of *L. reuteri* in the presence of 2% sucrose or 0.018 % isohumulone, or in the absence of cysteine or magnesium and manganese. Regardless of whether the incubation temperature was 37°C or was shifted from 25 to 37°C, the growth rate did not change. Only the addition of 4% NaCl caused a longer lag phase in *L. reuteri* Δ*rr431*Δ*cemK* compared to the other strains (Table 4-3). Organisms were cultured under different stress conditions to elucidate how these stressesors influenced cell growth. The final cell density was similar for all nine strains after 18 h of incubation at different temperatures, under aerobic conditions, with different initial pH values, and in media imposing osmotic stresses through the addition of NaCl, glucose, sucrose, or glycerol. The sensitivity of the wild type strain and the eight mutant strains to reutericyclin, isohumulone, or phenylethanol was similar. In summary, the *hk430*, *rr431*, or *cemAKR* deletion did not alter the phenotypes investigated in Table 4-3.

4.3.5 Relative qPCR analysis of hk430 *and* cemAKR *operons*

Expression of the operons *hk430* and *cemAKR* was quantified by qPCR to determine if an interaction was present between the two highly homologous two-component systems. The expression of *rr431* increased in *L. reuteri* strain Δ*hk430*, and the expression of *abc432* increased in strains Δ*hk430* and Δ*hk430*Δ*rr431*. The expression of *abc432* decreased in strain Δ*rr431* (Fig. 4-6). Disruption of *hk430* increased the expression of *cemK* and *cemR*, whereas disruption of *hk430* and *rr431* reduced the expression of *cemK*. In addition, an

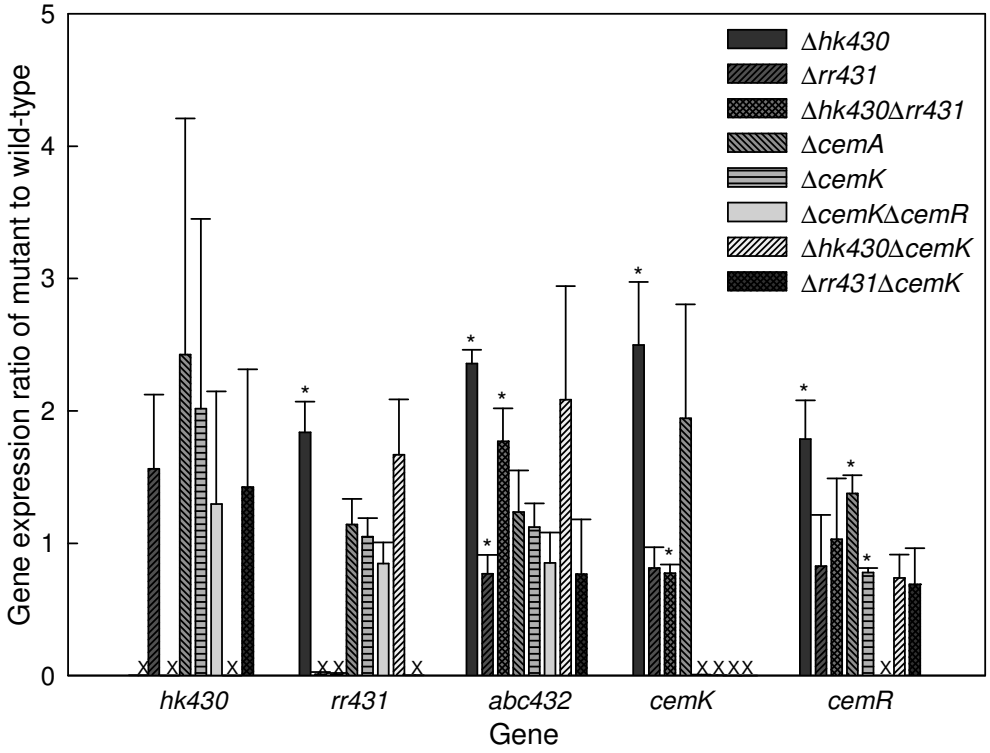


Figure 4-6 Relative quantification of gene expression in *L. reuteri* 100-23 and mutant strains. The expression of the *hk430* and *cemAKR* operons was determined by qPCR (the y-axis; linear scale) with primers specific to genes *hk430*, *rr431*, *abc432*, *cemK* and *cemR* (the x-axis). The *L. reuteri* isogenic strains used in this study are Δ*hk430*, Δ*rr431*, Δ*hk430*Δ*rr431*, Δ*cemA*, Δ*cemK*, Δ*cemK*Δ*cemR*, Δ*hk430*Δ*cemK*, and Δ*rr431*Δ*cemK*. x indicates a gene with expression below 0.01. A significant difference from the wild-type strain is indicated by * ($p < 0.05$). Results are shown as means ± standard deviations of three independent experiments.

increased expression of *cemR* was detected in strain Δ*cemA* but was reduced in strain Δ*cemK*. This data suggested a regulation between *hk430*, *rr431*, and *abc432* and a regulation relationship between *cemA*, *cemK*, and *cem*R. The increased expression of *rr431* and *cemR* in strain Δ*hk430* implied an interaction between *hk430*, *rr431*, and *cemR*.

4.3.6 Cell adherence characteristics of wild type and mutant strains

Although the experiments discussed above showed no change in the deletion mutants' cell morphology, growth rate, or stress assays, the results indicated that the response regulators of the LytR/AlgR family may have contributed to bacterial adhesion and biofilm formation (33). A quantitative adherence assay was conducted to assess *L. reuteri*'s ability to form biofilm (Fig. 4-7). When *L. reuteri* 100-23 and all eight mutants were grown in sucMRS, the optical density of cells adhering to polystyrene petri dishes was above 5.2, indicating that all strains formed biofilms in the presence of sucrose. Strains Δ*rr431*, Δ*hk430*Δ*rr431*, Δ*cemA*, andΔ*rr431*Δ*cemK* had even higher cell densities, with values nearing OD_{600nm} 10, which is significantly different from the behavior of the wild type strain. However, when grown in glucMRS, the optical density of adhering cells dropped from 3.3 to 3.9 for *L. reuteri* 100-23 and the mutant strains Δ*hk430*, Δ*rr431*, and Δ*hk430*Δ*rr431*. Only strains with a mutation of the *cemAKR* operon adhered better, having an optical density of 4.3 to 5.9, which indicates that *cemAKR* inhibits biofilm formation in in the presence of glucose but not sucrose.

4.4 Discussion

This study characterized the *tcsKR* in *L. reuteri* LTH2584, and the genetic loci of *hk430*, *rr431*, *abc432*, and *cemAKR* in *L. reuteri* 100-23 by bioinformatic analyses, and by phenotypic analysis of the mutatnts of *L. reuteri* LTH2584 and 100-23 performed with deletions of the single genes, *tcsK*, *tcsR*, *hk430*, *rr431*, *cemA*, and *cemK*, as well as the double genes, *hk430*/*rr431*, *cemKcemR*,

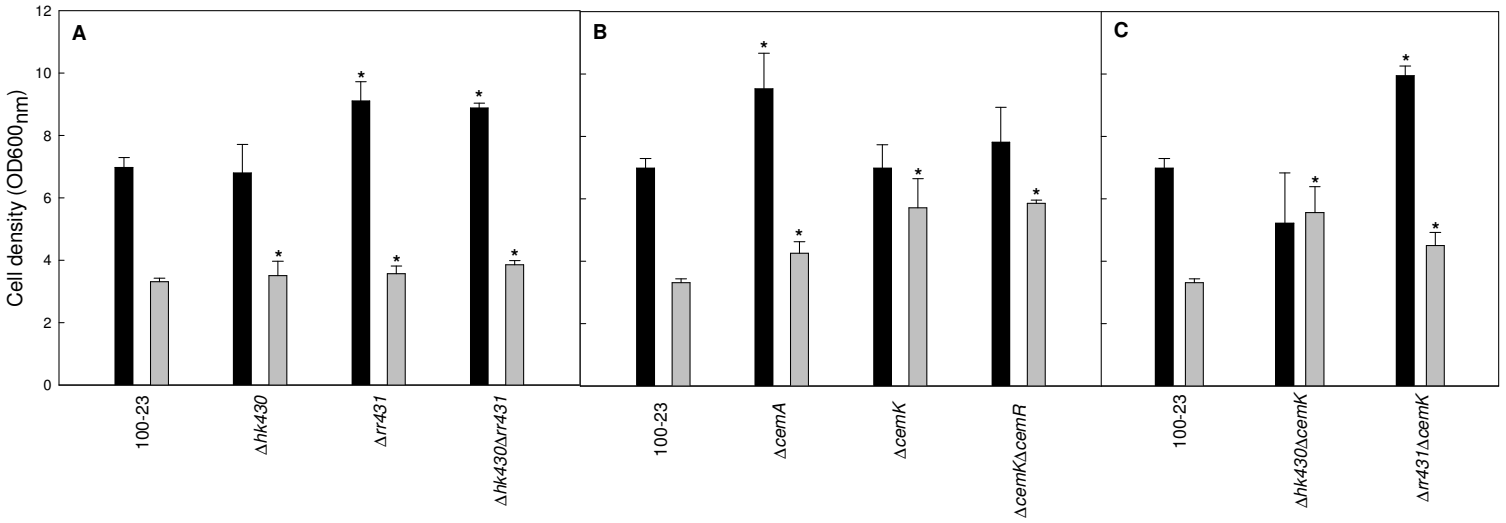


Figure 4-7 Quantification of adhered cells in plastic plate assay after 24 h of growth at 37°C in MRS broth containing 2% of sucrose (black color) or 2% of glucose (grey color). Adherence was measured by an optical density of 600 nm and shown in *L. reuteri* 100-23 and (A) mutants of the *hk430* operon, (B) mutants of the *cemAKR* operon, and (C) double-gene knockout mutants across operons *hk430* and *cemAK*. A significant difference from the wild-type strain is indicated by * ($p < 0.05$). Results are shown as means ± standard deviations of three independent experiments.

hk430/*cemK*, and *rr431*/*cemK*. In the studies with *L. reuteri*, gene disruption can be achieved by plasmid integration (4, 7, 13) or the double crossover method (Chapter 2, this thesis). The double crossover method generates an isogenic mutant by deleting specific target genes without introducing antibiotic resistance genes. Because no antibiotic resistance genes were present in the genome, subsequent deletions of other genes of interest could be achieved using the double crossover method. This facilitated the study of the metabolic traits and two-component regulatory systems of *L. reuteri* isolated from sourdough or intestinal ecosystems.

BLASTP analysis of the *tcsKR* operon in *L. reuteri* LTH2584 discovered the homologous operons *hk430/rr431* and *cemK/cemR* in *L. reuteri* rodent strain 100-23, and the bacteriocin-related two-component systems *abpKR* and *sppKR* (34, 35). *L. reuteri* LTH2584 is known to produce the antimicrobial substance reutericyclin, but not bacteriocins. (21) *L. reuteri* 100-23 is also not believed to produce bacteriocins (5). However, the operons *tcsKR*, *hk430*, and *cemAKR* also meet the criteria to be categorized as peptide-based quorum-sensing two-component systems in lactobacilli (36). The promoter prediction discovered the structure of an operator (direct repeats) in the promoter region of *rr431* and *cemR* (Fig. 4-3B, Table 4-3). The response regulators TcsR, RR431, and CemR are all closely related to response regulation in the LytR/AlgR family (37). The functions of the LytR/AlgR family are not well-defined, but this family is considered to be involved in the regulation of virulence factors and in the performance of

housekeeping functions, such as cell envelope maintenance, competence, and biofilm formation (33).

The genetic organization of *hk430/rr431/abc432* and *cemAKR* implies a cooperative regulation relationship between these two paralogous operons, which involves the sharing of the same auto-inducing peptides and ABC transporter. The fact that the protein sequences of RR431 and CemR are 76% similar, and their response regulator promoter sites are 81% similar, also suggests that these two operons work cooperatively. Moreover, the *cemAKR* operon harbors an auto-inducing peptide with a double-glycine (GG) type leader peptide (38), but lacks the corresponding ABC transporter. Therefore, the *cemAKR* operon may be complemented by the *hk430* operon, which harbors an ABC transporter with a peptidase C39 domain that recognizes the GG-leader peptide (39). The results of the qPCR analysis suggested cross-communication (Fig. 4-6). Moreover, the expressions of *rr431* and *cemR* were enhanced in the *hk430*-deletion mutant. Cross-communication was shown on some two-component systems with high levels of sequence similarity (40, 41, 42). The fact that the mutation of components in the cross-talk two-component systems did not abolish the possibility that regulation of phenotypes may be due to overlapping functions or asymmetrical/differential expressions. This may explain the lack of a clear phenotype in the mutants of this study because an isogenic response regulator mutant could not be obtained (Table 4-3; Fig. 4-4, 4-5).

Despite the functions of the LytR/AlgR family in cell envelope maintenace, adherence asccioated with biofilm formation was the only

distinguishable feature in the *L. reuteri* 100-23-derived mutants (Fig. 4-7). *L. reuteri* 100-23 attaches and forms dense layers of cells on the non-secretory stratified squamous epithelium of the murine forestomach where the glucose concentration is 10 – 20 g (kg dry weight)$^{-1}$ (27, 43). Although the sucrose concentration in the forestomach of mice is much lower, sucrose metabolism of *L. reuteri* 100-23 has been demonstrated *in vivo* (11, 43). The formation of biofilm by *L. reuteri* has also been determined using an *in vitro* assay of flowing cells, which indicates that sucrose contributes to biofilm formation (4). The role of *rr431* and *cemAKR* was investigated in this study using the adherence assay, implying that the function of *rr431* and *cemAKR* was to initiate the formation of biofilm (Fig. 4-7). In the presence of sucrose, adherence was enhanced in *L. reuteri* when *rr431* and *cemA* were disrupted, which supported the hypothesis that there is cross-regulation between the *hk430* and *cemAKR* operons (Fig. 4-6). Study of *in vivo* colonization of mice shows that *hk430* contributes to biofilm formation (19), whereas the mutation of *hk430* did not alter the adherence ability on the surface of plastic plates in the presence of sucrose. This may be the result of the difference in sensitivity of these approaches. The method that requires *in vivo* competition in the murine forestomach after 7 days of inoculation (19) has a higher sensitivity than the method that relies on *in vitro* adherence assayes for studies of the competitivness of *L. reuteri* in ecological niches.

In addition to adherence regulated by the *hk430* and *cemAKR* operons, two IS elements were identified in the upstream and downstream of the *hk430* operon in *L. reuteri* 100-23. This suggests that the *hk430* operon may be the result of a

lateral gene transfer in a manner similar to that of fructansucrase (*ftf*) in *L. reuteri* 100-23 (11), where the presence of mobile DNA elements adjacent to *ftf* results in a lateral gene transfer that allows *L. reuteri* to adapt to different environments (11). Taken together, the contribution of HK430 to colonization (11), the adherence factors CemA and RR431, and the cross-communication between the *hk430* and *cemAKR* operons suggested cooperation between these two operons, and indicates that they contribute to biofilm formation.

In conclusion, this study involved the generation of a series of deletion mutants to characterize the two-component systems *tcsKR* in *L. reuteri* LTH2584 and the *hk430* and *cemAKR* operons in *L. reuteri* 100-23. Deletion of *tcsK* and *tcsR* did not impact cell morphology or the production of reutericyclin, exopolysaccharides, or organic acids. Similarly, deletions of the *hk430* operon and the *cemAKR* operon in strain 100-23 had no influence on cell morphology, growth rate, or the sensitivity to various stressors. However, because of a possible co-regulation of these operons that may play a role in cell adherence, depending on the presence of different carbon resources, it is possible that the two-component systems *hk430/rr431/abc432* and *cemAKR* may be related to the fitness of *L. reuteri* in diverse ecological niches.

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Chapter Five: **Analysis of cell envelope architecture and biofilm formation in *Lactobacillus reuteri* rodent isolate 100-23**

5.1 Introduction

The metabolic traits contributing to the competitiveness of *Lactobacillus reuteri* in food fermentations and intestinal habitats include acid resistance, antimicrobial compound production, and exopolysaccharide production (for reviews, see 1 and 2). *L. reuteri* is frequently found in sourdoughs made of sorghum, maize, or wheat that has been fermented for more than two days (3, 4, 5). The final pH of this type of sourdough is usually below four, which causes acid stress on microbial cells. A similar situation exists in the gastrointestinal tract of humans and other animals where the pH of the stomach is less than three. Reutericyclin production contributed to the stable persistence of *L. reuteri* strains in industrial sourdoughs (6). In the human isolates of *L. reuteri*, a broad-spectrum antimicrobial compound reuterin (3-hydroxypropionaldehyde) is generated from glycerol fermentation. Reuterin production by *L. reuteri* in the intestinal tract is postulated to protect the host from pathogen invasions in the gut (7). Expression of glucansucrases and fructansucrases contributes to exopolysaccharide synthesis in sourdoughs and to biofilm formation in the murine intestines through the metabolism of sucrose, a major carbohydrate that is found in wheat and rye (8, 9).

The generation of biofilm involves a complex system of bacterial communities. Biofilm formation comprises four major steps: initial adherence of cells to surfaces, cell accumulation, clonal maturation, and formation of mixed

species biofilms (for reviews, see 10 and 11). In the first two steps, adhesins facilitate adherence to surfaces. In the latter two steps, quorum sensing, exopolysaccharide formation, coaggregation, and genetic exchange play an important role in biofilm maturation. Several large surface proteins are involved in colonization of the murine gastrointestinal tract by *L. reuteri* (12, 13). The mucus adhesion-promoting protein known as MapA, CnBP, CyuC, or BspA in combination with the mucus-binding protein Mub mediate the binding of *L. reuteri* to intestinal mucus or epithelial cells (14, 15). Mutation of *luxS* in *L. reuteri* 100-23 increased biofilm thickness but decreased *L. reuteri*'s competitiveness in the large intestine of mice (16). Fructansucrases of *L. reuteri* LTH5448 and 100-23 act as matrix-binding proteins (9, 17) while the extracellular polysaccharide matrix produced by reuteransucrase of *L. reuteri* TMW1.106 promotes biofilm formation in the murine forestomach (9). To build on the already completed studies of the mechanisms of adherence in *L. reuteri*, an intensive investigation on the regulation of colonization through the use of two-component systems in *L. reuteri* is needed to understand the maturation of biofilm formation.

Two-component signal transduction systems and quorum sensing systems regulate the development and dispersal of biofilms (11). In *Streptococcus mutans*, the *comCDE* system encodes a precursor competence-stimulating peptide, a histidine kinase, and its response regulator. ComCDE promotes plaque formation by positively mediating the expression of the genes encoding glucosyltransferase B/C/D (*gtfB/C/D*), fructosyltransferase (*ftf*), and the glucan-binding protein (*gbp*)

(18). In *Staphylococcus aureus* the accessory gene regulator locus (*agrBDCA*) encodes a quorum sensing system. The auto-inducing peptide AgrD is synthesized and processed into a cyclic peptide that triggers the activation of histidine kinase AgrC and response regulator AgrA. The activated AgrA indirectly regulates the extracellular proteases Aur and Spl that cause biofilm dispersion (19). Another two-component system in *S. aureus*, the *lytSR* system, activates the *lrg* operon that negatively regulates murein hydrolase activity, which causes biofilm dispersion (20).

A cross-communication between the paralogous *hk430* and *cemAKR* operons and their contribution to biofilm formation in *L. reuteri* 100-23 were observed previously (Chapter 4, this thesis). However, the mutation of genes in the *hk430* and *cemAKR* operons implied the possibility that these two operons have complementary functions. Supporting this hypothesis are the facts that these genes have a high homology between their promoter regions and the DNA sequences of their response regulators *rr431* and *cemR*. A comprehensive study on the cooperation of response regulators RR431 and CemR was needed to elucidate the regulon downstream of a signaling cascade. Mutations of *cemR*, *hk430*/*cemR*, and *rr431*/*cemR* were generated, and their influence on adherence and cell envelope formation was examined by biochemical characterization and quantitative PCR.

5.2 Materials and methods

5.2.1 Strains, media and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 5-1. *Escherichia coli* JM109 (Promega, Nepean, Canada) was cultured in Luria-Bertani (LB) broth with agitation at 37°C, or 30°C for the maintenance of the temperature-sensitive plasmid pJRS233 and its derived plasmids. *L. reuteri* 100-23 and its isogenic mutants were cultured at 37°C under micro-aerobic conditions (1% O_2, 5% CO_2, and 94% N_2) without agitation. *L. reuteri* was usually cultured at 37°C without agitation in deMan-Rogosa-Sharpe broth (MRS) (Difco, Becton Dickinson, Mississauga, Canada) (22). In addition to the unmodified MRS, three other types of modified MRS media were also used in this study. The first type of modified MRS medium was referred to as mMRS, and contained 1% maltose, 0.5% glucose, and 0.5% fructose (23). The second type was referred to as gluMRS, and contained 2% glucose. The third type was referred to as sucMRS, and contained 2% sucrose. Ampicillin (100 mg L^{-1}) and erythromycin (500 mg L^{-1}) were added to LB medium for plasmid maintenance in *E. coli*. 10 mg L^{-1} of erythromycin was added to the MRS medium for the growth of erythromycin-resistant *L. reuteri.*

5.2.2 DNA manipulation

Two milliliters of overnight cultures were harvested and washed with lysis buffer (20 mM Tris·Cl, pH 9.0; 2 mM sodium EDTA; 1.2% Triton X-100 (w/v)). Chromosomal DNA was extracted using the Blood & Tissue Kit (Qiagen, Mississauga, Canada) according to the manufacturer's instructions. DNA was suspended in 20 μL of 10 mM Tris-Cl (pH 8.5), and the concentrations of DNA were determined by UV spectrometry using a NanoDrop spectrophotometer ND-

Table 5-1 Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype	Source or reference
Strains		
Escherichia coli JM109	Cloning host for pGEMTeasy- and pJRS233-derivative plasmids	Promega
Lactobacillus reuteri		
100-23	Rodent isolate; wild type strain	(21)
Δ*cemK*Δ*cemR*	A deletion mutant of *L. reuteri* 100-23 on *cemK* and *cemR*	Chapter 4
Δ*cemR*	A deletion mutant of *L. reuteri* 100-23 on *cemR*	This study
Δ*hk430*Δ*cemR*	A deletion mutant of *L. reuteri* 100-23 on *hk430* and *cemR*	This study
Δ*rr431*Δ*cemR*	A deletion mutant of *L. reuteri* 100-23 on *rr431* and *cemR*	This study
Δ*cemRC*	A complemented mutant of *L. reuteri* 100-23 Δ*cemR* on *cemR*	This study
Plasmids		
pUC19	Cloning vector used in *E. coli*; 2.7 kb; Amp^r	New England Biolabs
pGEMTeasy	Cloning vector used in *E. coli*; 3.0 kb; Amp^r	Promega
pCemR-A	pUC19 containing 977 bp of the DNA sequence upstream of *cemR*; 3.7 kb; Amp^r	This study
pCemR-B	pGEMTeasy containing 909 bp of the DNA sequence downstream of *cemR*; 3.9 kb; Amp^r	This study
pCemR-14	pGEMTeasy containing 2.6 kb of the full length of *cemR* and its flanking sequences; 5.6 kb; Amp^r	This study
pJRS233	Shuttle vector used in the hosts *E. coli* and *L. reuteri* strains LTH2584 and 100-23; 6.0 kb; Erm^r	(24)
pKO-*hk430*-AB	pJRS233 containing 2.1 kb of the *hk430* flanking DNA sequences by ligating *Bam*HI/*Sal*I-cut 1.1 kb of pHK430-A with *Sal*I/*Pst*I-cut 1.0 kb of pHK430-B; 8.1 kb; Erm^r	Chapter 4
pKO-*rr431*-AB	pJRS233 containing 1.7 kb of the *rr431* flanking DNA sequences by ligating *Bam*HI/*Sal*I-cut 0.7 kb of pRR431-A with *Sal*I/*Pst*I-cut 1.0 kb of pRR431-B; 7.7 kb; Erm^r	Chapter 4
pKO-*cemR*-AB	pJRS233 containing 1.9 kb of the *cemR* flanking DNA sequences by ligating *Pst*I/*Sal*I-cut 977 bp of pCemR-A with *Sal*I/*Bam*HI-cut 909 bp of pCemR-B; 7.9 kb; Erm^r	This study
pKO-*cemR*-14	pJRS233 containing *Pst*I/*Bam*HI-cut 2.6 kb of the full length of *cemR* and its flanking sequences; 8.6 kb; Erm^r	This study

Amp^r, amplicillin resistance gene; Erm^r, erythromycin resistance gene

1000 (Thermo Fisher Scientific Inc., Wilmington, DE). Restriction enzymes (New England Biolabs, Pickering, Canada), T4 DNA ligase (Epicentre, Markham, Canada), and Taq DNA polymerase (Invitrogen, Burlington, Canada) were used for cloning. The oligonucleotides listed in Table 5-2 were purchased from Invitrogen (Burlington, Canada) and Integrated DNA Technologies (San Diego, CA). The Taq DNA polymerase was purchased from Invitrogen, and DNA sequencing was performed after PCR (MacrogenUSA, Rockville, MD).

5.2.3 Bioinformatic prediction of protein function

The genome sequence of *L. reuteri* 100-23 was retrieved from the DOE Joint Genome Institute (www.jgi.doe.gov) (25). BLASTP analysis was performed to find homologous proteins. Amino acid sequences were retrieved from UniProt (26) and then aligned to calculate their identity scores using MUSCLE alignment (Geneious version 5. 1. 6; Auckland, New Zealand).

5.2.4 Construction of deletion and complemented mutants

The double crossover method by homologous recombination has been described earlier (Chapters 2 and 4, this thesis). Briefly, the 5'- and 3'-flanking sequences of *cemR* were amplified by PCR. The amplicons were ligated with vectors resulting in pCemR-A and pCemR-B (Table 5-1), and then were cleaved with suitable restriction enzymes so they could be ligated into shuttle vector pJRS233 (24). Using this method, a knockout plasmid pKO-*cemR*-AB was generated. A single-gene knockout mutant was generated by temperature-impulse integration and a plasmid-curing test. The same strategy was used to generate a

Table 5-2 Primers used in this study

Primer name	Sequence (5' - 3')	Feature
hk430-KO-1a-*Bam*HI	CGGGATCCTTCATTACTTCGTCCATT	Sequencing primer for mutant Δ*hk430*Δ*cemR*
hk430-KO-4-*Pst*I	AACTGCAGAGCGGCTTCGACAATACCT	Sequencing primer for mutant Δ*rr431*Δ*cemR*
rr431-KO-2-*Sal*I	ACGCGTCGACTTACAGAATAATTGACGAC	Sequencing primer for mutant Δ*hk430*Δ*cemR*
abc432-KO-1-*Bam*HI	CGGGATCCTAAGACTAACCATGAGG	Sequencing primer for mutant Δ*rr431*Δ*cemR*
cemK-KO-3-*Bam*HI	CGGGATCCGAATAGAAAGGAATACTTACG	Sequencing primer for mutants Δ*cemR* and Δ*cemRC*
cemK-KO-4-*Bgl*II	GAAGATCTGAGTGAGAGGTTGTCTT	Sequencing primer for mutants Δ*cemR* and Δ*cemRC*
cemR-KO-1-*Pst*I	AACTGCAGCTCTACTATTACCATCCTCT	Forward primer for 5'-flanking sequence of *cemR*
cemR-KO-2-*Sal*I	ACGCGTCGACTCACATTTTAAGTAGTGTAGC	Reverse primer for 5'-flanking sequence of *cemR*
cemR-KO-3-*Sal*I	ACGCGTCGACTAAGACTGATGTAGATTAGCA	Forward primer for 3'-flanking sequence of *cemR*
cemR-KO-4-*Bam*HI	CGGGATCCCTGAAGGTGGCTCTG	Reverse primer for 3'-flanking sequence of *cemR*
RecA-qPCR-F2	CAACTATCCGGATGGAAATTCGTCG	qPCR forward primer for endogenous gene *recA*
RecA-qPCR-R2	TGTCAACTTCACAACGTTTGAATGGC	qPCR reverse primer for endogenous gene *recA*
pHK1745-qPCR-F1	CGGACTAGGCTATATTGGATCGTATT	qPCR forward primer for *hk430*
pHK1745-qPCR-R1	GTTGGATGCCCTTCGTTTGTA	qPCR reverse primer for *hk430*
RR1746-qPCR-F2	CTCAGCAAATTCAAAAAAAGCACCGT	qPCR forward primer for *rr431*
RR1746-qPCR-R2	ATCGCCGTTGCAATTTTCGTTG	qPCR reverse primer for *rr431*
ABC1747-qPCR-F3	ACTAAAGCCTGCAAAGTTGCGATGAT	qPCR forward primer for *abc432*
ABC1747-qPCR-R3	TTGTCCACCTGAAAGGGTAGTAGCATTTTC	qPCR reverse primer for *abc432*
70449-qPCR-F1	AGGACTTACTTTTGAACTTTTCACATTCTT	qPCR forward primer for *cemK*
70449-qPCR-R1	CATATTCCTTATGATTGGCTTAGGTTATAC	qPCR reverse primer for *cemK*
70450-qPCR-F2	CAGTCTAGCTTAATTAACTTACAAAATGTTGA	qPCR forward primer for *cemR*
70450-qPCR-R2	CGGCTTATTAAGTTTTCCAACAATG	qPCR reverse primer for *cemR*
AIP-qPCR-F2	TGATATATTTATGCAAAAACTATCAATTCATC	qPCR forward primer for *cemA*
AIP-qPCR-R2	TTATTTACAGACTTAAAAGTGAGTATATACCACCC	qPCR reverse primer for *cemA*
69269-qPCR762-F2	TGCAGTGAGTATCACCGATAGACA	qPCR forward primer for gene 2500069269

Table 5-2 –continued- Primers used in this study

Primer name	Sequence (5' - 3')	Feature
69269-qPCR836-R2	TTTCCTGGGATATGGTGATCATC	qPCR reverse primer for gene 2500069269
69270-qPCR365-F2	AGGATGATGCTACCAAAGCTAAGAG	qPCR forward primer for gene 2500069270
69270-qPCR-439-R2	TCGTTCGCTCGTCATTTTGA	qPCR reverse primer for gene 2500069270
69271-qPCR46-F2	ATGGGAATCTTTGCTGCAATTT	qPCR forward primer for gene 2500069271
69271-qPCR120-R2	AGGAACAACGAAACTCTTAGGAAAAA	qPCR reverse primer for gene 2500069271
69272-qPCR278-F2	CGCTTTATCGCCGGAATG	qPCR forward primer for gene 2500069272
69272-qPCR352-R2	TGAATCCACCCACAAATAATGTG	qPCR reverse primer for gene 2500069272
69363-qPCR261-F1	GGATTCACTAATTGCCGGTCTT	qPCR forward primer for gene 2500069363
69363-qPCR336-R1	CCGTTCAGGTGTCTGTGTAATATTG	qPCR reverse primer for gene 2500069363
69863-qPCR40-F4	AAGCAATGGATAACAGCTGCAA	qPCR forward primer for gene 2500069863
69863-qPCR114-R4	AGCTTGTGCCACACCTCCTAAA	qPCR reverse primer for gene 2500069863
70531-qPCR38-F1	AGGTTTCTGGTGGATGGAGTCTAT	qPCR forward primer for gene 2500070531
70531-qPCR119-R1	TGAGCCCATTTGTTCAAGGAA	qPCR reverse primer for gene 2500070531
70532-qPCR366-F1	TCGGGATGAATTTGGTCGTT	qPCR forward primer for gene 2500070532
70532-qPCR440-R1	TTTTGTGGCGTATATCCCTTAGC	qPCR reverse primer for gene 2500070532
70615-qPCR868-F2	GGCGGCTATGTACCTGGTCTT	qPCR forward primer for gene 2500070615
70615-qPCR943-R2	TTTCGATATCAGCGATTTGCA	qPCR reverse primer for gene 2500070615
70618-qPCR1234-F2	CTGTTAATTAGTAACGGGATGCAAAC	qPCR forward primer for gene 2500070618
70618-qPCR1308-R2	GGGATAAAGCATCGCTGCAA	qPCR reverse primer for gene 2500070618
70674-qPCR242-F1	GCGAAGTTGACGTTTATCCTGAT	qPCR forward primer for gene 2500070674
70674-qPCR316-R1	TCTTGCCAGTCCCCTTCTTTT	qPCR reverse primer for gene 2500070674
71010-qPCR1242-F1	CACTCTTCGTGATGCTCATGTTATC	qPCR forward primer for gene 2500071010
71010-qPCR1317-R1	CGTTCCAGTGTTTCCCTCAAA	qPCR reverse primer for gene 2500071010
71188-qPCR259-F1	CCAAGGTTTTTGCGGGATT	qPCR forward primer for gene 2500071188
71188-qPCR333-R1	AACAGCTCGGCTAAAGACTAAAACA	qPCR reverse primer for gene 2500071188

Table 5-2 –continued- Primers used in this study

Primer name	Sequence (5' - 3')	Feature
71258-qPCR638-F1	CAATCACTGCCGTAAAGAATGGT	qPCR forward primer for gene 2500071258
71258-qPCR712-R1	CCATTGTTATTCCCGCAACAG	qPCR reverse primer for gene 2500071258
70670-F36	CATCTTAGTAATCGCAGCAA	Forward primer for gene 2500070670
70670-R1802	GTCATTTTCCGCGCTT	Reverse primer for gene 2500070670
70671-F3	CAAAATGGACAATCTTATCAACG	Forward primer for gene 2500070671
70671-R1109	GTTGCCCCAATATCGC	Reverse primer for gene 2500070671
70672-F1	ATGAAGTATATGGTTACTGGTG	Forward primer for gene 2500070672
70672-R110	AAGGACAAGAGCAGCAA	Reverse primer for gene 2500070672
70673-F11	CAACCCGTTTTAGTGATAG	Forward primer for gene 2500070673
70673-R420	CTGTTTAACGCGAATAGTG	Reverse primer for gene 2500070673
70674-F38	GCATTGCTGGTTTACTAG	Forward primer for gene 2500070674
70674-R849	CCCGTCGACATTAACT	Reverse primer for gene 2500070674
70675-F3	GCTAGGGCAAATAATTCAA	Forward primer for gene 2500070675
70675-R632	CTGTCATGCTTTTGTACCT	Reverse primer for gene 2500070675
70676-F52	CAAACGGTGATTCCTGA	Forward primer for gene 2500070676
70676-R759	TTGCCTCCACAAATCTTG	Reverse primer for gene 2500070676
70677-F9	GTATTGGAATGAGAATTGGC	Forward primer for gene 2500070677
70677-R627	TACCCCTTTCCACCGA	Reverse primer for gene 2500070677
70678-F21	GAAATTAACGCCAATGCAATA	Forward primer for gene 2500070678
70678-R413	CTTAGATATTCTCCGTAGCC	Reverse primer for gene 2500070678
70679-F60	TTTAGGAAAACCATTCACCT	Forward primer for gene 2500070679
70679-R1331	TCATAAAGCGAAGGACGA	Reverse primer for gene 2500070679
70680-F17	GTAACGTAATCGCTGTTGTA	Forward primer for gene 2500070680
70680-R93	GTGTTTACCACCCACAA	Reverse primer for gene 2500070680

double-gene knockout mutant by transforming pKO-*hk430*-AB and pKO-*rr431*-AB into *L. reuteri* Δ*cemR* to disrupt *hk430* and *rr431*, respectively. *L. reuteri* Δ*cemR* was complemented with the following procedure. The inserted DNA fragment of pKO-*cemR*-14, containing the 5' and 3' flanking sequences of *cemR* and the full length of *cemR*, was amplified using *L. reuteri* 100-23 genomic DNA as a template with the primers *cemR*-KO-1-*Pst*I and *cemR*-KO-4-*Bam*HI. The complemented strain *L. reuteri* Δ*cemRC* was generated by transforming pKO-*cemR*-14 into *L. reuteri* Δ*cemR* using to the double crossover method described in Chapter 2 (this thesis). Erythromycin-sensitive *L. reuteri* Δ*cemRC* that contained the full length of *cemR* was selected by replicaing transformants on mMRS and mMRS-Erm agar plates. The mutants were all verified by PCR amplification and DNA sequencing.

5.2.5 Light microscope (LM) and scanning electron microscope (SEM) analysis of cell morphology

Refer to Chapter 4 (this thesis) for the cell growth conditions and the methods used to examine cell morphology.

5.2.6 Relative quantification of gene expression using quantitative PCR (qPCR)

Cultures that were grown overnight of *L. reuteri* 100-23, Δ*cemK*Δ*cemR,* and Δ*cemR* were washed and inoculated in gluMRS with a dilution factor of fifty at 37°C until the OD_{600nm} reached 0.4. This was followed by RNA extraction and cDNA synthesis, using the methods described earlier (Chapter 4, this thesis). Gene expression was quantified with cDNA using a SYBR Green-based assay

that has also been described earlier (Chapter 4, this thesis) (27). The analysis was performed in three independent experiments. The DNase I-treated RNA and genomic DNA were used as PCR negative and positive controls, respectively.

5.2.7 Adherence assay

The adherence assay method was performed as described previously (Chapter 4, this thesis).

5.2.8 Scanning electron microscope (SEM) analysis of biofilm specimens

L. reuteri 100-23 and Δ*cemR* were grown on polystyrene plates containing gluMRS or sucMRS broth at 37°C for 24 h under micro-aerobic conditions (1% of O_2, 5% of CO_2, and 94% of N_2). After incubation, cells were washed with buffer containing 50 mM NaH_2PO_4 (pH 6), and fixed with 2.5% glutaraldehyde in 10 mM of PBS buffer (pH 7.4) at 4°C overnight. Fixed cells were washed twice in PBS buffer, and then dehydrated by adding a series of one mL volumes of an increasingly concentrated ethanol in water solution (70, 85, 95, 100, and 100%, v/v) at room temperature. Cells were incubated in each ethanol concentration for 10 min. Hexamethyldisilazane (HMDS) (Sigma-Aldrich, Munich, Germany) was introduced into the cells by gradually increasing the concentration of HMDS in ethanol, with the following series: 75% ethanol/25% HMDS, 50% ethanol/50% HMDS, 25% ethanol/75% HMDS, and three volumes of 100% HMDS. Samples were air-dried overnight and then broken down to smaller pieces that were later mounted on SEM stubs, where they were immediately coated with Au/Pd on a sputter coater (Hummer 6.2) (Anatech, Union City, California). The examination

was performed using a scanning electron microscope XL30 (FEI, Hillsboro, Oregon) at an acceleration voltage of 20 kV.

5.2.9 Phenotypic analysis

The methods used to determine autoaggregation, membrane fluidity assay, minimum inhibitory concentration, and stress assays have been described earlier in Chapter 4 (this thesis).

5.2.10 Growth curves

L. reuteri was grown under the following conditions: in mMRS or sucMRS broth at 37°C; in mMRS broth with 32% (w/v) sucrose, 4% (w/v) NaCl or 0.018% (v/v) isohumulone; in mMRS medium without 0.05% (w/v) cysteine or without 0.01% (w/v) manganese and 0.001% (w/v) magnesium; and in mMRS broth incubated at 25°C for 20 h then 37°C for 24 h. The effect of heat shock (51°C for 15 min) on bacterial growth was evaluated in mMRS broth or mMRS broth with 4% NaCl. All cultures were incubated under micro-aerobic conditions (1% O_2, 5% CO_2, and 94% N_2), and the OD_{600nm} or OD_{630nm} was measured hourly.

5.2.11 Autolysis

Two methods of autolysis were used in this study. To determine the kinetics of autolysis, the method used in *L. helveticus* was modified (28). Cells of the wild type and the Δ*cemR* mutant were grown to an OD_{600nm} of 0.5 and an OD_{600nm} of 1.2, resuspended in 50 mM NaH_2PO_4 (pH 6) buffer, and incubated at 37°C with agitation. Autolysis was calculated by $A/A_{0,}$ where A is OD_{600nm} at a

given incubation time and A_0 is OD_{600nm} at time zero. To compare the autolyses of *L. reuteri* 100-23, Δ*cemK*Δ*cemR*, Δ*cemR*, Δ*hk430*Δ*cemR*, Δ*rr431*Δ*cemR*, and Δ*cemRC* after 5 h of inoculation, a previously used method was modified (29). A culture grown overnight was harvested and suspended in 50 mM of NaH_2PO_4 containing 0.05% (v/v) Triton X-100 (pH 6) and incubated at 37°C with agitation. Autolysis was calculated with the formula $A/A_{0,}$ where A is OD_{600} at 5 h incubation time and A_0 is OD_{600} at time zero. Five independent experiments were performed.

5.2.12 Transmission electron microscope (TEM) analysis

Isolated colonies on MRS agar containing 4% glucose were harvested after 24 h of incubation, and fixed with 2% glutaraldehyde in PBS buffer (pH 7.4) at 4°C for 3 h. Samples were washed three times in PBS buffer, and subjected to a post-fixation treatment with 2% osmic acid at 4°C for 2 h. After being washed twice in PBS buffer, the cells were dehydrated by incubation for 15 min in solutions containing increasingly concentrations of ethanol (20, 30, 40, 60, 80, 90, and 100%). Propylene oxide (Electron Microscope Sciences, Hatfield, PA) was used as a transitional solvent between dehydrant and spurr resin (Electron Microscope Sciences) to accelerate infiltration. The cells were infiltrated gradually by incubation in solutions containing 50% absolute ethanol (Electron Microscope Sciences)/50% propylene oxide, 100% propylene oxide, and 50% propylene oxide/50% spurr resin. The samples were embedded in pure spurr resin at 60°C for two days. The samples were cut into sections and were examined in an FEI MORGAGNI 268 electron microscope, and photographs were taken using a

CCD camera (Advanced Microscopy Facility, University of Alberta). The cells were examined at 71 000 and 56 000 magnifications. The thickness of the cell walls in a total of 11 or 13 cells was determined and 3 to 10 observations per cell were averaged.

5.2.13 Analysis of membrane fatty acids

L. reuteri 100-23 and *L. reuteri* Δ*cemR* were grown in mMRS broth and harvested at the early stationary phase (OD_{600nm} 2.0) by centrifugation. Analysis of membrane fatty acids was performed by the Pan Alberta Metabolomics Platform facility (PANAMP, Edmonton, Canada).

5.2.14 Sourdough fermentation

Sourdough fermentations were performed with *L. reuteri* 100-23, *L. reuteri* Δ*cemR*, and *L. reuteri* Δ*cemRC* to examine the effect of CemR on growth, acidification, and pH level. Doughs were prepared from 40 g of whole wheat flour (Rogers Food Ltd, Armstrong, Canada) and 40 mL of sterile tap water, inoculated to give 10^7 CFU g^{-1}, and incubated at 37°C. Samples were taken for pH measurement and viable cell counts after 0, 4, 12, 24, 48, 72, and 96 h of fermentation. Sourdough fermentations were carried out in two independent experiments and two samples of each strain were analyzed in each experiment.

5.2.15 Statistical analysis

All experiments were performed at least twice. Statistical analysis was performed using the *t*-test (SigmaPlot, version 11.0; Chicago, IL).

5.2.16 Sequences and accession numbers

The nucleotide sequence of *cemR* was retrieved from the GenBank database (accession number AAPZ02000001; region: 1100731..1101432). The protein sequence of CemR was retrieved from the GenPept database (NCBI Reference Sequence: ZP_03073361.1; GI:194467374). *L. reuteri* Δ*cemR*'s sequence is deposited with GenBank accession number JF339967, and the sequences of *L. reuteri* Δ*hk430*Δ*cemR*, Δ*rr431*Δ*cemR*, and Δ*cemRC* are deposited in Appendix D.

5.3 Results

5.3.1 The comparison of response regulators and transcriptional regulators to CemR of L. reuteri *100-23*

Previous studies on the *hk430* and *cemAKR* operons of *L. reuteri* 100-23 have shown their co-regulation through cross-talk, which may contribute to biofilm formation (Chapter 4, this thesis). To determine the functions of the *cemAKR* operon, the role of *cemR* in encoding a response regulator of the LytR/AlgR family was investigated. BLASTP analysis and the MUSCLE alignment revealed that protein CemR was similar to AgrA (30), SppR (31), PlnC (32), LamR, (33), ComE (34), and LytR (35, 36) (Table 5-3). In addition to proteins in the LytR/AlgR family, the predicted response regulator shares a 14 – 34% similarity with transcriptional regulators in the LytR-CpsA-Psr family (Table 5-3). Bioinformatic analysis suggested that CemR functioned as a response

Table 5-3 Protein alignment of response regulators and transcriptional regulators with similarity to CemR of *L. reuteri* 100-23

Uniprot	Name	Species	% Identity MUSCLE	Predictions and COG Family	Pfam domain
B3XMN9_LACRE	CemR	*Lactobacillus reuteri*	100	Putative response regulator	LytTr
B3XMM1_LACRE	RR431	*Lactobacillus reuteri*	75.5	Putative response regulator	LytTr
Q1WQY2_LACS1	AbpR	*Lactobacillus salivarius*	38.9	AbpR response regulator	LytTr
AGRA_STAAR	AgrA	*Staphylococcus aureus*	36.1	Accessory gene regulator protein A	LytTr
Q74KI5_LACJO		*Lactobacillus johnsonii*	34.2	Lactacin F response regulator	LytTr
YVHJ_BACSU	YvhJ	*Bacillus subtilis*	34.3	Putative transcriptional regulator YvhJ	LytR-CpsA-Psr
Q9EYK4_LACPL	PltR	*Lactobacillus plantarum*	33.5	Putative response regulator PltR	LytTr
Q6XVH6_LACSK	StxR	*Lactobacillus sakei*	31.0	Predicted response regulator StxR	LytTr
Q9REY4_CARML		*Carnobacterium maltaromaticum*	30.8	Predicted response regulator	LytTr
Q38Y65_LACSS	SppR	*Lactobacillus sakei*	30.0	Two-component system, response regulator SppR (Sakacin P production)	LytTr
Q48829_LACPL	PlnC	*Lactobacillus plantarum*	30.0	Response regulator	LytTr
Q8DNG0_STRR6	LytR	*Streptococcus pneumoniae*	29.4	Regulator of lytRABC operon	LytR-CpsA-Psr
Q88ZC3_LACPL	PlnD	*Lactobacillus plantarum*	29.4	Response regulator PlnD, repressor	LytTr
Q88T94_LACPL	LamR	*Lactobacillus plantarum*	29.2	Response regulator	LytTr
Q79CK7_STRPN	ComE	*Streptococcus pneumoniae*	27.7	Response regulator ComE	LytTr
Q9X474_ENTFC		*Enterococcus faecium*	27.7	Predicted AgrA type response regulator	LytTr
BRPA_STRMU	BrpA	*Streptococcus mutans*	27.7	Biofilm regulatory protein A	LytR-CpsA-Psr
Q8RMF2_9STRE	ComE	*Streptococcus infantis*	27.3	Response regulator ComE	LytTr

Table 5-3 –continued- Protein alignment of response regulators and transcriptional regulators with similarity to CemR of *L. reuteri* 100-23

Uniprot	Name	Species	% Identity MUSCLE	Predictions and COG Family	Pfam domain
Q9A1J5_STRP1	FasA	*Streptococcus pyogenes* serotype M1	25.6	Putative response regulator FasA	LytTr
YWTF_BACSU	YwtF	*Bacillus subtilis* subsp. *subtilis*	25.5	Putative transcriptional regulator YwtF	LytR-CpsA-Psr
Q81X17_BACAN	LytR	*Bacillus anthracis*	23.8	Transcription antiterminator, LytR family	LytR-CpsA-Psr
D2N6Y9_STAA5	MsrR	*Staphylococcus aureus* subsp. *aureus*	23.0	Cell envelope-related transcriptional attenuator	LytR-CpsA-Psr
LYTR_STAAN	LytR	*Staphylococcus aureu*	22.8	Autolysis response regulator LytR	LytTr
Q9S1I6_STRPN	BlpR	*Streptococcus pneumoniae*	22.5	Response regulator BlpR	LytTr
Q8DZD3_STRA5	CpsA	*Streptococcus agalactiae* serotype V	22.3	Capsular polysaccharide biosynthesis protein CpsA	LytR-CpsA-Psr
Q3K0S7_STRA1	CpsX	Group B streptococci	21.9	Regulatory protein CpsX	LytR-CpsA-Psr
Q88S62_LACPL	LamA	*Lactobacillus plantarum*	21.8	Response regulator; accessory gene regulator protein A	LytTr
LYTT_BACSU	LytT	*Bacillus subtilis*	21.4	Sensory transduction protein lytT	LytTr
LYTR_STRMU	LytR	*Streptococcus mutans*	20.2	Sensory transduction protein lytR	LytTr
CAPM_STAAU	CapM	*Staphylococcus aureus*	16.3	Capsular polysaccharide biosynthesis glycosyltransferase; PF00534. Glycos_transf_1.	LytR-CpsA-Psr
LYAT_BACSU	LytR	*Bacillus subtilis* subsp. *subtilis*	14.5	Membrane-bound transcriptional regulator LytR	LytR-CpsA-Psr

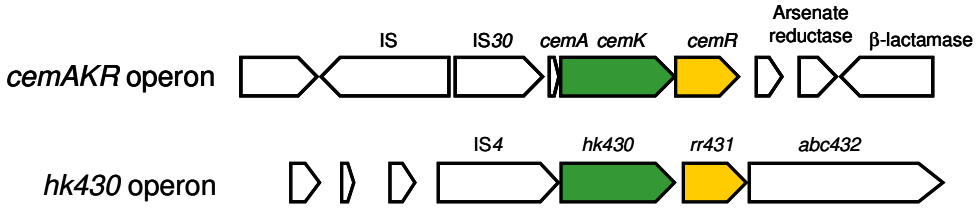

Figure 5-1 Organization of genes coding for the two-component regulatory systems *hk430* and *cemAKR* in *L. reuteri* 100-23. The *cemAKR* operon comprises a putative auto-inducing peptide (*cemA*), a histidine kinase (*cemK*), and a response regulator (*cemR*). The *hk430* operon consists of a histidine kinase (*hk430*), a response regulator (*rr431*), and an ATP-binding cassette transporter (*abc432*).

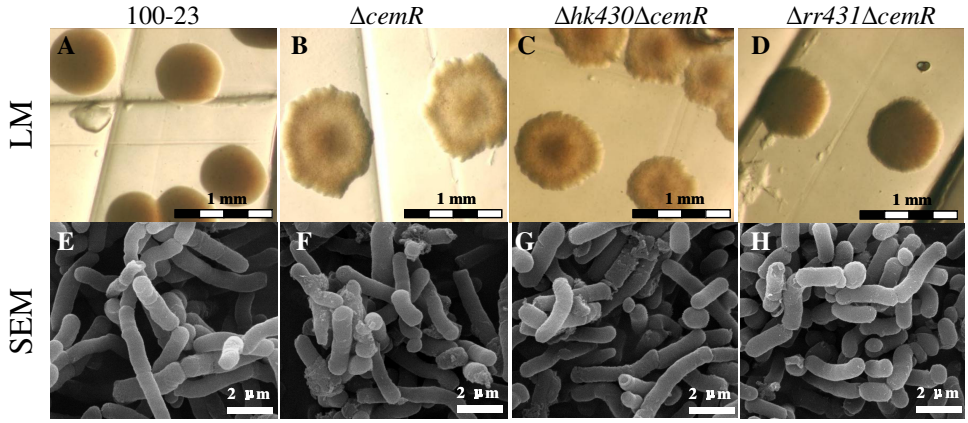

Figure 5-2 Morphology of *L. reuteri* 100-23, *L. reuteri* Δ*cemR*, *L. reuteri* Δ*hk430*Δ*cemR*, and *L. reuteri* Δ*rr431*Δ*cemR* grown on mMRS agar containing 2% glucose. The morphology was observed using light micrographs (LM) and scanning electron micrographs (SEM). The micrographs were taken from 6 fields in 2 independent experiments.

regulator controlled by an auto-inducing peptide, and was associated with either bacteriocin production or cell wall properties.

5.3.2 Cell morphology of L. reuteri *strains* ΔcemR, Δhk430ΔcemR, *and* Δrr431ΔcemR

To identify the functions and co-regulation roles of CemR (Fig. 5-1), deletions in *cemR*, *hk430*/*cemR*, and *rr431*/*cemR* were made using double crossover method. The colony morphology of *L. reuteri* strains Δ*cemR*, Δ*hk430*Δ*cemR*, and Δ*rr431*Δ*cemR* differed from that of *L. reuteri* 100-23 when grown on mMRS agar with 2% glucose at 37°C for 24 h. *L. reuteri* 100-23 had a circular and convex shape (Fig. 5-2A). *L. reuteri* Δ*cemR* and Δ*hk430*Δ*cemR* had a wavy and flat shape (Fig 5-2B and 5-2C), while *L. reuteri* Δ*rr431*Δ*cemR* had an irregular convex shape (Fig. 5-2D). Investigation of the cell morphology of *L. reuteri* mutants using scanning electron microscopy revealed that *L. reuteri* 100-23 had a smooth surface (Fig. 5-2E), while *L. reuteri* Δ*rr431*Δ*cemR* had a slightly rough surface (Fig. 5-2H). Many granules were observed on the surfaces of *L. reuteri* Δ*cemR* and Δ*hk430*Δ*cemR* (Fig. 5-2F and 5-2G).

5.3.3 Cross-regulation of hk430 *and* cemR *in biofilm formation and transcriptional expression*

The morphology of *L. reuteri* Δ*cemR*, Δ*hk430*Δ*cemR*, and Δ*rr431*Δ*cemR* showed that the properties of the cell envelope had been altered. To further examine phenotypes associated with the alteration of the cell envelope and biofilm formation, a quantitative adherence assay was performed (Fig. 5-3). When

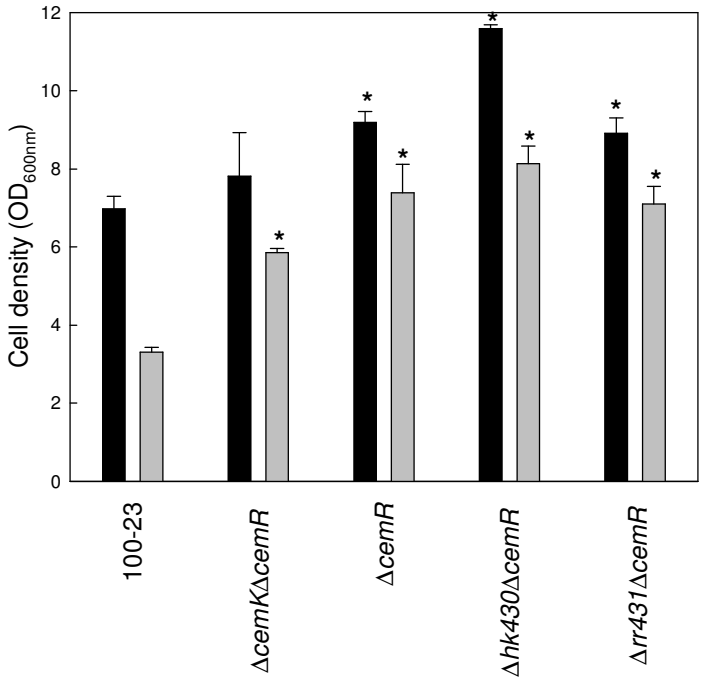

Figure 5-3 *In vitro* adherence assay of *L. reuteri* strains after 24 h of growth in sucMRS (black color) or in gluMRS (grey color). A significant difference from the wild-type strain is indicated by * ($p < 0.05$). Data shown are the mean of three independent experiments with standard deviation.

cultured in the presence of 2% sucrose, *L. reuteri* strains 100-23, Δ*cemK*Δ*cemR*, Δ*cemR*, Δ*hk430*Δ*cemR*, and Δ*rr431*Δ*cemR* reached an OD_{600nm} greater than 7, showing adherence occurred on the surfaces of plastic plates. When 2% sucrose was replaced with 2% glucose in the media, however, *L. reuteri* strain 100-23 showed reduced adherence compared to strains Δ*cemK*Δ*cemR*, Δ*cemR*, Δ*hk430*Δ*cemR*, and Δ*rr431*Δ*cemR*. To further examine the adherence of *L. reuteri* Δ*cemR*, scanning electron microscopy was used to visualize the structure of cells (Fig. 5-4). *L. reuteri* Δ*cemR* formed a thick, stacked structure of biofilm in gluMRS (Fig. 5-4C), whereas only a few attached cells were seen with *L. reuteri* 100-23 (Fig. 5-4A). In sucMRS, both strains developed biofilms

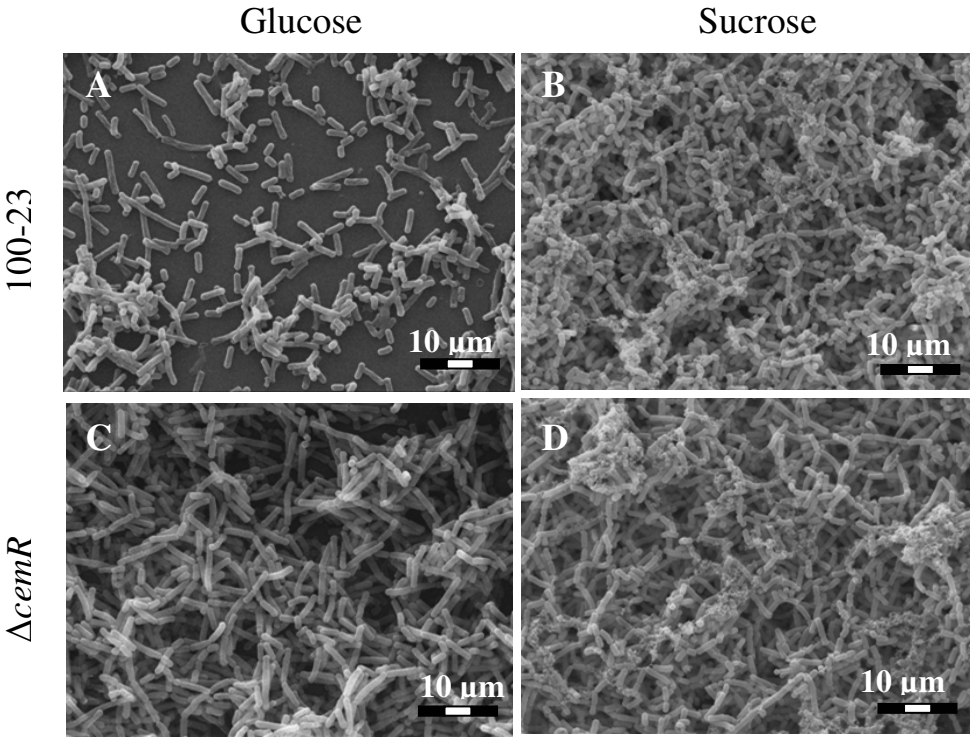

Figure 5-4 SEM micrographs of cells of *L. reuteri* 100-23 (A, B) and Δ*cemR* (C, D) on polystyrene surfaces. Cells in panels A and C were grown in MRS media containing 2% glucose, whereas cells in panels B and D were grown in MRS media containing 2% sucrose. Magnification, × 5000**.** Micrographs were taken from 6 different fields of 2 independent experiments.

(Fig. 5-4B and 5- 4D), implying that *L. reuteri* Δ*cemR* forms biofilm in MRS containing glucose and in the presence of sucrose.

The gene expression of the *hk430* and *cemAKR* operons was determined by quantitative PCR analysis to better understand the cooperation of the two-component systems in *L. reuteri* Δ*cemR*, Δ*hk430*Δ*cemR*, and Δ*rr431*Δ*cemR*. The gene expressions of *hk430*, *rr431*, *abc432*, and *cemK* in *L. reuteri* Δ*cemR* were not altered more than two-fold. *L. reuteri* Δ*hk430*Δ*cemR* over-expressed *rr431*, *abc432*, and *cemK* (Fig. 5-5). This result and this study's findings on the effect of

hk430 deletion (Chapter 4, this thesis), both supported the hypothesis that a cross-talk existed between the *hk430* and *cemAKR* operons.

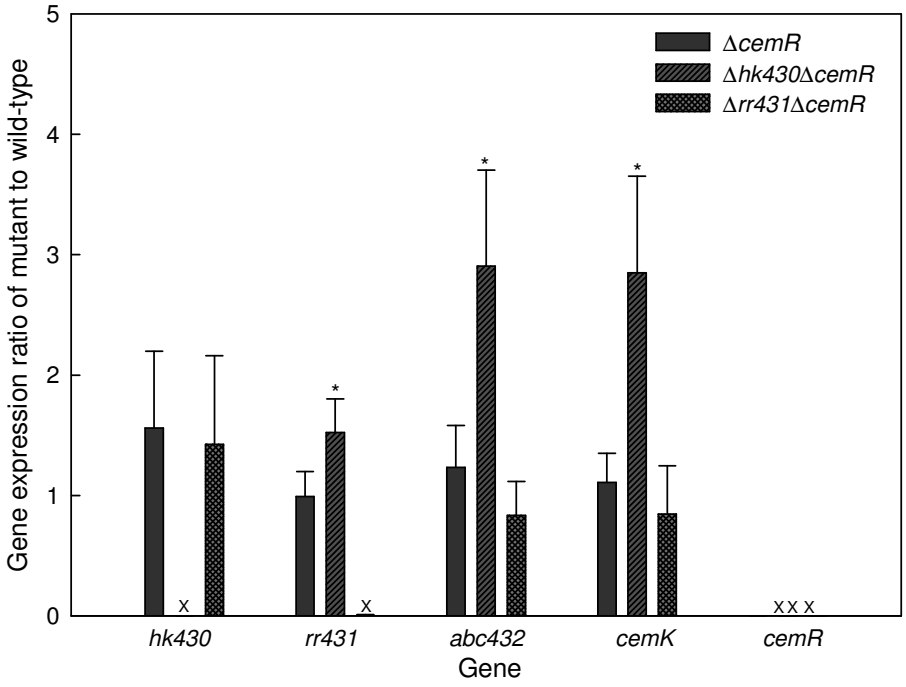

Figure 5-5 Relative quantification of gene expression in *L. reuteri* Δ*cemR*, Δ*hk430*Δ*cemR*, and Δ*rr431*Δ*cemR*. The expression of the *hk430* and *cemAKR* operons were quantified by qPCR (the y-axis; linear scale) with primers specific to genes *hk430*, *rr431*, *abc432*, *cemK*, and *cemR* (the x-axis). x indicates a gene with expression below 0.01. A significant difference from the wild-type strain is indicated by * ($p < 0.05$). Results are shown as means ± standard deviations of three independent experiments.

5.3.4 Phenotypic analysis

To evaluate other possible effects that may have been caused by the deletions of *cemR*, *hk430*, or *rr431*, a broad range of phenotypic analyses were performed (Table 5-4). Autoaggregation of *L. reuteri* 100-23 grown in mMRS

Table 5-4 Phenotypic analysis of *L. reuteri* Δ*cemR*, Δ*hk430*Δ*cemR*, and Δ*rr431*Δ*cemR*

	L. reuteri mutant strains compared to strain 100-23		
	Δ*cemR*	Δ*hk430*Δ*cemR*	Δ*rr431*Δ*cemR*
Autoaggregation			
mMRS grown cells in pH 4 buffer	5 min	5 min	5 min
sucMRS grown cells in pH 4 buffer	-	-	5 min
Membrane fluidity (Laurdan GP)	-	-	-
MIC assay			
mMRS (reutericyclin)	-	-	-
mMRS (isohumulone)	-	-	-
sucMRS (phenylethanol) WT: 128 mM	64 mM	64 mM	64 mM
Stress assays (OD_{630} after 18 h)			
mMRS (25°C)	-	-	-
mMRS (45°C)	-	-	-
Oxygen tolerance	-	-	-
mMRS pH 3, 4, 6, 8, 10	-	-	-
mMRS (4% NaCl)	-	-	-
mMRS (2- 10% glycerol)	-	-	-
mMRS (8% glucose) WT: 0.8	+ (1.2)	+ (1.2)	+ (1.1)
mMRS (10, 15 and 20% sucrose)	Higher	Higher	Higher
Growth curves			
mMRS (37°C)	-	-	-
sucMRS (37°C)	-	-	-
mMRS (0.018% isohumulone)	-	-	-
mMRS without cysteine	-	-	-
mMRS without Mn^{2+} and Mg^{2+}	-	-	-
mMRS (32% sucrose)	longer lag phase	longer lag phase	-
mMRS (4% NaCl)	-	-	shorter lag phase
mMRS (25°C for 20 h , 37°C for 24 h)	longer lag phase	longer lag phase	longer lag phase
mMRS (4% NaCl, 51°C for 15 min)	longer lag phase	longer lag phase	longer lag phase
mMRS heat shock (51°C for 15 min)	longer lag phase and lysis	longer lag phase	-

- indicates no significant change was observed in a mutant strain

was slower than that of the Δ*cemR*, Δ*hk430*Δ*cemR*, and Δ*rr431*Δ*cemR* cells in buffer at pH 4. *L. reuteri* Δ*rr431*Δ*cemR* that was cultured in sucMRS aggregated more rapidly than the wild-type strain, the Δ*cemR* mutant, and the Δ*hk430*Δ*cemR* mutant in this buffer. Laurdan generalized polarization was used to evaluate membrane fluidity in *L. reuteri* Δ*cemR*, Δ*hk430*Δ*cemR*, and Δ*rr431*Δ*cemR*, but no changes in membrane fluidity were detected. The sensitivity of strain 100-23 and the three mutant strains to reutericyclin and isohumulone was comparable. However, the MIC of phenylethanol was higher for *L. reuteri* 100-23 (128 mM) compared to *L. reuteri* Δ*cemR*, Δ*hk430*Δ*cemR*, and Δ*rr431*Δ*cemR* (64 mM). Various stressors were applied to assess their influence on the cell growth of *L. reuteri* 100-23 and the three mutants. The cell density after 18 h of incubation was equivalent for all strains at both 25°C and 45°C, under aerobic conditions, and with initial pH values ranging from 3 to 10. The effect of osmotic stress was examined by adding 4% NaCl, glycerol, glucose, or sucrose in the culture media. The former two stressors did not affect the final cell density of *L. reuteri* 100-23 and the three mutants. However, when grown in media containing 8% glucose or sucrose (10 to 20%) *L. reuteri* Δ*cemR*, Δ*hk430*Δ*cemR*, and Δ*rr431*Δ*cemR* all had a higher cell density than *L. reuteri* 100-23. The growth rate of *L. reuteri* Δ*cemR*, Δ*hk430*Δ*cemR*, and Δ*rr431*Δ*cemR* was not affected by the presence of an additional 2% sucrose or 0.018% isohumulone. The growth rate was also not affected when cysteine or magnesium and manganese were absent. The osmotic stress caused by the addition 32% sucrose in the media prolonged the lag phase of *L. reuteri* Δ*cemR* and Δ*hk430*Δ*cemR*. In contrast, *L. reuteri* Δ*rr431*Δ*cemR* had a

shorter lag phase when cultured in 4% NaCl. When a temperature shift (25 → 37 °C or 37 → 51°C) was imposed during the lag phase or early exponential phase of growth, *L. reuteri* Δ*cemR* and Δ*hk430*Δ*cemR* exhibited a longer lag phase compared to the wild-type strain. Exposing exponentially-growing cultures to a heat shock of 51°C for 15 min also induced cell lysis in *L. reuteri* Δ*cemR* (Fig. 5-6). In summary, the aggregation, osmotic stress, sensitivity to phenylethanol, and sensitivity to temperature shifts during growth of *L. reuteri* Δ*cemR*, Δ*hk430*Δ*cemR*, and Δ*rr431*Δ*cemR* was different than those of the wild-type strain. Because *L. reuteri* Δ*hk430*Δ*cemR* and *L. reuteri* Δ*rr431*Δ*cemR* were generated from *L. reuteri* Δ*cemR*, further analyses focused on *L. reuteri* Δ*cemR*.

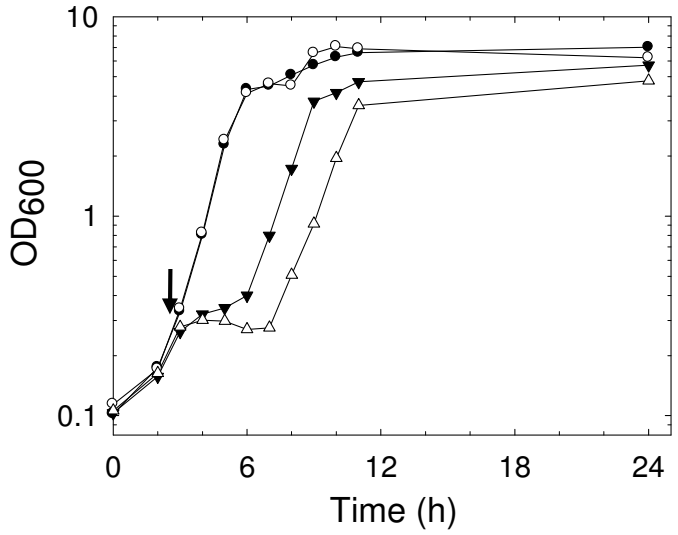

Figure 5-6 Growth of *L. reuteri* 100-23 (closed symbols) and *L. reuteri* Δ*cemR* (open symbols). Cells were incubated at 37°C (●, ○), or incubated at 37°C and then exposed to a heat shock of 51°C for 15 min at the time indicated by the arrow, followed by incubation at 37°C (▼, △). The data represents three independent experiments.

5.3.5 Autolysis and the architecture of cell walls in L. reuteri ΔcemR

The autolysis of exponentially-growing and early stationary cultures of *L. reuteri* Δ*cemR* was compared to that of the wild-type strain. After 4 h of incubation, the cell density of *L. reuteri* Δ*cemR* was reduced by 50%. In the wild-type strain, the same degree of autolysis was observed only after 11 h of incubation (Fig. 5-7).

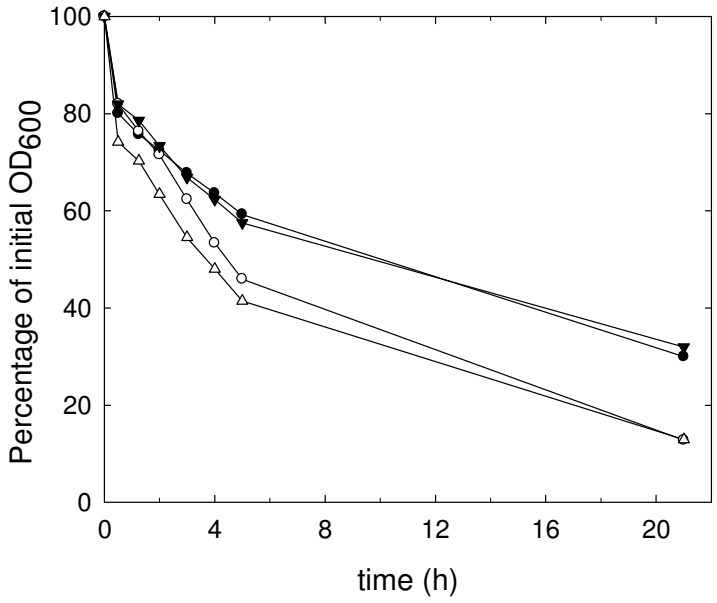

Figure 5-7 Autolysis of *L. reuteri* 100-23 (closed symbols) and *L. reuteri* Δ*cemR* (open symbols)**.** Cells were harvested at OD_{600nm} 0.5 (●, ○) or at OD_{600nm} 1.2 (▼, △), and suspended in 50 mM of NaH_2PO_4 (pH 6) for observation of autolysis. The figure shown describes the optical density as a percentage of the initial OD_{600nm}. The data represents three independent experiments.

The structure of the cell envelope was visualized by transmission electron microscopy (TEM). The TEM images showed that the cell wall had a trilaminar structure (Fig. 5-8). The peptidoglycan layer was present in an electron-dense

layer near the cell surface (Fig. 5-8 II). A significant difference ($p < 0.001$) in thicknesses of the peptidoglycan layers of *L. reuteri* Δ*cemR* and the wild-type strain was observed. The peptidoglycan layer of *L. reuteri* Δ*cemR* had an average thickness of 12.0 ± 1.7 nm in, compared to an average thickness of 18.6 ± 1.7 nm in the peptidoglycan wild-type strain (Fig. 5-8B II).

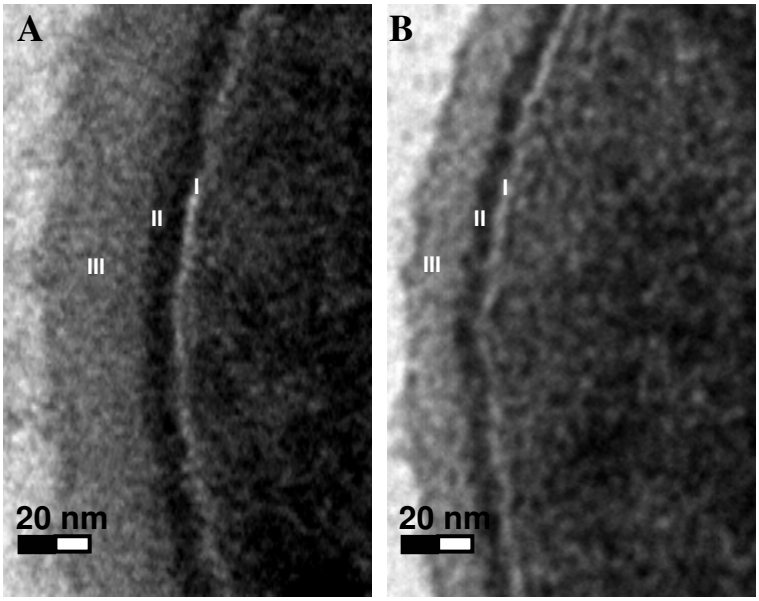

Figure 5-8 Transmission electron micrographs of cell walls of *L. reuteri* 100-23 (A) and *L. reuteri* Δ*cemR* (B) at 56 000-fold magnification**.** Layers corresponding to the cytoplasmic membrane (I), the peptidoglycan (II), and the non-peptidoglycan outer layers (III) are indicated. Micrographs were taken from at least three different fields in two independent experiments.

5.3.6 Analysis of membrane lipids

Properties of the cytoplasmic membrane were evaluated by the analysis of lipid composition. The ratio of saturated fatty acids to unsaturated fatty acids increased in the neutral lipid and glycolipid fractions in *L. reuteri* Δ*cemR* in

Table 5-5 Fatty acid compositions of membrane lipid in *L. reuteri* 100-23 and Δ*cemR*

Fatty acid (mol %)	**Total lipids**		**Neutral lipids**	
	100-23	Δ*cemR*	100-23	Δ*cemR*
Lauric acid (C12:0)	3.34 ± 1.05 *	0.96 ± 0.21 *	0.18 ± 0.01	0.14 ± 0.02
Myristic acid (C14:0)	2.09 ± 0.16	2.25 ± 0.58	2.73 ± 0.11	2.74 ± 0.14
Myristoleic acid (C14:1, *cis*-9)	0.20 ± 0.14	0.51 ± 0.17	0.69 ± 0.15	0.82 ± 0.59
Pentadecanoic acid (C15:0)	0.32 ± 0.07	0.35 ± 0.09	0.23 ± 0.02	0.20 ± 0.01
Palmitic acid (C16:0)	19.0 ± 4.26	19.8 ± 2.55	26.4 ± 4.52	28.6 ± 2.84
Palmitoleic acid (C16:1, *cis*-9)	7.03 ± 0.65	7.57 ± 0.60	5.72 ± 0.50	5.37 ± 0.45
Hexadecenoic acid (C16:1, *cis*-7)	1.42 ± 0.04	1.04 ± 0.38	0.88 ± 0.07	0.69 ± 0.22
Stearic acid (C18:0)	10.4 ± 2.81	11.3 ± 1.89	19.2 ± 3.69	20.9 ± 2.53
Oleic acid (C18:1, *cis*-9)	44.2 ± 6.40	43.2 ± 4.89	34.1 ± 7.04	30.3 ± 4.42
Elaidic acid (C18:1, *trans*-9)	0.86 ± 0.16	0.68 ± 0.05	0.77 ± 0.08	0.74 ± 0.05
Linoleic acid (C18:2, *cis*-9,12)	1.47 ± 0.27	1.49 ± 0.06	1.49 ± 0.32	1.24 ± 0.11
Linolelaidic acid (C18:2, *trans*-9,12)	1.22 ± 0.10 *	N.D.	N.D.	0.76 ± 0.08 *
Nonadecanoic acid (C19:0)	0.18 ± 0.06	0.36 ± 0.01	0.30 ± 0.14	0.29 ± 0.12
Cyclopropane (C19:0)	7.08 ± 0.29 *	8.30 ± 0.43 *	6.23 ± 0.86	5.87 ± 0.61
Arachidic acid (C20:0)	0.12 ± 0.02 *	0.46 ± 0.06 *	0.35 ± 0.08	0.33 ± 0.16
Eicosenoic acid (C20:1, *cis*-11)	1.35 ± 0.29	1.46 ± 0.09	0.63 ± 0.10	0.72 ± 0.09
Behenic acid (C22:0)	0.65 ± 1.02	0.36 ± 0.31	0.06 ± 0.02	0.09 ± 0.03
Erucic acid (C22:1, *cis*-13)	N.D.	0.15 ± 0.13	0.13 ± 0.03 *	0.21 ± 0.04 *
Ratio of saturated/unsaturated fatty acids	0.56	0.56	0.98	1.14

A significant difference from the wild-type strain is indicated by * ($p < 0.05$); N.D. not detected

Table 5-5 -continued- Fatty acid compositions of membrane lipid in *L. reuteri* 100-23 and Δ*cemR*

Fatty acid (mol %)	**Glycolipids**		**Phospholipids**	
	100-23	Δ*cemR*	100-23	Δ*cemR*
Lauric acid (C12:0)	0.11 ± 0.01	0.08 ± 0.02	0.12 ± 0.09	0.07 ± 0.05
Myristic acid (C14:0)	2.78 ± 0.22	2.67 ± 0.08	3.57 ± 0.30	4.29 ± 0.40
Myristoleic acid (C14:1, *cis*-9)	0.30 ± 0.03	0.26 ± 0.01	0.36 ± 0.07	0.30 ± 0.07
Pentadecanoic acid (C15:0)	0.52 ± 0.01	0.51 ± 0.02	0.39 ± 0.03	0.42 ± 0.03
Palmitic acid (C16:0)	16.6 ± 2.36	22.9 ± 0.33	8.57 ± 0.20	8.66 ± 0.30
Palmitoleic acid (C16:1, *cis*-9)	8.82 ± 1.21	8.74 ± 0.60	12.8 ± 3.44	7.31 ± 0.28
Hexadecenoic acid (C16:1, *cis*-7)	0.90 ± 0.01	1.25 ± 0.15	1.11 ± 0.10	1.02 ± 0.02
Stearic acid (C18:0)	7.17 ± 2.57	7.70 ± 1.56	3.37 ± 0.69	2.45 ± 0.10
Oleic acid (C18:1, *cis*-9)	47.4 ± 2.19 *	39.8 ± 0.60 *	52.8 ± 2.29 *	57.6 ± 0.39 *
Elaidic acid (C18:1, *trans*-9)	0.69 ± 0.09	0.62 ± 0.03	0.75 ± 0.08	0.65 ± 0.03
Linoleic acid (C18:2, *cis*-9,12)	1.54 ± 0.01	1.64 ± 0.25	1.48 ± 0.20	1.31 ± 0.10
Linolelaidic acid (C18:2, *trans*-9,12)	0.96 ± 0.04	0.97 ± 0.04	1.15 ± 0.13 *	0.86 ± 0.06 *
Nonadecanoic acid (C19:0)	N.D.	N.D.	N.D.	0.06 ± 0.01*
Cyclopropane (C19:0)	10.5 ± 1.37	11.3 ± 0.81	9.92 ± 1.42 *	13.4 ± 0.47 *
Arachidic acid (C20:0)	0.12 ± 0.04	0.16 ± 0.02	N.D.	0.03 ± 0.01
Eicosenoic acid (C20:1, *cis*-11)	1.35 ± 0.08 *	1.63 ± 0.05 *	1.48 ±0.23	1.51 ± 0.16
Behenic acid (C22:0)	0.06 ± 0.08	0.03 ± 0.01	N.D.	0.01 ± 0.00
Erucic acid (C22:1, *cis*-13)	0.16 ± 0.02 *	0.35 ± 0.03 *	0.07 ± 0.04	0.06 ± 0.01
Ratio of saturated/unsaturated fatty acids	0.38	0.51	0.20	0.19

A significant difference from the wild-type strain is indicated by * ($p < 0.05$); N.D. not detected

contrast to the wild-type strain (Table 5-5). Decreases in lauric acid and linolelaidic acid, along with increases in cyclopropane and arachidic acids were observed in the total lipids fraction. Linolelaidic acid was present in neutral lipids from *L. reuteri* Δ*cemR* but not in neutral lipids from the wild-type cells. The amount of erucic acid in the Δ*cemR* mutant was also larger than that in the wild-type strain. The amount of oleic acid, the major fatty acid in the glycolipids, was smaller in the Δ*cemR* mutant than that in the wild-type strain. In the glycolipid fraction, the amount of eicosenoic acid and erucic acid was slightly larger in the *L. reuteri* Δ*cemR* mutant than that in the wild-type cells. In the phospholipid fraction of *L. reuteri* Δ*cemR*, the amount of oleic acid, linolelaidic acid, nonadecanoic acid, and cyclopropane also increased. In summary, the increase of cyclopropane (a stress indicator) and decrease of linolelaidic acid (a polyunsaturated fatty acid) in the phospholipids fraction and the total lipids fraction of *L. reuteri* Δ*cemR* suggest that membrane compositions were altered.

5.3.7 qPCR analysis of genes related to the phenotypes observed in L. reuteri Δ*cemR*

Cell envelope structure, biofilm formation, and membrane composition of *L. reuteri* Δ*cemR* were altered. To better understand the changes to these properties of cells of *L. reuteri*, qPCR was carried out to determine the transcriptional expression of genes that may be associated with these new phenotypes observed in *L. reuteri* Δ*cemR* (Table 5-6, Fig. 5-9). The targeted genes (Table 5-6) included the genes that exhibit direct repeats similar to 5'-TGGTTACT in their promoter region (cell division regulator *ftsW* and acetate

Table 5-6 Genes which were related to phenotypes used for qPCR analysis

	Gene ID[a]	Annotation of protein sequence	Prediction
1	*cemA*	Predicted autoinducing peptide	Quorum-sensing two-component system
2	2500069269	LytS; histidine kinase; regulator of cell autolysis	Cell autolysis; two-component system
3	2500069270	LytR; response regulator of the LytR/AlgR family	Cell autolysis; two-component system
4	2500069271	Putative negative effector of murein hydrolase LrgA	Cell lysis; regulation of murein hydrolase activity
5	2500069272	Putative negative effector of murein hydrolase; LrgB-like holing/antiholin	Cell lysis; regulation of murein hydrolase activity
6	2500069363	Cystine transporter, amino acid ABC transporter substrate-binding protein, PAAT family; signal transduction systems, periplasmic component/domain	Homolog of BspA/CyuC/MapA/CnBP collagen binding protein
7	2500069863	Hypothetical protein	Homolog of glucansucrase/ reuteransucrase
8	2500070531	Bacteriocin-type signal sequence	Quorum sensing two-component system
9	2500070532	ABC-type bacteriocin transporter	Quorum sensing two-component system
10	2500070615	Cyclopropane-fatty-acyl-phospholipid synthase	Cyclopropane synthesis
11	2500070618	Acetolactate synthase, large subunit	Carbohydrate metabolism
12	2500070674	Glycine betaine/choline-binding (lipo)protein of an ABC-type transport system	Osmoprotectant binding protein
13	2500071010	Large surface protein with LPXTG-motif cell wall anchor domain	Homolog of FtfA/ levansucrase/ inulosucrase
14	2500071188	Cell division-specific peptidoglycan biosynthesis regulator FtsW	Cell division
15	2500071258	Acetate kinase	Carbohydrate metabolism

[a] This gene ID system is the same as that used in the DOE Joint Genome Institute databases

kinase). Genes known to be related to the production of auto-inducing peptides (*cemA*, 2500070531, and 2500070532), autolysis regulation (*lytSR* and *lrgAB*),

adhesion *(cyuC,* reuteransucrase, and levansucrase), cyclopropane synthesis, and lactate synthesis were chosen. Gene 2500070674, which is related to glycine betaine transport, was included because it was incorrectly identified as a mucus-binding protein. *L. reuteri* Δ*cemK*Δ*cemR* was chosen as a control strain for comparison with *L. reuteri* Δ*cemR*.

The expression of *cemA* was lower in *L. reuteri* Δ*cemK*Δ*cemR* than that in the wild-type strain (Fig. 5-9). A two-component system *lytS/lytR* (genes 2500069269 and 2500069270), which is related to autolysis, biofilm formation, and cell death, was had a slightly higher expression in *L. reuteri* Δ*cemR*, but not in the Δ*cemK*Δ*cemR* mutant. The reduced expression of fructansucrase and increased expression of acetate kinase were also detected in *L. reuteri* Δ*cemR*, but not in the Δ*cemK*Δ*cemR* mutant. The expression of *lrgA*, *lrgB*, *cyuC*, glucansucrase, the bacteriocin-type signal peptide, the bacterion-related ABC transporter, cyclopropane-fatty-acyl-phospholipid synthase, acetolactate synthase, and cell division regulator *ftsW* did not change significantly in *L. reuteri* Δ*cemR* and *L. reuteri* Δ*cemK*Δ*cemR*. However, the transcriptional level of gene 2500070674, which encodes a glycine betaine tansporter, was low in *L. reuteri* Δ*cemK*Δ*cemR* and was not detectable in *L. reuteri* Δ*cemR*, implying a genetic deficiency may occurr in *L. reuteri* Δ*cemR*.

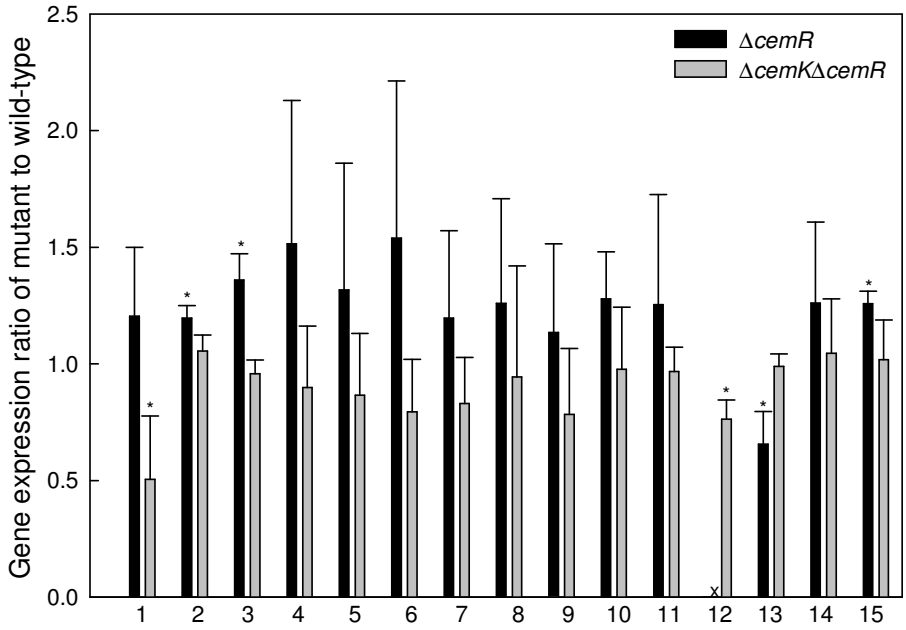

Figure 5-9 Transcriptional expression of genes related to phenotypes in *L. reuteri* Δ*cemR* (black color) in comparison with *L. reuteri* Δ*cemK*Δ*cemR* (grey color). The relative gene expression ratio (the y-axis; linear scale) is normalized to the endogenous gene *recA* of the wild-type strain 100-23 according to Paffal's model (27). Target genes (the x-axis) are as follows: 1, *cemA*; 2, *lytS*; 3, *lytR*; 4, *lrgA*; 5, *lrgB*; 6, *mapA*; 7, glucansucrase; 8, autoinducing peptide; 9, ABC transporter; 10, cyclopropane-fatty-acyl-phospholipid synthase; 11, acetolactate synthase; 12, glycine betaine tansporter (osmoprotectant binding protein); 13, fructansucrase; 14, cell devision regulator *ftsW*; and 15, acetate kinase. x indicates a gene with expression below 0.01. A significant difference from the wild-type strain is indicated by * ($p < 0.05$). Results are shown as means ± standard deviations of three indepedent experiments.

5.3.8 Identification of additional mutations in L. reuteri ΔcemR, Δhk430ΔcemR, Δrr431ΔcemR, *and* ΔcemRC

The phenotypes of *L. reuteri* Δ*cemR*, Δ*hk430*Δ*cemR*, and Δ*rr431*Δ*cemR* had no correlation to the of comparable phenotypes of *L. reuteri* Δ*cemK*Δ*cemR*, Δ*hk430*, and Δ*rr431* (Chapter 4, this thesis). These mismatched phenotypes implied the possibility of the occurrence of additional mutations in *L. reuteri* Δ*cemR*, which was used to generate *L. reuteri* Δ*hk430*Δ*cemR* and Δ*rr431*Δ*cemR*. Therefore, an *in cis* complemented mutant of *L. reuteri* Δ*cemR* was created and named "*L. reuteri* Δ*cemRC*." Although the results of PCR amplification (Fig. 5-10) and DNA sequencing confirmed the substitution of truncated *cemR* with the full-length *cemR* in *L. reuteri* Δ*cemRC*, the colony morphology of *L. reuteri* Δ*cemRC* was variable (data not shown).

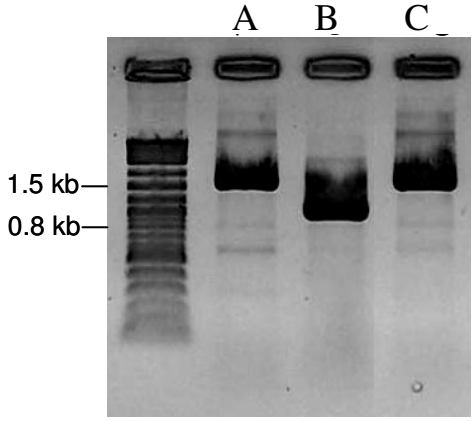

Figure 5-10 Verification of *L. reuteri* Δ*cemRC* by PCR analysis. The amplicon was carried out using primers *cemK*-KO-3-*Bam*HI and *cemK*-KO-4-*Bgl*II with sizes of 1.5 kb, 0.9 kb, and 1.5 kb for the 100-23 strain (A), Δ*cemR* mutant (B), and Δ*cemRC* mutant (C), respectively.

Moreover, the adherence of *L. reuteri* Δ*cemRC* in gluMRS and sucMRS did not return to the same level as *L. reuteri* 100-23 (Fig. 5-11). In addition, the growth kinetics of *L. reuteri* Δ*cemR* and Δ*cemRC* were similar over the 96 h of sourdough fermentation (Fig. 5-12A). The pH profiles of sourdough fermentation in *L. reuteri* Δ*cemR* and *L. reuteri* Δ*cemRC* were similar (Fig. 5-12B). Consequently, these results suggested that the phenotypes chosen for examination were not complemented in *L. reuteri* Δ*cemRC*. Unexpected mutations may have taken place in *L. reuteri* Δ*cemR* and Δ*cemRC*.

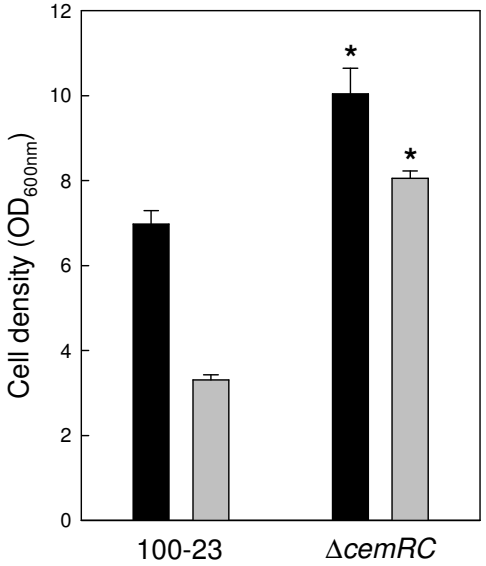

Figure 5-11 *In vitro* adherence assay of *L. reuteri* 100-23 and *L. reuteri* Δ*cemRC*. Quantification of cells adhered to the plastic plate assay after 24 h of growth at 37°C in MRS broth containing 2% sucrose (black color) or 2% glucose (grey color). Adherence was measured by an optical density of 600 nm. A significant difference from the wild-type strain is indicated by * ($p < 0.05$). Results are shown as means ± standard deviations of three indepedent experiments.

The mutagenesis of *L. reuteri* Δ*hk430*Δ*cemR*, Δ*rr431*Δ*cemR*, and Δ*cemRC* was originally derived from *L. reuteri* Δ*cemR*. Therefore, mutations identified in

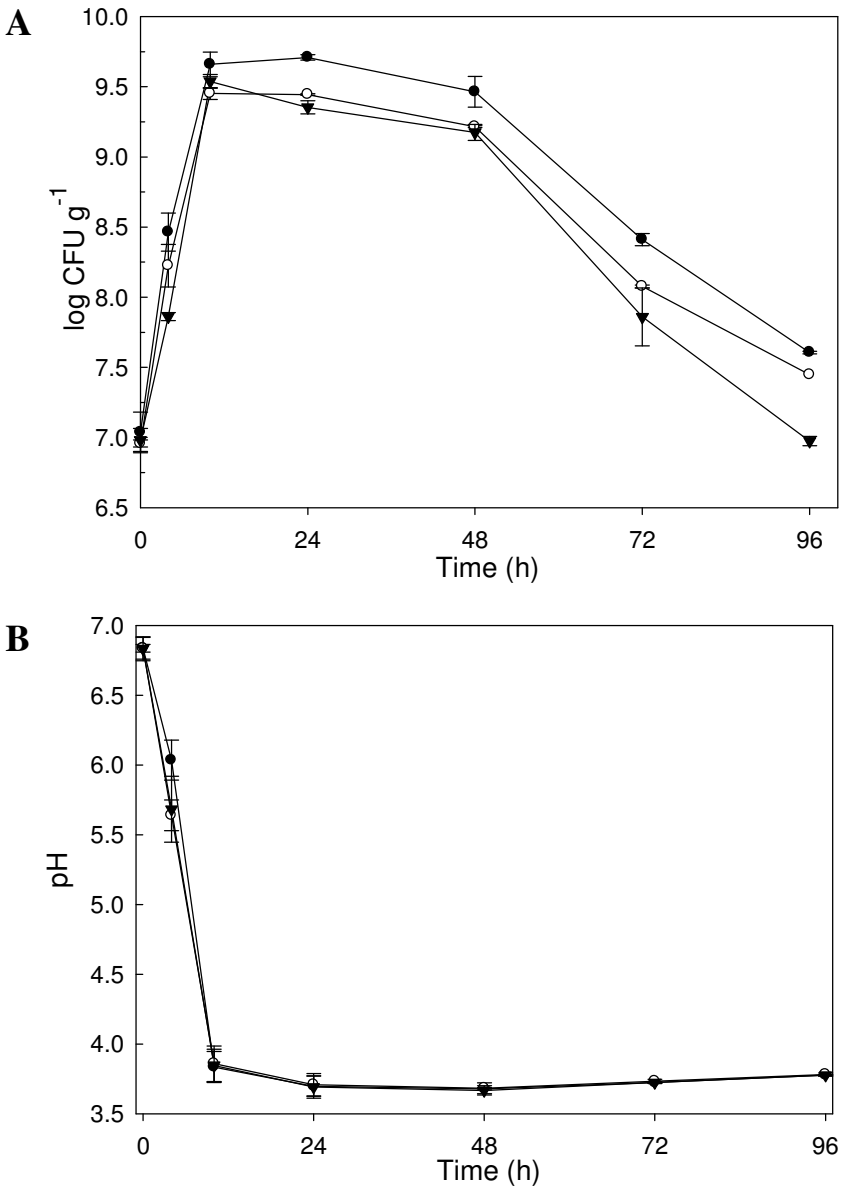

Figure 5-12 The cell growth and pH profile of *L. reuteri* 100-23, *L. reuteri* Δ*cemR*, and *L. reuteri* Δ*cemRC* in 96 h of sourdough fermentation. (A) The kinetic of cell growth in 96 h of sourdough fermentation cultured with *L. reuteri* 100-23 (●), *L. reuteri* Δ*cemR* (○), or *L. reuteri* Δ*cemRC* (▼). (B) The pH profile of acid production in *L. reuteri* 100-23 (●), *L. reuteri* Δ*cemR* (○), or *L. reuteri* Δ*cemRC* (▼) during sourdough fermentation over 96 h. Symbols indicate means ± standard deviation from quadruplicate determinations.

the genome of *L. reuteri* Δ*cemR* may also be found in these mutants. PCR analysis was performed to determine whether gene 2500070674 was present in *L. reuteri* Δ*cemR*, Δ*hk430*Δ*cemR*, Δ*rr431*Δ*cemR*, and Δ*cemRC*, as it was in the control strains *L. reuteri* 100-23 and *L. reuteri* Δ*cemK*Δ*cemR*. *L. reuteri* Δ*cemK*Δ*cemR* was generated independently from *L. reuteri* Δ*cemR*. The results of PCR amplification using the primers 70674-qPCR242-F1 and 70674-qPCR316-R1 with genomic DNA or cDNA revealed that gene 2500070674 was not detectable in *L. reuteri* Δ*cemR*, Δ*hk430*Δ*cemR*, Δ*rr431*Δ*cemR*, or Δ*cemRC* (data not shown). The results of autolysis assays also agreed with the PCR data. *L. reuteri* Δ*cemK*Δ*cemR* showed the lysis of cells in a manner similar to that of the wild-type strain, while *L. reuteri* strain Δ*cemR* and its derived strains Δ*hk430*Δ*cemR*, Δ*rr431*Δ*cemR*, and Δ*cemRC* enhanced cell lysis after 5 h of inoculation (Fig. 5-13).

To identitfy the mutation located in gene 2500070674 in *L. reuteri* Δ*cemR*, PCR analysis was performed. Primers were designed for approximately 9 kb of genetic loci coding from the 11 putative genes between 2500070670 and 2500070680 (Table 5-7). Amplicons of these 11 genes could not be obtained from the DNAs of *L. reuteri* Δ*cemR* and Δ*cemRC*, whereas the amplicons of the 11 putative genes were successfully obtained from strains *L. reuteri* 100-23 and Δ*cemK*Δ*cemR* (data not shown). This indicated an undefined deletion of genes occurred in *L. reuteri* Δ*cemR* where at least the 11genes between 2500070670 and 2500070680 were known to be missing.

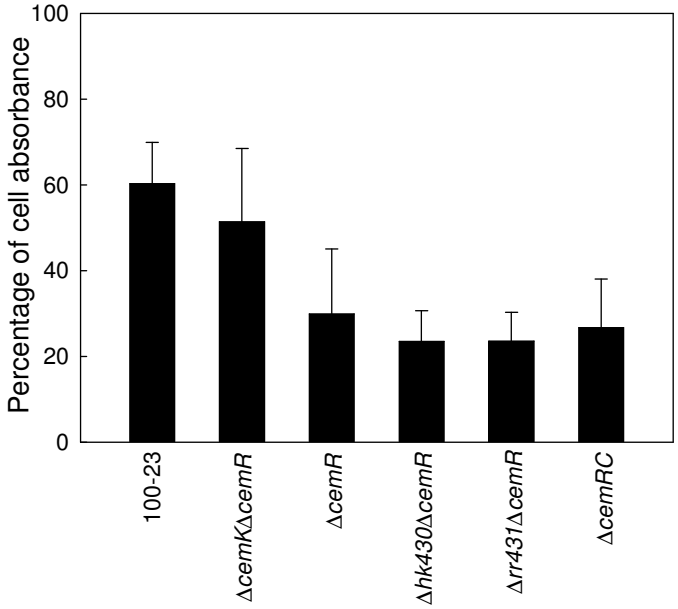

Figure 5-13 Autolysis of *L. reuteri* 100-23 and the mutant strains Δ*cemK*Δ*cemR*, Δ*cemR*, Δ*hk430*Δ*cemR*, Δ*rr431*Δ*cemR*, and Δ*cemRC*. Cells at the stationary phase were harvested and inoculated in 0.05% Triton X-100 buffer for 5 h at 37°C. The percentage of the final OD_{600} is shown from an average of three independent experiments.

5.4 Discussion

The aim of this study was to characterize the response regulator Δ*cemR*, and to assess its influence on the physiology and biofilm formation in the *L. reuteri* rodent isolate 100-23. However, this study was unable to clarify the function of *cemR* because of the presence of the additional mutations that were identified in *L. reuteri* Δ*cemR*. BLASTP analysis showed that there are several CemR homologs in the LytR/AlgR family of response regulators (Table 5-3). The protein sequence of CemR is similar to that of LamR, which is a response regulator involved in the regulation of adherence, biofilm formation, and cell envelope properties in *L. plantarum* (33). The phenotypes that were observed in

Table 5-7 Additional mutations identified in *L. reuteri* Δ*cemR* and Δ*cemRC*

Gene ID[a]	Annotation	Protein length (aa)	Homolog in genome
2500070670	Heavy metal-(Cd/Co/Hg/Pb/Zn)-translocating P-type ATPase	602	4
2500070671	Phosphopentomutase	396	2
2500070672	Hypothetical protein; predicted nucleoside-diphosphate-sugar epimerases (partial coverage)	38	0
2500070673	Transcriptional regulator, BadM/Rrf2 family	151	0
2500070674	Glycine betaine/choline-binding (lipo)protein of an ABC-type transport system (osmoprotectant binding protein)	299	0
2500070675	ABC-type proline/glycine betaine transport systems, permease component	216	1
2500070676	ABC-type proline/glycine betaine transport systems, ATPase components	256	35
2500070677	ABC-type proline/glycine betaine transport systems, permease component	212	1
2500070678	Methionine-R-sulfoxide reductase	142	0
2500070679	Chloride channel protein EriC	450	2
2500070680	Hypothetical protein	36	0

[a] This gene ID system is the same as that used in the DOE Joint Genome Institute databases

L. reuteri Δ*cemR* were similar to those caused by the deletion of *lamR* in *L. plantarum* and by the disruption of *lytR* in *S. aureus*. In *S. aureus*, LytR regulates biofilm formation, murein hydrolase activity, and autolysis (35). Likewise, disruption of the genes encoding transcriptional regulators in the LytR-CpsA-Psr families of *S. pneumoniae*, *S. mutans*, and *S. aureus* causes similar phenotype expressions to those of *L. reuteri* Δ*cemR*. In contrast to the LytR in *S. aureus*, the LytR in *S. pneumoniae* is not part of a two-component system, but is rather a transcriptional regulator in the LytR/CpsA/Psr family (37). LytR in *S. pneumoniae* has an important role in growth and cell division, and prevents premature autolysis. BrpA in *S. mutans* contributes to cell morphology and

autolysis and to the formation of glucose-based three-dimensional biofilm (38). MsrR in *S. aureus* regulates cell surface characteristics and virulence (39). The bioinformatic prediction of CemR suggested that CemR may be involved in the regulation of biofilm formation and cell envelope biogenesis. However, to test this hypothesis, it is necessary to mutate *cemR* in *L. reuteri* 100-23, and then verify it through a complementation of *cemR*.

The composition of the cytoplasmic membrane was changed in *L. reuteri* Δ*cemR*. Bacteria respond to different temperatures by adapting homeoviscously to modulate membrane fluidity through the cyclopropanation of double bonds to decrease proton permeability (40, 41). The total fatty acid profile in the membrane of *L. reuteri* Δ*cemR* contains less lauric acid, and a higher proportion of long-chain and cyclopropane fatty acids. However, the ratio of saturated to unsaturated fatty acids remained unchanged, as did the level of membrane fluidity. Changes in the chemical composition of the cytoplasmic membrane were a result of an increased sensitivity to upward shifts in temperature, while a lower inhibitory concentration of phenylethanol was the result of membrane property changes.

The phenotypes of *L. reuteri* Δ*cemR* that were observed were related to the structure of the cell envelope. In *L. reuteri* Δ*cemR*, the colony and cell morphology were altered when cultured in 2% glucose; the cell wall thickness was reduced when cultured in 4% glucose; autolysis of cells was increased; biofilm formation was enhanced in the presence of glucose and sucrose; the composition of cytoplasmic membrane was altered; and the sensitivity of exponentially growing cells to temperature shifts was increased. However, the

results of studies of autolysis, adherence, and the pH change in fermentations using the complemented strain *L. reuteri* Δ*cemRC* were not different from those obtained from the studies of *L. reuteri* Δ*cemR* (Fig. 5-11, 5-12, and 5-13). This suggested that the phenotypes shown in *L. reuteri* Δ*cemR* were not caused by a *cemR* mutation. Instead, they may result from the additional mutations that were identified in *L. reuteri* Δ*cemR*, because *L. reuteri* strains Δ*hk430*Δ*cemR* and Δ*rr431*Δ*cemR* were the derivatives of *L. reuteri* Δ*cemR*. Unsurprisingly, these two mutants also behaved similarly to *L. reuteri* Δ*cemR* in the studies of autoaggregation, autolysis, and biofilm formation (Table 5-4; Fig. 5-3, 5-13). These mutants also showed higher sensitivity to membrane-active phenylethanol, and displayed slower adaptation to osmotic stress and heat treatment (Table 5-4).

The additional mutations identified in *L. reuteri* Δ*cemR* include 11 genes (Table 5-7). Heavy metal-(Cd/Co/Hg/Pb/Zn)-translocating P-type ATPase is a membrane-bound heavy metal transporter responsible for metal homeostasis in bacteria, archaea, and eukaryotes (42). The P-type ATPases transport various cations across membranes with different affinities for essential metal and toxic metals (43). Phosphopentomutase catalyzes ribose 5-phosphate and ribose 1-phosphate, and participates in the pentose phosphate pathway and in purine metabolism (44). Transcriptional regulators in the BadM/Rrf2 family are usually repressors of genes related to the metabolism of nitrite, nitric oxide as well as iron (45, 46). ABC-type proline/glycine betaine/choline transport systems are membrane-bound osmosensors that sense and react to environmental osmotic stress. When extracellular osmotic pressure is high, the transporters import

organic osmolytes (glycine betaine) coupled with sodium ions, thus rehydrating cells to maintain membrane turgor (47, 48). Methionine-sulfoxide reductases are antioxidant repair enzymes that reduce methionine sulfoxide to methionine (49). Methionine-R-sulfoxide reductase is coded by *msrB* in *L. reuteri* 100-23 (50). Disruption of *msrB* has no effect on the ecological performance of *L. reuteri* in the colonization of the rodent forestomach or the presence in the jejunum and cecum. The chloride channel protein EriC belongs to the CIC superfamily of chloride ion channels, and there are two paralogous *eriC* genes in *L. reuteri* 100-23. In *Escherichia coli*, the EriC transporter promotes proton extrusion to cope with acid stress through the exchange of chloride ions (51).

One or more of the 11 genes disrupted in *L. reuteri* Δ*cemR*, *L. reuteri* Δ*cemRC*, and Δ*cemR*-derived mutants may contribute to the phenotypes observed in this study. Of these 11 genes, the ABC-type proline/glycine betaine/choline transport systems and the chloride channel protein EriC are likely associated with cell wall architecture and tolerance to osmotic stress. However, their exact roles remain unclear. Further investigation on the regulation of biofilm formation in *L. reuteri* Δ*cemR* is needed to understand this phenomenon.

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Chapter Six: **General Discussion and Conclusions**

This study aimed to understand the ecology of *Lactobacillus reuteri* LTH2584 and 100-23 in sourdough and the murine gut by investigating the metabolic traits, the physiological performance, and the regulatory signalling that contribute to *L. reuteri*'s persistence in these ecological niches. Comparative genomic studies of *L. reuteri* indicated that members of the species have evolved with their human, swine, poultry, and rodent hosts (1, 2). *L. reuteri* have adapted to the proximal gastrointestinal tract of their respective hosts to become members of the commensal microbiota, and contribute to the hosts' digestive processes. Genome comparison reveals a close evolutionary relationship between *L. reuteri* sourdough and rodent isolates, as was shown in this study. Both sourdough and rodent strains produce exopolysaccharides, form biofilms, colonize the forestomach of mice, and lack the *pdu-cbi-cob-hem* gene cluster responsible for reuterin and cobalamin synthesis. The ability of *L. reuteri* sourdough and rodent isolates to colonize the murine forestomach indicates that sourdough strain LTH2584 and rodent strain 100-23 may have evolved in similar environments, which is a theory that is supported by the results of the CGH analysis in this study. The furthur study of the sequence based phylogenetics of *L. reuteri* sourdough strains is needed to confirm the evolutionary traits of these strains. However, the *L. reuteri* sourdough strains LTH5331, LTH5448, and TMW1.106, which are all known to colonize the forestomach of the RLF mice, have all been found in German rye sourdoughs (3, 4). The hypothesis that *L. reuteri* sourdough strains were originally the result of contaminations in flour by rodents needs

further investigation, and these *L. reuteri* isolates from German rye sourdoughs need to be compared with isolates from different doughs and different geological areas, such as maize and sorghum fermentations in tropic climates (Sekwati-Monang, B., thesis in preparation).

The adaptation of *L. reuteri* that allows it to colonize in conditions of acid stress in the murine forestomach is also a focus of this study. The ecological competitiveness of *L. reuteri* has been observed in back-slopping sourdough fermentations and colonization of reconstituted-lactobacillus-free mice (5, 6). To investigate the contribution of genes to ecological performance, a gene-knockout mutant of *L. reuteri* was necessary. Current available methods of mutagenesis used in *L. reuteri* interrupt expression of a targeted gene by integrating a shuttle plasmid through homologous recombination (6, 7). However, the stability of integrated plasmids on target genes in the chromosome of *L. reuteri* has been a challenge for ecological studies (3, 6). Although the inactivation of *recA* on the replication origin of a plasmid inhibits its replication, transposition of the shuttle plasmids is uncontrollable. In addition to plasmid stability, supplementation of antibiotics is required to ensure the maintenance of antibiotic-resistant mutants, which restricts the application of insertional mutants in competitiveness studies. Moreover, other plasmid-derived genes may have undesirable influences on phenotypic analyses. In this study, a double crossover method was developed to generate deletion mutants of *L. reuteri*, which do not carry antibiotic-resistance genes or foreign genes from a shuttle plasmid. This approach was also applied to generate knockout mutants with multiple deletions, and to complement the

disrupted target gene by *in cis* gene replacement. The double crossover mutation system is a novel approach to ecological studies of *L. reuteri* because it omits the requirement of antibiotics and eliminates the problem of reversible mutagenesis that plagues insertional mutants of *L. reuteri* (6).

The contribution of exopolysaccharides and the arginine deiminase system to acid adaptation of *L. reuteri* has been investigated (8, 9). However, prior to this study an understanding of the glutamate decarboxylation system that allows the survival of *L. reuteri* under conditions of acid stress had not yet been established. Therefore, an isogenic deletion mutant *L. reuteri* Δ*gadB* was generated in this study. *L. reuteri* Δ*gadB* was unable to survive acidic environments (*in vitro*) and lost its competitiveness in back-slopping sourdough fermentation (*in vivo*). Analysis of amino acid utilization of *L. reuteri* 100-23 and Δ*gadB* in sourdough further indicated that there was a coupled metabolism of glutamine and glutamate contributing to acid resistance. This metabolism was confirmed by determining the role of glutamine in acid resistance of *L. reuteri* Δ*gadB* (Zhang, C., thesis in preparation). These studies together provide novel insights *L. reuteri*'s acid resistance mechanism, which involves glutamine coupled glutamate decarboxylation and allows competitiveness in acidic niches.

Biofilm formation in the murine intestine by *L. reuteri* has been attributed to production of exopolysaccharides and adhesins (4, 10). The contribution of two-component systems to biofilm formation was assessed in this study. Biofilm formation was influenced by the two-component systems *hk430* and *cemAKR*

operons, which are regulated by cross-communication. In addition, the availability of sources of carbon was also found to have an effect on biofilm formation. However, because the *rr431* or *cemR* regulons were not identified, and the unexpected mutations in *L. reuteri* Δ*cemR* may also be associated with biofilm regulation and cell envelope architecture, further studies are necessary to unravel the regulation of biofilm formation of *L. reuteri* in the murine intestine.

Probiotic strains are selected from viable cells that are isolated from humans and shown to have beneficial effects on human health. The criterion that insists that "probiotic" strains must originally be human isolates limits the number of food-associated lactobacilli that can be used as probiotics. There are numerous strains that could benefit host health even if they are isolated from sources other than the potential host. One obvious example is the sourdough isolates of *L. reuteri*, which have been shown to colonize the murine forestomach (3, 4, 12). My research has shown genetic and phenotypic similarities between the *L. reuteri* rat isolate 100-23 and sourdough isolate LTH 2584. The rat isolate colonizes in the murine forestomach, and lowers the pH levels of digesta, thus helping to preserve food. Because the sourdough isolate LTH 2584 shares numerous genetic and phenotypic similarities with the rat isolate, it is probable that it would have the same benefits to rodent health if it was introduced into rodent hosts. Similarly, there are likely bacteria strains in non-human enviroments that share genetic and phenotypic properties with currently used probiotic strains isolated from humans. Just as the non-rodent *L. reuturi* sourdough isolate LTH 2584 has the potential to benefit rodent hosts, there are non-human lactobacilli isolates that have the

potential to benefit human hosts. If the definition of probiotics was broadened, the number of food-associated lactobacilli used as probiotics could be increased for the benefit of the food industry and human health in general.

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Appendix A

A.1 Correlator program

%%

This code searches for selfsimilar patterns within a given DNA sequence. The search is based on the calculation of the partial autocorrelation (PA) sequence of the DNA sequence. The maximum value of the PA sequence as well as the index of the maximum value are then shown in the output. This procedure is repeated for different PA parameters, namely, the pattern length and the gap length.

%%

```
%%%%%%%%%%%%%% Loading the DNA sequence %%%%%%%%%%%%

fprintf('\nLoading the DNA sequence...\n');

fprintf('-----------------------------\n');

fid=fopen('DNA2500069269.txt');

dna=fread(fid);

fclose(fid);



%%%%%%%%%%% Mapping the DNA sequence to numbers %%%%%%%%%

dna(dna==65)=-3;

dna(dna==67)=-1;

dna(dna==71)=1;

dna(dna==84)=3;

dna((dna~=-3) & (dna~=-1) & (dna~=1) & (dna~=3))=[];

dnal=length(dna);
```

```
fprintf('\n\nDNA length = %d\n',dnal);



for pl=8:13     % the pattern length
   for gap=8:15    % the gap length

   corl=dnal-2*pl-gap+1;
   cor=zeros(1,corl);

   for i=1:corl
      s1=dna(i:i+pl-1);
      s2=dna(i+pl+gap:i+2*pl+gap-1);
      cor(i)=sum(s1.*s2)/sqrt(sum(s1.^2)*sum(s2.^2));
   end
   [maxcor,ind]=max(cor);
   fprintf('\n\nPattern length = %d\nGap length = %d\nPattern start index =
%d\nAutocorrelation coefficient (self similarity) = %g\n\n',pl,gap,ind,maxcor);
   end
end
fprintf('\nTask completed successfully.\n');
```

A.2 Crosscorrelator program

%%%

This code searches for a given pattern within a DNA sequence. The search is based on a similarity measure derived by crosscorrelating the pattern and the DNA sequence. The locations, in the DNA sequence, where the pattern is found (with a given minimum similarity level) are identified and the data before or after each of such locations are collected and saved in the output files.

%%%

%%%%%%%%%%%%%%%%%% Parameters %%%%%%%%%%%%%%%%%%

confidence=.9;   % minimum similarity (normalized crosscorrelation) in search for the pattern. It is a number between 0 and 1.

data_length=800;   % number of bases to be saved before or after each found pattern

data_direction=1;   % data_direction=1: the data after each found pattern is saved. data_direction=0: the data ahead of each found pattern is saved.

max_pattern_found=50;   % maximum number of output files allowed to be generated

DNA_file_name='genome2301424.txt';

%%%%%%%%%%%%%%%%% Pattern to search for %%%%%%%%%%%%%%%%

%pattern=[double('TGTTTACT'),zeros(1,10),double('TGGTTACT'),zeros(1,18),double('TTGAAT'),zeros(1,25),double('ATCTACT')].';   % pattern A1

pattern=
[double('AGTAGA'),zeros(1,25),double('ATTCAA'),zeros(1,18),double('AGTAACCA'),zeros(1,10),double('AGTAAACA')].';   % pattern A2

```
%pattern=[double('TGTATTTCAT'),zeros(1,11),double('TGTAACTGAT')].';  % pattern B1



%%%%%%%%% Mapping the pattern sequence to numbers %%%%%%%%%

pattern(pattern==65)=-3;

pattern(pattern==67)=-1;

pattern(pattern==71)=1;

pattern(pattern==84)=3;

pl=length(pattern);     % length of the pattern

p_var=sum(pattern.^2);  % power of the pattern. To be used in the calculation of the crosscorrelation.



%%%%%%%%%%% Loading the DNA sequence %%%%%%%%%%%%

fprintf('\nLoading the DNA sequence...\n');

fid=fopen(DNA_file_name);

dna_string=fread(fid);

fclose(fid);



%%%%%%%%% Mapping the DNA sequence to numbers %%%%%%%%%

dna_string((dna_string~=65) & (dna_string~=67) & (dna_string~=71) & (dna_string~=84))=[];

dna=zeros(size(dna_string));

dna(dna_string==65)=-3;
```

```
dna(dna_string==67)=-1;
dna(dna_string==71)=1;
dna(dna_string==84)=3;
dnal=length(dna);   % DNA length



%%%%%%%%%% Crosscorrelation calculation %%%%%%%%%%%%%%
if (exist ('old_dna','var')==1) && (exist ('old_pattern','var')==1)
   if (dnal==length(old_dna)) && (pl==length(old_pattern))
      if [(dna==old_dna);(pattern==old_pattern)]
         skip=1;
      else
         skip=0;
      end
   else
      skip=0;
   end
else
   skip=0;
end
if skip==0
   fprintf('\nCalculating the crosscorrelation...\n');
   crosscor=zeros(1,dnal-pl+1);
   for i=1:dnal-pl+1
      dna_section=dna(i:i+pl-1);
```

```
crosscor(i)=sum(dna_section.*pattern)/sqrt(sum(dna_section(pattern~=0).^2)*p_v
ar);
    end
    old_dna=dna;
    old_pattern=pattern;
end



%%%%%% Searching for the pattern (crosscorrelation >= confidence) %%%%%%
fprintf('\nSearching for the pattern...\n');
[max_crosscor,max_DNA_index]=max(crosscor);
if max_crosscor<confidence
    fprintf('\nThe confidence level is larger than the maximum crosscorrlation.\n');
    fprintf('It is automatically lowered to the maximum crosscorrlation value.\n');
    pattern_found=max_DNA_index;
else
    pattern_found=find(crosscor>=confidence);
end
pfl=length(pattern_found);  % number of locations where the pattern was found



%%%%%% Generating the output files (one file per pattern found) and finding
the best match %%%%%%

if pfl<=max_pattern_found
```

```
    folder_name='Output_Files';
    [status,message,messageid]=rmdir(folder_name,'s');
    [status,message,messageid]=mkdir(folder_name);
    fprintf('\nGenerating the output files...\n');
    for i=1:pfl
        if data_direction == 1
            data=dna_string(pattern_found(i):min(pattern_found(i)+data_length-
1,dnal));     % save the data after the pattern
        else
            data=dna_string(max(pattern_found(i)+pl-
data_length,1):pattern_found(i)+pl-1);   % save the data ahead of the pattern
        end
        data_length=length(data);
        formatted_data=[];
        for j=1:50:data_length
            formatted_data=[formatted_data;data(j:min(j+49,data_length));10];
        end
        file_name=strcat(folder_name,'/output',int2str(i),'.txt');
        fid=fopen(file_name,'w');
        fprintf(fid,'%c',['Crosscorrelation (similarity) =
',num2str(crosscor(pattern_found(i))),10,10]);
        fprintf(fid,'%c',formatted_data);
        fclose(fid);
    end
    [max_crosscor,max_file_index]=max(crosscor(pattern_found));
    fprintf('\n%d file(s) generated.\n\n',pfl);
```

```
    fprintf('\nMaximum crosscorrelation = %g\nIndex of maximum
crosscorrelation in the DNA sequence = %d',max_crosscor,max_DNA_index);

    fprintf('\nOutput file corresponding to the maximum match =
output%d.txt\n',max_file_index)

    fprintf('\nTask completed successfully.\n');

else

    fprintf('\nToo many patterns found. Output files are NOT generated\n');

end
```

Appendix B

B.1 DNA sequence of the *aip/tcsKR* operon in *L. reuteri* LTH2584

> DNA sequence of the *aip/tcsKR* operon in *L. reuteri* LTH2584

CTAATACGTTAAAATTCCACTAAAATTAGAAGAAAATTATGAAAAAA
GATAAATTATGCTATAGTAAAAACCAAACTTTTTAAGAGGAGTAAATT
AATGAATAGTAAACTTTTTCAATGGTTCTATAATATGCCCAAATTAAC
AAATCAAGATAATAAAGATTATATGGCGAAGATTAGTCTTAATATCGC
TGTTAGCTTATTTCTTTTTAACCTCGTTGCTGTTCTTATTTCCTTTTTCAT
TGTTACTACAACAGAGAATTATGAATTGTCTTTTAATATTTTAGCTGGG
GCGTTACTGACCTTTACTGTTTATATTGTTGGTGGCTATTCTATATATG
CAAAACGTAAAATTAATAGAGTCGTGACTCACGATAACACACATATTG
TAAAGAATAGTTTTGCCTCAGCTGTATATATAACACTGATTATTTACTT
AATTCTCTTACAAATGCTCTTAGTTTTCATACTTCACTCATTAGCGAAT
TAAAGAATCCCATGAGTTATATAATTCCAACTGTAATTGGGATTATAT
ATGGGGTGCTTATGGCTCTTATTACATGGTATAGGAGTAAATAATAAG
TATGTTTTTTATTAAGAAAGATTAACTGAAAGTAACTTCCTTATTGAGA
AGTTAAACAAAAATTTAACATTTATTATTTTTTGCTAACAGAATAATCG
GACTCATTTCTTACAGGATTTGAACCTTAAACAACTGCTTTTGATTATT
TATGATTTTTTTGATGCATCCTGTTAAAATAAATTACTGATAAAACAAT
AGGAGACATGATATATTTATGCAAAAACTATCAATTCATCAACTATCT
TTAATTAAGGGTGGCATATACTCACTTTTAAGTCTGTAAATAATAATA
CATATACTTACACTAATAAAATTATAAATAACTTGCAAAATATCTTCC
GTTAATTTTAATATGAATTTTTTTATTCAAATTATTTCAGTAATTCAAG
GACTTACTTTTGAACTTTTCACATTCTTTTATCTCGTAAAAATTAAGAT
TACTAAACGAGATATATTTTTCTTAACTATTATTTTTACTATCGTATTCC
TTATGATTGGCTTAGGTTATACAGGAGCATATCTCTCACCAATATTAG
AGGGCGGGTTCCTATATTTGTATTTTAAAAACAGAACCTCTAATCATA
AATTAATTGGTGCTATCCTATTAACCTTAACGACATCAATTGTAATTGA
TTTCTCAACCTTTACAATTGATAAAGTACTTTCACTACCAATCAATTAT
ATTCTATTTATTGATCCATTCTTTTACCTAATCACTTTTATTATTCTCTA
CTATTATCATCCTCTTGATAAAATAGAAGAAGATAATACAACTATTAA
TTTATGGATCTTATCCTATCTTTTTATTACCTTAAATATCGCAGGATTTA
CCATTTTTACAACTTCACGGGTAACCCCAGCTTTTATTCTTATCTTTTGC
TTACTGGTTCTTCAATGCAGTTTTACAGTCTTTGTATACCGAGTTAGTA
AACAAATTCAACAAAAGAAACTCCAACTTCAAGAAAAGAAGAACTTA
TTACTTCTTATTCAAAACCTAGAGGAAAATCAATCCAAGCTAAAAAGG
TTTAAACACGATTACCAAAACTTATTAAATAGCCTCAAACTAAGTGCT
TTAAATGGCGATAATCAAACTTTACTTAATAAATTAGAAGCTTATTCTC
GTGATAATCTTGGTGAAGATGCTCTTTGGCAGTTTCAAGATACTAAAA
ACATTAATAATGAAGTTTTGAGAAGTATTTTTATTAATAAATTAAATTC
AATTTATCAAAAAGATATTAACTATCATTTTGAGTGTTTAACTGAAATT
ACAAGTCTTCCTAATATAGATCTATTTGATTTAATTCGAATAATTGGGA
TTGTTTACGATAACGCCCTCGAAGAATGCATAAACTTAAGACACTCAG

GAATAAATGCTGTCGAAATCAATTCAATGCTATATCAAGACGCTCCTA
ATAAACTAGAATTTGAAATTAAAAATACCTGTCGAAATCAACTTATCA
CTAATAAACTCCACCAAGAAGGAATTACAAATAAAGCTAATCATGAA
GGGCTAGGATTAGCAACTGTAAAAAAATAGCTAACAAATACCGTAAC
GTATACATTGCTTACTCCTCTGATAATGGTTACTTTACATTCACCATCT
CAATCGAATAGAAAGGAATAAATATGGACTACCATTTACTTGTCTGCG
ATGATGACAGGACTCAAGCAAAAAATATCGCTACCCTACTTAAAATGT
CATTAATTATTCTTGAAGAACAAGATATTCATCCGACCATTGATTTGAT
TGCAACGGACGCAGAGAGTGTATTAGATTACCTTGAGTTTAATAGTGA
TTTAAATAATATTATTGCCTTTTTAGATATTCAGTTAGATGAAAATTCC
AGTTCAAAAGGCGGTCTAGATTTAGCTCAAAAAATAAAAACACTTAAC
GAAAAGGCACAAATAATCTTTATTACAACGCATGAAGAACTAGCTTTT
CTCACCTTTCAACGTCGAATTAATCCGATGGACTATATAACTAAAACT
ACAAACCAACGAGATTTACAAAGACGGCTAACCGAAACCCTAGAACT
AGCTATAAGGAAACTGAATGAATTTAACTATATTAAAAAAAATACATT
CAGTTATAAAGTTGGTACAAGGATTATAAATGTAAACTTCAGTAATAT
CCTTTATATCTCTACTACAAAATTCCCTCATAAATTAAAGATTGTTACA
TCTAATGGTCAAGGGGAATTCTCAGGTGATATTAAAACAATTGAGCAA
AATTATTCTATGTTTTTCAAGGCTTCTCAGTCTAGCTTAATTAACTTAC
AAAATGTTGAAACAATTAATACCAAAAAACGGCTTATTAAGTTTTCCA
ACAATGATTATCTTAAGTATTCTAGAAGCAGAGCTAAAGAATTAAATA
ATCTATATAAATCAAGTCAATAAGACTGATGTAGATTAGCAACAGTTC
TTTATAATTACGGCTCTAAAATTTCATTAGTAACTCTGCGAAAATTCAC
AAAAACCACTACTGATGGAACTACTGCACTTGGGATTGTAAAAGCAGC
AGAAAAGTTAAAAATGGATGTTAAAGCATATCAGTTTATTGATTCAAA
AGATGTAATTTATCCTTTTATTGCTCATCTCATGAAAAAGACAACCTCT
CACTCCCTAGA

Appendix C

C.1 Sequencing data of *L. reuteri* Δ*hk430*

> Sequencing data of *L. reuteri* Δ*hk430*

ACGACTGGCCTTTATCGTTGACGATACGTTAATTTCTCGTCCATACTCA
ACTAAAACTGAACTCTTGGCAAAAGTATATGATCATAATCAGGACAAA
TATATAACTGGTTACCGTAATTTAACAATTGGCTGGAGCGATGGTAAC
ACTTTTTTGCCAGTTAATTTTGCTTTAATGTCAACTAAAAATAGAGCCA
ACTTAGTTGGCACCAAGGCTTGTGTAACTGATCAACGAACGATTGCTG
GTCAACGACGCAACCAAGCGCAAAGAAAAATGAATGATGTGGTAATT
GAATTGATTCATCAAGCTCTTAAGTTTGGTATATCAGCTAAATACGTTC
TTTTTGATAGCTGGTATAGTTCTCCACGAATGTTTTGGCGGCTTAAAGA
ACTAGGATTAGATAGTGTAGCTATGCTTAAGCGAAGCTCTAAAGTTTA
TTACCGATATCGTGGACGTGCCTATAGTATCAAAGCATTATATTTACG
ACTACTTCATTCAAAACGCCATCAAGCTGAGAACTATCTTTATAGCAG
TAACGTTGAAGCTAACTTTCAGGGATATAGTTTTCCGCTAAAAGTTGTT
TTCGTAGCTAAAAAAGGTACCAAGAATCAATATTTGGTTCTAGCCTCA
ACTAACACTAAGCTAACACCACAGAAAATTATTCAATTATATAATCGC
CGATGGTCAATTGAGACTTACTTCAAAACAGCTAAACAATACTTACGA
CTTAATAAATCGCAAATTCAAAGTTATGATGGTCAGGTTGCACAAATT
ACGATTACTGCGCTGACTTTTATCCTATTAGCTTGGCAAGAACGTCAA
AGTAAGGACGATCGTACTTTAGGTGATTTGTTTTACCTGATGAATGAT
GCCCTTCCAGAAATTAAATTTATTGAAGCATTGGTCTACCTATTAAAA
ACACTAGAATCACACGAAACTGTTTTTTTTAATCAAACAATTAGTCAA
TTTATGAATTACTTACCTGAAAATATTCAAAACGCTCTTCATGAAGCTG
TTTGAAACTAATTTTAGTAAAGAAAAGCCAGAGCTGATAGCGGCTCCG
GTCCTTTAGGTCGACCCTCCGATAATGGTTACTTTACATTTACAATTTC
AATTGAATAGAAAGGAATACTTATGGACTACCATCTACTCGTTTGCGA
TGATGACCAGCTACAAGCTAAAAATAT

C.2 Sequencing data of *L. reuteri* Δ*rr431*

> Sequencing data of *L. reuteri* Δ*rr431*

AATAGTCTCAAACTAAGTGCCATCCCATGGTGATAATCAAGCCTTACT
CGATAAATTAGAAAACTATTTCNTCGAGATAACCTTGGTAAAGATTCT
CTTTGGCAATTCCAAGACACTAAAAAGAGATTTAAACATGATTATCAA
AACTTACTAAACATTAAAGATGATGTCTTGCGAAGTATCTTTATTAGT
AAATTAAATTCAATTTACCAAAAAGATATTAAATATCATTTTGAATGT
TTAACAGAAATTAATGATCTCCCAAATATCGATTTATTTGATTTAATTC
GAATAATTGGGATCGCTTATGATAATGCTCTTGAAGAATGTGAGGCTT
TAAAAAAGTTAGGAGCACGTACTGTAGAAATCAATTCAATGCTGTATC
AAAGTGCCCCTAACAAGTTAGAATTTGAAATTAAGAATACTTGTCGAA
AGCAGCTTAGCACCAATAAGCTTCATCAAGAAGGAATTACAAATAAG
ACTAACCATGAGGGACTTGGATTAGCAACGGCTCAAAAAATAGCTAA

CAAATACCACAACGTATATATTGTTTACTCCTCCGATAATGGTTACTTT
ACATTTACAATTTCAATTGAATAGAAAGGAATACTTATGGACTACCAT
CTACTCGTTTGCGATGATGACCAGCTACAAGCTAAAAATATTGCCACA
TTATTAAAAATGTCGTCAATTATTCTGTAAGTCGACCGGAGTCGAACA
AAAGAACTAAATAAAATCTATAAGGAGAAATTATAGATTAGTGGGGT
ATTTTTATGTATAACGCATTTAATAAATACTATGTAAGTCAAGTTGACG
AAGCAGATTGTGGAGTAGCCGCACTATCCATGATTCTTCGTAAATTTG
GCTCTAAAGTTTCACTTGCAACTTTAAGAAAAGCTACTAAAACAACTA
CTGAAGGTACAACTGCTTTAGGTATTGTCGAAGCCGCTAAAAAGTATA
AGCTTAACGTTGCTGCTTATCAAGCAGATATAAATCTTTTTAGCTCTAA
CGAAGCTTCTTACCCTTTTATTGCACATCTCATTAAAAAGGATACCGGC
TTATTACATTACTGTGTAGTCGTTAAAAACGCAAAAAATCATCTTATTA
TTGCAGATCCAAATCCAAACATAAAAATTCAAAAGGTATCTAAAGAA
GCTTTCTTTCAAGAATGGACCGGAATTGCAATTTTCGCTAATCCTGCTA
ATGATTATGAAATTATTCAAGAACAATCACCTACACTAAAGAGGTTAA
TCATTCCTGTCATAGAGCAAAAGAAGTTATTAATAGGCATTGTTACAT
CAGCATTTTTAGTAACATTTATCAATATAATAAGCTCATACTTTTTCCA
AGGCTTAATTGATAGATTGATCCCTTATAAATTACTAACACCTTTAAAC
ATAATTTGTATTGGATTACTAATAGCATATGTAGCAAATTCATTATTTA
ACTTTACAAGAGATTATCTATTAGCAACTCTAGGTCAAAGACTATCGA
AACTATTCTTCTAGATTACATTAAGCATGTTCTTAAACTCCCCATGGA

C.3 Sequencing data of *L. reuteri* Δ*hk430*Δ*rr431*

> Sequencing data of *L. reuteri* Δ*hk430*Δ*rr431*

CCTTTATCGTTGACGATACGTTAATTTCTCGTCCATACTCAACTAAAAC
TGAACTCTTGGCAAAAGTATATGATCATAATCAGGACAAATATATAAC
TGGTTACCGTAATTTAACAATTGGCTGGAGCGATGGTAACACTTTTTTG
CCAGTTAATTTTGCTTTAATGTCAACTAAAAATAGAGCCAACTTAGTT
GGCACCAAGGCTTGTGTAACTGATCAACGAACGATTGCTGGTCAACGA
CGCAACCAAGCGCAAAGAAAAATGAATGATGTGGTAATTGAATTGAT
TCATCAAGCTCTTAAGTTTGGTATATCAGCTAAATACGTTCTTTTTGAT
AGCTGGTATAGTTCTCCACGAATGTTTTGGCGGCTTAAAGAACTAGGA
TTAGATAGTGTAGCTATGCTTAAGCGAAGCTCTAAAGTTTATTACCGA
TATCGTGGACGTGCCTATAGTATCAAAGCATTATATTTACGACTACTTC
ATTCAAAACGCCATCAAGCTGAGAACTATCTTTATAGCAGTAACGTTG
AAGCTACTTTCAGGGATATAGTTTTCCGCTAAAAGTTGTTTTCGTAAGC
TAAAAAAGGTACCAAGAATCAATATTTGGTTCTAGCCTCAACTAACAC
TAAGCTAACACCACAGAAAATTATTCAATTATATAATCGCCGATGGTC
AATTGAGACTTACTTCAAAACACTGCTAAACAATACTTACGACTTAAT
AAATCGCAAATTCAAAGTTATGATGGTCAGGTTGCACAAATTACGATT
ACTGCGCTGACTTTTATCCTATTAGCTTGGCAAGAACGTCAAAGTAAG
GACGATCGTACTTTANGTGATTTGTTTTACCTGATGAATGATGCCCTTC
CAGAAATTAAATTTATTGAAGCATTGGTCTACCTATTAAAAACACTAG
AATCACACGAAACTGTTTTTCTTAATCAAACAATTAGTCAATTTATGA
ATTACTTACCTGAAAATATTCAAACGCTCTTCATGAAGCTGTTTGAAA

CTAATTTTAGTAAAGAAAAGCCAGAGCTGATAGCGGCTCCGGTCCTTT
AGGTCGACCGGAGTCGAACAAAAGAACTAAATAAAATCTATAAGGAG
AAATTATAGATTAGTGGGGTATTTTTATGTATAACGCATTTAATAAAT
ACTATGTAAGTCAAGTTGACGAAGCAGATTGTGGAGTAGCCGCACTAT
CCATGATTCTTCGTAAATTTGGCTCTAAAGTTTCACTTGCAACTTTAAG
AAAAGCTACTAAAACAACTCCTTTATCGTTGACGATACGTTAATTTCTC
GTCCATACTCAACTAAAACTGAACTCTTGGCAAAAGTATATGATCATA
ATCAGGACAAATATATAACTGGTTACCGTAATTTAACAATTGGCTGGA
GCGATGGTAACACTTTTTTGCCAGTTAATTTTGCTTTAATGTCAACTAA
AAATAGAGCCAACTTAGTTGGCACCAAGGCTTGTGTAACTGATCAACG
AACGATTGCTGGTCAACGACGCAACCAAGCGCAAAGAAAAATGAATG
ATGTGGTAATTGAATTGATTCATCAAGCTCTTAAGTTTGGTATATCAGC
TAAATACGTTCTTTTTGATAGCTGGTATAGTTCTCCACGAATGTTTTGG
CGGCTTAAAGAACTAGGATTAGATAGTGTAGCTATGCTTAAGCGAAGC
TCTAAAGTTTATTACCGATATCGTGGACGTGCCTATAGTATCAAAGCA
TTATATTTACGACTACTTCATTCAAAACGCCATCAAGCTGAGAACTAT
CTTTATAGCAGTAACGTTGAAGCTACTTTCAGGGATATAGTTTTCCGCT
AAAAGTTGTTTTCGTAAGCTAAAAAAGGTACCAAGAATCAATATTTGG
TTCTAGCCTCAACTAACACTAAGCTAACACCACAGAAAATTATTCAAT
TATATAATCGCCGATGGTCAATTGAGACTTACTTCAAAACACTGCTAA
ACAATACTTACGACTTAATAAATCGCAAATTCAAAGTTATGATGGTCA
GGTTGCACAAATTACGATTACTGCGCTGACTTTTATCCTATTAGCTTGG
CAAGAACGTCAAAGTAAGGACGATCGTACTTTANGTGATTTGTTTTAC
CTGATGAATGATGCCCTTCCAGAAATTAAATTTATTGAAGCATTGGTC
TACCTATTAAAAACACTAGAATCACACGAAACTGTTTTTCTTAATCAA
ACAATTAGTCAATTTATGAATTACTTACCTGAAAATATTCAAACGCTCT
TCATGAAGCTGTTTGAAACTAATTTTAGTAAAGAAAAGCCAGAGCTGA
TAGCGGCTCCGGTCCTTTAGGTCGACCGGAGTCGAACAAAAGAACTAA
ATAAAATCTATAAGGAGAAATTATAGATTAGTGGGGTATTTTTATGTA
TAACGCATTTAATAAATACTATGTAAGTCAAGTTGACGAAGCAGATTG
TGGAGTAGCCGCACTATCCATGATTCTTCGTAAATTTGGCTCTAAAGTT
TCACTTGCAACTTTAAGAAAAGCTACTAAAACAACT

C.4 Sequencing data of *L. reuteri* Δ*cemK*

> Sequencing data of *L. reuteri* Δ*cemK*

GCATCAGTATGNTTTCCGNAACAGCTACCGATGAAAGGNANCCGCCAT
CCCANGGNTATGTGGAACATCGTGTAAAGCTGGCCAATTAGGACGCA
GTATCTATCAACGATATCGTGATTTCCCCATTACCAACATGAATTTGGG
CACTTTGAAGCTGATACAGTTCAAGGTAAAGCTCACCGCGGAGCGGTA
ATGACGCTAGTAGAGCGACAATCCAAAGTAATGATTGTCCTTAATATT
CATCATAAAACAGACGAAGCAGTGAATTTCCAGCTTGATCAATGGCTC
GCTAAACTGCCACGTCACTTTGTTAAATCAATTACTTTTGATAACGGG
AAAGAATTTGCTGGATGGCGAGAAATAGCCAATAAGTATGATCTTCAC
ACCTATTTTGCGGAAGTCGGTGCTCCCAATCAACGAGGGTTAAACGAA
AATAATAACGGCCTCTTGCGTCGTGATGGTCTTAGTAAAAAGCTAGAT

TTTCGCTATTTACCAGATGAACTAGTCACTCAGCTAATGCATCGTCGCA
ACAATATCCCACGAAAATCTCTTAATTATCGTACACCATTAGAAGTAT
TCTTGAGTCATGTCACAGAAGAACAACTTTTACCTTTTTTTTAATTTAA
ATTGACATTTCAGGTTAAGAAAGATTAACTGAAAGTAACTTCCTTATT
GAGAAGTTAAACAAAAATTTAACATTTATCATTTTTTGCTAACAGAAT
AATCGGACTCATTTCTTACAGGATTTGAACCTTAAACAACTGCTTTTGA
TTATTTATGATTTTTTTGATGCATCCTGTTAAAATAAATTACTGATAAA
ACAATAGGAGACATGATATATTTATGCAAAAACTGTCAATTCATCAAC
TATCTTTAATTAAGGGTGGTATATACTCACTTTTAAGTCTGTAAATAAT
AATACATATACCTACACTAATAAAATTATAAATAACTTGCAAAATATC
TTCCGTTAATTTTAATATGAATTTTTTTATTCAAATTATTTCAGTAATTC
AATGAGTCGACGAATAGAAAGGAATACTTACGGACTACCATTTATTNT
GTGACAGGACTCAAGCAAAAAATATCGCTACACTACTTAAAATGTGG
GTCGACCCGTA

C.5 Sequencing data of *L. reuteri* Δ*cemK*Δ*cemR*

> Sequencing data of *L. reuteri* Δ*cemK*Δ*cemR*

GGCACTTTGAAGCTGATACAGTTCAAGGTAAAGCTCACCGCGGAGCG
GTAATGACGCTAGTAGAGCGACAATCCAAAGTAATGATTGTCTTANTA
TTCATCATAAAACAGACGAAGCAGTGAATTTCCAGCTTGATCAATGGC
TCGCTAAACTGCCACGTCACTTTGTTAAATCAATTACTTTTGATAACGG
GAAAGAATTTGCTGGATGGCGAGAAATAGCCAATAAGTATGATCTTCA
CACNTATTTTGCGGAAGTCGGTGCTCCCAATCAACGAGGGTTAAACGA
AAATAATAACGGCCTCTTGCGTCGTGATGGTCTTAGTAAAAAGCTAGA
TTTTCGCTATTTACCAGATGAACTAGTCACTCAGCTAATGCATCGTCGC
AACAATATCCCACGAAAATCTCTTAATTATCGTACACCATTAGAAGTA
TTCTTGAGTCATGTCACAGAAGAACAACTTTTACCTTTTTTCTAATTTA
AATTGACATTTCAGGTTAAGAAAGATTAACTGAAAGTAACTTCCTTAT
TGAGAAGTTAAACAAAAATTTAACATTTATTATTTTTTGCTAACAGAA
TAATCGGACTCATTTCTTACAGGATTTGAACCTTAAACAACTGCTTTTG
ATTATTTATGATTTTTTTGATGCATCCTGTTAAAATAAATTACTGATAA
AACAATAGGAGACATGATATATTTATGCAAAAACTGTCAATTCATCAA
CTATCTTTAATTAAGGGTGGTATATACTCACTTTTAAGTCTGTAAATAA
TAATACATATACCTACACTAATAAAATTATAAATAACTTGCAAAATAT
CTTCCGTTAATTTTAATATGAATTTTTTTATTCAAATTATTTCAGTAATT
CAATGAGTCGACTAAGACTGATGTAGATTAGCAACAGTTCTTTATAAT
TACGGCTCTAAAATTTCATTAGTAACTCTGCGAAAATTCACAAAAACC
ACTACCGATGGAACTACTGCACTTGGGATTGTAAAAGCAGCAAAAAA
GTTAAAAATGGATGTTGAAGCATATCAATTTATTTGATTCAAAAGATG
TAATTTATCTATTGCTCATCTTATGAAAAAAACAACCTCTCACTC

C.6 Sequencing data of *L. reuteri* Δ*hk430*Δ*cemK*

> Sequencing data of *L. reuteri* Δ*hk430*Δ*cemK*, using primers for *cemK* gene

ACCGATGAAAGGAAACCGCCATCCCAATGGCTATGTGGAACATCGTG
GTAAAGCTGGCCAATTAGGACGCAGTATCTATCAACGATATCGTGATT
TTCCCCATTACCAACATGAATTTGGGCACTTTGAAGCTGATACAGTTC
AAGGTAAAGCTCACCGCGGAGCGGTAATGACGCTAGTAGAGCGACAA
TCCAAAGTAATGATTGTCCTTAATATTCATCATAAAACAGACGAAGCA
GTGAATTTCCAGCTTGATCAATGGCTCGCTAAACTGCCACGTCACTTTG
TTAAATCAATTACTTTTGATAACGGGAAAGAATTTGCTGGATGGCGAG
AAATAGCCAATAAGTATGATCTTCACACCTATTTTGCGGAAGTCGGTG
CTCCCAATCAACGAGGGTTAAACGAAAATAATAACGGCCTCTTGCGTC
GTGATGGTCTTAGTAAAAAGCTAGATTTTCGCTATTTACCAGATGAAC
TAGTCACTCAGCTAATGCATCGTCGCAACAATATCCCACGAAAATCTC
TTAATTATCGTACACCATTAGAAGTATTCTTGAGTCATGTCACAGAAN
AACANCTTTTACCTTTTTTCTAATTTAAATTGACATTTCNNGTTAAGAA
AGA-TAACTGAAAGTANCTTCCTTAT-GAGA--GTAAACAAAAA-
NTAACANTTATCA-
TTTTTGCTNACAGANNATCGGACTCNTTCNTNCNGGNGTGGANCNTNA
ACACTGNTTTGATTATTTATGATTTTTTTGATGCATCCTGTTAAAATAA
ATTACTGATAAAACAATAGGAGACATGATATATTTATGCAAAAACTGT
CAATTCATCAACTATCTTTAATTAAGGGTGGTATATACTCACTTTTAAG
TCTGTAAATAATAATACATATACCTACACTAATAAAATTATAAATAAC
TTGCAAAATATCTTCCGTTAATTTTAATATGAATTTTTTTATTCAAATT
ATTTCAGTAATTCAATGAGTCGACGAATAGAAAGGAATACTTACGGAC
TACCATTTACTTGTCTGCGATGA

C.7 Sequencing data of *L. reuteri* Δ*rr431*Δ*cemK*

> Sequencing data of *L. reuteri* Δ*rr431*Δ*cemK*, using primers for *cemK* gene

AAGGAAACCGCCATCCCAATGGCTATGTGGAACATCGTGGTAAAGCT
GGCCAATTAGGACGCAGTATCTATCAACGATATCGTGATTTTCCCCAT
TACCAACATGAATTTGGGCACTTTGAAGCTGATACAGTTCAAGGTAAA
GCTCACCGCGGAGCGGTAATGACGCTAGTAGAGCGACAATCCAAAGT
AATGATTGTCCTTAATATTCATCATAAAACAGACGAAGCAGTGAATTT
CCAGCTTGATCAATGGCTCGCTAAACTGCCACGTCACTTTGTTAAATC
AATTACTTTTGATAACGGGAAAGAATTTGCTGGATGGCGAGAAATANC
CAATAAGTATGATCTTCACACCTATTTTGCGGAAGTCGGTGCTCCCAA
TCAACGAGGGTTAAACGAAAATAATAACGGCCTCTTGCGTCGTGATGG
TCTTAGTAAAAAGCTAGATTTTCGCTATTTACCANATGAACTAGTCNCT
CAGCTAATGCATCGTCNCAACAATATCCCACGAAAATCTCTTAATTAT
CGTACACCNTTAGAANTATTCTTGAGTCATGTCACANAAAAACAACTT
TTACCTTTTTTCTATTTTAAATTGACATTTCAGGTTAAGAAAGATTAAC
TGAAAGTAACTTCCTTATTGAGAAGTTAAACAAAAATTTAACATTTAT
CATTTTTTGCTAACAGAATAATCGGACTCATTTCTTACAGGATTTGAAC
CTTAAACAACTGCTTTTGATTATTTATGATTTTTTTGATGCATCCTGTTA
AAATAAATTACTGATAAAACAATAG-
GAGACATGATATATTTATGCAAAAACTGTCAATTCATCAACTATCTTT
AATTAAGGGTGGTATATACTCACTTTTAAGTCTGTAAATAATAATACA

TATACCTACACTAATAAAATTATAAATAACTTGCAAAATATCTTCCGTT
AATTTTAATATGAATTTTTTTATTCAAATTATTTCAGTAATTCAATGAG
TCGACGAATAGAAAGGAATACTTACGGACTACCATTTACTNGTCTGCG
ATGANGACAGGACTC

Appendix D

D.1 Sequencing data of *L. reuteri* Δ*hk430*Δ*cemR*

> Sequencing data of *L. reuteri* Δ*hk430*Δ*cemR*, using primers for *hk430* gene

ACTGGCCTTTATCGTTGACGATACGTTAATTTCTCGTCCATACTCAACT
AAAACTGAACTCTTGGCAAAAGTATATGATCATAATCAGGACAAATAT
ATAACTGGTTACCGTAATTTAACAATTGGCTGGAGCGATGGTAACACT
TTTTTGCCAGTTAATTTTGCTTTAATGTCAACTAAAAATAGAGCCAACT
TAGTTGGCACCAAGGCTTGTGTAACTGATCAACGAACGATTGCTGGTC
AACGACGCAACCAAGCGCAAAGAAAAATGAATGATGTGGTAATTGAA
TTGATTCATCAAGCTCTTAAGTTTGGTATATCAGCTAAATACGTTCTTT
TTGATAGCTGGTATAGTTCTCCACGAATGTTTTGGCGGCTTAAAGAAC
TAGGATTAGATAGTGTAGCTATGCTTAAGCGAAGCTCTAAAGTTTATT
ACCGATATCGTGGACGTGCCTATAGTATCAAAGCATTATATTTACGAC
TACTTCATTCAAAACGCCATCAAGCTGAGAACTATCTTTATAGCAGTA
ACGTTGAAGCTAACTTTCAGGGATATAGTTTTCCGCTAAAAGTTGTTTT
CGTAGCTAAAAAAGGTACCAAGAATCAATATTTGGTTCTAGCCTCAAC
TAACACTAAGCTAACACCACAGAAAATTATTCAATTATATAATCGCCG
ATGGTCAATTGAGACTTACTTCAAAACAGCTAAACAATACTTACGACT
TAATAAATCGCAAATTCAAAGTTATGATGGTCAGGTTGCACAAATTAC
GATTACTGCGCTGACTTTTATCCTATTAGCTTGGCAAGAACGTCAAAG
TAAGGACGATCGTACTTTAGGTGATTTGTTTTACCTGATGAATGATGCC
CTTCCAGAAATTAAATTTATTGAAGCATTGGNCNACCTATTAAAAACA
CTAGAATCACACGAAACTGNTTTTCTTATCAAACAATTAGTCAATTTAT
GAATTACTTACCTGAAATATTCAAAACGCTCTTCATGAAGCTGTTTGA
AACTAATTTAGTAAG

D.2 Sequencing data of *L. reuteri* Δ*hk430*Δ*cemR*

> Sequencing data of *L. reuteri* Δ*hk430*Δ*cemR*, using primers for *cemR* gene

CACACCTAGATGAAAATCAATCGAAGTTANGNCGNTTTAAACANGATT
ATCAAAACTTACTAAATAGCNTCAAATTAAGTGCCATCCATGGTGATA
ATCAAGCCTTACTCGATAAATTAGAAAACTATTCTCGAGATAACCTTG
GTAAAGATTCTCTTTGGCAATTCCAAGACACTAAAAACATTAAAGATG
ATGTCTTGCGAAGTATCTTTATTAGTAAATTAAATTCAATTTACCAAAA
AGATATTAAATATCATTTTGAATGTTTAACTGAAATTAATGATCTCCCA
AATATCGATTTATTTGATTTAATTCGAATAATTGGAATCGCTTATGATA
ATGCCCTTGAAGAATGCGAAGCCTTAAAAAAGTTAGGAGCACGTACT
GTAGAAATCAATTCAATGCTGTATCAAAGTGCCCCTAACAAGTTAGAA
TTTGAAATTAAGAATACTTGTCGAAAGCAGCTTAGCACTAATAAACTC

CACCAAGAAGGAATTACAAATAAAGCTAATCATGAAGGGCTAGGATT
AGCAACTGTAAAAAAAATAGCTAACAAATACCACAACGTATATATTG
CTTACTCCTCCAATAATGGTTACTTTACATTTACAATTTCAATTGAATA
GAAAGGAATACTTACGGACTACCATTTACTTGTCTGCGATGATGACAG
GACTCAAGCAAAAAATATCGCTACACTACTTAAAATGTGAGTCGACGC
GTCGACTAAGACTGATGTAGATTAGCAACAGTTCTTTATAATTACGGC
TCTAAAATTTCATTAGTAACTCTGCGAAAATTCACAAAAACCACTACT
GATGGAACTACTGCACTTGGGATTGTAAAAGCAGCAGAAAAGTTAAA
AATGGATGTTGAAGCATATCAGTTTATTTNNNCAAAAGATGTAATTTT
TCTTTTGCTCATCTTATGAAAAACACCACCCCTCCCTCCCTAATTTTGA
TGGTTTATCCCATAATTTATGGAAACGTTTCACAACAAAAAATTTAATT
AAGGAGCGAAATTTTGACCTTTGGAACCTATCAGCAAATTAAAAATGC
CCTAAAAAAATTCGGAATAACTGATTATACTGTGATTGATCCCCCCGC
TGCCCACACCACCGAAAAAACTGATGCATATTTTGCTGGTCATGAAAG
TGTTCCCACTAAAACCATGTTTCTGAAAGGGAAAAAAAAGAATTTCTT
CCTGGTTATTAAGGATGAAAAAAAACCAATGGACTTTCATGAATTTAT
GGCCCTAAAAGGTGGCAAAAAAGTCTCCATGGCCCGGCCC

D.3 Sequencing data of *L. reuteri* Δ*rr431*Δ*cemR*

> Sequencing data of *L. reuteri* Δ*rr431*Δ*cemR*, using primers for *rr431* gene

GATTAAACATGATTATCAAAACTTACTAAATAGTCTCAAACTAAGTGC
CATCCATGGTGATAATCAAGCCTTACTCGATAAATTTAAGAAAACTTT
TTCTTCGAGATAACCTTGGTAAAGATTCTCTTTGGCAATTCCAAGACAC
TAAAAACATTAAAGATGATGTCTTGCGAAGTATCTTTATTAGTAAATT
AAATTCAATTTACCAAAAAGATATTAAATATCATTTTGAATGTTTAAC
AGAAATTAATGATCTCCCAAATATCGATTTATTTGATTTAATTCGAATA
ATTGGGATCGCTTATGATAATGCTCTTGAAGAATGTGAGGCTTTAAAA
AAGTTAGGAGCACGTACTGTAGAAATCAATTCAATGCTGTATCAAAGT
GCCCCTAACAAGTTAGAATTTGAAATTAAGAATACTTGTCGAAAGCAG
CTTAGCACCAATAAGCTTCATCAAGAAGGAATTACAAATAAGACTAAC
CATGAGGGACTTGGATTAGCAACGGCTCAAAAAATAGCTAACAAATA
CCACAACGTATATATTGTTTACTCCTCCGATAATGGTTACTTTACATTT
ACAATTTCAATTGAATAGAAAGGAATACTTATGGACTACCATCTACTC
GTTTGCGATGATGACCAGCTACAAGCTAAAAATATTGCCACATTATTA
AAAATGTCGTCAATTATTCTGTAAGTCGACCGGAGTCGAACAAAAGAA
CTAAATAAAATCTATAAGGAGAAATTATAGATTAGTGGGGTATTTTTA
TGTATAACGCATTTAATAAATACTATGTAAGTCAAGTTGACGAAGCAG
ATTGTGGAGTAGCCGCACTATCCATGATTCTTCGTAAATTTGGCTCTAA
AGTTTCACTTGCAACTTTAAGAAAAGCTACTAAAACAACTACTGAAGG
TACAACTGCTTTAGGTATTGTCGAAGCCGCTAAAAAGTATAAGCTTAA
CGTTGCTGCTTATCAAGCAGATATAAATCTTTTTAGCTCTAACGAAGCT
TCTTACCCTTTTATTGCACATCTCATTAAAAAGGATACCGGCTTATTAC
ATTACTGTGTAGTCGTTAAAAACGCAAAAAATCATCTTATTATTGCAG
ATCCAAATCCAAACATAAAAATTCAAAAGGTATCTAAAGAAGCTTTCT
TTCAAGAATGGACCGGAATTGCAATTTTCGCTAATCCTGCTAATGATT

ATGAAATTATTCAAGAACAATCACCTACACTAAAGAGGTTAATCATTC
CTGTCATAGAGCAAAAGAAGTTATTAATAGGCATTGTTACATCAGCAT
TTTTAGTAACATTTATCAATATAATAAGCTCATACTTTTTCCAAGGCTT
AATTGATAGATTGATCCCTTATAAATTACTAACACCTTTAAACATAATT
TGTATTGGATTACTAATAGCATATGTAGCAAATTCATTATTTAACTTTA
CAAGAGATTATCTATTAGCAACTCTAGGTCAAAGACTATCGAAAACTA
TTCTTCTAGATTACATTAAGCATGTTCTTAAACTCCCCATGGANNCTTT
TCACCAGAAAACAGGAGATATAGTTTCAAGGTTTACTGATGCTANTAA
GTANGATGNTCTGTA

D.4 Sequencing data of *L. reuteri* Δ*rr431*Δ*cemR*

> Sequencing data of *L. reuteri* Δ*rr431*Δ*cemR*, using primers for *cemR* gene

TCGCAGATTAGCATTTTANANNCACGGGTAACCCCAGCTTTATNTATN
TTTGCTTACTGNTCTTCAATGCAGTTTACAGTCTTTGTATACCGAGTTA
GTAAACAAATTCAACAAAAAAAGCTCCAGCTTCAAGAAAAGAAAGAC
TTATTATTTCTCATTCAAAACCTAGAAGAAAATCAATCGAAGTTAAGA
CGATTTAAACATGATTATCAAAACTTACTAAATAGCCTCAAATTAAGT
GCCATCCATGGTGATAATCAAGCCTTACTCGATAAATTAGAAAACTAT
TCTCGAGATAACCTTGGTAAAGATTCTCTTTGGCAATTCCAAGACACT
AAAAACATTAAAGATGATGTCTTGCGAAGTATCTTTATTAGTAAATTA
AATTCAATTTACCAAAAAGATATTAAATATCATTTTGAATGTTTAACTG
AAATTAATGATCTCCCAAATATCGATTTATTTGATTTAATTCGAATAAT
TGGAATCGCTTATGATAATGCCCTTGAAGAATGCGAAGCCTTAAAAAA
GTTAGGAGCACGTACTGTAGAAATCAATTCAATGCTGTATCAAAGTGC
CCCTAACAAGTTAGAATTTGAAATTAAGAATACTTGTCGAAAGCAGCT
TAGCACTAATAAACTCCACCAAGAAGGAATTACAAATAAAGCTAATC
ATGAAGGGCTAGGATTAGCAACTGTAAAAAAAATAGCTAACAAATAC
CACAACGTATATATTGCTTACTCCTCCAATAATGGTTACTTTACATTTA
CAATTTCAATTGAATAGAAAGGAATACTTACGGACTACCATTTACTTG
TCTGCGATGATGACAGGACTCAAGCAAAAAATATCGCTACACTACTTA
AAATGTGAGTCGACGCGTCGACTAAGACTGATGTAGATTAGCAACAGT
TCTTTATAATTACGGCTCTAAAATTTCATTAGTAACTCTGCGAAAATTC
ACAAAAACCACTACTGATGGAACTACTGCACTTGGGATTGTAAAAGCA
GCAGAAAAGTTAAAAATGGATGTTGAAGCATATCAGTTTATTTGATCA
AAAGATGTAANTTATCTATGGTCAACTTATGAAAAAAACCACCCCTCA
CTCCCTAATTTTGATGGTTTATCCTATAATTAATGTAAACGTTTCACAA
CAAAAAATTTAATTAAGGAGCGAAATTATGACCTTTGGAACCTATCAA
CAAATTAAAAATGCCCTAAAAAAATTCGGAATAACTGATTATACTGGG
ATTGATCCCCCCGCTGCCCACACCACCGAAAAAACTGATGCATATATC
GCTGGTCATGAAAGTGTTCCCACTAAAACCATGTTTCTGAAAGGGAAA
AAAAAGAATTTCTACATGGTTATTAAGGAAGAAAAAAAACCAATGGA
CTTTCATGAATTTATGGCCCTAACAGGTGCCAAAAGAGTCTCAATGGC
AAAACCAAAACACCTTCAAGAATAACTTGGCTTAACTCCCGGTATTGT
TTCCCCATTCGGATTATTAAATAATGAAGCACATAACGTTTAAGTTTAT
TTTGACGAGGATATTGTCAATGAACCAATTCAGACTTTCCATCCTAAC

GAAAATACTCATACNANTTTTATTAAGACAGATGACTTGNTCAAATTT
CTTAAGCANATTGATTTTGAACCAGAAATTANTGATTTGNAACCCCCA
GTCGATTACCTATCNCGATTAGCTCCCNCTTTTTNNGGGGGGGANTTTT
TTTGATGGTTTATCCTATAATTAATGTAAACGTTTCACAACAAAAAATT
TAATTAAGGAGCGAAATTATGACCTTTGGAACCTATCAACAAATTAAA
AATGCCCTAAAAAAATTCGGAATAACTGATTATACTGGGATTGATCCC
CCCGCTGCCCACACCACCGAAAAAACTGATGCATATATCGCTGGTCAT
GAAAGTGTTCCCACTAAAACCATGTTTCTGAAAGGGAAAAAAAAGAA
TTTCTACATGGTTATTAAGGAAGAAAAAAAACCAATGGACTTTCATGA
ATTTATGGCCCTAACAGGTGCCAAAAGAGTCTCAATGGCAAAACCAA
AACACCTTCAAGAATAACTTGGCTTAACTCCCGGTATTGTTTCCCCATT
CGGATTATTAAATAATGAAGCACATAACGTTTAAGTTTATTTTGACGA
GGATATTGTCAATGAACCAATTCAGACTTTCCATCCTAACGAAAATAC
TCATACNANTTTTATTAAGACAGATGACTTGNTCAAATTTCTTAAGCA
NATTGATTTTGAACCAGAAATTANTGATTTGNAACCCCCAGTCGATTA
CCTATCNCGATTAGCTCCCNCTTTTTNNGGGGGGGANTTTTTTT

D.5 Sequencing data of *L. reuteri* Δ*cemRC*

> Sequencing data of *L. reuteri* Δ*cemRC*, using primers for *cemR* gene

ACTTTACATTTACAATTTCAATTGAATAGAAAGGAATAGTTACGGACT
ACCATTTACTNNTCTGCGATGATGACAGGACTCAAGCAAAAAATATCG
CTACACTACTTAAAATGTCATCAATTATTCTTGAAGAACAAGATATTC
ATCCGACCATTGATTTGATTGCAACCGACGCAGAGAGTGTATTAGATT
ACTTTGAGTTTAATAGTGATTTAAATAATATTATTGCCTTTTTAGATAT
TCAGTTAGATGAAAATTCTAATTCAAAAGGTGGTCTAGATTTAGCTCA
AAAGATAAAAACACTTAACGAAAAAGCACAAATAATCTTTATTACAA
CGCATGAAGAACTAGCTTTTCTCACCTTTCAACGTCGAATTAATCCGTT
GGACTATATAACTAAAAACTACAAACCAACGAGATTTACAAAGACGG
CTAACCGAAACCCTAGAACTAGCTATAAGGAAACTGAATGAATTTAAC
TATATTAAAAAAAATACATTCAGTTATAAAGTTGGTACAAGGATTATA
AATGTAACTTACTTCAGTAATATCCTTTATATCTCTACTACAAAATTCC
CTCATAAATTAAAGATTGTTACATCTAATGGTCAAGGGGAATTCTCAG
GTGATATTAAAACAATTGAGCAAAATTATTCTATGTTTTTCAAGGCTTC
TCAGTCTAGCTTAATTAACTTACAAAATGTTGAAACAATTAATACCAA
AAAACGGCTTATTAAGTTTCCTTTTCCAACAATGATTATCTTAAGTATT
CTAGAAGCAGAGCTAAAGAATTAAATAATCTATATAAATAACAAGTC
AATAAGACTGATGTAGATTAGCAACAGTTCTTTATGATTACGGCTCTA
AAATTTCATTAGTAA-CTC-TGCGAAAATTCACAAAAACCA-CTATT-
GATGGAACTACTGCACTT--GGGATTGT-
AAAGAAGCAGCAGAAAAGTTAAAAAT--GGATGTTGAAGCA-
TATCAGTTTATTTGN-TCAAAAGATGTAA