**Lactobacillus reuteri**-Specific Immunoregulatory Gene rsir Modulates Histamine Production and Immunomodulation by *Lactobacillus reuteri*

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Human microbiome-derived strains of *Lactobacillus reuteri* potently suppress proinflammatory cytokines like human tumor necrosis factor (TNF) by converting the amino acid L-histidine to the biogenic amine histamine. Histamine suppresses mitogen-activated protein (MAP) kinase activation and cytokine production by signaling via histamine receptor type 2 (H2) on myeloid cells. Investigations of the gene expression profiles of immunomodulatory *L. reuteri* ATCC PTA 6475 highlighted numerous genes that were highly expressed during the stationary phase of growth, when TNF suppression is most potent. One such gene was found to be a regulator of genes involved in histidine-histamine metabolism by this probiotic species. During the course of these studies, this gene was renamed the *Lactobacillus reuteri*-specific immunoregulatory (*rsir*) gene. The *rsir* gene is essential for human TNF suppression by *L. reuteri* and expression of the histidine decarboxylase (*hdc*) gene cluster on the *L. reuteri* chromosome. Inactivation of *rsir* resulted in diminished TNF suppression in vitro and reduced anti-inflammatory effects in vivo in a trinitrobenzene sulfonic acid (TNBS)-induced mouse model of acute colitis. A *L. reuteri* strain lacking an intact *rsir* gene was unable to suppress colitis and resulted in greater concentrations of serum amyloid A (SAA) in the bloodstream of affected animals. The *P_hdcAB* promoter region targeted by *rsir* was defined by reporter gene experiments. These studies support the presence of a regulatory gene, *rsir*, which modulates the expression of a gene cluster known to mediate immunomodulation by probiotics at the transcriptional level. These findings may point the way toward new strategies for controlling gene expression in probiotics by dietary interventions or microbiome manipulation.

Probiotics are defined as “living microorganisms, which when administered in adequate amounts confer a health benefit on the host” (1, 2). In 1907, Metchnikoff and Mitchell introduced the concept of beneficial microbes to the scientific community through their seminal discovery of the positive effects of fermented milk product consumption on the health and longevity of people in Eastern Europe (3). Since that time, probiotics have become increasingly popular as dietary supplements or functional foods. Investigations into the beneficial effects of probiotics and mechanisms of probiosis have demonstrated that several probiotic species produce metabolites that modulate the host’s mucosal immune system. For example, lactic acid production by *Lactobacillus casei* strain Shirota may work via Toll-like receptor 4 (TLR4) signaling to suppress indomethacin-induced myeloperoxidase activity and tumor necrosis factor (TNF) production by human myeloid (THP-1) cells in a rat model of small intestine injury (4). *Bifidobacterium breve* strain BbC50 and *Streptococcus thermophilus* strain S1065 also secrete small, digestive-enzyme-resistant metabolites that were found to be able to inhibit TNF production from lipopolysaccharide (LPS)-activated THP-1 cells (5). Several probiotic species convert dietary components into bioactive molecules that affect the host’s physiological functions. Many probiotics produce short-chain fatty acids (SCFAs) as a product of dietary fiber catabolism (6). SCFAs have anti-inflammatory effects on human immune cells and the gut through binding with G-protein-coupled receptor 43 (GPR43), and this interaction plays a key role in the resolution of several inflammatory conditions, such as arthritis, colitis, and asthma (7). Finally, a recent study demonstrated increased longevity in mice treated with *Bifidobacterium animalis* subsp. *lactis* LKM12 compared to control mice, possibly due to the anti-inflammatory effects of polyamines produced by the bacteria (8).

Amino acid decarboxylation and biogenic amine synthesis in bacteria (for example, the conversion of histidine to histamine) are proposed to have at least two major functions: maintaining intracellular pH homeostasis, especially in an acidic environment, and providing energy via proton motive force (9, 10). Histamine biosynthesis through decarboxylation of L-histidine has been extensively studied in both Gram-negative and Gram-positive bacteria. Two different families of histidine decarboxylase (HDC) enzymes have been identified and characterized: pyridoxal phosphate-dependent HDC and pyruvoyl-dependent HDC are present in Gram-negative bacteria and Gram-positive bacteria, respectively. The first HDC identified in lactobacilli was purified from *Lactobacillus saerimneri* ATCC 33222 (formerly known as *Lactobacillus* sp. strain 30a), an isolate from a horse’s stomach (11). Subsequently, several other *Lactobacillus* species were found to contain a functional *hdc* gene cluster, which consists of the histi-
dine decarboxylase pyruvolyt type (hdcA), a putative helper protein (hdcB), and a histidine/histamine antiporter (hdcP) (12). The hdcA and hdcB genes are cotranscribed as a single bicistronic mRNA, and hdcA and hdcB expression is coregulated under the P_hdcAB promoter, which lies directly upstream of hdcA (13, 14). Expression of hdcP is regulated by a different promoter. Factors affecting P_hdcAB promoter activity and the expression of genes in the hdc cluster have been identified in several Gram-positive bacteria, like Staphylococcus capitis IFJ12 (13), Lactobacillus saerimneri ATCC 33222, Lactobacillus sp. strain w53 (15), and L. hilgardii 464 (16, 17). These include acidic pH, supplemental L-histidine, histamine, and other molecules, like glucose, fructose, malic acid, and citric acid, in the growth medium. The exact regulatory mechanism of hdc gene cluster expression is still not well characterized.

The model prokaryotic organism L. reuteri ATCC PTA 6475 (L. reuteri 6475) also produces histamine (18). L. reuteri-derived histamine suppressed TNF production by TLR2-activated THP-1 cells via activation of the histamine receptor type 2 (H2) and inhibition of MEK/extracellular signal-regulated kinase mitogen-activated protein (MAP) kinase signaling. Supplementation of L-histidine in L. reuteri 6475 growth medium increased expression of the hdc gene cluster and production of TNF-inhibitory histamine (18).

In this study, we investigated the role of the Lactobacillus reuteri-specific immunoregulatory (rsir) gene, a novel regulator of genes involved in histidine-histamine metabolism, in L. reuteri-mediated immunomodulation and histamine production. We characterized the immunomodulatory phenotype of L. reuteri 6475 mutants deficient in Rsir compared to that of the wild type and investigated the regulatory role of Rsir in the expression of the hdc gene cluster and L. reuteri-derived histamine production. We found that Rsir was necessary for L. reuteri-mediated immunomodulation in vitro and in vivo. Inactivation of Rsir resulted in decreased expression of the hdc gene cluster and L. reuteri-derived histamine production compared to the levels for the wild type. Moreover, an Rsir-deficient mutant demonstrated defective up-regulation of hdc gene expression and histamine production in the presence of supplemental L-histidine. On the basis of the evidence presented in this report, Rsir regulates the expression of hdcA and hdcB genes at the transcriptional level.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** All bacterial strains used in this study are described in Table S1 in the supplemental material. L. reuteri strains were cultured under anaerobic conditions for 16 to 18 h in deMan, Rogosa, Sharpe (MRS) medium (Difco, Franklin Lakes, NJ) and inoculated into a semidefined medium, LDMIII (the optical density at 600 nm [OD600] was adjusted to 0.1), as previously described (18). Each LDMIII culture was incubated for 24 h at 37°C in an anaerobic workstation (MACS MG-500; Microbiology International, Frederick, MD) supplied with a mixture of 10% CO2, 10% H2, and 80% N2. At mid-exponential phase (6 to 8 h) or stationary phase (24 h), the cells were collected by centrifugation (4,000 × g, 10 min). Cell pellets and bacterial cell-free supernatants were further processed for TNF inhibition, histamine enzyme-linked immunosorbent assay (ELISA), RNA isolation, and GusA reporter assays.

**Cell line and reagents.** *In vitro* experiments were performed with THP-1 cells (human monocyteid cell line, ATCC number TIB-202; ATCC, Manassas, VA) maintained in RPMI (ATCC) and heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA) at 37°C in 5% CO2. All other reagents were obtained from Sigma (St. Louis, MO), unless otherwise stated.

**Analysis of CDNA microarray data.** We analyzed microarray data from data sets previously deposited under NCBI Gene Expression Omnibus (GEO) series accession number GSE24415. Data from three biological replicates of spotted two-color cDNA microarray experiments comparing the gene expression profiles of *L. reuteri* 6475 between mid-exponential phase (8 h) and stationary phase (24 h) (samples GSM601520, GSM601521, GSM601522, GSM601523, GSM601524, and GSM601525) were analyzed using the R- and Bioconductor-based web interface for microarray data analysis CARMAweh (19). Data preprocessing included the removal of flagged spots, background correction by use of the normexp algorithm, within-array normalization using the loess method, and between-array normalization with quantile normalization. Analysis of moderated t-statistics presented in Figure 2 with P-value correction was conducted using the Benjamini and Hochberg method provided by the limma package to identify differentially expressed genes.

**Inactivation of rsir in L. reuteri by targeted insertion mutagenesis (6475rsir).** All plasmids, primers, and oligonucleotides are described in Table S1 in the supplemental material. The Lactobacillus reuteri-specific immunoregulatory gene (*rsir*, HMREF0536_10683) was identified in the genome of *L. reuteri* 6475 (GenBank accession numbers NZ_ACGX02000001 through NZ_ACGX02000007). Inactivation of the *rsir* gene was targeted by site-specific integration of plasmid pORI28 (20) into the chromosome, as previously described (21, 22). Briefly, internal *rsir* gene fragments were PCR amplified (see Table S1 in the supplemental material) and cloned into pORI28. The resulting construct was integrated into *rsir* by site-specific homologous recombination. The targeted integration was confirmed by sequencing.

**Inactivation of rsir in L. reuteri by ssDNA recombinocering (6475rsir-Stop and 6475 Arsir).** The *rsir* gene was inactivated by single-stranded DNA (ssDNA) recombinocering as previously described (23, 24). Briefly, *L. reuteri* 6475/pJPO42 carrying recombinacering recT was cultured in MRS medium with 10 μg/ml erythromycin to mid-exponential phase (OD600 0.6) at 37°C. Expression of recT was induced by incubation with pSp peptide (125 μg/ml) at 37°C for 20 min. Recovered cells were plated for single colonies on 10 μg/ml chloramphenicol and incubated anaerobically at 37°C for 16 to 20 h. Colonies were passaged in MRS medium twice and screened by PCR using primers homologous to the stop codon mutations and the 3’ end of *rsir* (KJP33 and KJP16; see Table S1 in the supplemental material) or to the sequences flanking the deletion site (KJP15 and KJP16; see Table S1 in the supplemental material). Plasmids utilized in the recombinocering reaction were cured by continuous passaging until susceptibility to 10 μg/ml erythromycin and 10 μg/ml chloramphenicol was observed. Mutations were verified by sequencing.

**Complementation of 6475rsir mutant (6475rsir/pJKS104).** The Escherichia coli-L. reuteri shuttle vector (pKS100) was utilized for *rsir* complementation in the 6475rsir mutant strain (25). The *rsir* gene with its putative promoter was PCR amplified from purified wild-type strain 6475 genomic DNA (primers *rsir*-F and *rsir*-R; see Table S1 in the supplemental material) and cloned into pCR2.1-TOPO using a TOPO-TA cloning kit (Invitrogen, Carlsbad, CA) to create pCR2.1-rsir. Full-length *rsir* was subcloned from pCR2.1-rsir into pJKS100 using BstXI restriction sites, resulting in pJKS104 (see Fig. S2 in the supplemental material). The construct was introduced into the 6475-rsir insertion mutant via electroporation and confirmed by PCR amplification of the *cat* gene (see Table S1 in the supplemental material).

**Global transcriptomic analysis of L. reuteri.** Wild-type strain L. reuteri 6475 and the 6475rsir-Stop mutant strain were cultured in LDMIII as described above and harvested at 16 h post inoculation. Total RNA was isolated and used as the template to synthesize cDNA libraries using an Ovation prokaryotic RNA-seq system (Nugen Technologies, San Carlos, CA) for high-throughput sequencing on an Illumina HiSeq platform (Illumina, San Diego, CA). Coverage of sequence data was evaluated using the Artemis tool (Wellcome Trust Sanger Institute, Hinxton, United Kingdom). Comparative transcriptomic analyses were performed using the Bowtie (version 0.12.9) tool (26) for sequence mapping and align-
ment, followed by DNASTAR ArrayStar/QSeq 5 software (DNASTAR, Madison, WI) and the GFOld algorithm (27), with \textit{L. reuteri} 6475 draft genome contigs (GenBank accession numbers NZ\_AGCX02000001 through NZ\_AGCX02000007) used as a reference for transcript mapping. Data were normalized across all experiments by assigned reads per kilobase of feature per million mapped reads (RPKM), and the relative gene expression level was calculated for each open reading frame. Lists of genes whose expression was most affected by genetic inactivation of RsiR (>1.5-fold compared to wild type) were analyzed using subsequent functional clustering analysis with the DAVID bioinformatics tool (28, 29) to identify genetic enrichments of pathways that may be affected by RsiR mutation.

Gene expression studies of the \textit{L. reuteri} histidine decarboxylase cluster. Wild-type \textit{L. reuteri} 6475 and the 6475 ΔrsiR mutant strain were grown in LDMIII as described above in the presence or absence of 4 g/liter supplemental l-histidine and harvested at 16 h postinoculation. Isolation of RNA from collected cell pellets and cDNA synthesis from total RNA were performed as previously described (18). Expression of the hdcA, hdcB, and hdcP genes was analyzed using quantitative reverse transcription-PCR (RT-qPCR). All primers used in this study were designed using the Universal ProbeLibrary Assay Design Center (Roche Applied Science, Indianapolis, IN) and are described in Table S1 in the supplemental material. The RNA polymerase promoter sequence (rpOB) gene, which was unaffected by inactivation of rsiR (RNA-seq analysis; data not shown), was used as a reference gene. RT-qPCR mixtures included 2× FastStart Universal Probe Master (Roche; Roche Applied Science) and prepared cDNA as the template, along with the corresponding probes and primers at final concentrations of 100 nM and 200 nM, respectively. Serially diluted genomic DNA of wild-type \textit{L. reuteri} 6475 was also included to create the standard curve used in the analysis. PCRs were performed using a Viia® (version 7) real-time PCR system (Life Technologies, Grand Island, NY) with the cycling parameters described previously (18). The relative standard curve method (Viia® [version 7] data analysis software) was used to calculate relative changes in gene expression.

Quantification of histamine by ELISA. The production of histamine by \textit{L. reuteri} strains was measured by quantitative histamine ELISA, as previously described (18). Briefly, wild-type \textit{L. reuteri} 6475 and the 6475 ΔrsiR mutant strain were grown in LDMIII in the presence or absence of 4 g/liter supplemental l-histidine. Cultures were harvested at 24 h, centrifuged, and filter sterilized with 0.22-μm-pore-size-polyvinylidene difluoride filters (EMD Millipore, Billerica, MA). Histamine concentrations were determined using a histamine ELISA kit (Neogen, Lexington, KY) as per the manufacturer’s instructions. The absorbance was measured with a Bio-Rad Spectramax 340PC spectrophotometer. Data were normalized to those for the LDMIII culture at an OD of 1.0 and corrected with the values obtained from the background control.

β-Glucuronidase (GusA) promoter assay. The activity of the hdcAB promoter was tested in a β-glucuronidase (GusA) promoter assay. The putative promoter of the hdcAB genes (P\textsubscript{hdcAB}) from \textit{L. reuteri} 6475 was predicted using the Neural Network Promoter Prediction tool (30). The promoter DNA sequence was amplified by primers P\textsubscript{hdcAB}\_F and P\textsubscript{hdcAB}\_R (described in Table S1 in the supplemental material) and cloned into an expression vector, pJKS100, using KpnI and EcoRI restriction sites, replacing the original P\textsubscript{gusA} promoter. A hyperactive β-glucuronidase reporter gene, gusA3, from pGK12 (31) was cloned directly downstream from P\textsubscript{hdcAB} using an EcoRI site to create pPH-R1 (see Fig. S3 in the supplemental material). Wild-type \textit{L. reuteri} 6475 or the 6475 ΔrsiR mutant strain carrying pPH-R1 was grown anaerobically in LDMIII as described above in the presence or absence of 4 g/liter supplemental l-histidine to mid-exponential or stationary phase. Cell pellets were collected and assayed for GusA activity using a protocol from Axelsson et al. (32), with some modifications. Briefly, each pellet was resuspended in 200 μl of 30 mM NaH\textsubscript{2}PO\textsubscript{4}, pH 7.0. Fifty microliters of cell suspension was added to 450 μl of GUS buffer (50 mM NaH\textsubscript{2}PO\textsubscript{4}, pH 7.0, 10 mM β-mercaptoethanol, 1 mM EDTA, 0.1% Triton X-100). To this mixture, 12.5 μl of 0.1% sodium dodecyl sulfate and 25 μl of chloroform were added. After 5 min incubation at 37°C, 12.5 μl of 4 mg/ml of p-nitrophenyl-β-D-glucoronide was added. Each reaction mixture was incubated at 37°C for 3 min, and the reaction was stopped by adding 250 μl of 1 M Na\textsubscript{2}CO\textsubscript{3}. After centrifugation at 8,000 × g for 5 min, supernatants were transferred into cuvettes and optical densities at 420 nm were measured using a SmartSpec Plus spectrophotometer (Bio-Rad Laboratories, Hercules, CA). Data were converted to Miller units and analyzed using two-way analysis of variance (ANOVA) on GraphPad Prism (version 5) software (GraphPad Inc., La Jolla, CA).

TNF inhibition bioassay. A TNF inhibition bioassay and a TNF ELISA were performed as previously described (18). Briefly, supernatants from \textit{L. reuteri} LDMIII cultures were filter sterilized and size fractionated to select for factors smaller than 3 kDa in size. The filtrate was speed vacuum dried, resuspended in RPMI medium, and normalized by volume to an OD\textsubscript{595} of 1.5. Supernatants were tested for their ability to modulate TNF production in monocytes cells. THP-1 cells (5 × 10\textsuperscript{4} cells) were treated with \textit{L. reuteri} supernatant (5%, vol/vol) and subsequently activated by 100 ng/ml Pam3Cys-SKKKKX3 HCl (EMC Microcollections, Tübingen, Germany), as previously described (33). Cells were incubated at 37°C in 5% CO\textsubscript{2} for 3.5 h and then pelleted (3,000 × g × 5 min, 4°C). Quantitative ELISAs were used to determine the concentration of TNF in THP-1 cell supernatants according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN). Data were analyzed using an unpaired \textit{t} test on GraphPad Prism (version 5) software.

Mice. Female BALB/c mice (45 days old) were received from Harlan Laboratories (Houston, TX) and maintained under specific-pathogen-free (SPF) conditions. Mice were kept in filter-top cages (5 mice per cage) and had free access to distilled water and Harlan rodent chow 2918. After 10 days of acclimatization, mice were randomly divided into several experimental groups. All mouse experiments were performed in an SPF animal facility according to an Institutional Animal Care and Use Committee (IACUC)-approved mouse protocol at the Baylor College of Medicine, Houston, TX.

Preparation of supernatant from \textit{L. reuteri} strains and administration to mice. Bacterial supernatants from LDMIII cultures of wild-type \textit{L. reuteri} and the 6475ΔrsiR mutant were prepared as described above. All supernatants were filter sterilized, size fractionated, and concentrated to 20× with speed vacuum drying before administration to mice. Each mouse received two intraperitoneal (i.p.) injections of bacterial supernatant or medium control (0.1 ml each time), with the first dose given 18 h before a i.p. injection of the second dose given 2 min before the TNBS enema (described below) and the second dose given 2 min before the TNBS enema.

Induction of acute colitis by intrarectal instillation of TNBS. Two minutes after i.p. injection of the second dose of bacterial supernatant or medium control, mice were anesthetized by constant isoflurane inhalation. A 5% (vol/vol) TNBS (Sigma-Aldrich, St. Louis, MO) solution in water was diluted with an equal volume of absolute ethanol and administered intrarectally via a catheter (Braintree Scientific, Braintree, MA) at a dose of 100 mg/kg of body weight 4 cm distal to the anus. Mice were kept head down in a vertical position for 2 min after the enema to ensure complete retention of the enema in the colon. Procedure control mice received 50% ethanol in phosphate-buffered saline (PBS) as an enema and two i.p. injections of the medium control. Colitis-positive mice received a TNBS enema and two i.p. injections of the medium control, while test mice received a TNBS enema and two i.p. injections of the prepared bacterial supernatant.

Macroscopic assessment of TNBS-induced colitis. The colon was collected 48 h after colitis induction and opened longitudinally, and images were recorded with a digital camera. Colonic inflammation and damage in the distal colon were determined according to the Wallace criteria (34). In brief, the grading scale was as follows: score 0, normal/healthy appearance; score 1, focal hyperemia, slight thickening, and no ulcers; score 2, hyperemia, prominent thickening, and no ulcers; score 3, ulceration with inflammation at one site; score 4, ulceration with inflammation

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at two or more sites; score 5, major sites of damage extending >1 cm; scores 6 to 10, when the area of damage extended >2 cm, an increase of 1 score unit for each additional 1 cm of tissue involvement. Each colon was scored blindly by one individual.

**Plasma measurements of mouse SAA.** Blood samples were collected from mice via cardiac puncture, stored with anticoagulant, and centrifuged (10 min, 17,000 g) to isolate plasma. Serum amyloid A protein (SAA) concentrations in plasma were measured using an SAA ELISA kit (Alpco, Salem, NH) according to the manufacturer’s instructions.

### RESULTS

**Discovery of rsir gene and structural predictions.** Based on prior data showing robust TNF suppression from stationary-phase bacterial supernatants (35, 36), we hypothesized that genes upregulated in the stationary phase of growth would likely be involved in *L. reuteri*-mediated immunomodulation. We compared the gene expression profiles of *L. reuteri* 6475 between exponential phase (8 h) and stationary phase (24 h) from previously deposited sets of data for cDNA microarray experiments from our *L. reuteri* metabolic modeling study (35). The 20 most highly upregulated genes from this comparison are listed in Table 1. Of particular interest was a 354-bp open reading frame (GenBank gene locus tag HMPREF0536_11708) predicted to encode a 118-amino-acid hypothetical protein with a molecular mass of 12.9 kDa and a calculated isoelectric point of 8.76. The open reading frame is located on the minus strand of the chromosome, positioned between a 

**Table 1 The 20 most highly upregulated genes in L. reuteri 6475 in stationary phase compared to gene expression in mid-exponential phase**

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<th>Microarray identifier</th>
<th>Locus tag</th>
<th>Gene name</th>
<th>Corrected P value</th>
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These data represent microarray comparisons of a previously published study (35).

The expression of rsir in L. reuteri-suppressed TNF activity (strains ATCC PTA 4659, JCM 1112, DSM 20016) (36) (J. K. Spinler, unpublished data).

To elucidate the role of rsir in L. reuteri-mediated immunomodulation and in other bacterial metabolic pathways, we performed a global comparative analysis of stationary-phase transcriptomes from L. reuteri 6475 and 6475srsIR-Stop. Paired-end sequencing resulted in 162,737,873 and 195,032,924 100-bp reads from wild-type L. reuteri 6475 and 6475srsIR-Stop, respectively. The sequencing data demonstrated complete coverage of the en-
tire genome, with all predicted transcripts appearing at least once. All transcripts were mapped to L. reuteri 6475 draft genome contigs. After filtering out low-count transcripts (<200 transcripts per open reading frame), the gene expression profiles of the two strains were compared. We identified 195 genes (9.3% of the genome) that were downregulated more than 1.5-fold in 6475 rsiR mutant compared to wild-type (WT) L. reuteri 6475 strains. Expression ratios of each gene (rsiR mutant versus wild-type) were calculated, and results represent the mean ± SD (n = 3). P values were determined using a one-sample t test and are in comparison to the theoretical mean of 1.0. All RT-qPCR data were normalized to those for a reference gene, rpoB.

To validate the results of RNA sequence analysis and confirm the decreased expression of hdc cluster genes in the rsiR mutant, stationary-phase expression of the hdcA, hdcB, and hdcP genes in wild-type L. reuteri 6475 was compared to that in the 6475 ΔrsiR mutant using RT-qPCR. The expression of hdcA and hdcB in the 6475 ΔrsiR mutant was dramatically decreased compared to that in L. reuteri 6475, while the expression of hdcP was unaffected (Fig. 1A). In the presence of 4 mg/ml L-histidine, all 3 genes (hdcA, hdcB, and hdcP) in wild-type L. reuteri 6475 were upregulated compared to the level of expression in unsupplemented medium. However, the hdcA, hdcB, and hdcP genes were not affected in the 6475 ΔrsiR mutants, when cells were grown in the presence of L-histidine (Fig. 1B). Taken together, these data suggest that rsiR may play a role in the transcriptional regulation of genes involved in histamine biosynthesis.

Inactivation of rsiR diminished the expression of histamine biosynthesis genes (Fig. 1; see Table S2 in the supplemental material). DAVID functional clustering analysis of genes whose expression was most affected by RsiR inactivation highlighted several metabolic pathways with altered expression (see Table S4 in the supplemental material). In the rsiR mutant, genes involved in cysteine/methionine metabolism, fatty acid biosynthesis, and glycerolipid metabolism were most downregulated, while genes involved in cell redox homeostasis, glycerophospholipid metabolism, and cellular homeostasis were most upregulated. These results suggested that RsiR may regulate several essential metabolic functions in L. reuteri and may explain why it is conserved in some non-human-derived L. reuteri strains that lack the hdc gene cluster. Since hdcA was the most downregulated gene according to the RNA-seq analysis and histidine decarboxylation has been shown to be involved in L. reuteri-mediated immunomodulation (18), we decided to focus on the expression of hdc cluster genes in the rsiR mutant.

FIG 1 Effects of rsiR and L-histidine on hdc gene expression in L. reuteri. Gene expression in the histidine decarboxylase (hdc) cluster was suppressed in the rsiR mutant compared to its expression in wild-type (WT) L. reuteri. (A) RT-qPCR demonstrating decreased expression of the hdcA and hdcB genes in the RsiR-deficient mutant compared to wild-type strain 6475. Expression ratios of each gene (rsiR mutant versus wild-type) were calculated, and results represent the mean ± SD (n = 3). P values were determined using a one-sample t test and are in comparison to the theoretical mean of 1.0. All RT-qPCR data were normalized to those for a reference gene, rpoB.

FIG 2 Effects of rsiR and L-histidine on histamine production by L. reuteri. Histamine production was diminished in the rsiR mutant compared to that in wild-type L. reuteri even in the presence of supplemental L-histidine. The histamine produced by L. reuteri was quantified using ELISA. Data were analyzed by two-way ANOVA. Results represent the mean ± SD (n = 3). P was <0.0001 compared to wild-type 6475.
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FIG 3 Reporter assays defining the promoter region affected by rsiR. A mutation in rsiR resulted in decreased expression of the gusA3 reporter gene driven by the putative hdcAB promoter. Wild-type strain 6475 or the 6475 ΔrsiR mutant expressing reporter gene gusA3 was grown in the presence or absence of supplemental L-histidine. GusA3 activity was measured in Miller units. Data were analyzed using two-way ANOVA (P < 0.0001) and the Bonferroni posttest to compare the GusA3 activity between the wild type and mutant at each concentration of supplemental L-histidine (P < 0.0001 for both 0 and 4 mg/ml L-histidine).

FIG 4 Importance of rsiR in human TNF suppression by L. reuteri. The rsiR mutant could not suppress TNF production by activated THP-1 cells. Genetic inactivation of rsiR resulted in a complete loss of TNF suppression in THP-1 cells, which was not fully complemented in the presence of supplemental histidine. *** P = 0.0001 using an unpaired t test comparing wild-type strain 6475 and the 6475 ΔrsiR mutant.

inflammation in a mouse model. The in vivo effect of rsiR inactivation on intestinal and systemic inflammation was evaluated in a TNBS-induced colitis mouse model. Eight-week-old, female BALB/c mice received L. reuteri culture supernatants by intraperitoneal injection. Bacteria tested included wild-type L. reuteri 6475 and the 6475::rsiR mutant. TNBS was instilled intrarectally to induce acute intestinal inflammation. Colitis was evaluated by macroscopic examination of colons and quantified using the Wallace grade scoring system (34). Mice pretreated with control medium (LDMIII) followed by PBS rectal instillation (colitis-negative control) did not develop acute colitis, while mice instilled with TNBS (colitis-positive control) and lacking bacterial immunomodulatory factors developed significant colitis. Pretreatment with culture supernatants from wild-type L. reuteri 6475 significantly reduced the severity of acute colitis compared to that in colitis-positive control mice. However, protective effects were abolished in mice pretreated with culture supernatants from rsiR-deficient L. reuteri 6475. The alleviation of colitis was partially restored in mice receiving culture supernatants of L. reuteri with an intact rsiR gene on a plasmid (6475::rsiR/pJKS104) (Fig. 5A).

The plasma concentrations of acute-phase reactant protein SAA were measured (Fig. 5B). SAA is a biomarker of colonic mucosal inflammation, and its concentration correlates with the severity of pathology in the TNBS-induced colitis mouse model (41, 42). Elevated SAA concentrations were observed in colitis-positive control mice, while mice pretreated with L. reuteri 6475 culture supernatants yielded significantly reduced SAA concentrations (P < 0.005). The SAA concentrations in mice treated with culture supernatants from rsiR-deficient bacteria were not significantly different from the SAA concentrations in the colitis-positive control mice. Mice that received the culture supernatants from the complemented strain (6475::rsiR/pJKS104) demonstrated significant reductions in SAA concentrations compared to those in mice that received the same treatment with the rsiR mutant strain.

ional) and, coincidentally, histamine production (Fig. 2). Histamine concentrations in culture supernatants from L. reuteri 6475 grown to stationary phase were approximately 40 μg/ml and increased more than 2-fold in medium supplemented with 4 mg/ml L-histidine. In contrast, histamine production from the histidine mutant 6475 ΔrsiR mutant was significantly reduced, and supplementing the medium with L-histidine did not significantly increase the histamine concentration in the supernatants (Fig. 2).

Defining the hdcAB promoter region by reporter gene studies. We used the Neural Network Promoter Prediction tool (30) to identify a 117-bp region directly upstream of hdcAB (P_{hdcAB}) in wild-type L. reuteri 6475. The predicted sequence had 57.1% homology to the P_{hdcAB} promoter sequence previously identified in Staphylococcus capitis IFIJ12 (13). The activity of this putative β-glucuronidase reporter gene, gusA3 (31) (see Fig. S3 in the supplemental material). During stationary phase in the absence of supplemental histidine, wild-type L. reuteri containing pPH-R1 averaged 458.7 Miller units of GusA activity, while 6475 ΔrsiR produced very little GusA activity (Fig. 3). In the presence of 4 mg/ml supplemental L-histidine, wild-type L. reuteri 6475 yielded increased GusA reporter activity (P < 0.0001), while the GusA activity with the 6475 ΔrsiR mutant was unchanged.

The rsiR gene is essential for suppression of human TNF production by L. reuteri. We characterized the immunomodulatory role of rsiR by studying the effects of rsiR inactivation on suppression of TNF production by activated human myeloid (THP-1) cells. Bacterial culture supernatants from wild-type L. reuteri 6475 and the 6475 ΔrsiR mutant were evaluated for the ability to inhibit human TNF production by TLR2-activated THP-1 cells. Wild-type L. reuteri 6475 suppressed human production (P < 0.0001), while the mutant 6475 ΔrsiR strain yielded a diminished ability to inhibit TNF production (Fig. 4). Supplementation of growth medium with 4 mg/ml L-histidine was able to partially restore the TNF-inhibitory phenotype of the wild type. Other rsiR mutants (the 6475rsiR-Stop and 6475::rsiR mutants) also yielded a reduced ability to suppress TNF production (data not shown).

The rsiR gene is essential for L. reuteri to suppress intestinal inflammation. The rsiR gene on a plasmid (6475::rsiR/pJKS104) (Fig. 5A).
These observations suggest that the bacterial rsiR gene may play an important role in L. reuteri-mediated immunoregulation and amelioration of systemic and intestinal inflammation in vivo.

**DISCUSSION**

In this study, we characterized a hypothetical protein, RsiR, and investigated its role in immunomodulation, histamine production, and regulation of histidine decarboxylase gene cluster expression. Comparative transcriptomic analysis revealed downregulation of genes involved in histamine biosynthesis in RsiR-deficient mutants compared to their expression in the wild-type strain, and the finding was confirmed by quantitative reverse transcription-PCR. We found that L-histidine supplementation did not affect hdc cluster gene expression or histamine production in mutant L. reuteri deficient in RsiR. Promoter studies of P_hdcAB suggested that RsiR may regulate the expression of the hdc gene cluster at the transcriptional level. Inactivation of RsiR resulted in the relative inability to induce hdc gene expression, produce histamine, suppress TNF production by human myeloid cells, and protect animals in a TNBS colitis mouse model.

RsiR was predicted to be a transmembrane protein, and characterization of rsiR-deficient L. reuteri mutants demonstrated the regulatory role of rsiR in hdc gene cluster expression. In silico structural analysis of RsiR did not predict the presence of a DNA binding domain. We identified putative promoter regions for genes whose expression was most affected by RsiR inactivation (the 5 most downregulated genes and the 5 most upregulated genes) but failed to demonstrate significant homology among these sequences (data not shown). Transcriptional factors without a DNA binding domain have been previously characterized in many prokaryotes, for example, Spx found in Bacillus subtilis and other low-GC-content Gram-positive bacteria (43–45) and TsrA found in Vibrio cholerae (46). These proteins were global transcriptional regulators controlling expression of genes involved in the toxic stress response and virulence. Instead of binding to promoter DNA sequence, these proteins may interact with the α C-terminal domain (α-CTD) of RNA polymerases and suppress or induce gene expression (44, 47). Similarly, RsiR may indirectly regulate gene expression via interaction with other transcriptional factors. Protein-protein interaction studies, such as bacterium two-hybrid analysis or bimolecular fluorescence complementation (BiFC), would help identify the interactions between RsiR and other proteins and elucidate the mechanism of RsiR-dependent gene regulation. Results from protein function prediction indicated a role for RsiR in the metabolism and transport of folic acid and indole derivatives, which are physiologically relevant bacterial metabolites in the human gastrointestinal tract (48, 49). With the presence of transmembrane domains and a predicted role in transporting indole derivatives, it is possible that RsiR could form multimers and function as a histidine transporter. This hypothesis is unlikely to be true, since RsiR does not contain any known amino acid transporter domain with key residues that would be able to bind and transport amino acids. RsiR may instead play a role in the metabolism of folate or indole compounds that may be present in the intestinal milieu. Orally consumed nutrients, such as vitamins, amino acids, or other cofactors, may be metabolized by members of the intestinal microbiome and converted in the intestinal lumen to biologically active molecules (50), including short-chain fatty acids (SCFAs), biogenic amines (such as histamine), or other amino acid-derived metabolites, like serotonin, tryptophan, or gamma-aminobutyric acid (GABA). These small bacterial metabolites may be able to affect the physiological functions of the host, such as the immune system or the cardiovascular or central nervous system (51, 52).

The possible involvement of RsiR in amino acid metabolism and transport in L. reuteri is of particular interest, since these
processes have been suggested to play a role in intestinal physiology and immunology. Amino acid metabolism is an essential functional process that plays an important role in the biochemical pathways of prokaryotic cells, such as energy harvest and acid stress survival. In a recent DNA microarray transcriptomic study of *L. casei*, Zhang and colleagues identified 162 genes that were upregulated during soymilk fermentation (53). Approximately 38.4% of these genes are involved in amino acid metabolism and transport, especially histidine and lysine. Metagenomic analysis of the human intestinal microbiome has revealed the presence of genetic elements involved in amino acid biosynthesis and metabolism encoded in the genomes of bacteria that comprise the gut microbiota (54). A recent study demonstrated an association between amino acid malnutrition and susceptibility to chemical colitis in mice deficient in angiotensin I-converting enzyme 2 (Ace2), which facilitates amino acid transport (33). Ace2-deficient mice had an altered intestinal microbiota compared to that of wild-type mice. Transplantation of these altered microbial communities into germfree mice resulted in transcription of the susceptibility to developing severe colitis, which suggested the role of the gut microbiome in the regulation of intestinal inflammation. A recent metagenomic study in humans revealed a depletion of genes involved in the metabolism and biosynthesis of amino acids, especially histidine and lysine, in patients suffering from inflammatory bowel disease (28). This evidence suggests that bioactive molecules produced by intestinal microbes may be able to affect the integrity of the gut barrier and the proliferation of intestinal epithelial cells and to modulate the host immune response. RsiR may have a regulatory role in *L. reuteri*-mediated luminal conversion of certain cofactors that are involved in immunomodulation, as suggested by our *in vitro* and *in vivo* assays.

In order to characterize bacterial pathways that were affected by genetic inactivation of RsiR, we performed a comparative transcriptomic analysis using RNA-seq. To our knowledge, this was the first attempt at implementing RNA-seq in an *L. reuteri* transcriptomic study. Compared with DNA microarray analysis, whole-transcriptome sequencing has a more extensive detection range with no background and allows the absolute quantification of gene expression (55). Moreover, data obtained from different RNA-seq experiments can also be easily normalized and compared (56). Our analysis revealed several metabolic and regulatory pathways with altered expression in the *rsiR* mutant, which suggested that *rsiR* may possess a global regulatory function across many physiological pathways. A global regulatory role may underlie the conservation of *rsiR* across the *L. reuteri* strains sequenced to date. From our RNA-seq analysis, we identified *hdcA* and *hisS2* as highly downregulated genes in the *rsiR* mutant, along with a 2-fold downregulation of putative secondary 3-histidine transporter *lysP2*. A homologue of *lysP2* in *L. lactis* subsp. cremoris NZ9000 was proposed to play a role in histidine transport and was essential for growth in low concentrations of free 3-histidine (40). We also validated the results of the analysis using GFOOLD software (27), which gives more biologically meaningful results when no biological replicate is available. The results from the GFOOLD analysis were similar to those from an analysis with Qseq software (data not shown). The association between histidine metabolism and *L. reuteri*-mediated immunomodulation is supported by a recent study showing histamine to be one immunomodulatory factor produced by *L. reuteri* 6475 (18). Purified bacterium-derived histamine inhibited TNF production at the level of transcription. The loss of TNF inhibition in the *rsiR* mutant is most likely a result of impaired histamine production.

Quantitative histamine ELISA data demonstrated significantly diminished histamine production by the *rsiR* mutant even in the presence of supplemental histidine, the major substrate to the histidine decarboxylation pathway. Increased histamine production and increased expression of the *hdc* gene cluster in the presence of histidine have been described in other histaminogenic Gram-positive bacteria, such as *Lactobacillus hilgardii* ISE 5211 (57), *L. hilgardii* 464, *Pedococcus parvulus* P270, *Oenococcus oeni* 4042 (16), and *Streptococcus thermophilus* PR160 (12). However, the regulatory mechanisms affecting *hdc* gene expression in Gram-positive bacteria are currently unknown. The inability of *RsiR*-deficient mutants to increase production of histamine when supplemented with 3-histidine suggests that RsiR may have a regulatory role on histidine production, most likely via regulation of *hdc* gene expression. This finding was affirmed by the RT-qPCR results, which demonstrated significant downregulation of *hdcA* and *hdcB*, but not *hdcP*. In the presence of supplemental 3-histidine, *hdc* genes were not upregulated in the *rsiR* mutant compared to the level of expression in wild-type *L. reuteri*. It has previously been shown in histaminogenic lactobacilli, including *L. reuteri* 6475 (data not shown), that *hdcA* and *hdcB* are coregulated by a single common promoter (*P_{hdcAB}*) and transcribed as a bicistronic mRNA, while *hdcP* is regulated by a different promoter (14, 58).

To our knowledge, our *P_{hdcAB}* promoter study is the first report of a GusA promoter assay in *L. reuteri*. The low basal level of GusA activity, along with the lack of responsiveness to supplemental 3-histidine in the *rsiR* mutant, suggested a regulatory role of RsiR for genes under the control of the *P_{hdcAB}* promoter. However, RsiR could act as a global transcriptional activator and regulate other key metabolic pathways in *L. reuteri*; RNA-seq analysis along with the subsequent DAVID functional clustering analysis (28, 29) revealed downregulation of genes involved in other biological processes. Pathways highly downregulated in the *rsiR* mutant included cysteine and methionine metabolism, fatty acid biosynthesis, and glycerolipid metabolism (see Tables S2 and S3 in the supplemental material). Moreover, the loss of TNF suppression in human myeloid cells by the *RsiR*-deficient mutant strain was greater than what was seen in cells treated with culture supernatants from *hdcA*, *hdcB*, and *hdcP* mutants (18). These results suggest that genetic activation of RsiR may globally affect pathways involved in the *L. reuteri*-mediated production of other immunomodulatory factors besides histamine, ultimately resulting in a lack of TNF inhibition through multiple mechanisms. Further promoter analysis or a DNA-protein interaction study, such as by an *in vivo* chromatin immunoprecipitation (ChIP) assay, is needed in order to characterize the putative global regulatory role of RsiR in *L. reuteri*.

In addition to the hypothesis that the gene product of *rsiR* may function as a global transcriptional activator, it is possible that transcripts of *rsiR* may also function as small RNA (sRNA) regulators. Noncoding regulatory RNAs in bacteria, which can range from 50 to 500 nucleotides in size, have been extensively characterized and shown to regulate translation or the stability of target mRNAs (20, 59). Instead of increasing the degradation and inhibiting the translation of target genes, some sRNAs that contain partial complementarity (at least 6 to 8 contiguous base pairs) with target mRNAs enhance mRNA stability and prevent the for-
mation of inhibitory secondary RNA structures, allowing more efficient translation (60). A BLAST search of rsiR against the L. reuteri 6475 genome revealed seedling sequence complementarity of 10 bp or more between rsiR and genes that were differentially expressed in the 6475rsiR-Stop mutant compared to the wild type (see Table S5 in the supplemental material). rsiR transcripts may function as global trans-encoded base-pairing sRNAs and alter the stability of mRNAs involved in different metabolic pathways. Mutations in rsiR transcripts (like those in our 6475rsiR-Stop mutant) may affect its secondary structure and regulatory function, resulting in a decreased or increased mRNA stability of its target genes, with downstream effects on various biological processes.

As discussed above, the production of bioactive microbial metabolites that are products of amino acid conversion by human intestinal microbes may play an important role in interkingdom interactions and promote the health of the host. The current study has characterized the effects of supplemental L-histidine on the production of immunomodulatory histidine and has demonstrated a possible regulatory mechanism of histidine decarboxylation in L. reuteri, which is a member of the human intestinal microbiome. Studies of RsiR and similar transcriptional/translational regulators of the human microbiome may shed light on the connections between diet, the microbiome, and innate immunity. These potential connections are highlighted by a recent study in mice fed a Westernized fast-food diet, where the authors demonstrated a significant shift in the proinflammatory immune response in the host, which included a reduction in focal inflammation in abdominal fat and weight gain in animals treated with L. reuteri 6475, the same strain studied here (61). According to the authors, these effects were interleukin-10 dependent and may have been a result of Foxp3 regulatory T cell activation by the probiotic bacteria. By understanding how dietary components such as amino acids (e.g., L-histidine) regulate gene expression through specific regulators (e.g., RsiR), the gut microbiome and its effects on immunity could be modulated or manipulated by a combination of nutritional and probiotic interventions in the future.

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