Transcriptional and Metabolomic Consequences of *luxS* Inactivation Reveal a Metabolic Rather than Quorum-Sensing Role for LuxS in *Lactobacillus reuteri* 100-23

Charlotte M. Wilson,^a Raphael B. M. Aggio,^c Paul W. O'Toole,^b Silas Villas-Boas,^c and Gerald W. Tannock^a

Department of Microbiology and Immunology, University of Otago, Dunedin, New Zealand^a; Department of Microbiology and Alimentary Pharmabiotic Centre, University College Cork, Cork, Ireland^b; and Centre for Microbial Innovation, School of Biological Sciences, University of Auckland, Auckland, New Zealand^c

Autoinducer-2 (AI-2)-mediated quorum sensing has been extensively studied in relation to the regulation of microbial behavior. There are, however, two potential roles for the AI-2 synthase (LuxS). The first is in the production of AI-2 and the second is as an enzyme in the activated methyl cycle, where it catalyzes the conversion of S-ribosylhomocysteine to homocysteine. The by-product of the reaction catalyzed by LuxS is (S)-4,5-dihydroxy-2,3-pentanedione, which spontaneously forms the furanones known collectively as AI-2. The mammalian gut contains a complex collection of bacterial species so a method of interspecies communication might influence community structure and function. *Lactobacillus reuteri* 100-23 is an autochthonous inhabitant of the rodent forestomach, where it adheres to the nonsecretory epithelium, forming a biofilm. Microarray comparisons of gene expression profiles of the *L. reuteri* 100-23 wild type and a *luxS* mutant under different culture conditions revealed altered transcription of genes encoding proteins associated with cysteine biosynthesis/oxidative stress response, urease activity, and sortase-dependent proteins. Metabolomic analysis showed that the *luxS* mutation affected cellular levels of fermentation products, fatty acids and amino acids. Cell density-dependent changes (log phase versus stationary phase growth) in gene transcription were not detected, indicating that AI-2 was unlikely to be involved in gene regulation mediated by quorum sensing in *L. reuteri* 100-23.

Quorum sensing, in which gene transcription is regulated in response to a change in cell density, is facilitated by the synthesis, release, and detection of small signaling molecules (7). One candidate signaling molecule is AI-2, which is formed during the LuxS-catalyzed reaction in the activated methyl cycle (AMC) (19). The *luxS* gene is present in several Gram-positive and Gram-negative bacterial species, but few species have been definitively shown to use a LuxS/AI-2 quorum-sensing system (see reviews in references 10, 17, and 20). The demonstration of AI-2-dependent quorum sensing by a bacterial species is hindered by the difficulty in differentiating between the two potential roles of LuxS, the first as an enzyme in the AMC pathway and the second as the enzyme responsible for AI-2 production. Inactivation of the *luxS* gene prevents the AMC recycling intermediates to homocysteine as well as disrupting AI-2 production.

In previous work, a *luxS* mutant of *Lactobacillus reuteri* 100-23 did not grow differently from the wild type in rich growth medium but had a lower cellular ATP concentration (65%) than the wild-type strain (15). The mutant strain also differed from the wild type in the ability to form a biofilm. Thicker biofilms were formed by the mutant in a bioreactor and on the epithelial surface of the mouse forestomach than by the wild type (15). The addition of purified AI-2 from *Salmonella enterica* serovar Typhimurium to the *luxS* mutant culture in a bioreactor did not restore wild-type biofilm characteristics (15). The *luxS* mutant strain had poor competitive ability with another *Lactobacillus* strain in the murine large bowel, indicating that LuxS was an important enzyme in gut ecology of *L. reuteri* 100-23 (15). We sought to determine the effect of the *luxS* mutation on the transcriptome and metabolome of *L. reuteri* 100-23 and to evaluate whether the impacts of the mutation were likely to be due to AI-2-mediated quorum sensing.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *L. reuteri* 100-23C, a plasmid-free derivative of strain 100-23, was used in the experiments. The plasmids in 100-23 do not encode metabolic activities (GenBank accession no. GU108604.1 [U21859.1]). The *luxS* mutant of strain 100-23C was derived previously by site-specific integration of plasmid pORI28 into the *L. reuteri* chromosome (15). Cultures were grown anaerobically in filter-sterilized MRS medium (Becton Dickinson Co., Sparks, MD) at 37°C. Erythromycin (5 µg/ml) was included in the culture medium used to maintain the mutant but not in cultures used to harvest cells for RNA isolation or metabolomic assays.

Microarray analysis. Cells from 5-ml cultures grown in MRS for 4 h (AI-2 accumulation highest [15]) or 8 h (AI-2 levels returned to baseline [15]) were harvested by centrifugation (12,000 × g, 30 s, room temperature). Methods for RNA extraction from cultures and reverse transcription to generate cDNA are described in the supplemental material. The Cy-labeled cDNA samples were hybridized to a custom glass slide microarray (GEO submission GPL10986) using an Agilent Gene Expression Hybridization kit (part number 5188-5242) in an Agilent Hybridization oven (G2545A) at 65°C for 24 h. Further information on the microarray design is provided in the supplemental material. Microarrays were scanned using a GenePix 400B Scanner (Axon Instruments, USA) and GenePix Pro 6.0 software. The slides were scanned at 5 µm and a photomultiplier tube (PMT) setting of 720 for the red channel (635-nm laser measuring the Cy5-labeled nucleic acid) and 630 for the green channel (532-nm laser measuring the Cy3-labeled nucleic acid). A GenePix array

Received 6 October 2011 Accepted 4 January 2012

Published ahead of print 27 January 2012

Address correspondence to G. W. Tannock, gerald.tannock@otago.ac.nz.

Supplemental material for this article may be found at <http://jb.asm.org/>.

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doi:10.1128/JB.06318-11

TABLE 1 RT-qPCR of selected genes that were differentially expressed in microarray comparisons of the *luxS* mutant and the wild type during log and stationary growth phases

Genetic locus	Function	Fold change in transcription ^a			
		<i>luxS</i> mutant relative to wild type at log phase		<i>luxS</i> mutant relative to wild type at stationary phase	
		Microarray	RT-qPCR	Microarray	RT-qPCR
Lreu23DRAFT_4487	Predicted transcriptional regulator	0.11	0.07	0.12	0.19
Lreu23DRAFT_4533	Lipoprotein, YaeC family	37.54	40.57	10.63	12.29
Lreu23DRAFT_4688	Cysteine synthase	3.62	3.21	2.59	3.17
Lreu23DRAFT_4777	Predicted oxidoreductases	2.27	8.26	ND	ND
Lreu23DRAFT_4778	Predicted transcriptional regulator	ND	ND	3.35	5.07
Lreu23DRAFT_5059	Unknown function	0.04	0.04	0.07	0.06

^a Values for RT-qPCR were calculated by the comparative $2^{-\Delta\Delta CT}$ method using 16S rRNA as the normalizer for transcript abundance. ND, differential gene expression not determined.

list file was used for extraction of the microarray results. Feature extraction was performed using Agilent feature extraction software (version 9.5.3.1). Microarray data outliers were removed with the Grubbs test (4), and *P* values were calculated with the Cyber-*t* test (5). The expression patterns of a subset of genes were checked by reverse transcription-quantitative PCR (RT-qPCR) and used primers as described in Table S1 in the supplemental material.

Metabolomics. (i) Sample preparation and relative quantification of metabolites. An overnight culture of either the wild-type or the *luxS* strain of *L. reuteri* 100-23 was used to inoculate (0.01% [vol/vol] inoculum; three biological replicates of each strain) each prereduced and prewarmed 100-ml volume of MRS at a 1/5 concentration (diluted to reduce medium component interference in the metabolite analysis). On reaching an optical density at 600 nm of 0.4 (log phase), five lots of 5-ml aliquots of culture were centrifuged, and the supernatants were retained for extracellular metabolite (exometabolome) analysis. For intracellular metabolite (endometabolome) profiling, the remaining culture was quenched (13) using 12 5-ml aliquots in 20 ml of cold 60% (vol/vol) glycerol diluted in 0.9% (wt/vol) NaCl solution held at -23°C . After 3 to 5 min of acclimatization, the quenched samples were centrifuged for 20 min at $36,465 \times g$ at -20°C . The pellets were washed in 2 ml of cold 50% (vol/vol) glycerol diluted in 0.9% (wt/vol) NaCl solution at -23°C as described above. Three pellets were pooled to give four samples suspended in 2.5 ml of cold 50% (vol/vol) methanol diluted in H_2O at -20°C .

(ii) Analysis of metabolite content. The analysis of metabolite content of the *luxS* and wild-type *L. reuteri* 100-23C strains followed the procedure outlined by Smart et al. (13). Briefly, an internal standard (D-[2,3,3,3,3]-alanine) was added to quenched samples, which were further subjected to three freeze-thaw cycles to extract intracellular metabolites (endometabolome). After each cycle, the supernatant was recovered, and the cell pellet was suspended in 2.5 ml of cold 50% (vol/vol) methanol diluted in H_2O at -20°C . The supernatants were pooled together and stored at -80°C . For later normalization of each intracellular metabolite, the biomass of each sample was determined by quantifying the protein content of each remaining cell pellet (18). For exometabolome analysis, 10 ml of spent culture medium was filtered through a $0.2\text{-}\mu\text{m}$ -pore-size syringe-driven filter and stored at -20°C (13). Prior to gas chromatography-mass spectrometry (GC-MS) analysis, the internal standard (D-[2,3,3,3,3]-alanine) was added to the samples (endo- and exometabolome), and these were further concentrated by freeze-drying and derivatized using methyl chloroformate (MCF) (13). After analysis by GC-MS (13), the peak height of each MCF derivative was used to determine the relative concentrations of metabolites in the *luxS* samples relative to the wild-type samples. The MCF derivatives present in the endometabolome were normalized by the amount of protein-related biomass and internal standard, while the MCF derivatives present in the exometabolome were normalized by biomass content, internal standard, and the metabolite concentrations detected in the uncultured medium (13, 18). The identification and

quantification of metabolites was performed by AMDIS and Metab (1). Differences in metabolite abundance between the *luxS* mutant and wild-type strains were calculated, and the significance of this difference was determined by using the Mann-Whitney nonparametric test (6).

RESULTS AND DISCUSSION

The results of mutant and wild-type transcriptomic comparisons are shown in Table S2 in the supplemental material. Overall, 179 genes were differentially regulated. Nineteen genes were transcribed by >2 -fold in the *luxS* mutant compared to the wild type under all growth conditions. A total of 139 genes were either up-regulated or downregulated under all growth conditions but not above the 2-fold threshold in every case. The remaining 21 genes had variable transcription according to growth conditions. Clusters of genes associated with cysteine biosynthesis/oxidative stress response, sortase-dependent proteins, and urease activity exhibited differential expression (see Table S3 in the supplemental material). Differential gene expression observed in microarray exper-

TABLE 2 Differences in relative abundances of metabolites in endo- and exometabolomes of the wild-type and *luxS* mutant strains

Metabolite	Strain with greatest abundance ^a
Phenylalanine	WT***
Lactic acid	LuxS***
2,4-Bis(1,1-dimethylethyl)-phenol	LuxS***
Leucine	LuxS***
Docosanoic acid	LuxS**
Aspartic acid	LuxS***
Glutamic acid	LuxS***
Proline	LuxS***
Threonine	LuxS***
Myristic acid/tetradecanoic acid	LuxS*
3,5-Bis(1,1-dimethylethyl)-4-hydroxy-benzene propanoic acid	LuxS***
Azelaic acid	LuxS**
Methionine	LuxS***
Caprylate	LuxS**
Methoxysuccinate	LuxS*
10-Oxo-octadecanoic acid	LuxS**
Isoleucine	LuxS*
Citric acid (exometabolome)	LuxS*
Lactic acid (exometabolome)	LuxS*

^a The degree of significance is indicated: *, $P = 0.01$ to 0.05 ; **, $P = 0.001$ to 0.01 ; ***, $P \leq 0.001$. WT, wild type.

FIG 1 Overview of transcriptomic and metabolomic changes induced by insertional mutation of the *luxS* gene in *L. reuteri* 100-23C. Black solid arrows indicate the genes encoding the enzymes that catalyze these reactions were upregulated in the *luxS* mutant under all growth conditions. Black dashed arrows indicate the genes encoding the enzymes that catalyze these reactions were upregulated in the *luxS* mutant under some growth conditions. Gray solid arrows indicate the genes encoding the enzymes that catalyze these reactions were not affected in the *luxS* mutant. Gray dashed arrows indicate proposed effects/pathways. The metabolites in capital letters were more abundant in the *luxS* mutant than in the wild-type strain. SRH, S-ribosylhomocysteine. *, Glutaminase gene not above the 2-fold threshold for differential gene expression.

iments was confirmed using RT-qPCR in a subset of genes (Table 1). None of the transcriptional patterns were reversed between log and stationary growth phases. This observation suggested that cell density-dependent gene regulation (quorum sensing) had not occurred.

Endo- and exometabolome comparisons are recorded in Table 2. In summary, the levels of 17 metabolites were greater in the endometabolome of the *luxS* mutant than in the wild type. These metabolites included seven amino acids, six fatty acids, and lactic and citric acids.

We propose a simple model to explain the impact of LuxS inactivation on cell physiology (Fig. 1). The major effect of mutation of the *luxS* gene centers on the synthesis of cysteine. The transcription of genes (*cgl* and *cysK*) associated with the conversion of O-acetyl-serine, cystine, or cystathionine to cysteine were upregulated in the mutant (summarized in Table 3), as were genes (*cysABC*) known to be associated with cystine transport in other lactobacilli (16). These altered transcriptional activities probably arise because the incomplete AMC cycle in the mutant prevents sufficient production of cysteine by the reverse trans-sulfuration pathway. Similar transcriptional patterns have been observed in *L. reuteri* JCM112 grown in the absence of cysteine (11). These met-

TABLE 3 Summary of gene expression in relation to the proposed model of metabolic perturbation^a

Gene	Related function	Gene expression (up- or downregulated)
Lreu23DRAFT_5058	Sortase-dependent proteins	Down
Lreu23DRAFT_4486		Up
Lreu23DRAFT_5145	Disulfide stress	Up
Lreu23DRAFT_3422		Up
Lreu23DRAFT_3601		Up
Lreu23DRAFT_4110		Up
Lreu23DRAFT_4344		Up
Lreu23DRAFT_3336	Cystathione-gamma-lyase	Up
Lreu23DRAFT_4688	Cysteine synthase	Up
Lreu23DRAFT_3337	Cystine uptake	Up
Lreu23DRAFT_3338		Up
Lreu23DRAFT_3339		Up
Lreu23DRAFT_4512	Urease	Up
Lreu23DRAFT_4513		Up
Lreu23DRAFT_4514		Up
Lreu23DRAFT_3556	Glutaminase	Up

^a Further details are provided in Table S2 in the supplemental material.

abolic changes seem to have caused “disulfide stress” similar to that seen in *Bacillus subtilis* (9), where an Spx transcriptional regulator controls expression of genes encoding thioredoxin and oxidoreductase-related genes. Four homologous genes were upregulated in the *luxS* mutant, perhaps resulting in downregulation of 102 prophage genes (see Table S2 in the supplemental material). This type of stress regulation of prophage genes has been reported in *Lactobacillus plantarum* and *B. subtilis* (12, 14).

We propose that the accumulation of methionine in mutant cells reflects the derangement of the AMC. Glutamic acid probably accumulated from glutaminase-catalyzed reactions that would provide ammonia to balance the increased production of lactic acid within the cell. Glycolytic pathway genes were not differentially expressed between strains, suggesting that pyruvate was used to produce lactic acid by the action of cystathione- γ -lyase rather than by carbohydrate metabolism. Genes encoding urease components, upregulated in the *luxS* mutant, would also assist regulation of intracellular pH by the production of ammonia.

The fatty acid composition of the cell membrane of lactic acid bacteria can be altered under stressful growth conditions (2, 8). This may explain the differences in fatty acid levels between mutant and wild-type cells in our experiments. Genes encoding sortase-dependent proteins were differentially expressed between the strains. Lreu23DRAFT_5058 (SDP1) was downregulated and Lreu23DRAFT_4486 (SDP2) was upregulated in the mutant. Transcriptional regulators located near each of the SDPs (Lreu23DRAFT_5057 for SDP1 and Lreu23DRAFT_4487 for SDP2) were differentially expressed in a manner consistent with negative regulation since downregulation of the transcriptional regulator corresponded to upregulation of the corresponding SDP gene and vice versa. Hypothetical genes, regulated similarly to each SDP, were also differentially expressed at each locus. SDP2 has recently been identified in a comparative genome survey of *L. reuteri* strains as a cell surface protein specific to strains of *L. reuteri* that colonize the gut of rodents (3). We propose that altered expression of SDP1 and SDP2 might result in an altered cell surface charge or other properties that could influence biofilm formation.

The overwhelming majority of the data that we report here can be attributed to metabolic disturbance rather than impaired quorum sensing. We conclude from these experiments that quorum sensing mediated by AI-2 is unlikely to be important in *L. reuteri* 100-23 gene regulation under the conditions used in the present study.

ACKNOWLEDGMENTS

The research was supported by a grant from the Marsden Fund, administered by the Royal Society of New Zealand. The work at P.W.O.’s laboratory was supported by a Principal Investigator award (07/IN.1/B1780) from Science Foundation Ireland.

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