Novel two-component regulatory systems play a role in biofilm formation of *Lactobacillus reuteri* rodent isolate 100-23

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Abstract

This study characterized the two-component regulatory systems encoded by *bfrKRT* and *cemAKR*, and assessed their influence on biofilm formation by *L. reuteri* 100-23. A method for deletion of multiple genes was employed to disrupt genetic loci of two component systems. The operons *bfrKRT* and *cemAKR* show complementary organization. Genes *bfrKRT* code for a histidine kinase, a response regulator, and an ATP-binding cassette-type transporter with a bacteriocin processing peptidase domain, respectively. Genes *cemAKR* code for a signal peptide, a histidine kinase, and a response regulator, respectively. Deletion of single or multiple genes in the operons *bfrKRT* and *cemAKR* did not affect cell morphology, growth, or the sensitivity to various stressors. However, gene disruption affected biofilm formation, this effect was dependent on the carbon source. Deletion of *bfrK* or *cemA* increased sucrose-dependent biofilm formation *in vitro*. Glucose-dependent biofilm formation was particularly increased by deletion of *cemK*. The expression of *cemK* and *cemR* was altered by deletion of *bfrK*, indicating cross-talk between these two regulatory systems. These results may contribute to our understanding of the genetic factors related to the biofilm formation and competitiveness of *L. reuteri* in intestinal ecosystems.

Key words: *Lactobacillus reuteri*, two-component regulatory systems, biofilm formation, multi-deletion mutagenesis

Introduction

Lactobacillus reuteri a lactic acid bacterium that has adapted to the gastrointestinal tracts of vertebrates but also occurs in cereal fermentations (Vogel *et al*, 1999; Walter *et al.*, 2011). The competitiveness of *L. reuteri* in cereal fermentations is based on carbohydrate and amino acid metabolism matching the substrate supply in cereals (Schwab *et al.,* 2007; Su *et al.,* 2011; Vogel *et al.*, 1999). Sourdough isolates of *L. reuteri* share phylogenetic and physiological characteristics with intestinal strains of *L. reuteri* (Su *et al.,* 2012). *L. reuteri* colonizes the human gastrointestinal tract and the human female urogenital tract, and the upper intestinal tract of animals, including pigs, birds, and rodents (Fuller, 1973; Fuller & Brooker, 1974; Fuller *et al*., 1978; Savage *et al*., 1968; Wesney & Tannock, 1979; for review, see Walter, 2008). Because of its stable association with vertebrate hosts, *L. reuteri* has been used as a model organism to study host-microbe interaction and host-specific adaptation (Frese *et al.,* 2011; Walter *et al.*, 2011).

The colonization of animals by *L. reuteri* involved the formation of biofilms on non-secretory stratified squamous epithelia in the upper intestinal tract, e.g. the forestomach of mice and horses, and the crops of birds (Fuller & Brooker, 1974; Yuki *et al.*, 2000; Walter *et al.*, 2011). Biofilm formation is comprised of four major steps: adherence of cells to surfaces, cell accumulation, clonal maturation, and formation of mixed species biofilms (for reviews, see Karatan & Watnick, 2009; Nobbs *et al.,* 2009). In the first steps, adhesins facilitate adherence to surfaces. In the latter steps, quorum sensing,

exopolysaccharide formation, coaggregation, and genetic exchange play important roles. Adhesion of *L. reuteri* to the murine intestinal tract was mediated by a large surface protein (Frese *et al.,* 2011; Walter *et al.,* 2005). The mucus adhesion-promoting protein (referred to as MapA, CnBP, CyuC, or BspA) (Miyoshi *et al.*, 2006), and the mucus-binding protein (Mub) (Roos & Jonsson, 2002) mediated adherence of *L. reuteri* to porcine intestinal mucus or human epithelial cells. Extracellular polysaccharides produced by the reuteransucrase of *L. reuteri* promoted biofilm formation in the murine forestomach and fructansucrases of *L. reuteri* acted as matrix-binding proteins (Sims *et al.,* 2011; Walter *et al.,* 2008). Biofilm formation by *L. reuteri* thus shares similarity with the oral pathogen *Streptococcus mutans*, which is ecotypically and phylogenetically related to *L. reuteri* (Zhang *et al.,* 2011). In *S. mutans,* biofilm formation is mediated by glucansucrases and fructansucrases and their expression is dependent on signal transduction by two-component systems (Nobbs *et al.,* 2009; Quivey *et al.,* 2001; Senadheera *et al.,* 2007).

A typical two-component system consists of a histidine kinase that autophosphorylates in response to environmental stimuli and relays a phosphoryl group to its cognate response regulator. The response regulator then binds to DNA and alters gene expression (Mitrophanov & Groisman, 2008). A histidine kinase coded by gene *lr70430*, is unique to the rodent isolates of *L. reuteri* and contributes to the colonization of the rodent strain *L. reuteri* 100-23 in the murine forestomach (Frese *et al.,* 2011; Wesney & Tannock, 1979). However, the role of two component systems in the adaptation of *L. reuteri* to different hosts remains

to be elucidated. It was therefore the aim of this study to characterize the role of two-component systems in *L. reuteri* rodent isolate 100-23. A novel method for the multi-deletion mutagenesis in *L. reuteri* was employed. The genetic loci of two component systems were disrupted by homologous recombination. The phenotypes of deleted mutant strains, including their ability to adhere in the presence of glucose or sucrose, were characterized, and the regulatory signaling cascade was elucidated. Biofilm formation by *L. reuteri* 100-23 was compared to *L. reuteri* TMW1.106, a strain for which sucrose-dependent biofilm was described (Walter *et al.,* 2008).

Materials and methods

Bacterial growth

Bacterial strains and plasmids used in this study are listed in Table 1 and Table S1. *Escherichia coli* JM109 (Promega, Nepean, Canada) was cultured at 37°C in Luria-Bertani (LB) broth with agitation. *E. coli* harbouring pJRS233-derived plasmids was cultured in LB containing ampicillin (100 mg l^{-1}) and erythromycin (500 mg l^{-1}) at 30°C to maintain the plasmids. *L. reuteri* was cultured at 37°C under micro-aerobic conditions (1% O_2, 5% CO_2, and 94% N_2) in deMan-Rogosa-Sharpe broth (MRS) (Difco, Becton Dickinson, Mississauga, Canada) unless otherwise specified. MRS media were modified as follows for specific experiments: mMRS (Gänzle *et al.,* 2000) containing 2% glucose as sole carbon source (gluMRS), and mMRS containing 2% sucrose as sole carbon source (sucMRS). Erythromycin (10 mg l^{-1}) was added to MRS to grow erythromycin-resistant *L. reuteri.*

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 S2) 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).

Bioinformatic analyses

A web-based bacteriocin genome mining tool (BAGEL) (de Jong *et al.,* 2006) was used to predict sensing peptide-based two-component systems in lactobacilli. The similarity of nucleotide sequences was determined by pairwise sequence alignment using EMBOSS Water- Alignment. BLASTP analysis was performed to retrieve homologous proteins, which were further analyzed with Kyoto Encyclopedia of Genes and Genomes (KEGG) databases. Amino acid sequences were retrieved from UniProt (Bairoch *et al.,* 2005) and aligned to calculate their identity scores using MUSCLE pairwise alignment (Geneious version 5.6.6; Auckland, New Zealand). Protein function was predicted with the DAS program to define the transmembrane segments, the InterProscan program, Pfam, and the SMART method to find motifs and protein domains.

Generation of *L. reuteri* knockout mutants

133 The in-frame deletion method for generating *L. reuteri* knockout mutants has been
134 described in a previous study (Su *et al*., 2011). Plasmids and primers used are
135 listed in Table S2 and Table S3, respectively, of the online supplementary
136 material. In brief, the 5'- and 3'-flanking sequences of the target genes were
137 amplified by PCR, and were thereafter referred to as amplicon-A and amplicon-B,
138 respectively. Amplicon-A and amplicon-B were inserted separately into
139 pGEMTeasy vectors to produce pGene-A and pGene-B. Next, the restriction
140 enzymes (RE) were used to cut out two amplicons from pGene-A and pGene-B.
141 These two amplicons were then ligated into a pGEMTeasy vector using T4 DNA
142 ligase, which produced pGene-AB. The ligated DNA fragment AB was cut out of
143 pGene-AB with suitable RE, and inserted into the integration shuttle vector
144 pJRS233 (Perez-Casal *et al.*, 1993) to generate a knockout plasmid, pKO-*Gene*-
145 AB. After electro-transforming pKO-*Gene*-AB into the *L. reuteri* wild type strain,
146 a single-gene knockout mutant was generated by temperature-impulse integration
147 and a plasmid-curing test as described by Su *et al*. (2011). An antibiotic-sensitive
148 knockout mutant was identified by replica-plating onto mMRS and mMRS-
149 erythromycin agar plates. The truncation of the target gene in the derived deletion
150 mutant was confirmed by PCR with primer set *gene*-KO-1 and *gene*-KO-4, and
151 primer set *gene*-5-F and *gene*-6-R. The deletion of this region was confirmed by
152 Sanger sequencing using primers *gene*-5-F and *gene*-6-R. The same strategy was
153 used to generate double-gene knockout mutants and detailed in the supplementary
154 material.

155 **Scanning electron microscope (SEM) analysis of biofilm specimens**

156 *L. reuteri* 100-23, 100-23Δ*cemK*Δ*cemR*, TMW1.106, and TMW1.106Δ*gtfA* were
157 grown on polystyrene plates containing gluMRS or sucMRS broth. After
158 incubation, cells were washed with buffer containing 50 mM NaH_2PO_4 (pH 6),
159 and fixed with 2.5% glutaraldehyde in 10 mM of PBS buffer (pH 7.4) at 4°C
160 overnight. Fixed cells were washed twice in PBS buffer and dehydrated by adding
161 a series of one mL volumes of an increasingly concentrated ethanol in water
162 solution (70, 85, 95, 100, and 100%, v/v) at room temperature. Cells were
163 incubated in each ethanol concentration for 10 minutes. Hexamethyldisilazane
164 (HMDS) (Sigma-Aldrich, Munich, Germany) was introduced into the cells by
165 gradually increasing the concentration of HMDS in ethanol. The following series
166 of HMDS in ethanol solutions was used: 75% ethanol/25% HMDS, 50%
167 ethanol/50% HMDS, 25% ethanol/75% HMDS, and three volumes of 100%
168 HMDS. Samples were air-dried overnight and then broken down into smaller
169 pieces that were later mounted on SEM stubs, where they were immediately
170 coated with Au/Pd on a sputter coater (Hummer 6.2) (Anatech, Union City,
171 California). The examination was performed using a scanning electron
172 microscope XL30 (FEI, Hillsboro, Oregon) at an acceleration voltage of 20 kV.

173 **RNA extraction**

174 The MasterPure™ RNA Purification protocol (Epicentre Technologies,
175 Markham, Canada) was used with slight modifications. Overnight cultures of *L.*
176 *reuteri* were diluted fifty-fold in gluMRS broth and incubated at 37°C until an
177 OD_{600nm} of 0.4 was reached. Cells from 10 ml of culture were harvested and RNA
178 synthesis was halted by adding 1.25 ml of ice-cold ethanol/phenol Stop Solution

(5% acidic phenol in ethanol, pH < 7). Cells were harvested by centrifugation and lysed at 65°C for 15 min with 300 µl of Tissue and Cell Lysis Solution containing 5.5 µg of Proteinase K. After incubation 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 minutes. The supernatant was mixed with 500 µl of isopropanol and nucleic acids were collected by centrifugation. The resulting pellets were rinsed with a 75% ethanol solution, suspended in 30 µl of nuclease-free water (Ambion), and treated with RNase-free DNase I (Ambion, Streetsville, Canada) at 37°C for 2 h. The reaction was stopped by addition of 5 µl of 50 mM EDTA. Five units of SUPERase·In (RNase inhibitor, Ambion) were added and the RNA was stored at 4°C.

Complementary DNA synthesis

Two µg of RNA was used as a template for cDNA synthesis using Random Primers (Invitrogen), dNTPs (Invitrogen), and nuclease-free water (Ambion). The RNA/Primer mixture was incubated at 70°C for 10 minutes, then 25°C for 10 minutes, and finally chilled to 4°C. The reaction mix was prepared with the RNA/Primer mixture, 5× 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 minutes, 37°C for 1 h, 42°C for 1 h, and then 70°C for 10 minutes 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).

Relative quantification of gene expression using quantitative PCR (qPCR)

Gene expression was quantified with qPCR with cDNA as template. 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 method (Pfaffl, 2001). 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 (Pfaffl, 2001) with ABI software. The amplification program was 95°C for 2 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Data was collected at 60°C followed by a dissociation curve. Analysis was performed in triplicate technical repeats and three independent experiments. DNase I-treated RNA and genomic DNA were used as negative and positive controls, respectively.

Adherence assay

The adherence assay was based on the method of Loo *et al*. (Loo *et al.,* 2000) with modifications. 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, the supernatants were discarded, and wells were washed twice with 50 mM NaH_2PO_4 (pH 6) buffer. Cells adhering to the plate were scraped using plastic tips and resuspended in one milliliter of phosphate buffer. The cell density was determined by measuring the

225 OD_{600nm}. Analysis was performed in triplicate independent experiments with two
226 or three technical repeats per replicate.

227 **Statistical analysis**

228 Statistical analysis was performed using Student's *t*-test (SigmaPlot, version 11.0;
229 Chicago, IL).

230 **Sequences and accession numbers**

231 The nucleotide sequence of the *bfrKRT* and *cemAKR* operons were retrieved from
232 the GenBank database (accession number: NZ_AAPZ00000000.2 ; locus tag:
233 Lreu23DRAFT_4807 for *bfrK*; Lreu23DRAFT_4808 for *bfrR*;
234 Lreu23DRAFT_4809 for *bfrT*; Lreu23DRAFT_4825 for *cemK*; and
235 Lreu23DRAFT_4826 for *cemR*). The nucleotide sequence of *cemA* is 5'-
236 ATGCAAAAACTATCAATTCATCAACTATCTTTAATTAAGGGTGGTATA
237 TACTCACTTTTAAGTCTGTAAA-3', and the predicted protein sequence is
238 MQKLSIHQLSLIKGGIYSLLSL. The nucleotide sequences of *L. reuteri* mutant
239 strains were deposited to Genebank with the following accession numbers:
240 Δ*cemA*, JF339968; Δ*bfrK*, KF306072; Δ*bfrK*Δ*bfrR*, KF306073; Δ*cemK*,
241 KF306074; Δ*bfrR* KF306075; Δ*cemK*Δ*cemR,* KF306076; Δ*bfrR*Δ*cemK*,
242 KF306077; Δ*bfrK*Δ*cemK*, *bfrK* sequence, KF306078, *cemK* sequence, KF306079.

243 **Results**

244 ***In silico* prediction of the genetic loci *bfrKRT* and *cemAKR***

245 Inactivation of the gene *bfrK* (*lr70430*) impairs the ecological fitness of *L. reuteri*
246 100-23 in the intestinal tract of mice (Frese *et al.,* 2011) but the function of the

bfrKRT operon remains unclear. The two-component system *bfrKRT* was predicted to be a peptide-based quorum sensing two-component regulatory system. Analysis also identified *cemAKR*, a two-component system with high sequence homology to *bfrKRT* (Fig. 1). The *bfrKRT* operon consists of genes coding for a putative histidine kinase of the HPK_{10} subfamily, a response regulator of the LytR/AlgR family, and an ATP-binding cassette-type transporter with a bacteriocin processing peptidase C39 domain (Fig. 1). The *cemAKR* operon is composed of genes coding for an autoinducing peptide containing a conserved double-glycine (GG) motif in the leader peptide region, a histidine kinase of the HPK_{10} subfamily, and a response regulator of the LytR/AlgR family (Fig. 1). The BAGEL program identified the putative signal transduction peptide IYSLLSL as *cemA* in *L. reuteri* 100-23. The nucleotide sequences of *bfrK* and *cemK* are very similar, as are *bfrR* and *cemR* (Fig. 1). *BfrKR* and *cemKR* are similar to the bacteriocin-related two-component system *abpKR* in *Lactobacillus salivarius* UCC118; *bfrT* is similar to *abpT* coding for the cognate bacteriocin export accessory protein in *Lactobacillus salivarius* UCC118 (KEGG database). The complementary genetic organization of the two operons implies a co-operation of the *bfrKRT* and *cemAKR* operons. The response regulator *cemR* was compared to other members of the LytR/AlgR family by BLASTP analysis and MUSCLE alignment. CemR was similar to AgrA (Peng *et al.*, 1988), SppR (Brurberg *et al.*, 1997), PlnC (Diep *et al.*, 1996), LamR, (Fujii *et al.*, 2008), ComE (Ween *et al.*, 2002), and LytR (Brunskill & Bayles, 1996; Kuroda *et al.*, 2001) (Table S1 of the

online supplementary material), suggesting that CemR functions as a response regulator controlled by an autoinducing peptide.

Generation of *L. reuteri* single-gene and double-gene deletion mutants and the characterization of *bfrKRT* and *cemAKR* operons

To determine whether there is cooperative regulation between the *bfrKRT* and *cemAKR* operons, the single-gene deletion mutants Δ*bfrK*, Δ*bfrR*, Δ*cemA*, and Δ*cemK*, as well as the double-gene deletion mutants Δ*bfrK*Δ*bfrR*, Δ*cemK*Δ*cemR*, Δ*bfrK*Δ*cemK*, and Δ*bfrR*Δ*cemK* (Table 1), were generated using site-specific homologous recombination mutagenesis and verified by PCR and DNA sequencing (Table S2 and S3). Physiological properties of the resulting *L. reuteri* mutant strains were characterized by observation of cell morphology and colony morphology, determination of autoaggregation, membrane fluidity and autolysis, and by observation of growth in a diverse set of adverse environmental conditions (Table S4). The disruption of genes in the *bfrKRT* and / or the *cemAKR* operons did not alter morphological characteristics of cells or colonies, aggregation, autolysis, membrane fluidity, or growth at low pH, high osmotic pressure, or in the presence of membrane-active inhibitors (Table S4).

Cell adherence characteristics of *L. reuteri* wild type and mutant strains

Some response regulators of the LytR/AlgR family regulate biofilm formation (Galperin, 2008). Therefore, the ability of *L. reuteri* 100-23 and its mutant strains to form biofilms was evaluated in two *in vitro* adherence assays. *L. reuteri* TMW1.106, a strain for which biofilm formation was previously characterized *in*

291 *vitro* and *in vivo*, was used for comparison (Walter *et al.,* 2008). Scanning
292 electron microscopy was used to visualize the structure biofilms grown in
293 presence of glucose or sucrose (Fig. 2). In sucMRS, *L. reuteri* 100-23 and 100-
294 23Δ*cemK*Δ*cemR* formed thick, stack-structured biofilms [Fig. 2(a, c)]. Biofilm
295 formation was also observed with *L. reuteri* TMW1.106 (Fig. 2e). However, *L.*
296 *reuteri* TMW1.106Δ*gtfA* failed to form biofilms, owing to the disruption of
297 reuteransucrase, which produces extracellular glucan as biofilm matrix (Walter *et*
298 *al.,* 2008, Fig. 2g). In gluMRS, *L. reuteri* 100-23 did not form a biofilm (Fig. 2b),
299 while *L. reuteri* Δ*cemK*Δ*cemR* developed highly complex layers of biofilm (Fig.
300 2d). Biofilms formed by *L. reuteri* TMW1.106 in gluMRS (Fig. 2f) were more
301 dense when compared to *L. reuteri* TMW1.106Δ*gtfA* (Fig. 2h). These results
302 indicate that mechanisms of biofilm formation in *L. reuteri* are strain specific, and
303 specific for different carbon sources. The *cemAKR* operon appears to regulate
304 glucose-dependent biofilm formation in *L. reuteri* 100-23.
305 The microscopic observation of biofilm formation was verified by a quantitative
306 assay (Fig. 3). Consistent with the microscopic observation, *L. reuteri* TMW1.106
307 formed biofilms in gluMRS or sucMRS but *L. reuteri* TMW1.106Δ*gtfA* was
308 unable to form biofilm in either medium (Fig. 3). When grown in presence of
309 sucrose, *L. reuteri* 100-23 and all mutants except *L. reuteri* 100-23Δ*bfrK*Δ*cemK*
310 formed biofilms (Fig. 3a). Cell densities of *L. reuteri* 100-23Δ*bfrR*, 100-
311 23Δ*bfrK*Δ*bfrR*, 100-23Δ*cemA*, and 100-23Δ*bfrR*Δ*cemK* were higher when
312 compared to *L. reuteri* 100-23. The density of cells of *L. reuteri* 100-23 adhering
313 to the Petri dishes after growth in gluMRS was reduced in comparison to cells

after growth in sucMRS (Fig 3). When growing with glucose as carbon source, the adherence of all strains with mutations in *bfrR* or the *cemAKR* operon was higher when compared to the *L. reuteri* 100-23 and the cell densities were highest in strains with a mutation of *cemK* operon. The results of the quantitative determination of adherence and biofilm formation thus confirm that *cemAKR* regulates biofilm formation in the presence of glucose.

Interaction between of *bfrKRT* and *cemAKR* regulons: analysis of gene expression by quantitative RT-PCR

The expression of genes in the *bfrKRT* and *cemAKR* operons was determined by quantitative PCR to determine whether these two highly homologous two-component systems interact (Fig. 4). Expression of *bfrK* was unaffected by disruption of any other gene in the two operons; the expression of *bfrR* was significantly increased only by disruption of *bfrK* (Fig. 4). The transcription of *bfrT* increased in strains Δ*bfrK* and Δ*bfrK*Δ*bfrR* and decreased in strain Δ*bfrR* but remained unaffected by disruption of any of the genes in the *cemAKR* operon (Fig. 4). In contrast, the expression of genes in the *cemAKR* operon was influenced by the *bfrKRT* operon. The expression of *cemK* was increased by disruption of *bfrK* and decreased by disruption of both *bfrK* and *bfrR.* The expression of *cemR* was significantly altered by disruption of *bfrK, cemA*, or *cemK*. The influence of BfrK on the expression of CemK and CemR implies interaction between these two two-component systems.

Since the *cemAKR* operon seems to play a role in the downstream signaling cascade, the *L. reuteri* Δ*cemK*Δ*cemR* strain was examined by qPCR to investigate

the influence of gene disruption on the expression of genes that relate to the production of autoinducing peptides, adhesion, biofilm dispersal, or exopolysaccharide production (Table 2). Genes involved in cyclopropane synthesis (*lr70615*), carbohydrate metabolism (*lr70618* and *lr71258*), and a cell division regulator (*lr71258*) were also included in the screening. Gene *lr70674* was included because it was incorrectly annotated as a mucus-binding protein. Of these genes, only the expression of *cemA* and *lr70674* was significantly different in *L. reuteri* Δ*cemK*Δ*cemR* when compared to the wild-type strain (relative expression 0.76 ± 0.08 and 0.66 ± 0.14, respectively). This result indicates that *cemA* is controlled by CemK and CemR. Gene *lr70674* encodes an osmoprotectant binding protein related to glycine betaine transport. However, tolerance of *L. reuteri* Δ*cemK*Δ*cemR* to osmotic stress was not different when compared to the wild type strain (Supplementary Table S4).

Discussion

This study characterized the two-component regulatory systems *bfrKRT* and *cemAKR* operons, and assessed their influence on the physiology and biofilm formation of *L. reuteri* 100-23. The function of genes and their role in the two-component regulatory systems were deduced by comparison of isogenic knockout mutant strains with gene disruptions in one or two genes. Moreover, the hierarchical structure of the TCS signaling cascade was assessed based on the differential gene expressions of single-gene and double-gene knockout mutants.

In most previous studies of *L. reuteri*, gene disruption was achieved by plasmid integration (Hung *et al.*, 2005; Schwab *et al.,* 2007; Walter *et al.,* 2008). With

these methods, a plasmid-borne antibiotic-resistance gene cassette remains in the chromosome of the mutant strain and limits the subsequent inactivation of other genes of interest. Single-stranded DNA recombineering (Van Pijkeren *et al.*, 2012) or site-specific homologous recombination (Su *et al.,* 2011) are alternative approaches that were recently employed for genetic modification of *L. reuteri*. The multiple-deletion method employed in this study allowed the generation of double-gene knockout mutants.

Two-component regulatory systems are essential mechanisms in biofilm formation by *Streptococcus mutans* and *Staphylococcus aureus*. Two component systems known to regulate biofilm formation include the *comCDE* system of *S. mutans* (Senadheera & Cvitkovitch, 2008) and the *agrBDCA* system of *S. aureus* (Boles & Horswill, 2008). In these two systems, the response regulators ComE and AgrA were categorized as LytR/AlgR family proteins. Both are controlled by an autoinducing peptide (Nikolskaya & Galperin, 2002). Functions of regulatory proteins in the LytR/AlgR family include the regulation of virulence factors and in the performance of housekeeping functions, such as cell envelope maintenance, competence, and biofilm formation (Galperin, 2008). The response regulators BfrR and CemR investigated in this study are very similar to response regulation in the LytR/AlgR family (Table S1) and likely are controlled by the autoinducing peptide encoded by *cemA*.

The genetic organization of *bfrKRT* and *cemAKR* implies cooperative regulation between these two paralogous operons, which involves the sharing of the autoinducing peptide CemA and the ABC transporter BfrT. The *cemAKR*

operon harbors an autoinducing peptide with a double-glycine (GG) type leader peptide, but lacks the corresponding ABC transporter. The *bfrKRT* operon in turn harbors an ABC transporter with a peptidase C39 domain that recognizes the GG-leader peptide. Moreover, nucleotide sequences of *bfrK* and *cemK* are 83% similar and the sequences of *bfrR* and *cemR* are 75% similar. These two operons thus may work cooperatively, similar to the Agr-like TCSs in *L. plantarum* and CpxA-CpxR and EnvZ-OmpR in *E. coli* (Bijlsma & Groisman, 2003; Fujii *et al.*, 2008; Siryaporn & Goulian, 2008). Quantification of gene expression in *L. reuteri* 100-23 and its knockout mutants demonstrated that disruption of *bfrK* altered the expression of *cemK* and *cemR*. Disruption of genes in the *cemAKR* operon, however, did not alter the expression of the genes in the *bfrKRT* operon. This result indicates that BrfK has the highest place in the hierarchy in a signaling cascade that also includes proteins encoded by *cemAKR*.

L. reuteri 100-23 mutants with disruptions in the *bfrKRT* and / or the *cemAKR* operons were assessed with respect to a comprehensive set of phenotypic characteristics. This evaluation identified adherence associated with biofilm formation as their only phenotype distinguishing these mutants from the wild type. *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)$^{-1}$ (Schwab, 2006; Walter et al., 2008). Although the sucrose concentration in the forestomach of mice is much lower, sucrose metabolism and sucrose-dependent biofilm formation by *L. reuteri* 100-23 has been demonstrated *in vivo* (Schwab, 2006; Sims *et al.,* 2011). *L.*

406 *reuteri* TMW1.106 produces exopolysaccharide (reuteran) from sucrose with an
407 extracellular reuteransucrase (Schwab *et al.,* 2007). Disruption of the genes
408 coding for reuteransucrases and fructansucrases in *L. reuteri* reduced the
409 competitiveness of mutant strains in the forestomach of mice in competition with
410 the corresponding wild type strains but mutant strains colonized mice when
411 administered in pure culture (Walter *et al.,* 2008; Sims et al., 2011). However,
412 disruption of the reuteransucrase producing the extracellular biofilm matrix could
413 be complemented by co-colonization of a reuteran-producing wild type strain
414 (Walter et al., 2008). The *in vitro* biofilm formation is a simplified model system
415 for the *in vivo* situation because it uses an abiotic support, a constant carbon
416 supply (sucrose or glucose), and because competing microbiota are absent.
417 However, the *in vitro* biofilm formation and coaggregation by *L. reuteri*
418 TMW1.106, LTH5448 and 100-23 generally corresponded to competitiveness and
419 biofilm formation *in vivo* (Walter *et al.,* 2008, Sims et al., 2011, Frese et al., 2011;
420 this study).

421 Although the formation of biofilms by *L. reuteri* 100-23 and TMW1.106
422 *in vitro* and *in vivo* is supported by sucrose, the mechanisms of biofilm formations
423 exhibit strain specific differences. *L. reuteri* TMW1.106 but not 100-23 employs
424 an extracellular reuteransucrase to synthesise the biofilm matrix (Schwab *et al.,*
425 2007; Sims *et al.,* 2011; Walter *et al.,* 2008). Analysis of the genetic locus of
426 *bfrKRT* in *L. reuteri* 100-23 identified two IS elements upstream and downstream
427 of *bfrKRT*. The presence of *bfrKRT* in the rodent lineage strains of *L. reuteri* is
428 variable but the operon is rodent specific (Frese *et al.,* 2011). It is likely that the

bfrKRT operon results of lateral gene transfer in a manner similar to that of fructansucrase (*ftf*) in *L. reuteri* 100-23 (Sims *et al.,* 2011) and allows *L. reuteri* to adapt to different environments.

The quantitative assessment of biofilm formation that was performed in this study indicated that *L. reuteri* 100-23 formed less dense biofilm with glucose as sole carbon source when compared to *L. reuteri* TMW1.106. With glucose as the sole carbon source, biofilm formation of *L. reuteri* 100-23 was repressed by proteins coded by the *cemAKR* operon. *BfrK* is overexpressed by *L. reuteri* 100-23 during colonization of the murine forestomach epithelium (Frese et al., 2013) and disruption of *bfrK* impaired *in vivo* colonization of mice (Frese *et al.,* 2011). However, disruption of *bfrK* did not reduce *in vivo* biofilm formation, suggesting that the gene may be functionally redundant (Frese et al., 2013). This study confirmed and extended the *in vivo* results by demonstration that *bfrK* did not substantially alter adherence and biofilm formation ability of *L. reuteri* 100-23 with sucrose or glucose as carbon source (this study). Moreover, cooperative gene regulation through the *bfrKRT* and *cemAKR* operons may account for a partial overlap of the functions of the histidine kinases BfrK and CemK.

L. reuteri has adapted to specific vertebrate hosts. This adaptation allowed the identification of genetic or metabolic traits that are required for colonization of intestinal ecosystems. Genetic traits of *L. reuteri* contribute to biofilm formation and the ecological fitness in rodents were recently reviewed (Frese *et al*., 2013; Frese *et al.,* 2011; Walter, 2008) and are depicted in Figure 5. Biofilm formation contributes to the competitiveness of *L reuteri* in intestinal ecosystems. However,

452 the proteins that are involved in attachment and biofilm formation by *L. reuteri*
453 are strain-specific and have not been fully elucidated (Frese *et al.,* 2011; Walter *et*
454 *al.,* 2008). Attachment of *L. reuteri* to intestinal epithelia is mediated by the large
455 surface protein coded by *lsp* (Walter *et al.,* 2005), the mucus adhesion-promoting
456 protein MapA or the mucus-binding protein Mub (Miyoshi *et al.*, 2006**;** Roos &
457 Jonsson, 2002). The D-alanine-D-ananyl carrier protein ligase (DltA) also affects
458 the ability of *L. reuteri* ability to adhere and to resist acid stress (Walter *et al.*,
459 2007). The present study demonstrated that disruption of *bfrKRT* enhanced
460 sucrose-dependent biofilm formation of *L. reuteri* 100-23, and altered expression
461 of the *cemAKR* operon (Fig. 5). Disruption of genes in the *cemAKR* operon
462 enhanced glucose-dependent biofilm formation. Unlike the the *comCDE* system
463 of *S. mutans* and the *agrBDCA* system of *S. aureus*, the *bfrKRT* and *cemAKR*
464 systems of *L. reuteri* 100-23 did not influence the expression of genes encoding
465 exopolysaccharide formation, biofilm dispersal (the *lytSR* system and the *lrg*
466 operon), or another quorum-sensing autoinducing peptide and ABC transporter
467 (*lr70531*, *lr70532*). Data presented in this study link the *bfrKRT* and *cemAKR*
468 systems of *L. reuteri* to biofilm formation.

469 In conclusion, this study characterized several single and multiple deletion
470 mutants to characterize the two-component systems *bfrKRT* and *cemAKR* in *L.*
471 *reuteri* 100-23. Deletion of the histidine kinase BfrK impairs ecological fitness of
472 *L. reuteri* 100-23 in mice but the function of the operons remained unknown
473 (Frese *et al.,* 2011). Deletion of single or multiple genes in the operons *bfrKRT*
474 and *cemAKR* did not affect cell morphology, growth rate, or the sensitivity to

various stressors. However, several mutants exhibited increased adherence and biofilm formation *in vitro*. The effect of gene disruption on adherence and biofilm formation was dependent on the carbon source. Moreover, quantification of gene expression indicated cross talk between these two operons. The study thus links the contribution of *bfrK* on the competitiveness of *L. reuteri in vivo* to biofilm formation and adherence to the forestomach epithelium. However, the genes required for formation of the extracellular biofilm matrix in *L. reuteri* 100-23 remain unknown. The networks regulating of biofilm formation by *L. reuteri* and the specific contribution of the *bfrKRT* and *cemAKR* operons to biofilm formation *in vitro* and *in vivo* thus remain to be elucidated.

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642 **Figure legends:**

643 **Figure 1.** Schematic representation of the two-component regulatory systems *bfrKRT* and
644 *cemAKR* operons and their protein sequence analysis. Bioinformatic analysis of histidine
645 kinases BfrK and CemK reveals an amino-terminal transmembrane domain and a carboxyl
646 terminal ATPase-like ATP-binding domain, where the specific homology boxes H, X, N,
647 and G assign features to the HPK_{10} subfamily. The functional domains of response
648 regulators BfrR and CemR are predicted as CheY-like superfamily receiver with conserved
649 aspartate (D) and lysine (K) residues at the amino-terminus, along with LytTR DNA-
650 binding domain of the LytR/AlgR family at the carboxyl-terminus. The functional
651 prediction of BfrT shows a bacteriocin processing peptidase C39 domain, transmembrane
652 segments, and ABC transporter-like domain. CemA was identified using the BAGEL
653 program, which also revealed it was a bacteriocin-like autoinducing peptide, which
654 contains a conserved double-glycine (GG) motif in the leader peptide region. Numbers
655 indicate the protein identity of the histidine kinases and response regulators.

656 **Figure 2.** SEM micrographs of *L. reuteri* cells on polystyrene surfaces. *L. reuteri* 100-23
657 (a, b), Δ*cemK*Δ*cemR* (c, d), TMW1.106 (e, f), and Δ*gtfA* (g, h) were grown in MRS media
658 containing either 2% sucrose (a, c, e, and g) or 2% glucose (b, d, f, and h). Micrographs
659 were taken from six different fields of two independent experiments. Magnification is ×
660 5000.

661 **Figure 3.** Adherence ability of *L. reuteri* on polystyrene plates. Quantitative analysis of
662 cells adhered to polystyrene plates was carried out using cells that were grown over 24 h,
663 which were inoculated in MRS broth containing 2% sucrose (black bar, panel A) or 2%

glucose (grey bar, panel B). Adherence ability was measured by an optical density of 600 nm. A significant difference between the adherence ability of the mutant strains and the wild-type strains is indicated by asterisk (*) ($p < 0.05$). Data shown are the means of three independent experiments with standard deviations.

Figure 4. Relative quantification of gene expression in *L. reuteri* 100-23 and its derived mutant strains grown in gluMRS. The expressions of the *bfrKRT* and *cemAKR* operons were determined by qPCR (the y-axis; linear scale) with primers specific to genes *bfrK*, *bfrR*, *bfrT*, *cemK* and *cemR* (the x-axis). The *L. reuteri* isogenic strains used in this study are Δ*bfrK*, Δ*bfrR*, Δ*bfrK*Δ*bfrR*, Δ*cemA*, Δ*cemK*, Δ*cemK*Δ*cemR*, Δ*bfrK*Δ*cemK*, and Δ*bfrR*Δ*cemK*. A significant difference from the wild-type strain (relative gene expression = 1) is indicated by asterisk (*) ($p < 0.05$). The results are shown as means ± the standard deviations of three independent experiments performed in triplicate. Primers targeting deleted genes in mutant strains yielded no amplicons in RT-qPCR reactions; these controls are indicated by the letter "X".

Figure 5. Schematic overview of the TCS regulatory signaling cascade of the *bfrKRT* and *cemAKR* operons, and its relationship to the genes related to ecological performance of *L. reuteri* in the intestinal tract of mice. Dashed lines indicate relationships that were established on the basis of quantification of gene expression. The TCS involves signal sensing by a histidine kinase (BfrK, CemK), followed by histidine (H) phosphorylation and phosphotransfer to an aspartate (D) of a response regulator (BfrR, CemR). Disruptions in *bfrKR* affect expression of *bfrT* as well as genes in the *cemAKR* operon when glucose is the sole carbohydrate source in the growth media; disruption of *cemRK* does not affect expression of *brfKRT* but alters expression of *cemA* and *lr70674* (osmoprotectant binding

687 protein). Because a transport enzyme is not part of the *cemAKR* system, the maturation of
688 the autoinducing peptide CemA may be processed and transported by the ABC transporter
689 BfrT. Mutations in the *bfrKRT* system as well as *cemA* affected predominantly biofilm
690 formation with sucrose as carbon source while disruption of genes in the *cemAKR* system
691 has a stronger effect on glucose-dependent regulons of biofilm formation.

692 Adherence of *L. reuteri* to mouse epithelial cells or mediated by *lsp* (Walter *et al.,* 2005).
693 The membrane protein D-alanine-D-ananyl carrier protein ligase (DltA) affects *L. reuteri*'s
694 ability to adhere and to resist acid stress (Walter *et al.*, 2007). Extracellular glucansucrases
695 and levansucrase contribute to biofilm formation *in vitro* and ecological fitness *in vivo*
696 (Walter *et al.,* 2008). Disruption of the *secA2* operon and ABC transporters (*lr70458*,
697 *lr70532*) also impaired colonization of mice by *L. reuteri* (Frese *et al.,* 2011).

698 Methionine-R-sulfoxide reductase, coded by *msrB* in *L. reuteri* 100-23, is an antioxidant
699 repair enzyme reducing methionine sulfoxide to methionine. Disruption of *msrB* has
700 impairs colonisation of mice by *L. reuteri* (Walter *et al.,* 2005). Disruption of *luxS* in *L.*
701 *reuteri* 100-23 is associated with the metabolic conversion of S-ribosyl homocysteine to
702 homocysteine but not AI-2 quorum-sensing regulation (Wilson *et al.*, 2012). Genes *gadB*
703 (Su *et al.,* 2011) and *dltA* (Walter *et al.*, 2007) increase acid resistance of *L. reuteri*.

704

705 **Table 1.** Bacterial strains used in this study

Strains	Relevant genotype or description	Source or reference
Escherichia coli JM109	Cloning host for pGEMTeasy- and pJRS233-derived plasmids	Promega
Lactobacillus reuteri		
TMW1.106	Type II sourdough isolate; wild type strain producing glucan and fructan	Schwab et al. (2007)
TMW1.106Δ*gtfA*	TMW1.106 Δ*gtfA*::pORI28; non-glucan producing strain; Erm^r	Walter *et al.*, (2008)
100-23	Rodent isolate; wild type strain producing levan	Wesney & Tannock (1979)
100-23Δ*bfrK*	Truncation of *bfrK*	This study
100-23Δ*bfrR*	Truncation of *bfrR*	This study
100-23Δ*bfrK*Δ*bfrR*	Truncation of *bfrK* and *bfrR*	This study
100-23Δ*cemA*	Truncation of *cemA*	This study
100-23Δ*cemK*	Truncation of *cemK*	This study
100-23Δ*cemK*Δ*cemR*	Truncation of *cemK* and *cemR*	This study
100-23Δ*bfrK*Δ*cemK*	Truncation of *bfrK* and *cemK*	This study
100-23Δ*bfrR*Δ*cemK*	Truncation of *bfrR* and *cemK*	This study

706 Erm^r, erythromycin resistance gene

707 **Table 2.** Quantitative PCR primers used in this study

Primer name	Sequence (5' - 3')	Target gene's locus_tag	Features of putative protein
recA-qPCR-F2	CAACTATCCGGATGGAAATTCGTCG	Lreu23DRAFT_3582	RecA; endogenous protein; DNA recombination
recA-qPCR-R2	TGTCAACTTCACAACGTTTGAATGGC		
bfrK-qPCR-F1	CGGACTAGGCTATATTGGATCGTATT	Lreu23DRAFT_4807	BfrK; histidine kinase of the HPK_{10} family; two-component system
bfrK-qPCR-R1	GTTGGATGCCCTTCGTTTGTA		
bfrR-qPCR-F2	CTCAGCAAATTCAAAAAAAGCACCGT	Lreu23DRAFT_4808	BfrR; response regulator of the LytR/AlgR family; two-component system
*bfr*R-qPCR-R2	ATCGCCGTTGCAATTTTCGTTG		
bfrT-qPCR-F3	ACTAAAGCCTGCAAAGTTGCGATGAT	Lreu23DRAFT_4809	BfrT; ABC-type bacteriocin transporter; two-component system
bfrT-qPCR-R3	TTGTCCACCTGAAAGGGTAGTAGCATTTTC		
cemK-qPCR-F1	AGGACTTACTTTTGAACTTTTCACATTCTT	Lreu23DRAFT_4825	CemK; histidine kinase of the HPK_{10} family; two-component system
cemK-qPCR-R1	CATATTCCTTATGATTGGCTTAGGTTATAC		
cemR-qPCR-F2	CAGTCTAGCTTAATTAACTTACAAAATGTTGA	Lreu23DRAFT_4826	CemR; response regulator of the LytR/AlgR family; two-component system
cemR-qPCR-R2	CGGCTTATTAAGTTTTCCAACAATG		
*cem*A-qPCR-F2	TGATATATTTATGCAAAAACTATCAATTCATC	N.D.	CemA; autoinducing peptide of peptide-based quorum sensing two-component system
*cem*A -qPCR-R2	TTATTTACAGACTTAAAAGTGAGTATATACCA CCC		
lr69269-qPCR762-F2	TGCAGTGAGTATCACCGATAGACA	Lreu23DRAFT_3257	LytS; histidine kinase; cell autolysis; two-component system
lr69269-qPCR836-R2	TTTCCTGGGATATGGTGATCATC		
lr69270-qPCR365-F2	AGGATGATGCTACCAAAGCTAAGAG	Lreu23DRAFT_3258	LytR; response regulator of the LytR/AlgR family; cell autolysis; two-component system
lr69270-qPCR-439-R2	TCGTTCGCTCGTCATTTTGA		
lr69271-qPCR46-F2	ATGGGAATCTTTGCTGCAATTT	Lreu23DRAFT_3259	Putative negative effector of murein hydrolase LrgA; cell lysis; regulation of murein hydrolase activity
lr69271-qPCR120-R2	AGGAACAACGAAACTCTTAGGAAAAA		
lr69272-qPCR278-F2	CGCTTTATCGCCGGAATG	Lreu23DRAFT_3260	Putative negative effector of murein hydrolase; LrgB-like holing/antiholin; cell lysis; regulation of murein hydrolase activity
lr69272-qPCR352-R2	TGAATCCACCCACAAATAATGTG		

Table 2. –continued- Quantitative PCR primers used in this study

Primer name	Sequence (5' - 3')	Target gene's locus_tag	Features of putative protein
lr69363-qPCR261-F1	GGATTCACTAATTGCCGGTCTT	Lreu23DRAFT_3339	Homolog of BspA/CyuC/MapA/CnBP collagen binding protein; cystine transporter, amino acid ABC transporter substrate-binding protein, PAAT family; signal transduction systems, periplasmic component/ domain
lr69363-qPCR336-R1	CCGTTCAGGTGTCTGTGTAATATTG		
lr69863-qPCR40-F4	AAGCAATGGATAACAGCTGCAA	Lreu23DRAFT_4288	Homolog of glucansucrase/ reuteransucrase; exopolysaccharide synthesis
lr69863-qPCR114-R4	AGCTTGTGCCACACCTCCTAAA		
lr70531-qPCR38-F1	AGGTTTCTGGTGGATGGAGTCTAT	Lreu23DRAFT_4899	Bacteriocin-type signal sequence; quorum sensing two-component system
lr70531-qPCR119-R1	TGAGCCCATTTGTTCAAGGAA		
lr70532-qPCR366-F1	TCGGGATGAATTTGGTCGTT	Lreu23DRAFT_4900	ABC-type bacteriocin transporter; quorum sensing two-component system
lr70532-qPCR440-R1	TTTTGTGGCGTATATCCCTTAGC		
lr70615-qPCR868-F2	GGCGGCTATGTACCTGGTCTT	Lreu23DRAFT_4979	Cyclopropane-fatty-acyl-phospholipid synthase; cyclopropane synthesis
lr70615-qPCR943-R2	TTTCGATATCAGCGATTTGCA		
lr70618-qPCR1234-F2	CTGTTAATTAGTAACGGGATGCAAAC	Lreu23DRAFT_4982	Acetolactate synthase, large subunit; carbohydrate metabolism
lr70618-qPCR1308-R2	GGGATAAAGCATCGCTGCAA		
lr70674-qPCR242-F1	GCGAAGTTGACGTTTATCCTGAT	Lreu23DRAFT_5027	Glycine betaine/choline-binding (lipo)protein of an ABC-type transport system; osmoprotectant binding protein
lr70674-qPCR316-R1	TCTTGCCAGTCCCCTTCTTTT		
lr71010-qPCR1242-F1	CACTCTTCGTGATGCTCATGTTATC	Lreu23DRAFT_3826	Large surface protein with LPXTG-motif cell wall anchor domain; homolog of FtfA/ levansucrase/ inulosucrase; exopolysaccharide synthesis
lr71010-qPCR1317-R1	CGTTCCAGTGTTTCCCTCAAA		
lr71188-qPCR259-F1	CCAAGGTTTTTGCGGGATT	Lreu23DRAFT_3689	Cell division-specific peptidoglycan biosynthesis regulator FtsW; cell division
lr71188-qPCR333-R1	AACAGCTCGGCTAAAGACTAAAACA		
lr71258-qPCR638-F1	CAATCACTGCCGTAAAGAATGGT	Lreu23DRAFT_3623	Acetate kinase; carbohydrate metabolism
lr71258-qPCR712-R1	CCATTGTTATTCCCGCAACAG		

708 N.D., not determined.

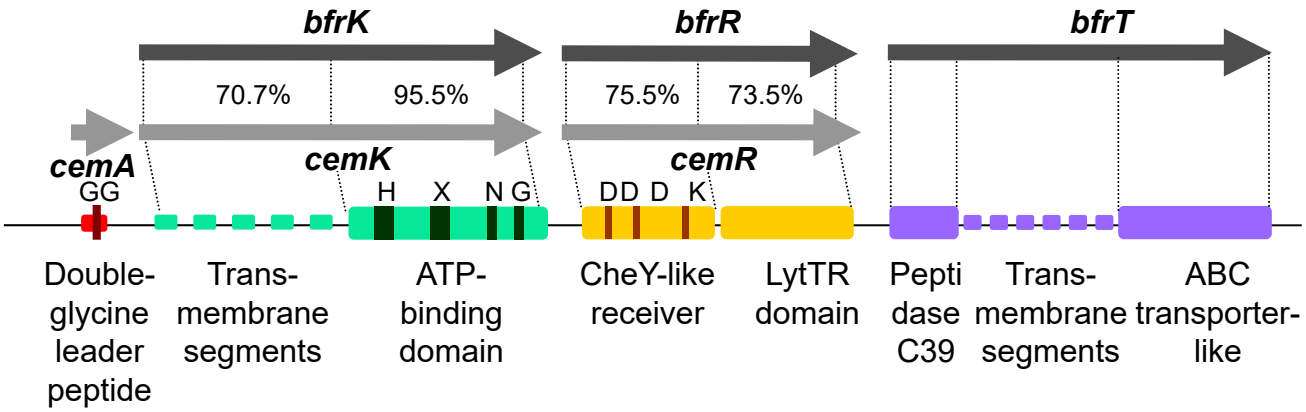
bfrK
bfrR
bfrT
70.7%
95.5%
75.5%
73.5%
cemA
GG
cemK
H
X
N G
cemR
DD D
K
Double-glycine leader peptide
Trans-membrane segments
ATP-binding domain
CheY-like receiver
LytTR domain
Pepti dase C39
Trans-membrane segments
ABC transporter-like

Figure 1. Schematic representation of the two-component regulatory systems *bfrKRT* and *cemAKR* operons and their protein and nucleotide sequence analysis. Bioinformatic analysis of histidine kinases BfrK and CemK reveals an amino-terminal transmembrane domain and a carboxyl terminal ATPase-like ATP-binding domain, where the specific homology boxes H, X, N, and G assign features to the HPK_{10} subfamily. The functional domains of response regulators BfrR and CemR are predicted as CheY-like superfamily receiver with conserved aspartate (D) and lysine (K) residues at the amino-terminus, along with LytTR DNA-binding domain of the LytR/AlgR family at the carboxyl-terminus. The functional prediction of BfrT shows a bacteriocin processing peptidase C39 domain, transmembrane segments, and ABC transporter-like domain. CemA was identified using the BAGEL program, which also revealed it was a bacteriocin-like autoinducing peptide, which contains a conserved double-glycine (GG) motif in the leader peptide region. Numbers indicate the protein identity of the histidine kinases and response regulators.

Figure 2. Su and Gänzle

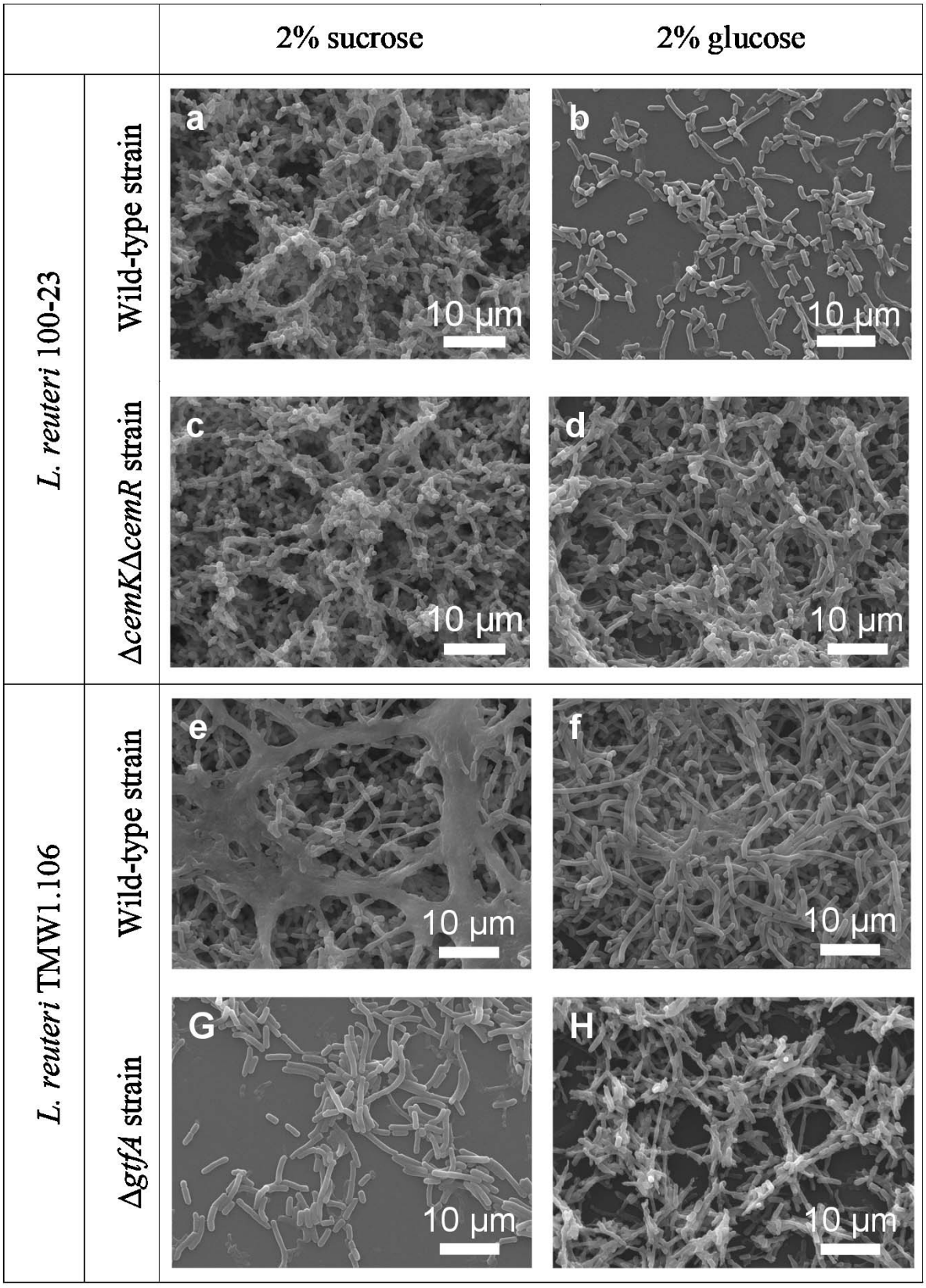

Figure 2. SEM micrographs of *L. reuteri* cells on polystyrene surfaces. *L. reuteri* 100-23 (a, b), Δ*cemK*Δ*cemR* (c, d), TMW1.106 (e, f), and Δ*gtfA* (g, h) were grown in MRS media containing either 2% sucrose (a, c, e, and g) or 2% glucose (b, d, f, and h). Micrographs were taken from six different fields of two independent experiments. Magnification is × 5000.

1

2 **Figure 3.** Su and Gänzle

3

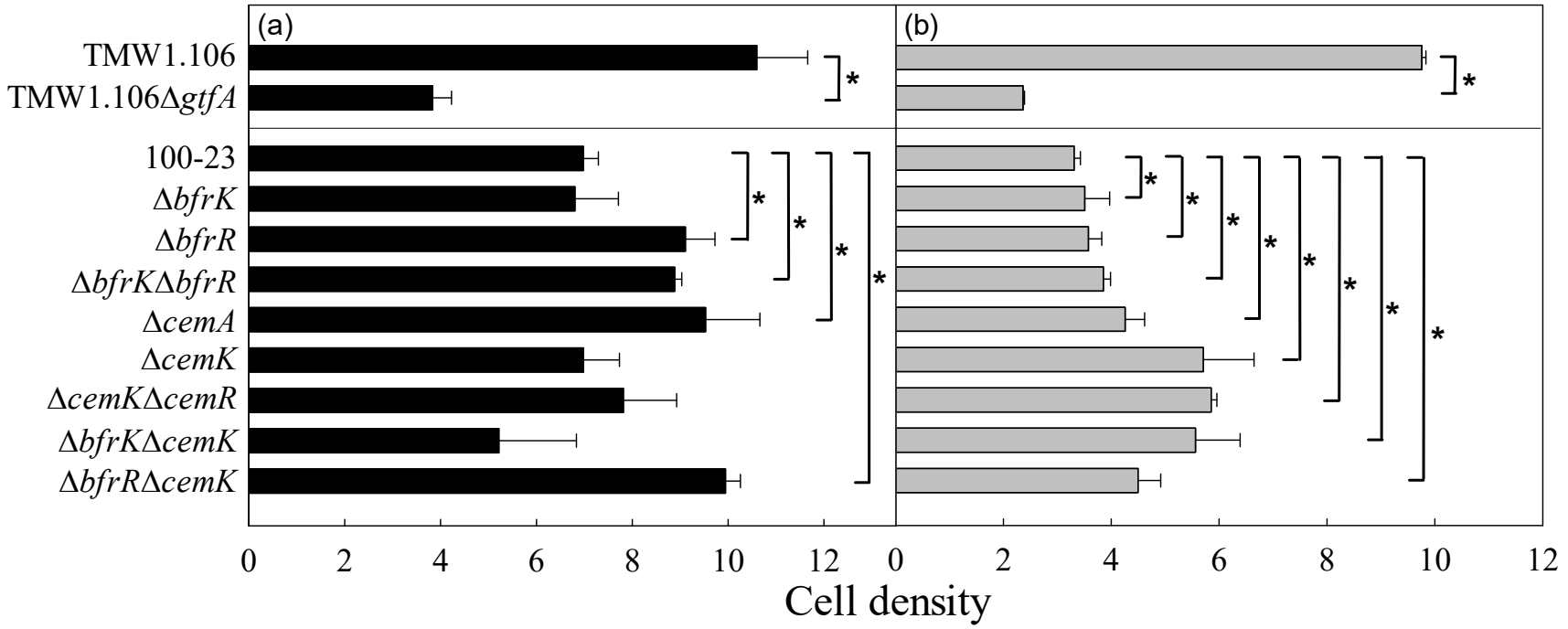

Figure 3. Adherence ability of *L. reuteri* on polystyrene plates. Quantitative analysis of cells adhered to polystyrene plates was carried out using cells that were grown over 24 h, which were inoculated in MRS broth containing 2% sucrose (black bar, a) or 2% glucose (grey bar, b). Adherence ability was measured by an optical density of 600 nm. A significant difference between the adherence ability of the mutant strains and the wild-type strains is indicated by asterisk (*) ($p < 0.05$). Data shown are the means of three independent experiments with standard deviations.

Figure 4. Su and Gänzle

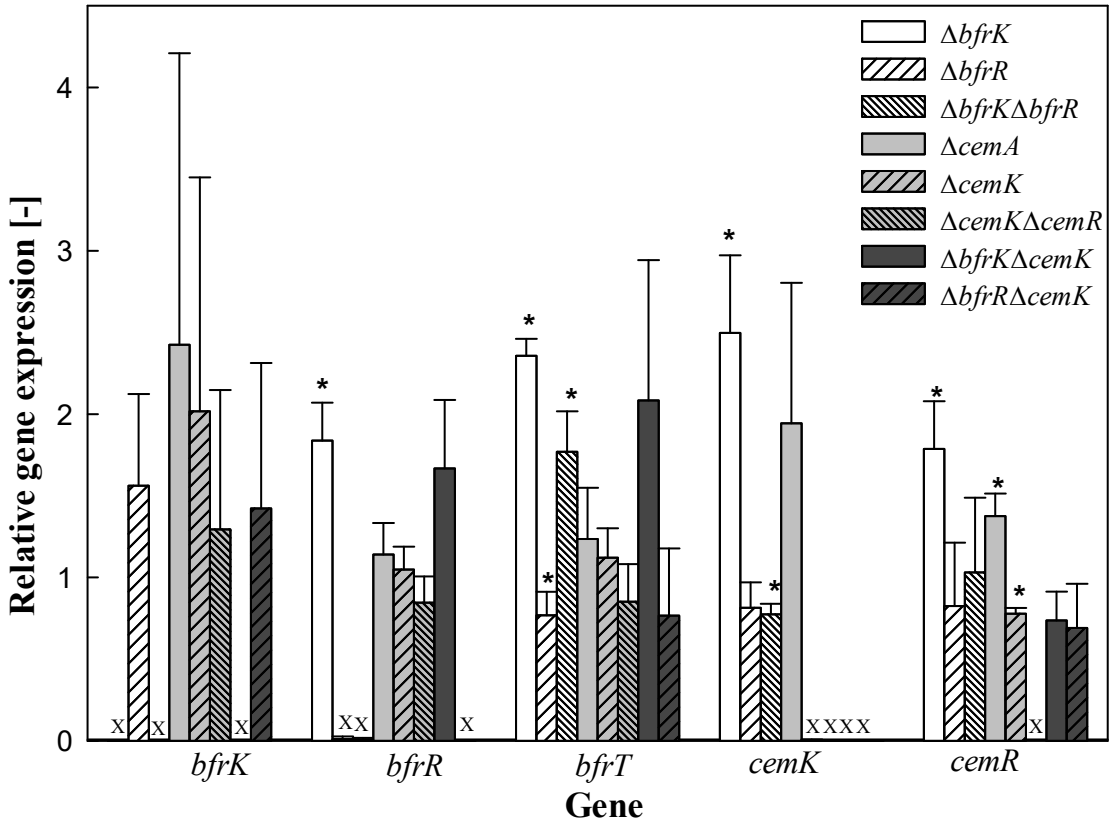

Figure 4. Relative quantification of gene expression in *L. reuteri* 100-23 and its derived mutant strains grown in gluMRS. The expressions of the *bfrKRT* and *cemAKR* operons were determined by qPCR (the y-axis; linear scale) with primers specific to genes *bfrK*, *bfrR*, *bfrT*, *cemK* and *cemR* (the x-axis). The *L. reuteri* isogenic strains used in this study are Δ*bfrK*, Δ*bfrR*, Δ*bfrK*Δ*bfrR*, Δ*cemA*, Δ*cemK*, Δ*cemK*Δ*cemR*, Δ*bfrK*Δ*cemK*, and Δ*bfrR*Δ*cemK*. A significant difference from the wild-type strain (relative gene expression = 1) is indicated by asterisk (*) ($p < 0.05$). The results are shown as means ± the standard deviations of three independent experiments performed in triplicate. . Primers targeting deleted genes in mutant strains yielded no amplicons in RT-qPCR reactions; these controls are indicated by the letter "X".

15 **Figure 5.** Su and Gänzle

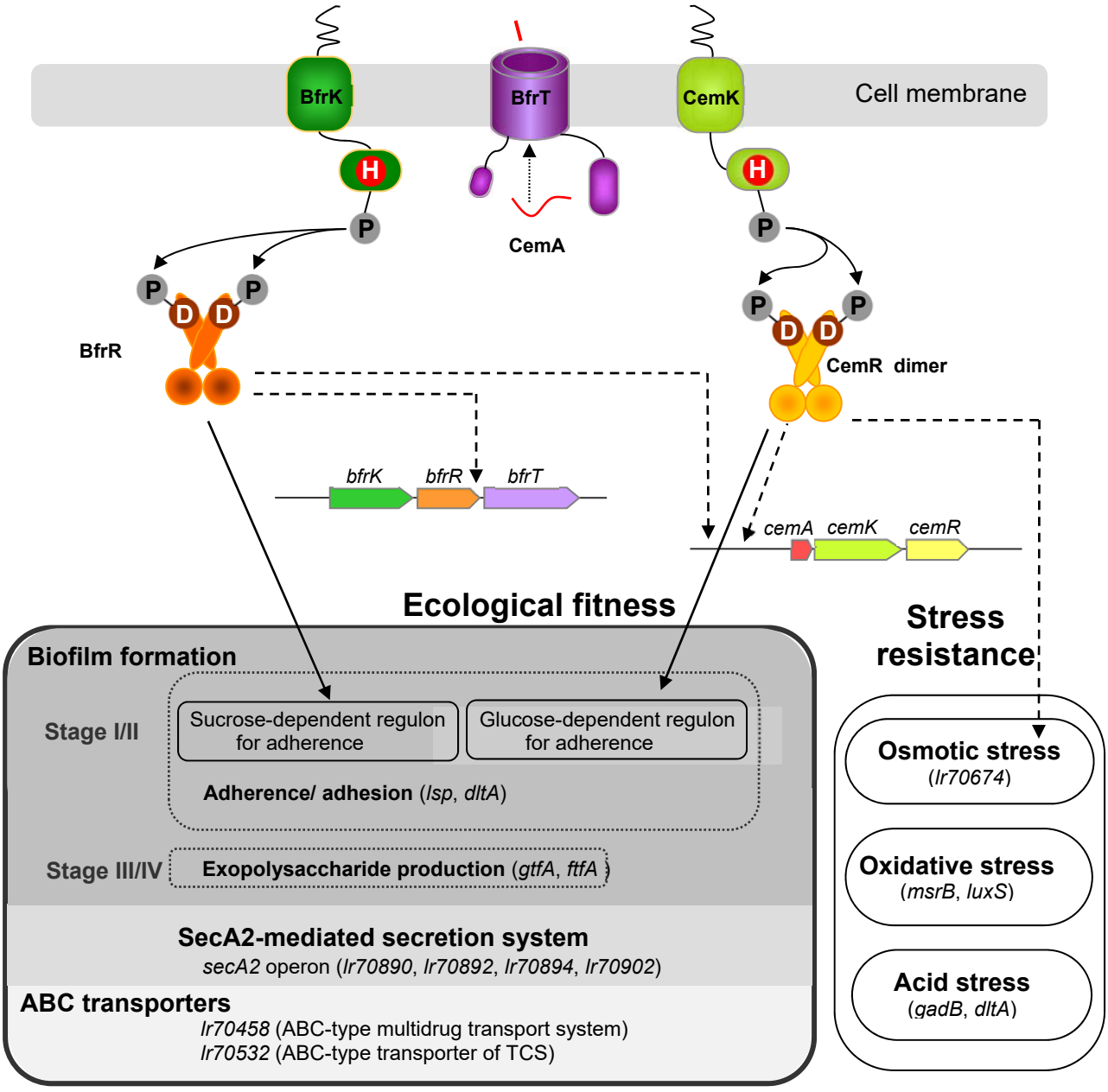

Figure 5. Schematic overview of the TCS regulatory signaling cascade of the *bfrKRT* and *cemAKR* operons, and its relationship to the genes related to ecological performance of *L. reuteri* in the intestinal tract of mice. Dashed lines indicate relationships that were established on the basis of quantification of gene expression. The TCS involves signal sensing by a histidine kinase (BfrK, CemK), followed by histidine (H) phosphorylation and phosphotransfer to an aspartate (D) of a response regulator (BfrR, CemR). Disruptions in *bfrKR* affect expression of *bfrT* as well as genes in the *cemAKR* operon when glucose is the sole carbohydrate source in the growth media; disruption of *cemRK* does not affect expression of *brfKRT* but alters expression of *cemA* and *lr70674* (osmoprotectant binding protein). Because a transport enzyme is not part of the *cemAKR* system, the maturation of the autoinducing peptide CemA may be processed and transported by the ABC transporter BfrT. Mutations in the *bfrKRT* system as well as *cemA* affected predominantly biofilm formation with sucrose as carbon source while disruption of genes in the *cemAKR* system has a stronger effect on glucose-dependent regulons of biofilm formation.

Adherence of *L. reuteri* to mouse epithelial cells or mediated by *lsp* (Walter *et al.,* 2005). The membrane protein D-alanine-D-ananyl carrier protein ligase (DltA) affects *L. reuteri*'s ability to adhere and to resist acid stress (Walter *et al.*, 2007). Extracellular glucansucrases and levansucrase contribute to biofilm formation *in vitro* and ecological fitness *in vivo* (Walter *et al.,* 2008). Disruption of the *secA2* operon and ABC transporters (*lr70458*, *lr70532*) also impaired colonization of mice by *L. reuteri* (Frese *et al.,* 2011).

Methionine-R-sulfoxide reductase, coded by *msrB* in *L. reuteri* 100-23, is an antioxidant repair enzyme reducing methionine sulfoxide to methionine. Disruption of *msrB* has impairs colonisation of mice by *L. reuteri* (Walter *et al.,* 2005). Disruption of *luxS* in *L. reuteri* 100-23 is associated with the metabolic conversion of S-ribosyl homocysteine to homocysteine but not AI-2 quorum-sensing regulation (Wilson *et al.*, 2012). Genes *gadB* (Su *et al.,* 2011) and *dltA* (Walter *et al.*, 2007) increase acid resistance of *L. reuteri*.