FOIC2-mediated folate metabolism contributes to suppression of inflammation by probiotic Lactobacillus reuteri

Carissa M. Thomas1,a, Delphine M. A. Saulnier2,3,a,*, Jennifer K. Spinler2,3, Peera Hemarajata4, Chunxu Gao2,3, Sara E. Jones1, Ashley Grimm2,3, Miriam A. Balderas2,3, Matthew D. Burstein5, Christina Morra1,3, Daniel Roeth6, Markus Kalkum6 & James Versalovic2,3

1Integrative Molecular and Biomedical Sciences (IMBS), Baylor College of Medicine, One Baylor Plaza, Houston, Texas 77030
2Department of Pathology & Immunology, Baylor College of Medicine, Houston, Texas
3Department of Pathology, Texas Children’s Hospital, 1102 Bates Ave, Houston, Texas 77030
4Department of Molecular Virology and Microbiology, Baylor College of Medicine, Houston, Texas
5Structural and Computational Biology and Molecular Biophysics Graduate Program, Baylor College of Medicine, Houston, Texas
6Department of Molecular Immunology, Beckman Research Institute of the City of Hope, 1500 E Duarte Rd., Duarte, California 91010

© 2016 The Authors. MicrobiologyOpen published by John Wiley & Sons Ltd.
This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

Abstract

Bacterial-derived compounds from the intestinal microbiome modulate host mucosal immunity. Identification and mechanistic studies of these compounds provide insights into host–microbial mutualism. Specific Lactobacillus reuteri strains suppress production of the proinflammatory cytokine, tumor necrosis factor (TNF), and are protective in a mouse model of colitis. Human-derived L. reuteri strain ATCC PTA 6475 suppresses intestinal inflammation and produces 5,10-methenyltetrahydrofolic acid polyglutamates. Insertional mutagenesis identified the bifunctional dihydrofolate synthase/folylpolyglutamate synthase type 2 (FOIC2) gene as essential for 5,10-methenyltetrahydrofolic acid polyglutamate biosynthesis, as well as for suppression of TNF production by activated human monocytes, and for the anti-inflammatory effect of L. reuteri 6475 in a trinitrobenzene sulfonic acid-induced mouse model of acute colitis. In contrast, FOIC encodes the enzyme responsible for folate polyglutamylation but does not impact TNF suppression by L. reuteri. Comparative transcriptomics between wild-type and mutant L. reuteri strains revealed additional genes involved in immunomodulation, including previously identified hdc genes involved in histidine to histamine conversion. The FOIC2 mutant yielded diminished hdc gene cluster expression and diminished histamine production, suggesting a link between folate and histidine/histamine metabolism. The identification of genes and gene networks regulating production of bacterial-derived immunoregulatory molecules may lead to improved anti-inflammatory strategies for digestive diseases.

Keywords
Colitis, folate, FOIC2, histamine, immunomodulation, Lactobacillus reuteri.

Correspondence
James Versalovic, Department of Pathology, Texas Children’s Hospital, Feigin Center, Suite 830, 1102 Bates Avenue, Houston, TX 77030. Tel: +1 832-824-3710; Fax: 832-825-1165; E-mail: jamesv@bcm.edu

Funding Information
This work was supported by the National Institutes of Health to JV, including the National Institute of Diabetes, Digestive and Kidney Diseases (R01 DK065075), Texas Medical Center Digestive Disease Center (P30 DK56338), National Cancer Institute (U01 CA170930), and National Center for Complementary and Alternative Medicine (R01 AT004326). Additionally, the use of facilities at City of Hope was partially supported by the National Institutes of Health National Cancer Institute (P30 CA33572).

Received: 25 December 2015; Revised: 20 March 2016; Accepted: 30 March 2016

MicrobiologyOpen 2016; 5(5): 802–818
doi: 10.1002/mbo3.371

*aCo-first authors

*Current address: Microbiome and Inflammation Start up Lab, Department of Gastrointestinal Microbiology, German Institute of Human Nutrition, Potsdam-Rehbrücke, Germany
Introduction

*Lactobacillus reuteri* is a vertebrate symbiont found in the gastrointestinal (GI) tract of a variety of mammalian species and considered indigenous to the human gut (Reuter 2001; Walter et al. 2011). Several *L. reuteri* strains are probiotics, “viable microorganisms that confer a health benefit to the host when administered in adequate amounts” (FAO/WHO, 2006). Selective deficiencies of intestinal lactobacilli have been described in patients with inflammatory bowel disease (IBD) (Giaffer et al. 1991; Vigsnaes et al. 2012; Zella et al. 2011), and oral or intrarectal supplementation with various probiotic *Lactobacillus* species effectively ameliorates intestinal inflammation in patients with pouchitis (Bibiloni et al. 2005; Gupta et al. 2000) and in rodent colitis models (Foligne et al. 2006; Holma et al. 2001; Moller et al. 2005; Pena et al. 2005; Schreiber et al. 2009; Satish Kumar et al. 2015; Liu et al. 2011; Peran et al. 2007). In vitro studies have demonstrated that lactobacilli possess species-specific, potent immunosuppressive activities, such as modulation of murine dendritic cell-induced differentiation of Th1 and Th2 cells (Christensen et al. 2002), increasing production of IL-10 from dendritic cells and macrophages (Livingston et al. 2010; Bleau et al. 2010), inhibiting production of TNF from lipopolysaccharide (LPS)-stimulated monocytes (Kim et al. 2008), and driving development of IL-10-producing regulatory T cells (Smits et al. 2005; Zhao et al. 2013). The immunosuppressive functions of probiotics, like *L. reuteri*, could be harnessed to make new therapeutics for chronic autoimmune or inflammatory disorders.

The reduction in biologically active, circulating TNF by neutralizing antibodies has been an effective treatment strategy for patients with IBD (Peyrin-Biroulet 2010) and trinitrobenzene sulfonic acid (TNBS)-challenged rats (Triantafillidis et al. 2005). Anti-TNF strategies, however, are complicated by secondary deficiencies in antimycobacterial immunity and possible sensitization or development of antibodies to these therapies (Hoentjen and van Bodegraven 2009; Jauregui-Amezaga et al. 2013; Ungar et al. 2014). Additionally, anti-TNF therapies have been associated with reactivation of hepatitis B virus and a slightly increased risk of melanoma (Chebli et al. 2014). These side effects make anti-TNF strategies less desirable as long-term therapeutics. In contrast to systemic antibody-based strategies, luminal bacteria in the intestine may be able to suppress inflammation and proinflammatory cytokine activities in a gut-specific manner. Bacterial-derived, cell-free culture supernatants of human-derived *L. reuteri* ATCC PTA 6475 (6475) and *L. reuteri* CRL1098 suppressed TNF production by primary monocyte-derived macrophages from patients with Crohn’s disease and activated myeloid cell lines (Lin et al. 2008; Pena et al. 2005) and activated peripheral blood mononuclear cells (Mechoud et al. 2012). *L. reuteri* 6475 biofilms were capable of suppressing TNF production by LPS-activated monocyte cells (Jones and Versalovic 2009). A combination of *L. reuteri* 6475 and *L. paracasei* reduced colonic TNF as well as intestinal inflammation in an IL-10-deficient, *Helicobacter hepaticus*-induced IBD mouse model (Pena et al. 2005), and a mixture of four *L. reuteri* strains was protective in a dextran sodium sulfate (DSS)-induced colitis rat model (Schreiber et al. 2009). These previously published results suggest that *L. reuteri* strains may be effective immunoregulatory probiotics, and autochthonous components of the gut microbiome may affect the biology of the mucosal immune system.

Probiotic supernatants and cell-derived factors inhibit cytokine production and suppress inflammatory signaling in macrophages and other immune cells (Grangette et al. 2005; Thomas and Versalovic 2010), but a paucity of bacterial genes and products required for immunomodulation have been identified (Grangette et al. 2005; Yasuda et al. 2008). Recently the biogenic amine, histamine, was identified as a TNF-inhibitory factor produced by *L. reuteri* 6475. Histamine is produced by the decarboxylation of l-histidine, and *L. reuteri*-mediated histamine production can be increased by histidine supplementation in the growth medium (Thomas et al. 2012). *L. reuteri* also synthesizes the essential B-complex vitamin, folate, when a precursor para-aminobenzoic acid (pABA), is provided in the medium (Spinler et al. 2014; Rossi et al. 2011; Santos et al. 2008). In selected microorganisms, folate may catalyze one-carbon units into histidine, suggesting that folate may be involved in histidine biosynthesis (Broquist 1957). Crosstalk between these microbial metabolic pathways may result in the coregulation of histamine and folate biosynthesis. Additionally, in eukaryotic cells, it is known that oxidation of amino acids, including histidine, is linked to folate metabolism. Folate plays a key role in the reduction in NAD+ to NADH and NADP+ to NADPH in the oxidation–reduction reactions necessary for one-carbon metabolism (Brosnan et al. 2015). Similar reactions may occur in prokaryotes, reinforcing the possible coregulation of histamine and folate biosynthesis in *L. reuteri*.

Greater understanding of bacterial immunomodulatory gene networks and mechanistic studies of immunomodulatory compounds should improve selection of effective probiotics for specific therapeutic applications. In this era of microbiome science, functional linkages between different microbial metabolic pathways may elucidate mechanisms of probiosis and immunoregulation by gut microbes. The goal of this study was to identify immunomodulatory genes and regulatory networks present in TNF-inhibitory *L. reuteri* 6475. These studies demonstrated a novel role for the dihydrofolate synthase/folylpolyglutamate synthase...
gene type 2 (folC2) in TNF suppression and colitis attenuation, and demonstrate a potentially important link between folate metabolism and histamine production.

**Experimental Procedures**

**Bacterial strains and culture conditions**

All bacterial strains and plasmids used in this study are described in Table S2. *L. reuteri* strains ATCC PTA 6475, ATCC 6475 folC2::pORI28, ATCC 6475 folC::pORI28, and ATCC 55730, are referred to as strains 6475, 6475::folC2, 6475::folC, and 55730, respectively. *L. reuteri* strains were cultured 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 for 16–18 h in de Man–Rogosa–Sharpe (MRS) medium (Difco, Franklin Lakes, NJ), and then inoculated into a defined medium, LDMIII (OD600 adjusted to 0.1), which has been described previously (Jones and Versalovic 2009). At stationary phase (24 h), the cells were pelleted (4000g, 10 min). Insertion mutants were cultured in the presence of 10 μg/mL erythromycin.

**Construction folC2 and folC insertion mutants (L. reuteri 6475::folC2 and 6475::folC)**

Bifunctional dihydrofolate synthase/folypolyglutamyl synthase type 2 (folC2) and bifunctional dihydrofolate synthase/ folylypolyglutamyl synthase (folC) genes were identified in the whole draft genome sequence of *L. reuteri* 6475 (GenBank NZ_ACGX02000001-007; HMPREF0536_11260 and HMPREF0536_10555, respectively). Inactivation of these genes was achieved by site-specific integration of plasmid pORI28 into the *L. reuteri* 6475 chromosome as described previously (Walter et al. 2005). Briefly, internal gene fragments were amplified by PCR (outlined in Table S2) and directionally cloned into pORI28. Site-specific homologous recombination of target-specific pORI28 vectors was performed as detailed by Walter et al. (2005). Site-specific insertional mutagenesis was confirmed by dideoxy DNA sequencing.

**Tetrahydrofolate acid compound analysis by MALDI mass spectrometry**

Cell pellets normalized by weight from wild-type and mutant *L. reuteri* strains were washed with ice-cold PBS and water. Folates were extracted by washing with 50% acetonitrile/0.1% v/v TFA. The cell suspension was centrifuged for 10 min, 4000g at 4°C. Samples were prepared with α-cyano-4-hydroxy-cinnamic acid as MALDI matrix, spotted onto a sample plate, dried, and analyzed on a pROTOF2000 MALDI mass spectrometer (PerkinElmer/Sciex, Boston, MA) or on a SimulTOF Combo 200 MALDI mass spectrometer (Virgin Instruments Marlborough, MA). For MS/MS fragmentation analysis, the same MALDI sample plate was subsequently transferred into a self-built MALDI quadrupole ion trap that was based on a modified LCQ DECA iontrap (Thermo Waltham, MA) (Krutchnisky et al. 2001). Tetrahydrofolate ions were fragmented with a 4 Da selection window, 30% collision energy, a fixed ion trap injection time of 200 msec, and a 2–10-hz laser repetition rate.

**Assessment of TNF inhibition by ELISA**

Bacterial supernatants from a 24 h LDMIII culture were filter-sterilized using polyvinylidene fluoride membrane filters (0.22 μm pore size, Millipore, Bedford, MA) and size-fractionated with Amicon Ultra-15 centrifugal filter units using ultral-3 membrane (Millipore). The filtrate was speed vacuum-dried and resuspended in RPMI medium. All supernatants were normalized by volume to OD600 = 1.5. Supernatants and cell pellet washes were tested for their ability to modulate TNF production. In vitro experiments were performed with THP-1 cells (human monocytoid 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, with 5% CO2. THP-1 cells (5 × 10⁴ cells) were stimulated to produce TNF by the addition of 100 ng/mL Pam3Cys-SKKKK x 3 HCl (EMC Microcollections, Tuebingen, Germany) as previously described (Pena et al. 2004). *L. reuteri* supernatant or cell pellet wash was added to the activated THP-1 cells (5% v/v). Plates were incubated at 37°C and 5% CO2 for 3.5 h. THP-1 cells were pelleted (3000g, 5 min, 4°C), and quantitative ELISAs were used to determine TNF quantities in THP-1 cell supernatants according to the manufacturer’s instructions (R&D Systems, Minneapolis, MN).

**TNF gene expression studies by qPCR**

THP-1 cells were treated with *L. reuteri* cell-free supernatant and PCK as described above. RNA isolation was performed with the AllPrep DNA/RNA mini kit from Qiagen (Valencia, CA) according to manufacturer’s instructions. RNA quantity and quality was assessed, and only RNA with RIN greater than or equal to 9 was used in the subsequent assays. Gene expression was analyzed using the RT² Profiler PCR Array (innate and adaptive immune response) from Qiagen according to manufacturer’s instructions. In brief, cDNA was prepared from purified RNA with the RT² First Strand kit. The cDNA was added to RT² SYBR green mastermix, and aliquoted into the
96-well RT² Proiler PCR Array of interest. All PCR reactions were performed using the Stratagene Mx3005P PCR System. Cycling parameters were as follows: program 1; One cycle of 95°C for 10 min, program 2; 40 two-step cycles of 95°C for 15 sec, 60°C for 1 min, program 3; hold at 4°C. Fluorescence was detected after the extension step in each cycle. The 2^−ΔΔCT method was used to calculate relative changes in gene expression.

Transcriptomics comparisons of L. reuteri mutants

L. reuteri 6475, 6475::folC2, and 6475::folC were cultured in LDMIII to stationary phase (24 h). For expression analyses, three biological replicates were performed with dye-swap experiments for each strain/mutant. Following mRNA isolation, cDNA synthesis, labeling, and hybridization were performed as previously described (Wall et al. 2007; Yang et al. 2005). Information regarding the microarray platforms is at the NCBI Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/) under GEO platform GPL754. The complete set of microarray data for 6475::folC2 (formerly known as 6475::thfs) and 6475::folC (formerly known as 6475::thfs2) can be found under the GEO series accession GSE32971 and GSE32972, respectively. Microarray data analysis was performed as previously described (Maguin et al. 1992) utilizing the array package in R 2.12.1. The gene set of interest (GSI) was the list of genes potentially contributing to the immunomodulatory phenotype of strain 6475. The GSI was refined by analyzing subsequent layers of gene expression data. The GSEA v2.07 analysis was performed using the online analysis tool (http://www.broadinstitute.org/gsea/index.jsp). The 402 genes significantly up-regulated in stationary phase of 6475 that were missing a homolog or not significantly up-regulated in stationary phase of 55,730 were used as the input Gene Set. The ranked list of 6475::folC2 expression pattern via fold change was used as the preranked input. A weighted enrichment statistic was calculated using 1000 permutations. The core enriched and down-regulated 6475::folC2 genes were further refined by removing genes significantly down-regulated in 6475::folC. These potential immunoregulatory genes were sorted by a combined DEDS statistic which placed equal weight on 6475::folC2 and 6475 stationary phase linear fold changes (Yang et al. 2005). DEDS represents the selected differential expression measures from the previous analyses as a multivariate point cloud with multiple dimensions associated with different input statistics. The scalar distance to the most extreme combination of these input statistics, following permutation, is the final metric by which genes are ranked and displayed here.

Gene expression studies of the L. reuteri hdc cluster

L. reuteri 6475 and 6475::folC2 were grown as described above in LDMIII or LDMIII + 4 mg/mL l-histidine. At 16 h post-inoculation, the cultures were harvested. RNA isolation and cDNA synthesis from total RNA were performed as previously described (Thomas et al. 2012). Expression of the hdcA, hdcB, hdcP, and narl genes was analyzed using quantitative real-time PCR. All primers were designed using the Universal Probe Library Assay Design Center (Roche Applied Science, Indianapolis, IN) and are described in Table S2. The RNA polymerase β-subunit (rpoB) gene was used as a reference gene. PCR reactions were set up using 2x FastStart Universal Probe Master (RoX) (Roche Applied Science) and the cDNA described above, with final concentrations of 200 nmol/L for each primer and 100 nmol/L for each probe. All PCR reactions were performed using the ViiA 7 Real-Time PCR System (Life Technologies, Carlsbad, CA). Cycling parameters were as follows: program 1; One cycle of 25°C for 2 min, one cycle of 95°C for 10 min, program 2; 50 three-step cycles of 95°C for 15 sec, 60°C for 1 min and 72°C for 1 min, program 3; hold at 4°C. Fluorescence was detected after the extension step in each cycle. The 2^−ΔΔCT method was used to calculate relative changes in gene expression.

Quantification of histamine by ELISA

Wild-type L. reuteri 6475 and 6475::folC2 were grown as described above in LDMIII or LDMIII + 4 mg/mL l-histidine. Cultures were harvested at 24 h, centrifuged (1500g), and filter-sterilized with 0.22-μm PVDF filters. Histamine concentrations were determined as previously described (Thomas et al. 2012) using the Histamine ELISA kit (Neogen, Lexington, KY). Absorbance was measured with a Spectramax 340PC (Molecular Devices, Sunnyvale, CA), and data were analyzed using GraphPad Prism 5 software. Data were corrected with values obtained from the background control.

Preparation of bacterial supernatants and administration to mice

Bacterial supernatants were prepared as described for the TNF inhibition bioassay above, filter-sterilized, and concentrated 20× with speed vacuum drying. Administration to mice was as described previously (Hemarajata et al. 2013). In brief, each mouse received two intraperitoneal injections of bacterial supernatant or medium control, with the first dose at 18 h before TNBS rectal enema (described below) and the second dose at 2 min before TNBS enema. All mouse experiments were performed in a Specific Pathogen-Free (SPF) animal facility, according to an Institutional
Animal Care and Use Committee (IACUC)-approved mouse protocol at Baylor College of Medicine, Houston, TX.

Induction of acute colitis using TNBS rectal enema

Female Balb/c mice (45-day old) were received from Harlan Laboratories (Houston, TX) and maintained under specific pathogen-free conditions. Animals were provided standard chow and water and allowed to feed ad libitum under a 12 h daylight cycle. Mice acclimated post-shipment for 10 d. Induction of TNBS colitis, determination of colitis severity, and protection conferred by probiotic compounds was performed according to established protocols with minor modifications as described previously (Foligne et al. 2006; Hemarajata et al. 2013). In brief, mice were anesthetized by constant isoflurane inhalation. A 5% v/v TNBS (Sigma-Aldrich, St. Louis, MO) solution in water was diluted with equal volume of absolute ethanol and administered intrarectally via catheter at a dose of 100 mg/kg body weight, 4 cm distal to the anus. Mice were kept head down in a vertical position for 2 min after enema to ensure complete retention of enema in the colon. Procedure control mice received 50% ethanol in PBS as an enema and two IP injections of the medium control. Colitis-positive mice received a TNBS enema and two IP injections of the medium control, while treated mice received a TNBS enema and two IP injections of the prepared bacterial supernatant. Mice were weighed immediately prior to TNBS enema and again 48 h after TNBS enema. Percent weight loss was calculated based on differences between these measurements.

Macroscopic assessment of TNBS-induced colitis

Colons were collected 48 h after induction of TNBS colitis and opened longitudinally. Colonic inflammation and damage were determined according to the Wallace criteria (Morris et al. 1989). In brief, the grading scale was: 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 at two or more sites; Score 5: major sites of damage extending >1 cm; Score 6–10: when area of damage extends >2 cm, the score is increased by each additional cm of tissue involvement. Each colon was scored blindly by one individual.

Plasma measurements of mouse serum amyloid protein A (SAA)

Blood samples were collected from mice via cardiac puncture, stored with anticoagulant, and centrifuged (10 min, 17000g) to isolate plasma. SAA levels in plasma were measured using ELISA kits from ALPCO (Salem, NH) according to the manufacturer’s instructions.

Results

Identification of 5,10-CH = THF polyglutamate compounds produced by TNF-inhibitory L. reuteri 6475

L. reuteri cell pellets from stationary phase cultures were treated with 0.1% trifluoroacetic acid (TFA)-acidified water to collect a concentrated solution of extracellular compounds loosely associated with the bacterial cell surface. TFA-treated cell pellets from TNF-inhibitory L. reuteri 6475 and the non-TNF-inhibitory strain 55730 were analyzed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry. Differences in the composition of TFA-treated cell pellets were observed. Multiple peaks (labeled MGlu_n) differing by m/z 129, the expected mass of glutamate (Glu), were identified in strain 6475 (Fig. 1A). Two of these peaks were also identified in strain 55730, but the peak intensity was much less compared to strain 6475 (Fig. 1B). These peaks were also identified in bacterial cell-free culture supernatants from strains 6475 and 55730 (data not shown). The observed masses did not match the predicted masses for simple polyglutamate homopolymers, indicating the presence of a covalently linked compound (M). MS/MS fragmentation analysis in both L. reuteri strains 6475 and 55730 at m/z 1101.4 (MGlu_5) (Fig. 1C) indicated that the core compound M was 5,10-methenyltetrahydrofolic acid (5,10-CH = THF) with a covalently linked, polyglutamate homopolymer tail (5,10-CH = THF polyglutamate). Neither 5,10-CH = THF nor 5,10-CH = THF polyglutamate were produced by the folC2 mutant (Fig. 1D), and only 5,10-CH = THF is
produced by the folC mutant (Fig. 1E). The structure of 5,10-CH = THF is depicted in Fig. 1E.

The folC2 gene in L. reuteri is required for human TNF suppression

Targeted mutagenesis was used to construct mutants defective in bifunctional dihydrofolate synthase/folylpolyglutamate synthase type 2 (folC2) and bifunctional dihydrofolate synthase/folylpolyglutamate synthase (folC), 6475::folC2 and 6475::folC, respectively. The ability of the mutants to modulate TNF levels was compared to wild-type strain 6475. Wild-type L. reuteri 6475 TFA-treated cell pellet washes containing 5,10-CH = THF and 5,10-CH = THF polyglutamate significantly inhibited TNF production by activated human monocytoid cells stimulated with a Toll-like receptor 2 (TLR2) agonist (P < 0.05) (Fig. 2). Inactivation of folC had no significant effect on TNF inhibition by strain 6475 (Fig. 2); however, inactivation of folC2 in strain 6475 resulted in abrogation of the TNF-inhibitory phenotype (Fig. 2), suggesting that folC2 is a part of the immunomodulatory gene network. Similar results were obtained with the bacterial cell-free culture supernatants of the same L. reuteri strains (Fig. S1A), indicating that the immunomodulins were actively secreted and associated with the bacterial cell surface. In TLR2-activated human monocytoid cells, TNF gene expression was decreased by L. reuteri 6475 compared to the medium control (Fig. S1B), confirming prior results that L. reuteri suppressed human TNF at the transcriptional level (Lin et al. 2008). The 6475::folC2 mutant did not suppress TNF gene expression compared to the medium control (Fig. S1B), indicating that the folC2 gene was important for human TNF suppression by L. reuteri 6475 in vitro.

FolC2 is necessary for 5,10-CH = THF and 5,10-CH = THF polyglutamate production and immunomodulation

Production of 5,10-CH = THF and 5,10-CH = THF polyglutamate in L. reuteri 6475::folC2 and 6475::folC was determined by MALDI-TOF mass spectrometry. Analysis revealed the absence of both 5,10-CH = THF and 5,10-CH = THF polyglutamate in the 6475::folC2 mutant, which lacks the TNF-inhibitory phenotype (Fig. 1D). Analysis of the 6475::folC mutant, which retains the TNF-inhibitory phenotype demonstrated the presence of 5,10-CH = THF (m/z 456.2), but not 5,10-CH = THF polyglutamate (Fig. 1E). Strain 6475::folC inhibited TNF production (Fig. 2 and S1A), indicating that polyglutamylation of 5,10-CH = THF was not necessary for TNF inhibition by L. reuteri.

Additional L. reuteri genes potentially involved in immunomodulation were identified by comparative transcriptomics between wild-type and mutant L. reuteri strains 6475 and 55730, representing the two known human clades II and VI, respectively (Spinler et al. 2014) (Fig 3A). L. reuteri immunomodulins (TNF-inhibitory factors) were only detected in strain 6475 cultures grown to stationary phase (Lin et al. 2008). The gene expression profile of strain 6475 in stationary phase (24 h) was compared to the same strain in early log phase (8 h), and 461 significantly up-regulated genes (P < 0.05) were identified as genes potentially important for immunomodulin production (Saulnier et al. 2011). These 461 up-regulated genes comprised the initial gene set of interest. L. reuteri 55730 does not inhibit TNF, allowing the gene set of interest to be restricted to up-regulated genes that were unique to strain 6475 or not significantly up-regulated in strain 55730 in stationary phase (402 total genes). A Gene Set Enrichment Analysis (GSEA) demonstrated that these 402 genes were significantly enriched in 6475::folC2 down-regulated genes (P < 0.001, Fig. 3B) (Subramanian et al. 2005).
Figure 3. Comparative transcriptomics analysis revealed gene set encoding potential immunomodulins. (A) Flowchart of the comparative transcriptomic analysis employed in this study. \( N = 3 \) for each wild-type and mutant \( L. \) reuteri strain that was included in the comparative analysis. (B) GSEA showed significant enrichment of strain 6475 genes up-regulated in stationary phase in the down-regulated genes of 6475::\textit{folC2}. (C) The yellow circle indicates genes significantly down-regulated in 6475::\textit{folC2} that were up-regulated in wild-type 6475 (stationary phase). The blue circle indicates genes that were not down-regulated in 6475::\textit{folC} that were up-regulated in wild-type 6475 (stationary phase). The red circle indicates genes that were included in the GSEA “core enrichment.” The overlap between these three gene sets revealed a final gene set of interest including 125 genes potentially involved in immunomodulin production by \( L. \) reuteri 6475. GSEA, A Gene Set Enrichment Analysis.
Lactobacillus reuteri Immunomodulatory Genes

Table 1. Bacterial genes of interest.

| Gene ID     | Description                  | Functional Group       |
|-------------|------------------------------|------------------------|
| NT01L1336   | Esterase                     | Central intermediary   |
| NT01L1981   | L1016                        | Unclassified           |
| NT01L0282   | Conserved hypothetical protein| Hypothetical protein   |
| NT01L1905   | Conserved membrane protein    | Cell envelope          |
| NT01L0849   | Hypothetical protein         | Hypothetical protein   |
| NT01L0279   | Hypothetical protein         | Hypothetical protein   |
| NT01L1786   | Respiratory nitrate reductase | Energy metabolism      |
| NT01L0128   | Amidohydrolase family, putative| Central intermediary   |
| NT01L1242   | Histidine/histamine antiporter| Transport and binding  |
| NT01L1034   | Hypothetical protein         | Hypothetical protein   |

The differential expression via distance synthesis (DEDS) statistic represents the selected differential expression measures from the previous analyses as a multivariate point cloud on as many dimensions as there are input statistics. The scalar distance to the most extreme combination of these input statistics, following permutation, was the final metric by which genes are ranked and displayed here.

Expression of the hdc gene cluster and histamine production were diminished in L. reuteri 6475::folC2

Comparative transcriptomics analysis identified 125 genes that may be responsible for immunomodulation by L. reuteri 6475 (Table 1 and Table S1). Two identified genes, hdcA and hdcP, are L. reuteri genes known to be involved in conversion of histidine to histamine, a compound that suppresses TNF production by myeloid cells (Thomas et al. 2012). Quantitative RT-PCR validated the comparative transcriptomics studies by confirming changes in gene expression in 6475::folC2 for three of the 10 genes listed in Table 1: hdcA, hdcP, and respiratory nitrate reductase gamma subunit (narI). All three genes had diminished gene expression in the 6475::folC2 mutant compared to wild-type 6475 (Fig. 4A). Expression of the complete hdc gene cluster (hdcA, hdcB, and hdcP) was examined in wild-type L. reuteri and 6475::folC2. Expression of the entire hdc gene cluster was increased in wild-type L. reuteri 6475 grown in l-histidine-supplemented medium compared to unsupplemented medium (Fig. 4B), while expression was not significantly changed in 6475::folC2 in the presence of additional histidine (Fig. 4C). Histamine production by L. reuteri 6475 and 6475::folC2 strains was measured with a histamine-specific ELISA. Production of histamine was significantly increased in L. reuteri 6475 when grown in medium supplemented with l-histidine (Fig. 4D). The folC2 mutant produced significantly less histamine compared to wild-type L. reuteri even in the presence of media supplementation with l-histidine (Fig. 4D).

The folC2 gene contributes to suppression of intestinal inflammation by L. reuteri 6475 in vivo

To investigate whether the folC2 gene contributes to anti-inflammatory effects in vivo, L. reuteri strain 6475 and 6475::folC2 bacterial cell-free supernatants were tested in a TNBS-induced mouse model of acute colitis. An 8-week-old female inbred Balb/c mice received two intraperitoneal (IP) injections of concentrated bacterial supernatant (18 h apart) followed by induction of colitis by TNBS instillation. Mice that received IP injections of the medium control and were challenged with TNBS (colitis-positive mice) or phosphate-buffered saline (PBS) (colitis-negative mice) were studied as controls. Weight loss, which reflects the overall health status of mice, and a Wallace scoring system, which assesses the relative extent of macroscopic colon injury and inflammation, were measured to evaluate colitis severity. Serum amyloid A (SAA), an acute phase protein that serves as a plasma biomarker of intestinal mucosal inflammation in mice
and correlates with severity of colitis, (de Villiers et al. 2000; Uhlar and Whitehead 1999) was quantified in plasma. Colitis-negative mice showed no evidence of spontaneous disease (Fig. 5A–B). Colitis-positive mice developed moderate colitis characterized by weight loss and macroscopic intestinal inflammation (Fig. 5A–B). As expected, these mice had significantly elevated concentrations of SAA ($P < 0.001$) compared to colitis-negative mice (Fig. 5C). IP injection with \textit{L. reuteri} 6475 supernatant reduced macroscopic inflammation (Fig. 5B). Treatment with \textit{L. reuteri} 6475 supernatant also resulted in diminished weight loss 48 h post-treatment compared to colitis-positive mice (Fig. 5A), and reduced quantities of SAA in peripheral blood serum (Fig. 5C). Treatment with bacterial supernatants from the \textit{L. reuteri} 6475::folC2 strain did not attenuate colitis as indicated by no significant change in body weight or macroscopic colonic inflammation (Fig. 5A–B). Additionally, the 6475::folC2 supernatant did not reduce SAA compared to colitis-positive mice (Fig. 5C).

**Discussion**

Probiotic \textit{Lactobacillus} species secrete a variety of organic compounds that may regulate host immune responses (Lin et al. 2008). Our results show that specific strains of
Lactobacillus reuteri are capable of producing abundant 5,10-CH = THF and polyglutamylated 5,10-CH = THF have been reported in other lactic acid bacteria cultures (Sybesma et al. 2003), this study demonstrates that production of these compounds in human-derived L. reuteri strains correlates with the ability to inhibit TNF production in vitro. Mutagenesis of the L. reuteri folC2 gene and subsequent lack of 5,10-CH = THF is associated with loss of TNF-inhibitory activity in vitro. L. reuteri 6475 attenuates murine TNBS-induced colitis and the folC2 gene is necessary for L. reuteri’s protective and anti-inflammatory activities in vivo. Additional studies demonstrate decreased L. reuteri hdc gene expression and histamine production in 6475::folC2, linking the pathways responsible for 5,10-CH = THF production to histamine production and immunomodulation (Fig. 6A–B).

This manuscript provides insights into genes and pathways involved in folate metabolism by human-associated microbes. Varied chain length 5,10-CH = THF polyglutamates (up to nine glutamate residues) were identified in cell wall-associated compounds and secreted factors of TNF-inhibitory L. reuteri strain 6475. Production of 5,10-CH = THF and 5,10-CH = THF polyglutamate of any chain length by strain 6475 required folC2. In contrast, 6475::folC maintained production of 5,10-CH = THF but without the polyglutamate tail. Based on these results, folC2 appears to be necessary for production of 5,10-CH = THF, and folC appears to encode the enzyme involved in polyglutamation of 5,10-CH = THF in L. reuteri. To date, activities of FolC2 have not been well characterized in lactobacilli. FolC2 and FolC are not orthologs and are included in different Clusters of Orthologous Groups (COGs), COG1478 and COG0285, respectively (de Crecy-Lagard 2014). However, both FolC and FolC2 are considered...
Figure 6. Folate synthesis appears to be linked to histamine production in L. reuteri 6475. (A) Folate synthesis in wild-type L. reuteri 6475 with FolC2 necessary for production of dihydrofolate and FolC responsible for addition of a polyglutamate tail to tetrahydrofolate. Folate synthesis contributes to histamine production and the anti-inflammatory effect of L. reuteri. (B) Inhibition of folate synthesis in L. reuteri 6475::folC2 leads to reduced histamine production and loss of anti-inflammatory activity. (C) Inhibition of folate polyglutamylation in L. reuteri 6475::folC does not impact histamine production and L. reuteri anti-inflammatory activity is preserved. Hdc, histidine decarboxylase.

to be bifunctional enzymes with dihydrofolate synthase and folylpolyglutamate synthase activity (Bogner and Shane 1986; Toy and Bogner 1990; Kimlova et al. 1991; Murata et al. 2000). Dihydrofolate synthase is needed for the synthesis of dihydrofolate by coupling glutamate with dihydropteroate. Conversion of dihydrofolate to tetrahydrofolate (THF) is performed as an intermediate reaction by dihydrofolate reductase, which is encoded by the folA gene (Fig. 6A). Presence of folA is ubiquitous in Lactobacillus strains (Magnusdottir et al. 2015), and consequently should not play a major role in differential folate production in lactobacilli. Folylpolyglutamate synthase adds a glutamyl tail to THF (Fig. 6A). Many lactobacilli possess folC in their genome, however, prevalence of folC2 is restricted to fewer species (PATRIC database, https://www.patricbrc.org/). Whether or not these genes are fully functional in all species and strains is currently unknown. FolC2 is found in human-derived Lactobacillus strains (L. reuteri, L. iners), as well as in lactobacilli commonly found in fermented foods (L. plantarum, L. delbrueckii, L. sakei, L. pentosus, L. buchneri, and L. hilgardii). These genes have been poorly characterized at the functional level and more research is needed in this area.

The 5,10-CH = THF compound that we detected in L. reuteri 6475 can be synthesized in two successive reactions from THF (Fig. 6A). THF is converted to 10-formyl tetrahydrofolate (10-CHO = THF) via formate tetrahydrofolate dehydrogenase. 10-CHO = THF is then transformed into 5,10-CH = THF by the bifunctional enzyme methylenetetrahydrofolate dehydrogenase/methylenetetrahydrofolate cyclohydrolase. The genes encoding these two enzymes are present in the L. reuteri strains included in this report. The conversion of 10-CHO = THF into 5,10-CH = THF also occurs spontaneously at a very low pH (Eto and Krumdieck 1980; Arnold and Reilly 2000), however, our data suggest that 5,10-CH = THF was synthesized by L. reuteri.

The generation of tetrahydrofolate compounds by L. reuteri strains contributes to immunoregulation (TNF suppression) through interconnected metabolic pathways involved in folate and histidine metabolism. Even if lactobacilli can produce abundant, long-chain 5,10-CH = THF, the TNF-inhibitory phenotype relies primarily on the ability of specific strains to convert l-histidine to histamine and several histamine metabolites (Spinler et al. 2014). Folate compounds mediate the interconversion of serine and glycine, and play a role in histidine biosynthesis and catabolism (Broquist 1957). The folC2 gene may exert its immunomodulatory effects by regulating the production of histamine, a known L. reuteri immunomodulin (Figs 6A and B). The folC gene plays a role in adding the polyglutamate tail to 5,10-CH = THF, but unlike folC2, this gene does not affect the production of histamine nor the ability of L. reuteri to suppress production of proinflammatory cytokines (Fig. 6C). Histamine production as well as the expression of genes involved in histamine production were decreased in the 6475::folC2 mutant, and parallel changes in 5,10-CH = THF and histamine production suggest that folate metabolism and specifically the folC2 gene may be important for the conversion of l-histidine to histamine. The synchronous changes in gene expression affecting histamine and folate metabolism link these two pathways, contributing to the immunomodulatory phenotype of L. reuteri. Further studies will continue to elucidate biologic connections between histamine, a known TNF-inhibitory factor, l-histidine metabolism, and other compounds produced by L. reuteri 6475 including 5,10-CH = THF.

Tetrahydrofolic acid and its derivatives (5,10-CH = THF, 10-CHO = THF) are essential cofactors that facilitate the
transfer of single-carbon units from donor molecules into important biosynthetic pathways leading to methionine, purine, and pyrimidine biosynthesis (Ragsdale 2008; Fowler 2001). Histidine is a known end product of purine biosynthesis (Allen et al. 2002). Additionally, the oxidation of amino acids, including histidine, has been linked to the role of folate in generating reducing agents, NADH and NADPH, in eukaryotes (Brosnan et al. 2015). Our gene expression data suggest that this function of folate reviewed by Brosnan et al. may also occur in prokaryotes. In the folC2 mutant, there is down-regulation of several genes encoding enzymes that require NADPH as a substrate (i.e., thioredoxin reductase, nitrate reductase, methionine-S-reductase). These enzymes are involved in oxidative stress response, making it possible that the redox state of L. reuteri plays a role in its immunosuppressive function. Preliminary studies demonstrate that growth of L. reuteri in anaerobic versus aerobic conditions (different redox states) affects folate production and immunomodulation. The studies presented here were performed under standard anaerobic conditions. When L. reuteri is grown under aerobic conditions, the composition of folate compounds is different and the ability of L. reuteri to inhibit TNF production is reduced (data not shown). Further studies are needed to understand the metabolic pathways of L. reuteri and how they are regulated to produce histamine and 5,10-CH = THF, resulting in the immunosuppression phenotype of L. reuteri.

Secreted factors produced by wild-type L. reuteri 6475 can significantly ameliorate the intestinal pathology and inflammation in a TNBS-induced mouse model of colitis. Previous studies demonstrated that delivery of pharmacological agents via the IP route is an efficient and successful method, especially when the target is within the peritoneal cavity (Chaudhary et al. 2010). We administered L. reuteri-derived secreted factors in a concentrated form inside the peritoneal cavity and observed significantly diminished ulceration, local inflammation, and mucosal biomarkers of inflammation. Our study demonstrates that secreted factors produced by L. reuteri are important for preventing colitis, enabling alternative strategies that could supplant or supplement delivery of intact viable bacteria or bacterial colonization in the gut. These findings have important implications for future human clinical trials. Studies of various L. reuteri mutants in this model have provided insights into candidate genes that may be essential for suppression of intestinal inflammation by the model commensal L. reuteri strain 6475. In this study, we demonstrated the role of the Lactobacillus gene, folC2, as a potential key regulatory gene in the microbiome involved in protection from or amelioration of colitis. Our data support the conclusion that L. reuteri strain 6475 protects mammals from severe intestinal inflammation by production and secretion of potent immunosuppressive compounds locally in the gut lumen.

Deficiencies in bacterial-derived micronutrients such as folate can lead to immune dysregulation highlighting the interdependence between diet, commensal bacteria, and the host mucosal immune system (Spencer and Belkaid 2012). Diminished TNF production by M1 macrophages has been observed when these cells were grown in the presence of folic acid (Samaniego, 2014). Immune cells of the GI tract such as regulatory T cells express high levels of the folate receptor 4 (FR4) (Yamaguchi et al. 2007). Interestingly, it has been demonstrated that folic acid can prevent TNBS-induced colitis by maintaining regulatory T cells via suppression of apoptosis and subsequent prevention of colonic inflammation. Mice fed a folic acid-free diet had significantly increased colonic inflammation, weight loss, and mortality rate after exposure to TNBS compared to mice fed a normal chow diet (Kinoshita et al. 2012). Protection against colitis could be restored if FR4-expressing regulatory T cells were transferred into folic acid-free diet mice prior to induction of colitis (Kinoshita et al. 2012). Folate is also able to activate mucosal-associated invariant T cells (MAIT) in the GI tract by acting as antigens on infected cells (Chua and Hansen 2012; Kjer-Nielsen et al. 2012). In our TNBS-induced colitis studies, decreased production of 5,10-CH = THF by L. reuteri 6475::folC2 may contribute to the diminished protective effect by reduced TNF suppression as well as loss or dysfunction of regulatory T cells in the colon.

A comprehensive understanding of gene networks and gene regulation in beneficial gut microbes is critical to understanding the interaction of bacterial metabolites with the host. This knowledge may enhance the ability of the scientific community to select or engineer commensal gut bacterial strains that can suppress mucosal inflammation. Combining a genome-scale metabolic model of Bacteroides thetaiotaomicron (iAH991) with a mouse metabolic model demonstrated the essential host–microbe symbiosis that occurs in the GI tract (Heinken et al. 2013). Modeling metabolic interactions between a gut microbe and its host has enabled identification of metabolites that are exchanged between the two organisms, influences on growth fitness for both organisms, and the ability of commensal bacteria to rescue lethal enzyme deficiencies in the host (Heinken et al. 2013). The recent identification of multiple biological pathways and genes involved in suppression of proinflammatory cytokine production in L. reuteri provides opportunities for combining such discoveries in future therapies and disease prevention strategies. For example, the potential dietary contribution of the amino acid l-histidine to bacterial histamine generation in combination with
methods to enhance production of tetrahydrofolic acid compounds may result in nutritional and immunomodulatory benefits for the mammalian host. As metabolic pathways and modules become linked together in human microbiome research (Hmp 2012), nutritional and medical interventions may promote healthy whole body metabolism and immune function in partnership with the gut microbiome. Future probiotic strategies will benefit from the identification of biochemical compounds and genes required for healthy intestinal physiology and probiotic-mediated immunomodulation.

Acknowledgments

This work was supported by the National Institutes of Health to JV, including the National Institute of Diabetes, Digestive and Kidney Diseases (R01 DK065075), Texas Medical Center Digestive Disease Center (P30 DK56338), National Cancer Institute (U01 CA170930), and National Center for Complementary and Alternative Medicine (R01 AT004326). Additionally, the use of facilities at City of Hope was partially supported by the National Institutes of Health, National Cancer Institute (P30 CA33572). We thank Eamonn Connolly (BioGaia AB, Stockholm) for providing the L. reuteri strains, Oscar Ayala and Sujata Gosh for culturing the strains, Fan Zhang for his efforts in designing the TNBS mouse model, and Toni-Ann Mistrutta and Ruth Ann Luna for assistance with the statistical analysis of the data.

Conflict of Interest

JV receives unrestricted research support from BioGaia AB.

References

Allen, S., J. L. Zilles, and D. M. Downs. 2002. Metabolic flux in both the purine mononucleotide and histidine biosynthetic pathways can influence synthesis of the hydroxymethyl pyrimidine moiety of thiamine in Salmonella enterica. J. Bacteriol. 184:6130–6137.

Arnold, R. J., and J. P. Reilly. 2000. Observation of tetrahydrofolylpolyglutamic acid in bacteria cells by matrix-assisted laser desorption/ionization mass spectrometry. Anal. Biochem. 281:45–54.

Bibiloni, R., R. N. Fedorak, G. W. Tannock, K. L. Madsen, P. Gionchetti, M. Campieri, et al., 2005. VSL#3 probiotic-mixture induces remission in patients with active ulcerative colitis. Am. J. Gastroenterol. 100:1539–1546.

Bleau, C., A. Monges, K. Rashidan, J. P. Laverdure, M. Lacroix, M. R. Van Calsteren, et al., 2010. Intermediate chains of exopolysaccharides from Lactobacillus rhamnosus RW-9595M increase IL-10 production by macrophages. J. Appl. Microbiol. 108:666–675.

Bognar, A. L., and B. Shane. 1986. Bacterial folylpoly(gamma-glutamate) synthase-dihydrofolate synthase. Methods Enzymol. 122:349–359.

Broquist, H. P. 1957. Evidence for the involvement of folic acid in histidine synthesis in microorganisms. Arch. Biochem. Biophys. 70:210–216.

Brosnan, M. E., L. MacMillan, J. R. Stevens, and J. T. Brosnan. 2015. Division of labour: how does folate metabolism partition between one-carbon metabolism and amino acid oxidation? Biochem. J. 472:135–146.

Chaudhary, K., S. Haddadin, R. Nistala, and C. Papageorgio. 2010. Intra­peritoneal drug therapy: an advantage. Curr. Clin. Pharmacol. 5:82–88.

Chebli, J. M., P. D. Gaburri, L. A. Chebli, T. C. da Rocha Ribeiro, A. L. Pinto, O. Ambrogini Junior, et al. 2014. A guide to prepare patients with inflammatory bowel diseases for anti-TNF-alpha therapy. Med. Sci. Monit. 20:487–498.

Christensen, H. R., H. Frokiaer, and J. J. Pestka. 2002. Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. J. Immunol. 168:171–178.

Chua, W. J., and T. H. Hansen. 2012. Immunology: vitamins prime immunity. Nature 491:680–681.

de Crecy-Lagard, V. 2014. Variations in metabolic pathways create challenges for automated metabolic reconstructions: examples from the tetrahydrofolate synthesis pathway. Comput. Struct. Biotechnol. J. 10:41–50.

Eto, I., and C. L. Krumdieck. 1980. Determination of three different pools of reduced one-carbon-substituted folates. 1. A study of the fundamental chemical reactions. Anal. Biochem. 109:167–184.

FAO/WHO. 2006. Probiotics in Food: health and nutritional properties and guidelines for evaluation. Report of the Joint Food and Agriculture Organization (FAO) of the United Nations / World Health Organization (WHO) Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria. Report of the Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food.

Foligne, B., S. Nutten, L. Steidler, V. Dennin, D. Goudercourt, A. Mercenier, et al. 2006. Recommendations for improved use of the murine TNBS-induced colitis model in evaluating anti-inflammatory properties of lactic acid bacteria: technical and microbiological aspects. Dig. Dis. Sci. 51:390–400.

Fowler, B. 2001. The folate cycle and disease in humans. Kidney Int. Suppl. 78:S221–229.

Giaffer, M. H., C. D. Holdsworth, and B. I. Duerden. 1991. The assessment of faecal flora in patients with...
inflammatory bowel disease by a simplified bacteriological technique. J. Med. Microbiol. 35:238–243.

Granette, C., S. Nutten, E. Palumbo, S. Morath, C. Hermann, J. Dewulf, et al., 2005. Enhanced anti-inflammatory capacity of a Lactobacillus plantarum mutant synthesizing modified teichoic acids. Proc. Natl Acad. Sci. USA 102:10321–10326.

Gupta, P., H. Andrew, B. S. Kirschner, and S. Guandalini. 2009. Lactobacillus reuteri Immunomodulatory Genes and anti-inflammatory properties as a potential treatment for inflammatory bowel disease in Crohn's disease? Results of a preliminary, open-label study. J. Pediatr. Gastroenterol. Nutr. 31:453–457.

Hemmink, A., S. Sahoo, R. M. Fleming, and I. Thiele. 2013. Systems-level characterization of a host-microbe metabolic symbiosis in the mammalian gut. Gut. Microbes 4:28–40.

Hemarajata, P., C. Gao, K. J. Pfughoeft, C. M. Thomas, D. M. Saulnier, J. K. Spinler, et al. 2013. Lactobacillus reuteri-specific immunoregulatory gene rsiR modulates histamine production and immunomodulation by Lactobacillus reuteri. J. Bacteriol. 195:5567–5576.

Hmp, H. M. P. C. 2012. Structure, function and diversity of the healthy human microbiome. Nature 486:207–214.

Hoentjen, F., and A. A. van Bodegraven. 2009. Safety of anti-tumor necrosis factor therapy in inflammatory bowel disease. World J. Gastroenterol. 15:2067–2073.

Holma, R., P. Salmenpera, J. Lohi, H. Vapaatalo, and R. Korpela. 2001. Effects of Lactobacillus rhamnosus GG and Lactobacillus reuteri R2LC on acetic acid-induced colitis in rats. Scand. J. Gastroenterol. 36:630–635.

Jauregui-Amezaga, A., F. Turon, I. Ordas, M. Gallego, F. Feu, E. Ricart, et al. 2013. Risk of developing tuberculosis under anti-TNF treatment despite latent infection screening. J. Crohns. Colitis 7:208–212.

Jones, S. E., and J. Versalovic. 2009. Probiotic Lactobacillus reuteri biofilms produce antimicrobial and anti-inflammatory factors. BMC Microbiol. 9:35.

Kim, H. G., N. R. Kim, M. G. Gim, J. M. Lee, S. Y. Lee, M. Y. Ko, et al., 2008. Lipoteichoic acid isolated from Lactobacillus plantarum inhibits lipopolysaccharide-induced TNF-alpha production in THP-1 cells and endotoxin shock in mice. J. Immunol. 180:2553–2561.

Kimlová, L. J., C. Pyne, K. Keshavjee, J. Huy, G. Beebakhee, and A. L. Bognar. 1991. Mutagenesis of the folC gene encoding folylpolyglutamate synthetase-dihydrofolate synthetase in Escherichia coli. Arch. Biochem. Biophys. 284:9–16.

Kinoshita, M., H. Kayama, T. Kusu, T. Yamaguchi, J. Kunisawa, H. Kiyono, et al., 2012. Dietary folic acid promotes survival of Foxp3 + regulatory T cells in the colon. J. Immunol. 189:2869–2878.

Kjer-Nielsen, L., O. Patel, A. J. Corbett, J. Le Nours, B. Meehan, L. Liu, et al., 2012. MR1 presents microbial vitamin B metabolites to MAIT cells. Nature 491:717–723.

Knutson, L., C. H. Thibodeau, J. A. Pena, G. D. Ferry, and J. Versalovic. 2008. Probiotic Lactobacillus reuteri suppress proinflammatory cytokines via c-Jun. Inflamm. Bowel Dis. 14:1068–1083.

Liu, Y. W., Y. W. Su, W. K. Ong, T. H. Cheng, and Y. C. Tsai. 2011. Oral administration of Lactobacillus plantarum K68 ameliorates DSS-induced ulcerative colitis in BALB/c mice via the anti-inflammatory and immunomodulatory activities. Int. Immunopharmacol. 11:2159–2166.

Livingston, M., D. Loach, M. Wilson, G. W. Tannock, and M. Baird. 2010. Gut commensal Lactobacillus reuteri 100-23 stimulates an immunoregulatory response. Immunol. Cell Biol. 88:99–102.

Magnudottir, S., D. Ravcheev, V. de Crecy-Lagard, and I. Thiele. 2013. Systematic genome assessment of B-vitamin biosynthesis suggests co-operation among gut microbes. Front. Genet. 6:148.

Maguin, E., P. Duwat, T. Hege, D. Ehrlich, and A. Gruss. 1992. New thermosensitive plasmid for gram-positive bacteria. J. Bacteriol. 174:5633–5638.

Mechoud, M. A., M. V. Mateos, G. F. de Valdez, J. Villena, G. A. Salvador, and A. V. Rodriguez. 2012. Lactobacillus reuteri CRL1098 soluble factors modulate tumor necrosis factor alpha production in peripheral blood mononuclear cells: involvement of lipid rafts. Int. Immunopharmacol. 14:446–453.

Moller, P. L., A. Paerregaard, M. Gad, N. N. Kristensen, and M. H. Claesson. 2005. Colitic scid mice fed Lactobacillus spp. show an ameliorated gut histopathology and an altered cytokine profile by local T cells. Inflamm. Bowel Dis. 11:814–819.

Morris, G. P., P. L. Beck, M. S. Herridge, W. T. Depew, M. R. Szewczuk, and J. L. Wallace. 1989. Hapten-induced model of chronic inflammation and ulceration in the rat colon. Gastroenterology 96:795–803.

Murata, T., A. L. Bognar, T. Hayashi, M. Ohnishi, K. Nakayama, and Y. Terawaki. 2000. Molecular analysis of the folC gene of Pseudomonas aeruginosa. Microbiol. Immunun. 44:879–886.

Pena, J. A., S. Y. Li, P. H. Wilson, S. A. Thibodeau, A. J. Szary, and J. Versalovic. 2004. Genotypic and phenotypic studies of murine intestinal lactobacilli: species differences in mice with and without colitis. Appl. Environ. Microbiol. 70:558–568.

Pena, J. A., A. B. Rogers, Z. Ge, V. Ng, S. Y. Li, J. G. Fox, et al. 2005. Probiotic Lactobacillus spp. diminish Helicobacter hepaticus-induced inflammatory bowel disease in interleukin-10-deficient mice. Infect. Immun. 73:912–920.
ecotypes of *Lactobacillus reuteri* have diverse probiotic functions. Genome Biol. Evol. 6:1772–1789.

Subramanian, A., P. Tamayo, V. K. Mootha, S. Mukherjee, B. L. Ebert, M. A. Gillette, et al., 2005. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc. Natl Acad. Sci. USA 102:15545–15550.

Sybesma, W., M. Starrenburg, L. Tijseling, M. H. Hoefnagel, and J. Hugenholtz. 2003. Effects of cultivation conditions on folate production by lactic acid bacteria. Appl. Environ. Microbiol. 69:4542–4548.

Thomas, C. M., and J. Versalovic. 2010. Probiotics-host communication: modulation of signaling pathways in the intestine. Gut. Microbes 1:148–163.

Thomas, C. M., T. Hong, J. P. van Pijkeren, P. Hemarajata, D. V. Trinh, W. Hu, et al., 2012. Histamine derived from probiotic *Lactobacillus reuteri* suppresses TNF via modulation of PKA and ERK signaling. PLoS ONE 7:e31951.

Toy, J., and A. L. Bognar. 1990. Cloning and expression of the gene encoding Lactobacillus casei folypolyglutamate synthetase in *Escherichia coli* and determination of its primary structure. J. Biol. Chem. 265:2492–2499.

Triantafillidis, J. K., A. E. Papalois, A. Parasi, E. Anagnostakis, S. Burnazos, A. Gikas, et al., 2005. Favorable response to subcutaneous administration of infliximab in rats with experimental colitis. World J. Gastroenterol. 11:6843–6847.

Uhlar, C. M., and A. S. Whitehead. 1999. Serum amyloid A, the major vertebrate acute-phase reactant. Eur. J. Biochem. 265:501–523.

Ungar, B., Y. Chowers, M. Yavzori, O. Picard, E. Fudim, O. Har-Noy, et al., 2014. The temporal evolution of antidrug antibodies in patients with inflammatory bowel disease treated with infliximab. Gut 63:1258–1264.

Vignaes, L. K., J. Brynskov, C. Steenholdt, A. Wilcks, and T. R. Licht. 2012. Gram-negative bacteria account for main differences between faecal microbiota from patients with ulcerative colitis and healthy controls. Benef. Microbes 3:287–297.

de Villiers, W. J., G. W. Varilek, F. C. de Beer, J. T. Guo, and M. S. Kindy. 2000. Increased serum amyloid A levels reflect colitis severity and precede amyloid formation in IL-2 knockout mice. Cytokine 12:1337–1347.

Wall, T., K. Bath, R. A. Britton, H. Jonsson, J. Versalovic, and S. Roos. 2007. The early response to acid shock in *Lactobacillus reuteri* involves the ClpL chaperone and a putative cell wall-altering esterase. Appl. Environ. Microbiol. 73:3924–3935.

Walter, J., P. Chagnaud, G. W. Tannock, D. M. Loach, F. Dal Bello, H. F. Jenkinson, et al., 2005. A high-molecular-mass surface protein (Lsp) and methionine sulfoxide reductase B (MsrB) contribute to the ecological
performance of *Lactobacillus reuteri* in the murine gut. Appl. Environ. Microbiol. 71:979–986.

Walter, J., R. A. Britton, and S. Roos. 2011. Host-microbial symbiosis in the vertebrate gastrointestinal tract and the *Lactobacillus reuteri* paradigm. Proc. Natl Acad. Sci. USA 108(Suppl 1):4645–4652.

Yamaguchi, T., K. Hirota, K. Nagahama, K. Ohkawa, T. Takahashi, T. Nomura, et al. 2007. Control of immune responses by antigen-specific regulatory T cells expressing the folate receptor. Immunity 27:145–159.

Yang, Y. H., Y. Xiao, and M. R. Segal. 2005. Identifying differentially expressed genes from microarray experiments via statistic synthesis. Bioinformatics 21:1084–1093.

Yasuda, E., M. Serata, and T. Sako. 2008. Suppressive effect on activation of macrophages by *Lactobacillus casei* strain Shirota genes determining the synthesis of cell wall-associated polysaccharides. Appl. Environ. Microbiol. 74:4746–4755.

Zella, G. C., E. J. Hait, T. Glavan, D. Gevers, D. V. Ward, C. L. Kitts, et al. 2011. Distinct microbiome in pouchitis compared to healthy pouches in ulcerative colitis and familial adenomatous polyposis. Inflammm. Bowel Dis. 17:1092–1100.

Zhao, H. M., X. Y. Huang, Z. Q. Zuo, Q. H. Pan, M. Y. Ao, F. Zhou, et al., 2013. Probiotics increase T regulatory cells and reduce severity of experimental colitis in mice. World J. Gastroenterol. 19:742–749.

**Supporting Information**

Additional supporting information may be found in the online version of this article:

Figure S1. FocC2 was necessary for suppression of TNF production at protein and mRNA levels. (A) *L. reuteri* cell-free supernatants (normalized to an OD₆₀₀ of 1.5) were tested for the ability to inhibit TNF production by TLR2-activated THP-1 cells. THP-1 cells were treated with 100 ng/mL PCK (TLR2 agonist) in the presence of *L. reuteri* for 3.5 h and TNF production was monitored by ELISA. As seen with the cell pellets, wild-type 6475 significantly inhibited TNF compared to medium control. The 6475::focC2 mutant yielded significantly reduced ability to inhibit TNF production compared to wild-type 6475. There was no significant difference between 6475 and 6475::focC in terms of effects on human TNF production. Data were analyzed with one-way analysis of variance with Bonferroni's multiple comparison test correction, mean ± SD, n = 3, *P < 0.05 compared to medium control #P < 0.05 compared to 6475. (B) TNF gene expression was determined in THP-1 cells treated with a TLR2 agonist plus medium control, 6475, or 6475::focC2 cell-free supernatants. Quantitative real-time PCR demonstrated down-regulation of human TNF gene expression by *L. reuteri* strain 6475. No significant effects on human TNF gene expression were seen when THP-1 cells were treated with 6475::focC2. Gene expression data were normalized using five housekeeping genes, b2 m, hprt1, rpl13A, gapdh, and actb. Expression ratios of *tnf* (*L. reuteri* strain/medium control) were calculated, and results represent the mean ± SD, n = 3, *P < 0.05 compared to the theoretical mean of 1.0.

Table S1. Final Gene Set of Interest – 125 genes potentially involved in immunomodulation by wild-type 6475.

Table S2. Bacterial strains, vectors, and primers used in this study.