Host lysozyme-mediated lysis of *Lactococcus lactis* facilitates delivery of colitis-attenuating superoxide dismutase to inflamed colons

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Beneficial microbes that target molecules and pathways, such as oxidative stress, which can negatively affect both host and microbiota, may hold promise as an inflammatory bowel disease therapy. Prior work showed that a five-strain fermented milk product (FMP) improved colitis in *T-bet*$^{-/-}$ *Rag2*$^{-/-}$ mice. By varying the number of strains used in the FMP, we found that *Lactococcus lactis* I-1631 was sufficient to ameliorate colitis. Using comparative genomic analyses, we identified genes unique to *L. lactis* I-1631 involved in oxygen respiration. Respiration of oxygen results in reactive oxygen species (ROS) generation. Also, ROS are produced at high levels during intestinal inflammation and cause tissue damage. *L. lactis* I-1631 possesses genes encoding enzymes that detoxify ROS, such as superoxide dismutase (SodA). Thus, we hypothesized that lactococcal SodA played a role in attenuating colitis. Inactivation of the *sodA* gene abolished *L. lactis* I-1631's beneficial effect in the *T-bet*$^{-/-}$ *Rag2*$^{-/-}$ model. Similar effects were obtained in two additional colonic inflammation models, *Il10*$^{-/-}$ mice and dextran sulfate sodium-treated mice. Efforts to understand how a lipophobic superoxide anion (O_2^-) can be detoxified by cytoplasmic lactoccocal SodA led to the finding that host antimicrobial-mediated lysis is a prerequisite for SodA release and SodA's extracytoplasmic O_2^- scavenging. *L. lactis* I-1631 may represent a promising vehicle to deliver antioxidant, colitis-attenuating SodA to the inflamed intestinal mucosa, and host antimicrobials may play a critical role in mediating SodA's bioaccessibility.

Lactococcus lactis | oxidative stress | lysozyme | colitis | probiotics

Inflammatory bowel disease (IBD) pathophysiology is driven by both host genetic mutations and the gut microbiota. Immune dysregulation in IBD can result from deficiencies in acute inflammatory response pathways (1) or impaired counterregulation of immune responsiveness (2). Host production of reactive oxygen species (ROS) is an evolutionarily conserved response to microbes (3). However, chronic and excessive ROS up-regulate host inflammatory pathways (4, 5) and result in oxidative stress. Chronic intestinal inflammation and oxidative stress affect not only the host but also the microbiota. Oxidative stress within the lumen is a fitness challenge for gut anaerobic bacteria. IBD patient fecal microbiomes reflect a pattern of response to oxidative stress with enrichments in genes for sulfate transport and cysteine and glutathione metabolism (6). In IBD, oxidative stress contributes to chronic inflammation and dysbiosis, and modulating oxidative stress may help to restore intestinal homeostasis.

Beneficial microbes hold promise for IBD inflammation and dysbiosis (7). However, human clinical trials have shown mixed results (8, 9) because of variations in microbes under study and patient heterogeneity. Preclinical studies that use model systems that recapitulate key features of the human disease are needed to elucidate the mechanism of action of beneficial microbes on hosts and their microbiota. Such information facilitates clinical trial design by identifying patients with the host and microbial features most likely to benefit from the bioactivity of a beneficial microbe. Identifying microbes that target molecules and pathways such as oxidative stress—which negatively affects both host and microbiota—affords opportunities for new IBD therapies.

Building on prior studies examining how a five-strain fermented milk product (FMP) affected the gut microbiome in a preclinical model of colitis and human subjects (10–12), herein we focused on how individual bacterial strains in the FMP affected host response in several preclinical colitis models. One of the five strains, *Lactococcus lactis* subsp. *lactis* CNCM I-1631 (*L. lactis* I-1631), reduced

Significance

Microbes hold promise as an inflammatory bowel disease (IBD) therapy. *Lactococcus lactis*, which has not been appreciated as a beneficial microbe, attenuated colitis in three preclinical mouse IBD models. Neither colonization nor an intact bacterium throughout the colon per se was required. Rather, host lysozyme-mediated lysis in an inflamed colon led to *L. lactis*'s release of its superoxide dismutase, which was necessary for its colitis-attenuating and oxidative stress-reducing activity. Overall, these findings unveil a mechanism by which a bacterium offers benefits to the host but requires the host for targeted release of this beneficial activity. Furthermore, because *L. lactis* is generally regarded as safe, it represents an opportunity for rapid bench-to-bedside testing in IBD.

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gut oxidative stress and attenuated colitis in three mouse models of colonic inflammation: BALB/c *T-bet*$^{-/-}$ *Rag2*$^{-/-}$ mice, BALB/c *Il10*$^{-/-}$ mice, and BALB/c wild-type mice treated with dextran sulfate sodium (DSS), a colitogenic mucosal disruptant. The colitis-attenuating activity of *L. lactis* I-1631 was dependent on *L. lactis* I-1631 superoxide dismutase A (SodA), which reduced colonic epithelial ROS. Our data also support that host factors, increased at sites of inflammation, facilitated targeted delivery of *L. lactis* I-1631 effects. Specifically, lysis of *L. lactis* I-1631 by the host peptidoglycan hydrolase and antimicrobial lysozyme-1 appeared to mediate *L. lactis* I-1631 SodA release and reduction in host oxidative stress.

Results

An *L. lactis* FMP Attenuates Colitis in *T-bet*$^{-/-}$ *Rag2*$^{-/-}$, *Il10*$^{-/-}$, and DSS-Treated Mice. Unraveling the specific bioactivities that beneficial microbes afford to a host is challenging. Prior work suggested that a five-strain FMP attenuated colitis in an innate immune model of colitis (10) and furnished the opportunity to determine the contributions of these strains to attenuating colitis. We began by varying the number of strains present in the five-strain FMP fed to *T-bet*$^{-/-}$ *Rag2*$^{-/-}$ mice (13). Two single-strain FMPs prepared with either *Bifidobacterium animalis* subsp. *lactis* I-2494 (*B. animalis* I-2494) or *L. lactis* I-1631 ameliorated colitis to a level comparable to the five-strain FMP (Fig. 1*A*). This was not unexpected for *B. animalis* I-2494, because this probiotic species has antiinflammatory activities in various animal models (14, 15). However, in our experiments, the *L. lactis* I-1631 FMP was highly effective in reducing colitis (Fig. 1*A*) and, heretofore, *L. lactis* has not been widely recognized as a beneficial microbe.

Using endoscopic imaging of *T-bet*$^{-/-}$ *Rag2*$^{-/-}$ mice fed the *L. lactis* I-1631 FMP, we observed a marked reduction in colonic inflammation versus sham-handled mice, which were fed an equal volume of water (Fig. 1*B*, representative distal colon endoscopic images). With subsequent evaluation using microscopy, *L. lactis* I-1631 FMP-fed mice showed no evidence of colitis in contrast to sham-handled mice (Fig. 1*B*, representative micrographs).

L. lactis I-1631 is a bacterium used in the dairy industry, because it imparts desirable properties to food. There is limited knowledge that it possesses antiinflammatory bioactivities. In in vitro assays used to screen beneficial microbes, such as attenuation of LPS-induced TNF-α production or amplification of IL-10 production (16, 17), *L. lactis* I-1631 did not exhibit these activities (Fig. S1). To mitigate concerns that this strain's effects were exclusive to the *T-bet*$^{-/-}$ *Rag2*$^{-/-}$ model and to evaluate whether its effects would extend to other intestinal inflammation models, we tested the *L. lactis* I-1631 FMP in *Il10*$^{-/-}$ mice and wild-type mice treated with DSS. *L. lactis* I-1631 FMP attenuated colitis in *Il10*$^{-/-}$ (Fig. 1*C*) and DSS-treated wild-type mice (Fig. 1*E* and Table S1) compared with controls. Endoscopic imaging of the distal colons from *Il10*$^{-/-}$ (Fig. 1*D*) and DSS-treated mice (Fig. 1*F*) demonstrated a marked reduction in colonic inflammation in *L. lactis* I-1631 FMP-fed mice compared with sham-treated mice (Fig. 1 *D* and *F*).

***L. lactis* Ameliorates Colitis in a SodA-Dependent Manner.** Given the phylogenetic and metabolic differences between *B. animalis* I-2494 and *L. lactis* I-1631, we hypothesized that their effects on intestinal inflammation derived from distinct mechanisms. We explored the metabolic differences distinguishing these two species by projecting on the KEGG (Kyoto Encyclopedia of Genes and Genomes) metabolic map the functions ascribed to the clusters of orthologous groups (COGs) of proteins encoded by the *B. animalis* I-2494 genome. Predicted functions present in *L. lactis* I-1631 but absent from *B. animalis* I-2494 were also projected on the same map (Fig. 2*A*). Out of the 792 nonredundant COGs assigned to *B. animalis* I-2494, 639 (~80%) were shared with *L. lactis* I-1631. Out of the 1,230 nonredundant COGs assigned to the *L. lactis*

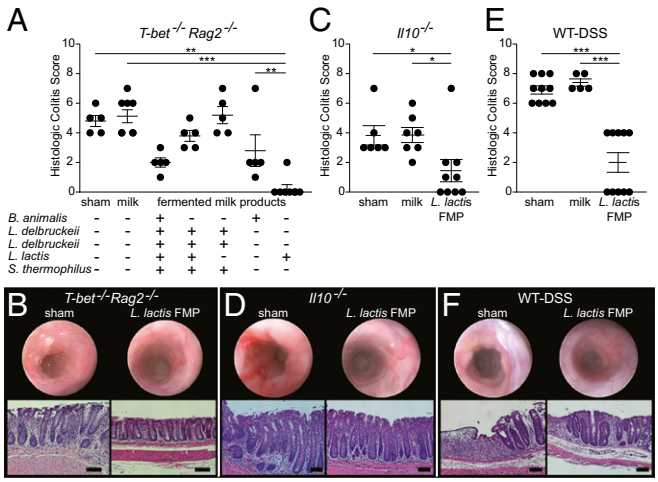

Fig. 1. *L. lactis* FMP attenuates colitis in *T-bet*$^{-/-}$ *Rag2*$^{-/-}$, *Il10*$^{-/-}$, and DSS-treated BALB/c wild-type mice. (*A*) Histologic colitis scores from *T-bet*$^{-/-}$ *Rag2*$^{-/-}$ mice treated as labeled. Symbols represent data from individual mice from three experiments. (*B*) Endoscopic distal colon images (*Upper*) and H&E section photomicrographs from distal colons (*Lower*) of *T-bet*$^{-/-}$ *Rag2*$^{-/-}$ mice treated as labeled. (*C*) Histologic colitis scores from *Il10*$^{-/-}$ mice, treated as labeled. Symbols represent data from individual mice from three experiments. (*D*) Endoscopic distal colon images and H&E section photomicrographs from distal colons of *Il10*$^{-/-}$ mice treated as labeled. (*E*) Histologic colitis scores from DSS-exposed mice treated as labeled. Symbols represent data from individual mice from three experiments. (*F*) Endoscopic distal colon images and H&E section photomicrographs from distal colons of DSS-treated wild-type mice treated as labeled. Error bars indicate mean ± SEM; Kruskal–Wallis test with post hoc Dunn's comparison test. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001. (Scale bars, 100 μm.)

I-1631 genome, 591 COGs were not detected in *B. animalis* I-2494. Consideration of *L. lactis* I-1631's unique COGs led us to pathways and functions involved in oxygen utilization/respiration (e.g., cytochrome *bd* and menaquinone pathways) and oxygen radical detoxification (e.g., SodA) (Fig. 2*A*). SodA converts the superoxide anion (O_2^-) into hydrogen peroxide.

To test whether *L. lactis* I-1631's capacity to detoxify superoxide contributes to its ability to reduce colitis in mice, we generated an isogenic deletion mutant lacking *sodA*. As expected, the *sodA* deletion (Δ*sodA*) mutant displayed compromised growth kinetics in vitro when cultivated aerobically on solid medium or in milk (Fig. 2*B*). The complemented strain (Δ*sodA*/p*sodA*$^+$) was able to restore aerobic growth under both conditions (Fig. 2 *B* and *C*). This growth defect did not affect survival of the Δ*sodA* mutant in vivo in *T-bet*$^{-/-}$ *Rag2*$^{-/-}$ mice fed single-strain FMPs, using *L. lactis* Δ*sodA* versus *L. lactis* I-1631 or *L. lactis* Δ*sodA*/p*sodA*$^+$, by comparing culturable counts of these strains plated from stool and proximal and distal colon homogenates (Fig. 2*D*). Thus, we were able to test whether these single-strain FMPs attenuated inflammation in *T-bet*$^{-/-}$ *Rag2*$^{-/-}$, *Il10*$^{-/-}$, and DSS-exposed wild-type mice. Loss of *sodA* abrogated the colitis-attenuating effects of *L. lactis* I-1631, whereas the fermented milk with the complemented strain restored the inflammation-dampening effects in all three models (Fig. 2*E* and Tables S2 and S3).

***L. lactis* I-1631 Reduces Primary Colonic Epithelial Cell Superoxide Levels.** Oxidative stress occurs when there is an imbalance between ROS production and scavenging. To determine whether *L. lactis* I-1631 was lowering oxidative stress in vivo, we measured superoxide levels in colonic epithelial cells (CECs) isolated from *T-bet*$^{-/-}$ *Rag2*$^{-/-}$ mice that were fed *L. lactis* I-1631 fermented milk or sham-handled. Levels of colonic epithelial superoxide

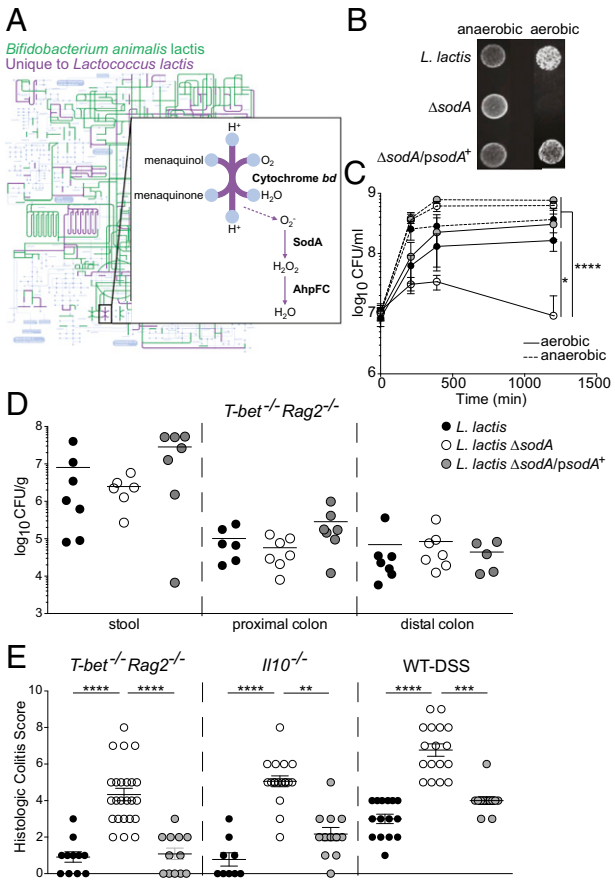

Fig. 2. *L. lactis* I-1631 ameliorates colitis in a SodA-dependent manner. (*A*) iPath projection of KEGG metabolic pathways for predicted functions of *B. animalis* I-2494 (green) and predicted functions present in *L. lactis* I-1631 and absent from *B. animalis* I-2494 (purple). The KEGG global map (gray) is in the background layer. (*Inset*) Details and adaptation of the oxidative phosphorylation pathway present in *L. lactis* I-1631 and absent from *B. animalis* I-2494, with superoxide generation indicated by the dashed arrow. (*B*) Photograph of *L. lactis* I-1631, the Δ*sodA* mutant, and the Δ*sodA*/p*sodA*$^+$ strain grown on plates under anaerobic and aerobic conditions. (*C*) Growth curves of *L. lactis* I-1631, the Δ*sodA* mutant, and the Δ*sodA*/p*sodA*$^+$ strain grown in milk under anaerobic and aerobic conditions. Data represent mean ± SD; two-way ANOVA with post hoc Bonferroni's multiple comparison test (MCT). (*D*) *L. lactis* I-1631, Δ*sodA* mutant, and Δ*sodA*/p*sodA*$^+$ strain enumerations in the stool and large intestine of *T-bet*$^{-/-}$ *Rag2*$^{-/-}$ mice. Symbols represent data from individual mice. (*E*) Histologic colitis scores from *T-bet*$^{-/-}$ *Rag2*$^{-/-}$, *Il10*$^{-/-}$, and DSS-exposed mice treated as labeled. Symbols represent data from individual mice from three experiments. Error bars indicate mean ± SEM; Kruskal–Wallis test with post hoc Dunn's comparison test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

radicals were lowest in mice that received the *L. lactis* I-1631 FMP compared with controls (Fig. 3*A*).

To evaluate the ability of *L. lactis* I-1631 to lower colonic epithelial superoxide radicals, we established an in vitro assay that used primary CECs and stimulation of ROS production using xanthine and xanthine oxidase. Lysates from *L. lactis* I-1631 lowered superoxide levels in stimulated CECs in a SodA-dependent manner (Fig. 3*B*).

The antiinflammatory activity of SOD has been explored for several inflammatory diseases; however, efficacy has been inconsistent, attributable to its short half-life secondary to rapid hydrolysis and proteolysis. Chemical modification with polyethylene glycol (PEG) can improve its stability (18). However, PEG is an osmotic laxative, which is problematic from a symptom perspective in preclinical models of intestinal inflammation and IBD patients. Aware of this caveat, we undertook a pilot experiment using DSS wild-type and *T-bet*$^{-/-}$ *Rag2*$^{-/-}$ mice fed either PEG or PEG coupled to superoxide dismutase. The differences in colitis scores were not statistically significant between the experimental and control groups (Fig. S2), and all of the mice had very loose stools. These data support the concept that how SodA is delivered to a host may affect SodA's ability to attenuate colitis.

Lysozyme-Mediated Lysis of *L. lactis* I-1631 Is Required for Colitis Attenuation. Although our data supported that the colitis-attenuating effects of *L. lactis* I-1631 were SodA-dependent (Fig. 2) and that *L. lactis* I-1631 lowered oxidative stress in the colonic mucosa and superoxide levels within colonic epithelial cells (Fig. 3), it was unclear how lactococcal SodA enzyme would encounter host-generated O_2^-, because lactococcal SodA is predicted to be cytoplasmic by PSORT (www.psort.org). Because *L. lactis* lacks a secretion system that would enable targeted delivery of SodA to host cells, we hypothesized that lactococcal lysis was a required step for SodA release into the intestinal lumen. Host-derived peptidoglycan hydrolases (PGHs), such as lysozyme-1 (encoded by *lyz1*) or PGLYRP-2, could contribute to *L. lactis* I-1631 lysis within the intestine (19). Expression levels of *lyz1* were detected in colonic tissue and significantly elevated in inflamed versus healthy colonic mucosa from *T-bet*$^{-/-}$ *Rag2*$^{-/-}$ mice (Fig. 4*A*), consistent with prior findings in mouse models of colitis and IBD patients (20, 21). However, expression levels of *pglyrp-2* were below the level of detection. Protein-based determinations of colonic tissue lysozyme-1 levels from *T-bet*$^{-/-}$ *Rag2*$^{-/-}$ and *Il10*$^{-/-}$ mice confirmed that lysozyme-1 levels were markedly elevated in inflamed versus noninflamed colonic tissues (Fig. 4*B*). Collectively, these data support that colonic inflammation may contribute to lactococcal lysis, providing a mechanism by which SodA could reach the host.

To further interrogate this hypothesis, we questioned whether impeding *L. lactis*'s lysis would impair SodA release and diminish *L. lactis* I-1631's colitis-attenuating effect. O-acetylation is a peptidoglycan modification that confers lysozyme resistance to gram-positive bacteria (19). In the *L. lactis* MG1363 strain, the regulator SpxB up-regulates the expression of *oatA*, the gene encoding the *O*-acetyltransferase responsible for peptidoglycan O-acetylation, and *spxB* overexpression leads to lysozyme resistance (19). We transformed *L. lactis* I-1631 with a multiple-copy plasmid containing the *spxB* gene from *L. lactis* MG1363 (p*spxB*$^+$) or the empty plasmid (p*Vector*). As expected, the p*spxB*$^+$-transformed strain was resistant to lysozyme compared

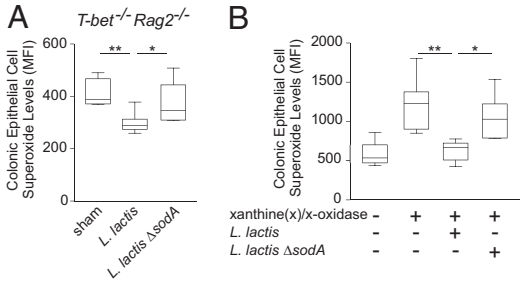

Fig. 3. *L. lactis* I-1631 reduces primary CEC superoxide levels in vivo and in vitro. (*A*) Superoxide levels in CECs isolated from *T-bet*$^{-/-}$ *Rag2*$^{-/-}$ mice and stained with DHE. *T-bet*$^{-/-}$ *Rag2*$^{-/-}$ mice were treated as labeled. Box and whiskers plot; data reflect samples from four experiments; unpaired *t* test. (*B*) Superoxide levels in CECs isolated from wild-type mice and stained with DHE. Cells were stimulated with xanthine and xanthine oxidase or unstimulated. Additional treatment with *L. lactis* I-1631 or *L. lactis* Δ*sodA* culture lysates is as labeled. Box and whiskers plot; data reflect six experiments. One-way ANOVA with post hoc Bonferroni's MCT. **P* < 0.05 and ***P* < 0.01.

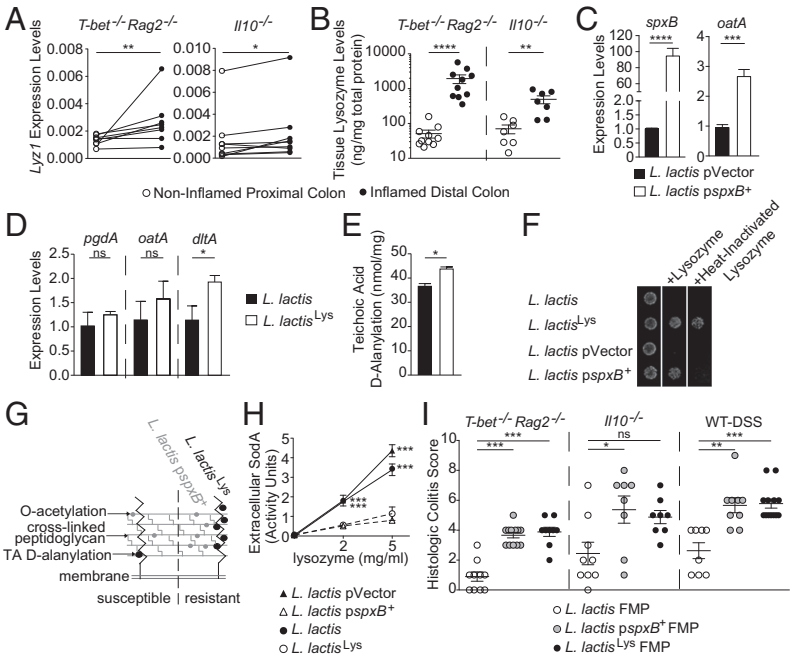

Fig. 4. Lysozyme-mediated lysis of *L. lactis* I-1631 is required for colitis attenuation. (*A*) Colonic tissue *lyz1* expression levels from *T-bet*$^{-/-}$ *Rag2*$^{-/-}$ and *Il10*$^{-/-}$ mice. Wilcoxon matched-pairs test (*T-bet*$^{-/-}$ *Rag2*$^{-/-}$; non-Gaussian data distribution); paired *t* test (*Il10*$^{-/-}$). (*B*) Colonic tissue lysozyme-1 protein levels from *T-bet*$^{-/-}$ *Rag2*$^{-/-}$ *and Il10*$^{-/-}$ mice. Symbols represent data from individual mice from three experiments. Mean ± SEM; Mann–Whitney test. (*C* and *D*) mRNA expression levels of genes contributing to lactococcal lysozyme resistance. Mean ± SEM of three experiments; unpaired *t* test. (*E*) D-alanylation of teichoic acids in the lysozyme-resistant mutant *L. lactis*^{Lys} and its parent. Mean ± SEM of three experiments; unpaired *t* test. (*F*) Lysozyme-resistant *L. lactis* I-1631 mutants grown on media with lysozyme or heat-inactivated lysozyme. (*G*) Model of the lactococcal peptidoglycan layer with two lysozyme resistance mechanisms. (*H*) Extracellular SodA levels of strains grown in lysozyme. Mean ± SEM of three experiments; two-way ANOVA with post hoc Bonferroni's MCT. (*I*) Histologic colitis scores from *T-bet*$^{-/-}$ *Rag2*$^{-/-}$, *Il10*$^{-/-}$, and DSS-exposed mice treated as labeled. Symbols represent data from individual mice from three experiments. Mean ± SEM; Kruskal–Wallis test with post hoc Dunn's comparison test. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001; ns, not significant.

with the p*Vector*-carrying strain (Fig. 4*F*). We confirmed that expression of *spxB* and *oatA* was increased (20- and 2-fold, respectively) in the *L. lactis* I-1631 p*spxB*$^{+}$ strain, similar to what was observed with *L. lactis* MG1363 (Fig. 4*C*) (19). In line with these transcription results, the *L. lactis* p*spxB*$^{+}$ strain had a higher percentage of O-acetylated muropeptides compared with the p*Vector* control strain (4.60 ± 0.54% and 4.21 ± 0.21%; Fig. S3). In agreement with a previous report, we observed that a modest difference in PG O-acetylation has a strong effect on lysozyme susceptibility (19).

We also isolated a spontaneous lysozyme-resistant mutant derived from *L. lactis* I-1631 (*L. lactis*^{Lys}) and characterized its lysozyme-resistant phenotype. Three genes contribute to *L. lactis* lysozyme resistance (*oatA*, *pgdA*, *dltA*) (19, 22). Of those, only *dltA* showed a statistically significant increase in expression (~1.8-fold) in *L. lactis*^{Lys} compared with *L. lactis* I-1631 (Fig. 4*D*). The *dltA* gene belongs to the *dltABCD* operon, which encodes the machinery for teichoic acid (TA) D-alanylation (22). We quantified the TA D-alanylation and observed an average 9.3% increase of TA D-alanylation in *L. lactis*^{Lys} compared with *L. lactis* I-1631 (Fig. 4*E*). Increasing TA D-alanylation decreases the negative charge of the cell wall, conferring resistance to cationic antimicrobial peptides (CAMPs) (22). Lysozyme exerts its antimicrobial effects via its PGH and/or CAMP activities (23). Heat inactivation of lysozyme abolishes its enzymatic activity whereas conserving its CAMP potential (24). Using heat-inactivated lysozyme, we found that *L. lactis*^{Lys} is resistant to the CAMP activity of lysozyme, consistent with its increased TA D-alanylation (Fig. 4*F*). In contrast, resistance of *L. lactis* p*spxB*$^{+}$ to lysozyme was lost with heat activation compared with its isogenic control (Fig. 4*F*), suggesting that its lysozyme resistance was dependent on lysozyme's hydrolytic activity, consistent with its up-regulation of the O-acetylation pathway (Fig. S3). Thus, lysozyme resistance of these strains was dependent upon two distinct mechanisms (Fig. 4*G*).

To test our hypothesis of lysozyme-mediated lactococcal SodA release, we measured extracellular SodA release with lysozyme exposure in vitro. SodA release was markedly decreased in both lysozyme-resistant strains (Fig. 4*H*). To ensure that the observed phenotype was not due to altered *sodA* expression, we measured *sodA* expression levels (Fig. S4*A*) and intracellular SodA levels (Fig. S4*B*) in the lysozyme-resistant mutants and found that they were equal or higher compared with their respective parental strains. We tested FMPs of the mutants compared with *L. lactis* I-1631 in the three models of intestinal inflammation and observed that the lysozyme-resistant strains were less effective in reducing colitis than the lysozyme-sensitive wild-type strain (Fig. 4*I*) and empty vector control [*T-bet*$^{-/-}$ *Rag2*$^{-/-}$: $n = 11$; 3 ± 0.3 (mean colitis score ± SEM); DSS wild type: $n = 8$; 4 ± 0.5]. These data support that lysozyme-mediated lysis is a critical step for *L. lactis* I-1631's colitis attenuation.

Discussion

We identify a bacterium that exhibits a host-beneficial activity facilitated by a host factor. We found that an *L. lactis* strain, which was not appreciated as a beneficial microbe and that naturally produces superoxide dismutase, attenuates colitis in three different mouse models and lowers colonic epithelial oxidative stress. Neither colonization nor an intact bacterium throughout the colon per se is required. Rather, lysozyme-mediated lysis at inflamed colonic sites contributes to *L. lactis*'s release of its cytoplasmic SodA, which is necessary for *L. lactis*'s colitis-attenuating activity. Collectively, our results suggest that a bacterium used in FMPs may have an activity that ameliorates intestinal inflammation and that targeted delivery of this beneficial activity need not be synthetically engineered.

Probiotic strains have been explored as a tool to combat oxidative stress. In vitro experiments suggest that *L. lactis* has antioxidant properties (25). Two studies expressed *L. lactis* catalase and SOD in *Lactobacillus casei* BL23. The catalase-expressing *L. casei* strain was protective against trinitrobenzenesulfonic acid (TNBS)-induced colitis in rats (26), and the *L. casei sodA*-expressing strain reduced intestinal inflammation in DSS-exposed mice (27). Also, a recombinant *L. lactis* strain expressing lactococcal SOD from another strain improved TNBS-induced colitis in rats (28). In another study, a *Lactobacillus gasseri* strain expressing *Streptococcus thermophilus* MnSOD reduced inflammation in *Il10*$^{-/-}$ mice (29). Our studies, in contrast to the aforementioned, tested the nonengineered *L. lactis* I-1631 in three genetically distinct colitis models and used a *sodA* deletion mutant and its complement to demonstrate that SodA was necessary and sufficient to reduce colitis.

Our experiments support that *L. lactis* I-1631 susceptibility to lysozyme-mediated lysis is required to reduce inflammation. Peptidoglycan hydrolases, degrading the peptidoglycan of bacterial cell walls, may be derived from the host or bacteria. Lysozyme-1 is a host-produced peptidoglycan hydrolase. Whereas many studies have focused on lysozyme production by small intestinal Paneth cells, lysozyme-1 levels are increased in the inflamed gastrointestinal tracts and feces of IBD patients versus healthy controls (20, 30). In the mouse models of chronic colitis used in this study, lysozyme-1 was elevated at sites of colonic inflammation. *lyz1* encodes an epithelial-expressed lysozyme, which is what we examined, as opposed to Lyz2, which is produced by lamina propria myeloid cells. The use of lysozyme-resistant *L. lactis* I-1631 mutants in three models of colitis enabled us to test the hypothesis that lysozyme-mediated lysis was a key step in the targeted release of SodA from *L. lactis* I-1631 to inflamed regions of the colon.

Bacteria, including members of the gut microbiota, may be another source of peptidoglycan hydrolytic activity required for *L. lactis* I-1631 lysis. Bacterial growth and division require peptidoglycan hydrolysis so that bacteria can change shape, polarity, and their growth dynamics (31, 32). Such PGH can be released into bacterial culture media and can even affect cell-wall rigidity of other species, as shown previously (31). We tested the ability of members of a "healthy" human gut microbiota (*Faecalibacterium prausnitzii*, *Eubacterium rectale*, *Enterococcus faecalis*, *Bacteroides fragilis*, *Ruminococcus torques*, and *Bifidobacterium longum*) to produce PGH using the conventional *Micrococcus luteus* assay. *E. rectale* and *E. faecalis* digested *M. luteus* cell wall, and *E. faecalis* digested *L. lactis* I-1631 cell wall (Fig. S5). Thus, PGH produced by human gut microbiota members could participate in *L. lactis* I-1631 lysis. Some PGHs are associated with prophages that can be induced by inflammatory conditions, suggesting that colitis may tune resident gut microbes' PGH activity and potentially lyse *L. lactis* I-1631 (33, 34). However, the relative contribution and importance of bacterial compared with host lysozyme-1 remains to be elucidated and represents a topic for future investigation.

Both bacterial and host cells have evolved several strategies to lyse bacteria. Some bacteria can kill or lyse each other via bacteriocin-mediated killing or type VI secretion systems. Both epithelial and myeloid cell subsets also possess a range of antimicrobial molecules, including c-type lectins, defensins, and cathelicidins. Because myeloid cells contribute to the inflammatory infiltrates in colitis, they represent a source for antimicrobials that may potentially lyse *L. lactis*. In this study, however, we focused on lysozyme-1, not lysozyme-2, which we did not detect in the inflamed colon and which is produced by myeloid cells.

We questioned whether *L. lactis* I-1631 and its lysis were necessary to deliver SOD to the colon and investigated delivering SOD to mice without bacteria. The protein was PEGylated, which is required for its stability (18). Delivering superoxide dismutase in this way could help standardize dosage and efficacy and appears a more direct approach than using bacteria. However, PEG–SOD did not improve colitis to the same degree as SodA delivered by *L. lactis* I-1631, and caused diarrhea and weight loss. Bacterial delivery, besides ensuring that the active compound is released directly at the site of inflammation, may afford additional benefits. The use of bacteria that produce, lyse, and release SOD may circumvent issues of oxidative damage caused by the accumulation of intermediate metabolites, such as hydrogen peroxide, observed in a study that examined using lecithinized SOD in DSS-exposed mice (35). *L. lactis* I-1631 also possesses alkyl hydroperoxide reductase (AhpC), which is predicted to reduce hydrogen peroxide and is produced during superoxide reduction, obviating the need for enzymes from other sources to detoxify such intermediate metabolites. For these reasons, *L. lactis* I-1631 is appealing.

However, we wondered whether members of the human gut microbiota had SOD genomic potential similar to *L. lactis* I-1631. We searched the genomes of 497 strains, sequenced under the Human Microbiome Project, for the presence of SOD activities, as well as four *Akkermansia* sp. available from the National Center for Biotechnology Information. Of 501 genomes, we found 330 with predicted SOD function (Fig. S6*A*). Predicted SOD function was absent from Archaea and from Fusobacteria and Synergistetes. Bacteroidetes, Proteobacteria, and Verrumicrobia were predominantly composed of species encoding at least one SOD enzyme. For Firmicutes and Actinobacteria, the distribution was more genus- and species-specific.

Because SOD localization appears critical for its bioavailability, we predicted the localization of the SOD proteins using PSORT. We found that SOD was not predicted to be secreted extracellularly but was either cytoplasmic or periplasmic in the case of bacteria with an outer membrane (Fig. S6*B*). This observation supports our hypothesis that lysis of SOD-carrying bacteria is key to scavenging host-produced ROS. We also investigated the gut microbiome's ability to express SOD-encoding genes by interrogating metatranscriptomics data obtained from gnotobiotic mice colonized with a 20-strain consortium (11). SOD was highly expressed in Bacteroidetes species compared with other members of the consortium (Fig. S6*C*), suggesting that Bacteroidetes might be a source of bacterial SOD in vivo.

Using bacteria as vehicles to deliver biologically active molecules is a promising approach for the treatment of many diseases. Our experiments support that the nonengineered *L. lactis* I-1631 strain reduces colitis in three murine models, does so in a SodA-dependent fashion, and requires host lysozyme to facilitate delivery. Our study challenges the perception that beneficial microbes need to remain viable throughout the gastrointestinal tract to confer their health benefit. Our findings also delineate a mechanism by which beneficial microbial strains offer up benefits to the host and highlight how specific characteristics of the host may be necessary to promote a bacterium's beneficial activity.

Materials and Methods

Growth Conditions and Bacterial Strains. For bacterial strains and plasmids used in this study, see Table S4. For routine cultures, *L. lactis* I-1631 strains were grown at 37 °C under aerobic conditions in M17 broth (BD Biosciences) supplemented with 0.5% lactose (Sigma-Aldrich), M17L media. For all other strains and culture conditions, see *SI Materials and Methods*.

Study Products. FMPs were prepared before each experiment. The five-strain FMP, described previously (10), was provided by Danone Nutricia Research. For details, see *SI Materials and Methods*.

Animal Husbandry. Specified pathogen-free BALB/c wild-type, BALB/c *T-bet*$^{-/-}$ *Rag2*$^{-/-}$ (13), and BALB/c *Il10*$^{-/-}$ mice were weaned and randomized into experimental cages between postnatal days 21 and 25. Mice were fed standard mouse chow [PicoLab Mouse Diet 20 (5058); LabDiet] and housed in the barrier facility at the Harvard School of Public Health under a 12-h light cycle. Animal experiments were approved and conducted in accordance with the Harvard Medical School Standing Committee on Animals and National Institutes of Health guidelines. For details, see *SI Materials and Methods*.

Histology. Sections were examined and the degree of colitis was scored as previously described (13). Image acquisition was performed on a Nikon Eclipse Ni-U, equipped with a Nikon digital sight Fi2 color camera using NIS-Elements basic research software.

Mouse Colonoscopy. Rigid colonoscopy was performed on mice using a miniendoscope (Karl Storz). For details, see *SI Materials and Methods*.

Bioinformatics. Genes of *B. animalis* subsp. *lactis* I-2494 and *L. lactis* subsp. *lactis* CNCM I-1631 were organized into COGs using the CD-Search Tool (www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi). Retrieved COGs were projected on the global map of KEGG metabolism using iPath v1 software (pathways.embl.de).

Gene Inactivation and Complementation. The *sodA* gene was deleted by using the pG+h9 gene replacement system (36). The Δ*sodA* mutant was in *cis* complemented by the chromosomal insertion of p*sodA*. For details, see *SI Materials and Methods*.

Enumeration of *L. lactis* in the Digestive Tract. Absolute enumeration of *L. lactis* I-1631 strains was conducted by administering FMP (150 mg) to BALB/c *T-bet*$^{-/-}$ *Rag2*$^{-/-}$ mice 4–5 h before stool and/or tissue collection. For details, see *SI Materials and Methods*.

Colonic Epithelial Cell Isolation. For procedural details, see *SI Materials and Methods*.

Dihydroethidium Assay. CECs were isolated and incubated with dihydroethidium (DHE) (3 μM; Calbiochem) in RPMI (Corning) for 20 min at 37 °C, followed by staining and flow cytometry. For details and bacterial lysate coincubation experiments, see *SI Materials and Methods*.

Quantitative RT-PCR. Quantitative (q) real-time PCR was performed using a Stratagene Mx3005P instrument (Agilent Technologies). For details, see *SI Materials and Methods*. All primers used for expression analysis are listed in Table S5.

ELISA. Samples were processed using the Mouse Lysozyme (LZM) ELISA Kit (MyBioSource). Total protein levels were determined by the BCA assay (Pierce). Lysozyme levels were normalized to the total protein content of the respective samples. For details, see *SI Materials and Methods*.

Spontaneous Mutant Selection. For selection of lysozyme-resistant *L. lactis* clones, see *SI Materials and Methods*.

D-Alanylation of Teichoic Acids. D-Ala was quantified by HPLC. For details, see *SI Materials and Methods*.

Superoxide Dismutase A Activity Assay. The SodA activity assay was adapted from ref. 37. For details, see *SI Materials and Methods*.

Statistical Analysis. All statistical analysis was performed using Prism 5 (GraphPad).

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