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

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This study characterized the two-component regulatory systems encoded by bfrKRT and cemAKR, and assessed their influence on biofilm formation by *Lactobacillus reuteri* 100-23. A method for deletion of multiple genes was employed to disrupt the genetic loci of two-component systems. The operons bfrKRT and cemAKR showed complementary organization. Genes bfrKRT encode 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.

INTRODUCTION

*Lactobacillus reuteri* is 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 a review see Walter, 2008). Because of its stable association with vertebrate hosts, *L. reuteri* has been used as a model organism for the study of host-microbe interaction and host-specific adaptation (Frese et al., 2011; Walter et al., 2011).

The colonization of animals by *L. reuteri* involves 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 comprises four major steps: adherence of cells to surfaces, cell accumulation, clonal maturation and formation of mixed species biofilms (for reviews see Karatan & Watnick, 2009, and 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 found to be 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) mediate adherence of *L. reuteri* to porcine intestinal mucus or...
human epithelial cells. Extracellular polysaccharides produced by the reuteransucrase of *L. reuteri* promote biofilm formation in the murine forestomach and fructansucrases of *L. reuteri* act 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 *Strep. 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 encoded by gene lr70430 is unique to 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 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 signalling 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).

### METHODS

**Bacterial growth.** The bacterial strains and plasmids used in this study are listed in Tables 1 and S2, available in the online Supplementary Material. *Escherichia coli* JM109 (Promega) was cultured at 37 °C in Luria–Bertani (LB) broth with agitation. *E. coli* harbouring pJR233-derived plasmids was cultured in LB broth 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–Birch–Moller–Elsden (MBE) broth. The bacterial strains and plasmids used in this study are listed in Tables 1 and S2, available in the online Supplementary Material.

**DNA isolation and manipulation.** Genomic DNA was isolated using a Blood & Tissue kit (Qiagen) according to the protocol provided by the manufacturer. Oligonucleotides (Table S3) were purchased from Integrated DNA Technologies. Restriction enzymes (New England Biolabs), T\(_4\) DNA ligase (Epigence) and Taq DNA polymerase (Invitrogen) were used for cloning. DNA sequencing was performed after PCR cloning (using TA vector; Invitrogen) (Macrogen).

**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 analysed with the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Amino acid sequences were retrieved from UniProt (Bairoch et al., 2005) and aligned to calculate their identity scores using MUSCLE pairwise alignment (Gonçalves, 5.6.6; Biomatters). Protein function was predicted with the IAS 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.** The in-frame deletion method for generating *L. reuteri* knockout mutants has been described in a previous study (Su et al., 2011). Plasmids and primers used are listed in Tables S2 and S3, respectively. In brief, the 5′- and 3′-flanking sequences of the target genes were amplified by PCR, and referred to as amplicon-A and amplicon-B, respectively. Amplicon-A and amplicon-B were inserted separately into pGEtMEasy vectors to produce pGene-A and pGene-B. Next, restriction enzymes were used to cut out the two amplicons from pGene-A and pGene-B. These two amplicons were then ligated into a pGEtMEasy vector using T\(_4\) DNA ligase, which produced pGene-AB. The ligated DNA fragment AB was cut out of pGene-AB with suitable restriction enzymes, and inserted into the integration shuttle vector pJR233 (Perez-Casal et al., 1993) to generate a knockout plasmid, pKO-Gene-AB. After electroporation of pKO-Gene-AB into the *L. reuteri* wild-type strain, a single-gene knockout mutant was generated by temperature-impulse integration and a plasmid-curing test as described by Su et al. (2011). An antibiotic-sensitive knockout mutant was identified by replica-plating onto mMRS and mMRS-erythromycin agar plates. The truncation of the target gene in the derived deletion mutant was confirmed by PCR with primer set gene-KO-1 and gene-KO-4, and primer set gene-5-F and gene-6-R. The deletion of this region was confirmed by Sanger sequencing using primers gene-5-F and gene-6-R. The same strategy was used to generate double-gene knockout mutants and is detailed in the supplementary material.

**Scanning electron microscope (SEM) analysis of biofilm specimens.** *L. reuteri* 100-23, 100-23ΔcemKΔcemR, TMW1.106 and TMW1.106ΔgfpA were grown on polystyrene plates containing gluMRS or sucMRS broth. After incubation, cells were washed with buffer containing 50 mM Na\(_2\)HPO\(_4\) (pH 6), and fixed with 2.5 % glutaraldehyde in 10 mM PBS buffer (pH 7.4) at 4 °C overnight. Fixed cells were washed twice in PBS buffer and dehydrated by adding a series of 1 ml volumes of an increasingly concentrated ethanol in water solution (70, 85, 95 and 100 %, v/v) at room temperature. Cells were incubated in each ethanol concentration for 10 min. Hexamethyldisilazane (HMDS) (Sigma-Aldrich) was introduced into the cells by gradually increasing the concentration of HMDS in the solution. The following series of HMDS in ethanol solutions was used: 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 into 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). The examination was performed using a SEM XL30 (FEI) at an acceleration voltage of 20 kV.

**RNA extraction.** The MasterPure RNA purification protocol (Epicentre Technologies) was used with slight modifications. Overnight cultures of *L. reuteri* were diluted 50-fold in gluMRS.
Table 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype or description</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. coli</td>
<td>Cloning host for pGEMTeasy- and pJRS233-derived plasmids</td>
<td>Promega</td>
</tr>
<tr>
<td>JM109</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. reuteri</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TMW1.106</td>
<td>Type II sourdough isolate; wild-type strain producing glucan and fructan</td>
<td>Schwab et al. (2007)</td>
</tr>
<tr>
<td>TMW1.106ΔgftA</td>
<td>TMW1.106 ΔgftA::pORI28; non-glucan-producing strain; Erm&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Walter et al., (2008)</td>
</tr>
<tr>
<td>100-23</td>
<td>Rodent isolate; wild-type strain producing levan</td>
<td>Wesney &amp; Tannock (1979)</td>
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<td>100-23ΔbfrK</td>
<td>Truncation of bfrK</td>
<td>This study</td>
</tr>
<tr>
<td>100-23ΔbfrR</td>
<td>Truncation of bfrR</td>
<td>This study</td>
</tr>
<tr>
<td>100-23ΔbfrKΔbfrR</td>
<td>Truncation of bfrK and bfrR</td>
<td>This study</td>
</tr>
<tr>
<td>100-23ΔcemA</td>
<td>Truncation of cemA</td>
<td>This study</td>
</tr>
<tr>
<td>100-23ΔcemK</td>
<td>Truncation of cemK</td>
<td>This study</td>
</tr>
<tr>
<td>100-23ΔcemKΔcemR</td>
<td>Truncation of cemK and cemR</td>
<td>This study</td>
</tr>
<tr>
<td>100-23AbfrKΔcemK</td>
<td>Truncation of bfrK and cemK</td>
<td>This study</td>
</tr>
<tr>
<td>100-23AbfrRΔcemK</td>
<td>Truncation of bfrR and cemK</td>
<td>This study</td>
</tr>
</tbody>
</table>

Erm<sup>f</sup>, Erythromycin-resistance gene.

broth and incubated at 37 °C until an OD<sub>600</sub> of 0.4 was reached. Cells from 10 ml of culture were harvested and RNA synthesis was halted by adding 1.25 ml 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 tissue and cell lysis solution containing 5.5 µg of proteinase K. After incubation on ice for 5 min, 175 µl MPC protein precipitation reagent was added to denature proteins, followed by centrifugation at 10 000 g for 10 min. The supernatant was mixed with 500 µl 2-propanol and nucleic acids were collected by centrifugation. The resulting pellets were rinsed with 75% ethanol solution, suspended in 30 µl nuclease-free water (Ambion) and treated with RNase-free DNase I (Ambion) at 37 °C for 2 h. The reaction was stopped by addition of 5 µl 50 mM EDTA. Five units SUPERase.In (RNase inhibitor; Ambion) were added and the RNA was stored at 4 °C.

**Complementary DNA synthesis.** RNA (2 µg) 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 min, then 25 °C for 10 min and finally chilled to 4 °C. The reaction mix was prepared with the RNA/primer mixture, 5 x first 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).

**Relative quantification of gene expression using quantitative PCR (qPCR).** Gene expression was quantified with qPCR with cDNA

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**Fig. 1.** Schematic representation of the two-component regulatory systems bfrKRT and cemAKR operons and their protein sequence analysis. Bioinformatic analysis of histidine kinases BfrK and CemK revealed an amino-terminal transmembrane domain and a carboxyl-terminal ATPase-like ATP-binding domain, where the specific homology boxes H, X, N and G indicate features of the HPK<sub>10</sub> subfamily. The functional domains of response regulators BfrR and CemR are predicted as a CheY-like superfamily receiver with conserved aspartate (D) and lysine (K) residues at the amino-terminus, along with a 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 an 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. The percentages indicate the protein identity of the histidine kinases and response regulators.
as the template. Gene-specific primers were designed to have amplicons of 90–150 bp in size using Primer Express software 3.0 (Applied Biosystems) (Tables 2). PCR was carried out with custom SYBR Green master mix (MBSU Facility, University of Alberta, Canada) in a 7500 fast real-time PCR instrument (Applied Biosystems). The calculation of the relative gene expression was carried out according to the $\Delta\Delta$Ct method (Pfaffl, 2001). Exponentially growing cells of \textit{L. reuteri} 100-23 were used as a reference condition, and \textit{recA} was used as endogenous gene control. The PCR efficiencies of the primers were experimentally determined with serial dilutions of the cDNA of \textit{L. reuteri} 100-23 and calculated as described elsewhere (Pfaffl, 2001) 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 were 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. (2000) with modifications. Cells from cultures grown overnight were washed, subcultured in 2 ml gluMRS or sucMRS media and incubated in 35 × 10 mm polystyrene Petri dishes. After 24 h incubation, the supernatants were discarded and the wells were washed twice with 50 mM NaH$_2$PO$_4$ (pH 6) buffer. Cells adhering to the plate were scraped using plastic tips and resuspended in 1 ml phosphate buffer. The cell density was determined by measuring the OD$_{600}$. Analysis was performed in triplicate independent experiments with two or three technical repeats per replicate.

**Statistical analysis.** Statistical analysis was performed using Student’s $t$-test (SigmaPlot; version 11.0).

**Sequences and accession numbers.** The nucleotide sequence of the \textit{bfrKRT} and \textit{cemAKR} operons were retrieved from the GenBank database (accession number NZ_AAPZ00000000.2, locus tags: Lreu23DRAFT\_4807 for \textit{bfrK}; Lreu23DRAFT\_4808 for \textit{bfrR}; Lreu23DRAFT\_4809 for \textit{bfrT}; Lreu23DRAFT\_4825 for \textit{cemK}; and Lreu23DRAFT\_4826 for \textit{cemR}). The nucleotide sequence of \textit{cemA} is 5'-ATGCAAAAACATATCATCATTCACTATCTTTTAAAAATGGGG-TGGTTATATACGTAGTTAATGCGTTAATAGCTCTAGAAA-3', and the predicted protein sequence is MQKLSIHQLSLIKGGIGYSL. The nucleotide sequences of the \textit{L. reuteri} mutant strains were deposited in GenBank with the following accession numbers: \textit{ΔcemA}, JF339968; \textit{ΔbfrK}, KF306072; \textit{ΔbfrKΔcemA}, KF306073; \textit{ΔcemK}, KF306074; \textit{ΔbfrR}, KF306075; \textit{ΔcemKΔcemR}, KF306076; \textit{ΔcemKR}, KF306077; \textit{ΔcemKΔcemR}, KF306078; \textit{ΔcemKΔcemR}, KF306079.

**RESULTS**

**In silico prediction of the genetic loci \textit{bfrKRT} and \textit{cemAKR}**

Inactivation of the gene \textit{bfrK} (lr70430) impairs the ecological fitness of \textit{L. reuteri} 100-23 in the intestinal tract of mice (Frese et al., 2011), but the function of the \textit{bfrKRT} operon remains unclear. The two-component system \textit{bfrKRT} was predicted to be a peptide-based quorum-sensing two-component regulatory system. Analysis also identified \textit{cemAKR}, a two-component system with high sequence homology to \textit{bfrKRT} (Fig. 1). The \textit{bfrKRT} operon consists of genes encoding 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 \textit{cemAKR} operon is composed of genes encoding 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 \textit{BAGEL} program identified the putative signal transduction peptide IYSLLSL as \textit{cemA} in \textit{L. reuteri} 100-23. The nucleotide sequences of \textit{bfrK} and \textit{cemK} are very similar, as are \textit{bfrR} and \textit{cemR} (Fig. 1). \textit{bfrKR} and \textit{cemKR} are similar to the bacteriocin-related two-component system \textit{abpBR} in \textit{Lactobacillus salivarius} UCC118; \textit{bfrT} is similar to \textit{abpT} encoding the cognate bacteriocin export accessory protein in \textit{L. salivarius} UCC118 (KEGG database). The complementary genetic organization of the two operons implies a co-operation of the \textit{bfrKRT} and \textit{cemAKR} operons. The response regulator \textit{cemR} was compared to other members of the LytR/AlgR family by BLAST analysis and MUSCLE alignment. CemR was similar to AgrA (Peng et al., 1988), SppR (Brurberg et al., 1997), PnC.
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), 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 ΔbfrK, ΔbfrR, ΔcemA and ΔcemK, as well as the double-gene deletion mutants ΔbfrKΔbfrR, ΔcemKΔcemR, ΔbfrKΔcemK and ΔbfrRΔcemK (Table 1), were generated using site-specific homologous recombination mutagenesis, and verified by PCR and DNA sequencing (Tables 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

![Fig. 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 (a) or 2% glucose (b). Adherence ability was measured by the optical density at 600 nm. A significant difference between the adherence ability of the mutant strain and the wild-type strain is indicated by an asterisk (P<0.05). Data shown are the means of three independent experiments with sds.](http://mic.sgmjournals.org)

![Fig. 4. Relative quantification of gene expression in L. reuteri 100-23 and its derived mutant strains grown in gluMRS. The expression of the bfrKRT and cemAKR operons was 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 were ΔbfrK, ΔbfrR, ΔbfrKΔbfrR, ΔcemA, ΔcemK, ΔcemKΔcemR, ΔbfrKΔcemK and ΔbfrRΔcemK. A significant difference from the wild-type strain (relative gene expression=1) is indicated by an asterisk (P<0.05). The results are shown as means±sds of three independent experiments performed in triplicate. Primers targeting deleted genes in mutant strains yielded no amplicons in reverse transcription (RT) qPCRs; these controls are indicated by the letter ‘X’.](http://mic.sgmjournals.org)
Table 2. qPCR primers used in this study

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’→3’)</th>
<th>Target gene’s locus tag</th>
<th>Features of putative protein</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>recA</em>-qPCR-F2</td>
<td>CAACTATCCGGATGAAATTTGTCG</td>
<td>Lreu23DRAFT_3582</td>
<td>RecA; endogenous protein; DNA recombination</td>
</tr>
<tr>
<td><em>recA</em>-qPCR-R2</td>
<td>TGTCAACCTTGCCACCAATGGTTGAAGGC</td>
<td>Lreu23DRAFT_4807</td>
<td>BfrK; histidine kinase of the HPK&lt;sub&gt;10&lt;/sub&gt; family; two-component system</td>
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<td><em>bfrK</em>-qPCR-F1</td>
<td>CCGGACTAGGCTATTTGGATGCTATT</td>
<td>Lreu23DRAFT_4808</td>
<td>BfrR; response regulator of the LytR/AlgR family; two-component system</td>
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<tr>
<td><em>bfrK</em>-qPCR-R1</td>
<td>GTGGAATGCCCTGGCTTGTGA</td>
<td>Lreu23DRAFT_4809</td>
<td>BfrT; ABC-type bacteriocin transporter; two-component system</td>
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<td><em>bfr</em>-qPCR-F2</td>
<td>ACTAAGACCTGCAATGTTGCGATGAT</td>
<td>Lreu23DRAFT_4825</td>
<td>CemK; histidine kinase of the HPK&lt;sub&gt;10&lt;/sub&gt; family; two-component system</td>
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<td><em>bfr</em>-qPCR-R2</td>
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<td>Lreu23DRAFT_4826</td>
<td>CemR; response regulator of the LytR/AlgR family; two-component system</td>
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<td>cemK-qPCR-F1</td>
<td>AGGACTTACTTTTGAACTTTGCAACATTCTT</td>
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<td>CemA; autoinducing peptide of peptide-based quorum-sensing two-component system</td>
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<td>LytS; histidine kinase; cell autolysis; two-component system</td>
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<td>cemA-qPCR-F2</td>
<td>TGGATATATTGACATCAATGTCATCG</td>
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<td>Homologue of BspA/CyuC/MapA/CnBP collagen-binding protein; cystine transporter, amino acid</td>
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<td>TTAATTCACAGACCTAAAGTGAATACTACCC</td>
<td>Lreu23DRAFT_4288</td>
<td>ABC transporter; amino acid ABC transporter substrate-binding protein, PAAT family; signal transduction systems, periplasmic component/domain</td>
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<td>lr69269-qPCR762-F2</td>
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<td>Bacteriocin-type signal sequence; quorum-sensing two-component system</td>
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<td>lr69269-qPCR836-R2</td>
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<td>Lreu23DRAFT_4979</td>
<td>Cyclopropane-fatty-acyl-phospholipid synthase; cyclopropane synthesis</td>
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<td>lr69271-qPCR46-F2</td>
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<td>AGGAACACAGGAACACTCTGAGGAAAAA</td>
<td>Lreu23DRAFT_5027</td>
<td>Glycine betaine/choline-binding (lipo)protein of an ABC-type transport system; osmoprotectant-binding protein</td>
</tr>
<tr>
<td>lr69272-qPCR278-F2</td>
<td>CGCTTTATCGCCGGATAAG</td>
<td>Lreu23DRAFT_3826</td>
<td>Large surface protein with LPXTG-motif cell wall anchor domain; homologue of Fta/levansucrase/inulosucrase; exopolysaccharide synthesis</td>
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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 vitro* and *in vivo*, was used for comparison (Walter et al., 2008). Scanning electron microscopy was used to visualize the structure of biofilms grown in the presence of glucose or sucrose (Fig. 2). In sucMRS, *L. reuteri* 100-23 and 100-23ΔcemKΔcemR formed thick, stack-structured biofilms (Fig. 2a, c). Biofilm formation was also observed with *L. reuteri* TMW1.106 (Fig. 2e). However, *L. reuteri* TMW1.106ΔgtfA failed to form biofilms, owing to the disruption of reuteransucrase, which produces extracellular glucan as biofilm matrix (Walter et al., 2008; Fig. 2g). In gluMRS, *L. reuteri* 100-23 did not form a biofilm (Fig. 2b), while *L. reuteri* ΔcemKΔcemR developed highly complex layers of biofilm (Fig. 2d). Biofilms formed by *L. reuteri* TMW1.106 in gluMRS (Fig. 2f) were more dense when compared to *L. reuteri* TMW1.106ΔgtfA (Fig. 2h). These results indicate that mechanisms of biofilm formation in *L. reuteri* are strain specific, and specific for different carbon sources. The *cemAKR* operon appears to regulate glucose-dependent biofilm formation in *L. reuteri* 100-23.

The microscopic observation of biofilm formation was verified by a quantitative assay (Fig. 3). Consistent with the microscopic observation, *L. reuteri* TMW1.106 formed biofilms in gluMRS or sucMRS, but *L. reuteri* TMW1.106ΔgtfA was unable to form biofilm in either medium (Fig. 3). When grown in presence of sucrose, *L. reuteri* 100-23 and all mutants except *L. reuteri* 100-23ΔbfrKΔcemK formed biofilms (Fig. 3a). Cell densities of *L. reuteri* 100-23ΔbfrR, 100-23ΔbfrKΔbfrR, 100-23ΔcemA and 100-23ΔbfrRΔcemK were higher when compared to *L. reuteri* 100-23. The density of cells of *L. reuteri* 100-23 adhering to the Petri dishes after growth in gluMRS (Fig. 3b) was reduced in comparison to cells adhered in sucMRS. When growing with glucose as the 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 the *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 *bfrKRT* and *cemAKR* regulons: analysis of gene expression by RT qPCR

The expression of genes in the *bfrKRT* and *cemAKR* operons was determined by RT-qPCR 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 ΔbfrK and ΔbfrKΔbfrR, decreased in strain ΔbfrR, 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 signalling cascade, the *L. reuteri* ΔcemKΔcemR 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* ΔcemKΔcemR 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, the tolerance of *L. reuteri* ΔcemKΔcemR to osmotic stress was not different when compared to the wild-type strain (Table S4).

### DISCUSSION

This study characterized the two-component regulatory systems *bfrKRT* and *cemAKR* operons, and assessed their

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5’→3’)</th>
<th>Target gene’s locus tag</th>
<th>Features of putative protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>lr71188-qPCR259-F1</td>
<td>CCAAGGTTTTGGCCGGATT</td>
<td>Lreu23DRAFT_3689</td>
<td>Cell division-specific peptidoglycan biosynthesis regulator FtsW; cell division</td>
</tr>
<tr>
<td>lr71188-qPCR333-R1</td>
<td>AACAGCTCGGCTAAAGACTAAAAC</td>
<td>Lreu23DRAFT_3623</td>
<td>Acetate kinase; carbohydrate metabolism</td>
</tr>
<tr>
<td>lr71258-qPCR638-F1</td>
<td>CAATCACTGCGTTAAAGAGGTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>lr71258-qPCR712-R1</td>
<td>CCATTGGTTATCCCGCAACAG</td>
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</tr>
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</table>

ND, Not determined.
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 two-component system signalling 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 Strep. mutans and Staphylococcus aureus. Two-component systems known to regulate biofilm formation include the comCDE system of Strep. mutans (Senadheera & Cvitkovich, 2008) and the agrBDDCA system of Staph. 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 the mechanisms of biofilm formation exhibit strain-specific differences. The formation of biofilms by L. reuteri and TMW1.106 in vitro and in vivo is supported by sucrose, a constant carbon supply (sucrose or glucose) and because competing microbiota are absent. However, the in vitro biofilm formation and coaggregation by L. reuteri TMW1.106, LTH5448 and 100-23 generally corresponded to competitive-ness and biofilm formation in vivo (Walter et al., 2008, Sims et al., 2011, Frese et al., 2011; this study).

Although the formation of biofilms by L. reuteri 100-23 and TMW1.106 in vitro and in vivo is supported by sucrose, the mechanisms of biofilm formation exhibit strain-specific differences. L. reuteri TMW1.106 but not 100-23 employs an extracellular reuteransucrase to synthesize the biofilm matrix (Schwab et al., 2007; Sims et al., 2011; Walter et al., 2008). Analysis of the genetic locus of bfrKRT in L. reuteri 100-23 identified two IS elements upstream and downstream of bfrKRT. The presence of bfrKRT in rodent lineage strains of L. reuteri is variable but the operon is rodent specific (Frese et al., 2011). It is likely that the bfrKRT operon results from lateral gene transfer in a manner similar to that of fructansucrase (ffr) 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 the sole carbon source.
source when compared to \textit{L. reuteri} TMW1.106. With glucose as the sole carbon source, biofilm formation of \textit{L. reuteri} 100-23 was repressed by proteins encoded by the \textit{cemAKR} operon. \textit{BfrK} is overexpressed by \textit{L. reuteri} 100-23 during colonization of the murine forestomach epithelium (Frese \textit{et al.}, 2013) and disruption of \textit{bfrK} impaired \textit{in vivo}

**Fig. 5.** Schematic overview of the two-component system regulatory signalling cascade of the \textit{bfrKRT} and \textit{cemAKR} operons, and its relationship to the genes related to ecological performance of \textit{L. reuteri} in the intestinal tract of mice. Dashed lines indicate relationships that were established on the basis of quantification of gene expression. The two-component system 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 \textit{bfrKR} affect expression of \textit{bfrT}, as well as genes in the \textit{cemAKR} operon, when glucose is the sole carbohydrate source in the growth media; disruption of \textit{cemRK} does not affect expression of \textit{bfrKRT}, but alters expression of \textit{cemA} and \textit{lr70674} (osmoprotectant-binding protein). Because a transport enzyme is not part of the \textit{cemAKR} system, the maturation of the autoinducing peptide CemA may involve processing and transportation by the ABC transporter BfrT. Mutations in the \textit{bfrKRT} system, as well as \textit{cemA}, affected predominantly biofilm formation with sucrose as the carbon source, while disruption of genes in the \textit{cemAKR} system has a stronger effect on glucose-dependent regulons of biofilm formation. Adherence of \textit{L. reuteri} to mouse epithelial cells is mediated by \textit{lsp} (Walter \textit{et al.}, 2005). The membrane protein \textit{d}-alanine-\textit{d}-ananyl carrier protein ligase (DltA) affects the ability of \textit{L. reuteri} to adhere and to resist acid stress (Walter \textit{et al.}, 2007). Extracellular glucansucrases and levansucrase contribute to biofilm formation \textit{in vitro} and ecological fitness \textit{in vivo} (Walter \textit{et al.}, 2008). Disruption of the \textit{secA2} operon and ABC transporters (\textit{lr70458}, \textit{lr70532}) also impaired colonization of mice by \textit{L. reuteri} (Frese \textit{et al.}, 2011). Methionine-\textit{R}-sulfoxide reductase, encoded by \textit{msrB} in \textit{L. reuteri} 100-23, is an antioxidant repair enzyme reducing methionine sulfoxide to methionine. Disruption of \textit{msrB} impairs colonization of mice by \textit{L. reuteri} (Walter \textit{et al.}, 2005). Disruption of \textit{luxS} in \textit{L. reuteri} 100-23 is associated with the metabolic conversion of \textit{S}-ribosyl homocysteine to homocysteine, but not AI-2 quorum-sensing regulation (Wilson \textit{et al.}, 2012). Genes \textit{gadB} (Su \textit{et al.}, 2011) and \textit{dltA} (Walter \textit{et al.}, 2007) increase the acid resistance of \textit{L. reuteri}.
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 the adherence and biofilm formation ability of L. reuteri 100-23 with sucrose or glucose as the carbon source. 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 has allowed the identification of genetic or metabolic traits that are required for colonization of intestinal ecosystems. Genetic traits of L. reuteri that contribute to biofilm formation and the ecological fitness in rodents were recently reviewed (Frese et al., 2011, 2013; Walter, 2008) and are depicted in Fig. 5. Biofilm formation contributes to the competitiveness of L. reuteri in intestinal ecosystems. However, the proteins that are involved in attachment and biofilm formation by L. reuteri are strain specific and have not been fully elucidated (Frese et al., 2011; Walter et al., 2008). Attachment of L. reuteri to intestinal epithelia is mediated by the large surface protein coded by lsp (Walter et al., 2005), the mucus adhesion-promoting protein MapA or the mucus-binding protein Mub (Miyoshi et al., 2006; Roos & Jonsson, 2002). The D-alanine-D-ananyl carrier protein ligase (DltA) also affects the ability of L. reuteri to adhere and to resist acid stress (Walter et al., 2007). The present study demonstrated that disruption of bfrKRT enhanced sucrose-dependent biofilm formation of L. reuteri 100-23, and altered expression of the cemAKR operon (Fig. 5). Disruption of genes in the cemAKR operon enhanced glucose-dependent biofilm formation. Unlike the comCDE system of Strep. mutans and the agrBDCA system of Staph. aureus, the bfrKRT and cemAKR systems of L. reuteri 100-23 did not influence the expression of genes encoding exopolysaccharide formation, biofilm dispersal (the ltySR system and the lrg operon) or another quorum-sensing autoinducing peptide and ABC transporter (lr70531, lr70532). Data presented in this study link the bfrKRT and cemAKR systems of L. reuteri to biofilm formation.

In conclusion, this study characterized several single and multiple deletion mutants to characterize the two-component systems bfrKRT and cemAKR in L. reuteri 100-23. Deletion of the histidine kinase BfrK impairs ecological fitness of L. reuteri 100-23 in mice, but the function of the operons remained unknown (Frese et al., 2011). Deletion of single or multiple genes in the operons bfrKRT 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 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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Two-component systems and biofilms in L. reuteri


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