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Epithelial IL-22RA1-Mediated Fucosylation Promotes Intestinal Colonization Resistance to an Opportunistic Pathogen

Tu Anh N. Pham,[1] Simon Clare,[1] David Goulding,[1] Julia M. Arasteh,[1] Mark D. Stares,[1] Hilary P. Browne,[1] Jacqueline A. Keane,[1] Andrew J. Page,[1] Natsuhiko Kumasaka,[1] Leanne Kane,[1] Lynda Mottram,[1] Katherine Harcourt,[1] Christine Hale,[1] Mark J. Arends,[2] Daniel J. Gaffney,[1] The Sanger Mouse Genetics Project,[1] Gordon Dougan,[1] and Trevor D. Lawley[1,*]

[1]Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, CB10 1SA, UK
[2]Department of Pathology, University of Cambridge, Addenbrooke's Hospital, Cambridge, CB2 0QQ, UK
*Correspondence: tl2@sanger.ac.uk
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SUMMARY

Our intestinal microbiota harbors a diverse microbial community, often containing opportunistic bacteria with virulence potential. However, mutualistic host-microbial interactions prevent disease by opportunistic pathogens through poorly understood mechanisms. We show that the epithelial interleukin-22 receptor IL-22RA1 protects against lethal *Citrobacter rodentium* infection and chemical-induced colitis by promoting colonization resistance against an intestinal opportunistic bacterium, *Enterococcus faecalis*. Susceptibility of *Il22ra1*$^{-/-}$ mice to *C. rodentium* was associated with preferential expansion and epithelial translocation of pathogenic *E. faecalis* during severe microbial dysbiosis and was ameloriated with antibiotics active against *E. faecalis*. RNA sequencing analyses of primary colonic organoids showed that IL-22RA1 signaling promotes intestinal fucosylation via induction of the fucosyltransferase *Fut2*. Additionally, administration of fucosylated oligosaccharides to *C. rodentium*-challenged *Il22ra1*$^{-/-}$ mice attenuated infection and promoted *E. faecalis* colonization resistance by restoring the diversity of anaerobic commensal symbionts. These results support a model whereby IL-22RA1 enhances host-microbiota mutualism to limit detrimental overcolonization by opportunistic pathogens.

INTRODUCTION

The mammalian gastrointestinal tract harbors a dense and diverse microbial community that is essential for host nutrient acquisition, immune development, and pathogen defense. In health, the microbiota consists mainly of obligate anaerobes and can act as an effective barrier to prevent colonization and access of pathogenic microbes to host tissues. Colonization resistance to enteric pathogens is thought to involve mutualistic interactions between the host immune system and the commensal microbiota through mechanisms that remain poorly understood. Moreover, while many host-commensal associations are considered symbiotic, the microbiota also contains indigenous opportunistic microbes, which can potentially trigger intestinal or systemic pathology particularly during perturbations of the intestinal ecosystem (Stecher and Hardt, 2008). In most healthy individuals, however, these opportunistic pathogens fail to replicate to sufficient levels to colonize the mucosal surface or to invade the host systemic organs, suggesting the importance of mechanisms to restrict their overcolonization and virulence expression.

Interleukin-22 (IL-22) is an important cytokine for maintaining homeostasis at various mucosal barriers, including the gastrointestinal tract (Sonnenberg et al., 2011). During infection with enteropathogens such as *Salmonella* Typhimurium and *Citrobacter rodentium*, IL-22 is highly upregulated, leading to induction of multiple antimicrobial and inflammatory factors (Raffatellu et al., 2008; Zheng et al., 2008). The protective function of IL-22 is evident by the observations that *Il22*$^{-/-}$ mice are susceptible to Gram-negative pathogens, including *C. rodentium* and *Klebsiella pneumoniae* (Aujla et al., 2008; Zheng et al., 2008). In contrast, IL-22 deficiency is not associated with enhanced susceptibility to *Neisseria gonorrhoea*, *Listeria monocytogenes*, or the pathogenic protozoan *Toxoplasma gondii* (Feinen and Russell, 2012; Graham et al., 2011; Wilson et al., 2010), and IL-22 can even promote *S.* Typhimurium colonization (Behnsen et al., 2014). These findings suggest that the effects of IL-22 on pathogen colonization resistance are highly complex and varied depending on the inflammatory stimuli and the indigenous microbiota.

IL-22 signals through a heterodimeric receptor complex consisting of a ubiquitously expressed IL-10Rβ subunit and an IL-22RA1 subunit, which has a remarkably tissue-specific distribution. IL-22RA1 expression is limited to a few organs (e.g., liver, kidney) and barrier surfaces such as the skin and gastrointestinal tract, where it is thought to mediate the effects of IL-22 in maintaining barrier homeostasis (Wolk et al., 2004; Zheng et al., 2008). Given the complex antimicrobial function of IL-22, in the present study we set out to elucidate the impact of IL-22RA1 on the resident microbiota and the maintenance of pathogen colonization resistance.

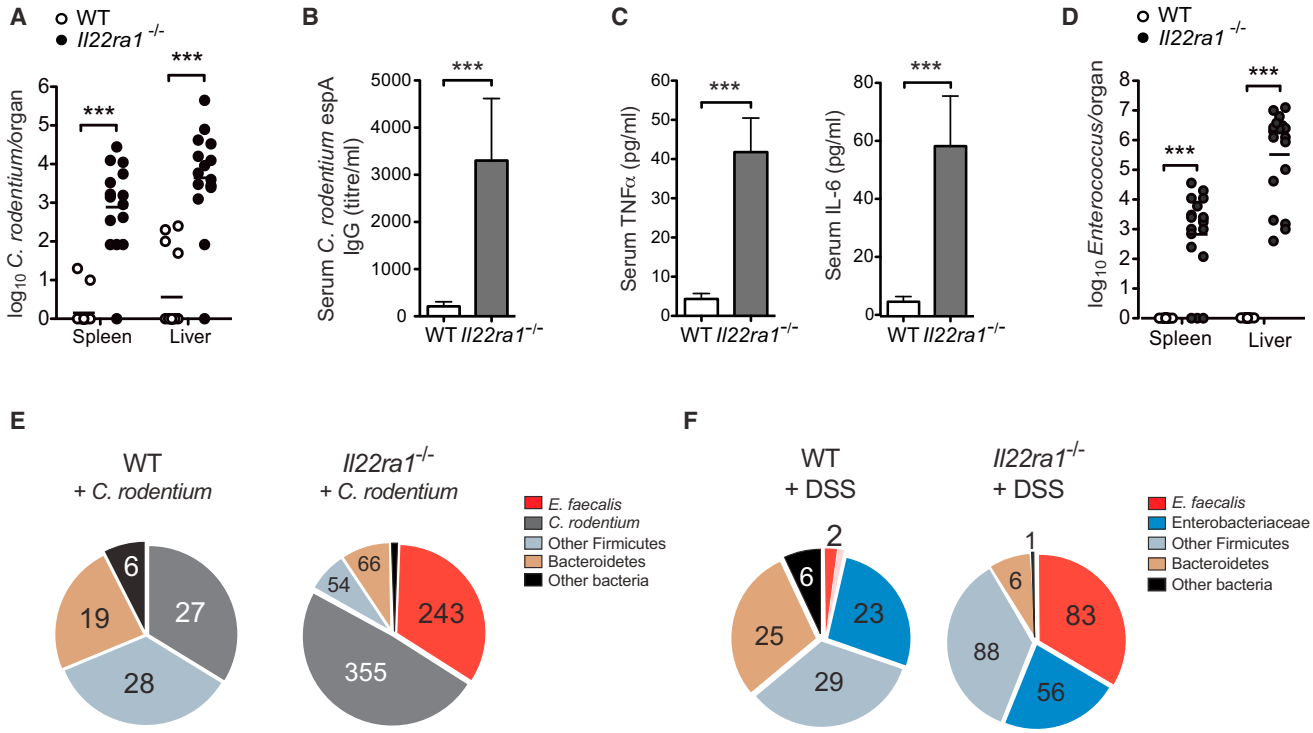

Figure 1. IL-22RA1 Limits Systemic Bacterial Dissemination during Intestinal Disease

(A) *C. rodentium* cfus in systemic organs.

(B) Serum anti-*C. rodentium* EspA IgG titer.

(C and D) TNF-α and IL-6 cytokines (C) and *E. faecalis* cfus (D) in systemic organs of infected WT and *Il22ra1*$^{-/-}$ mice. Shown are mean ± SEM in five independent infections (n = 4–6 each); ***p < 0.0001.

(E and F) Profile of systemic bacterial isolates from (E) WT (n = 10) and *Il22ra1*$^{-/-}$ mice infected with *C. rodentium* (n = 28) and (F) DSS-treated WT and *Il22ra1*$^{-/-}$ mice (n = 8–10). Numbers indicate total number of isolates assigned to a particular bacterial taxon in 3–8 independent experiments. See also Figure S2 and Tables S1 and S2.

RESULTS

IL-22RA1 Limits Systemic Bacterial Dissemination during Intestinal Inflammation

We generated *Il22ra1*$^{-/-}$ mice homozygous for the targeted *Il22ra1*$^{tm1a(KOMP)Wtsi}$ allele (Skarnes et al., 2011) and confirmed that within the gastrointestinal tract, endogenous *Il22ra1* expression is restricted to the epithelium (Figures S1A and S1B, available online). By a global phenotyping approach (White et al., 2013), we found no obvious systemic or intestinal pathology in specific pathogen-free (SPF) *Il22ra1*$^{-/-}$ mice (Figures S1C–S1E). 16S rRNA gene pyrosequencing revealed no significant difference in the diversity and community structure of the baseline microbiota between wild-type (WT) and *Il22ra1*$^{-/-}$ mice (Figures S1F and S1G). Next, we infected *Il22ra1*$^{-/-}$ mice with pathogens to discern the role of IL-22RA1 in systemic and intestinal host defenses. IL-22RA1 is not required for protection against systemic *Salmonella* Typhimurium, as all *Il22ra1*$^{-/-}$ mice survived intravenous *Salmonella* infection and were able to clear the bacteria in a manner similar to that of WT mice (Figures S2A–S2C). By contrast, similarly to *Il22*$^{-/-}$ mice (Zheng et al., 2008), *Il22ra1*$^{-/-}$ mice were highly susceptible to the attaching-effacing enteropathogen *C. rodentium* (Figures S2D and S2E).

We next examined the impact of IL-22RA1 on mucosal immunity to *C. rodentium*. Infected *Il22ra1*$^{-/-}$ mice had significantly higher transcript and protein expression of proinflammatory Th1-type cytokines (interferon gamma [IFNγ], IL-1β, IL-6, tumor necrosis factor alpha [TNF-α]) and Th17-type cytokines (IL-17a, IL-17f, IL-21, IL-22) in the cecal tissues and isolated colonic lamina propria (cLP) leukocytes (Figure S3A). This is consistent with an ~3-fold accumulation of IFNγ- and IL-17a-producing CD4$^+$ T cells in the cLP of infected *Il22ra1*$^{-/-}$ mice (Figures S3B–S3D), whereas B cells, Foxp3$^+$ regulatory T cells, neutrophils, and inflammatory macrophages were at frequencies similar to that of WT (Figures S3E and S3F). We also detected similar titers of total and *Citrobacter*-specific immunoglobulin A (IgA) in the intestine of *Il22ra1*$^{-/-}$ mice compared to WT mice at day 9 postinfection (p.i.) (Figure S3G). Thus, susceptibility of *Il22ra1*$^{-/-}$ mice to *C. rodentium* is not associated with an insufficient mucosal immune response.

Notably, infected *Il22ra1*$^{-/-}$ mice showed a high systemic burden of *C. rodentium*, accompanied by elevated serum titers of *Citrobacter*-specific IgG (Figures 1A and 1B). Furthermore, serum levels of the proinflammatory cytokines IL-6 and TNF-α were significantly increased (Figure 1C), suggesting dissemination of gut bacteria and systemic inflammation. To characterize this microbial dissemination, we performed nonselective,

unbiased aerobic and anaerobic culturing together with 16S rRNA sequencing to identify bacterial isolates from the systemic organs. Interestingly in addition to *C. rodentium*, we identified substantial and consistent breakthrough of *Enterococcus faecalis*—a normal commensal of the mouse and human microbiota (Figures 1D and 1E and Table S1). Dissemination of *E. faecalis* was detectable from day 5 p.i., and at day 9 the systemic burden of *E. faecalis* was comparable to that of *C. rodentium* among infected *Il22ra1*$^{-/-}$ mice (Figures 1D and 1E and Table S1). Importantly, *E. faecalis* was not recovered from the organs of infected WT mice (Table S1) or of infected *Il22ra1*$^{-/-}$ mice without signs of morbidity (data not shown).

We further examined the dissemination of enterococci in *Il22ra1*$^{-/-}$ mice using a noninfectious model of intestinal inflammation, which involves treatment with dextran sodium sulfate (DSS). DSS-treated *Il22ra1*$^{-/-}$ mice showed significantly higher weight loss and susceptibility compared to WT littermates (Figures S2F and S2G). Similarly to the *C. rodentium* infection model, we noted a high *E. faecalis* bacterial load in the livers and spleens of DSS-treated *Il22ra1*$^{-/-}$ mice (Figure 1F and Table S2). Our data suggest that IL-22RA1 plays a role in controlling the systemic dissemination of intestinal bacteria, particularly a potential opportunistic *E. faecalis* species, during infectious and noninfectious intestinal injuries.

An Opportunistic *E. faecalis* Species Displays In Vivo and Genetic Virulence Potential

To examine the process of enterococcal dissemination from the gut of *Il22ra1*$^{-/-}$ mice, we performed transmission electron microscopy and immunogold labeling to identify bacteria at the intestinal mucosa during *C. rodentium* infection. At day 9 p.i., enterococci were found in contact with the epithelial surface of *Il22ra1*$^{-/-}$ mice and within membrane-bound compartments of enterocytes (Figures 2A and 2B). By contrast, *C. rodentium*-infected WT mice showed no epithelial association or invasion of *E. faecalis* (Figure 2A). This result suggests that *E. faecalis* can directly invade the intestinal epithelium of *Il22ra1*$^{-/-}$ mice during inflammation.

Next, we determined the systemic virulence of the *E. faecalis* species recovered from *Il22ra1*$^{-/-}$ mice (termed mEF) in vivo, using a previously established enterococcal peritonitis model (Bourgogne et al., 2008). Intraperitoneal administration of mEF led to a higher mortality rate and a greater systemic inflammatory response (characterized by elevated serum IL-6 and TNF-α levels) compared to equivalent administration of *C. rodentium* (Figures 2C and S4A). This finding led us to determine the relative contribution of enterococci to the susceptibility of *Il22ra1*$^{-/-}$ mice to *C. rodentium* infection by administering clinically relevant doses of ampicillin intraperitoneally from day 5 p.i. Ampicillin is an antibiotic commonly used for enterococcal septicemia in humans and is active against mEF (minimum inhibitory concentration [MIC] = 1.6 μg/ml), but not *C. rodentium* (MIC > 125 μg/ml). Ampicillin-treated *Il22ra1*$^{-/-}$ mice showed substantially less weight loss and improved survival, associated with decreased intestinal colonization and systemic dissemination of *E. faecalis* (Figures 2D, S4B, and S4C), despite no alteration in the colonization levels of *C. rodentium* (Figure S4D). This suggests that dissemination of opportunistic *E. faecalis* can contribute to the susceptibility of *Il22ra1*$^{-/-}$ mice.

The ability of human pathogenic *E. faecalis* isolates to cause infections has been shown to involve virulence genes that enhance host colonization and adherence (Jett et al., 1994). We sequenced and annotated the complete mEF genome by comparative analysis with the previously sequenced *E. faecalis* pathogenicity island (Shankar et al., 2002) and the genome of *E. faecalis* V583, a hospital outbreak isolate (Figure S4E) (Paulsen et al., 2003). Interestingly, we identified multiple virulence factors implicated in the pathogenesis of invasive enterococcal infections (Figure S4E and Table S3). Notably, the mEF genome includes a chromosome-encoded cytolysin operon (Figures 2E and S4E), which encodes a membrane-targeting toxin often expressed by nosocomial *E. faecalis* isolates (Huycke et al., 1991). Next, using the multilocus sequence typing (MLST) of previously sequenced *E. faecalis* isolates from diverse human and animal origins (McBride et al., 2007), we assessed the genetic relatedness of mEF to 43 representative commensal and pathogenic *E. faecalis* strains (Table S4A). This analysis revealed a close phylogenetic relationship between mEF and several hospital-associated strains, including *E. faecalis* V583 (Figure 2F). Taken together, our results suggest that a genetically distinct *E. faecalis* species in *Il22ra1*$^{-/-}$ mice possesses the genetic virulence potential to cause systemic infection.

IL-22RA1 Restricts Expansion of Opportunistic *E. faecalis* during Intestinal Dysbiosis

Microbial dysbiosis often occurs during intestinal inflammation and can potentially impair colonization resistance to pathogens (Stecher and Hardt, 2011). We hypothesized that during *C. rodentium*-induced dysbiosis, IL-22RA1 could prevent dissemination of *E. faecalis* by restricting its intestinal overgrowth. By 16S rRNA pyrosequencing, we analyzed the microbiota composition of WT and *Il22ra1*$^{-/-}$ mice during infection. While the microbiota of WT and *Il22ra1*$^{-/-}$ mice were similarly diverse at baseline, *C. rodentium* infection resulted in a global reduction of species richness and diversity (shown by the Shannon index and rarefaction curves) that was significantly exacerbated in *Il22ra1*$^{-/-}$ mice (Figures 3A and S5A). Clustering analysis further showed that the microbial community structure of WT and *Il22ra1*$^{-/-}$ mice underwent distinct shifts during infection (Figure 3B). Unlike WT equivalents, the microbiota of infected *Il22ra1*$^{-/-}$ mice was characterized by a much higher population of *C. rodentium* (54% ± 34.9% compared to 11.4% ± 7.3% in WT controls; p value < 10^{-7}) and severe depletion of the health-associated Lactobacillaceae, Bifidobacteriaceae, and Ruminococcaceae families (Figures 3B and S5B). Notably, all infected *Il22ra1*$^{-/-}$ mice displayed a clear and consistent overgrowth of *Enterococcus* spp., which was not detectable in WT equivalents (Figure 3B).

Using group-specific qPCR and selective plating, we independently confirmed that *Il22ra1*$^{-/-}$ mice were colonized by a higher abundance of Enterobacteriaceae and *Enterococcus* spp., both within the intestinal lumen and at the mucosa (Figures S5C and S5D). Immunofluorescence labeling further revealed greater adherence of these pathogens to the colonic crypts of infected *Il22ra1*$^{-/-}$ mice (Figure 3C). Notably, by monitoring *Enterococcus* colonization over the course of *C. rodentium* infection, we detected a remarkable (~3 log-fold) increase among *Il22ra1*$^{-/-}$ mice compared to WT equivalents (Figure 3D). Similarly, during

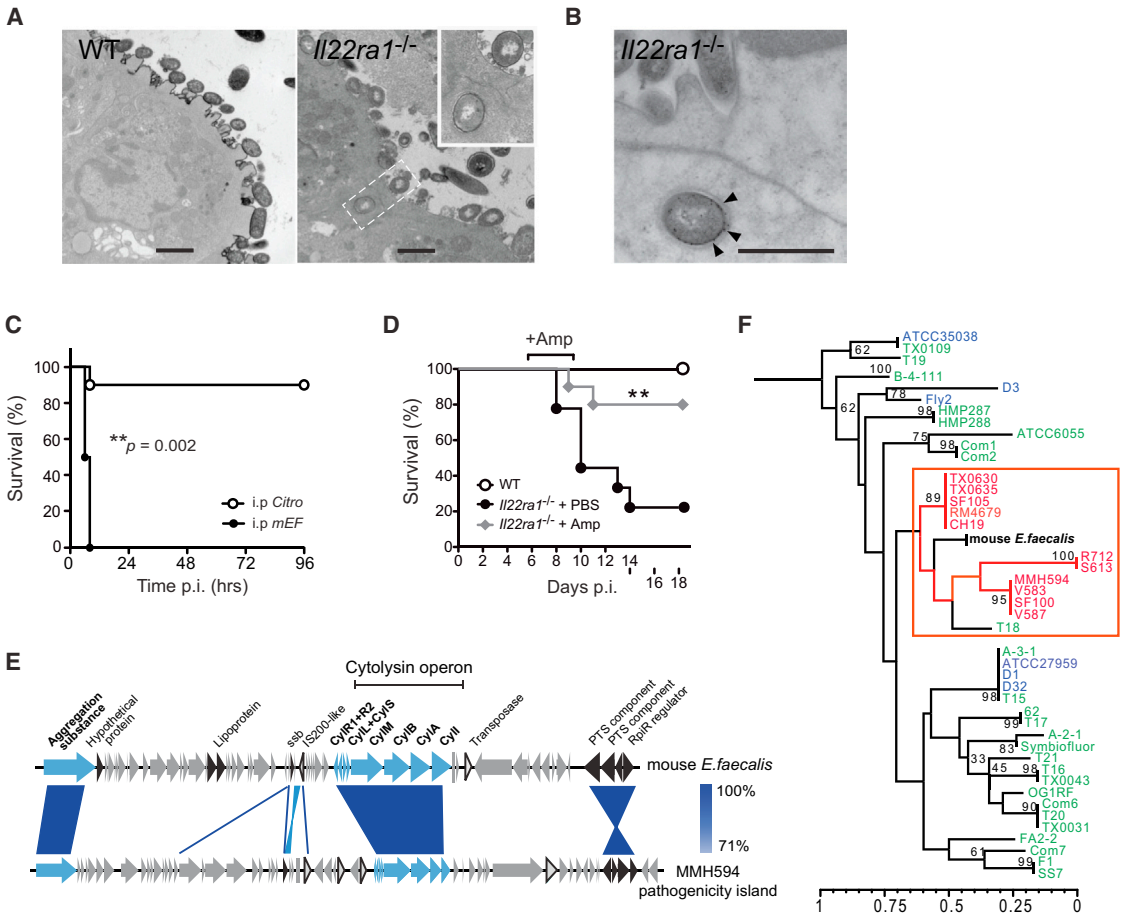

Figure 2. A Pathogenic *Enterococcus faecalis* Isolate Harbors Virulence Factors, Translocates Intracellularly in *Il22ra1*-Deficient Mice, and Causes Lethal Septicemia

(A) Transmission electron micrographs of *C. rodentium*-infected WT and *Il22ra1*$^{-/-}$ mice cecal tissues, showing translocation of a coccal bacterium.

(B) *Enterococcus*-specific immunogold labeling (arrows) of an intracellular bacterium. Scale bar, 500 nm.

(C) Survival of WT mice infected i.p. with either *C. rodentium* or *E. faecalis* (n = 10–12).

(D) Survival *C. rodentium*-infected WT mice and *Il22ra1*$^{-/-}$ mice given i.p. ampicillin or PBS. Log-rank p value of two independent experiments (n = 5 each).

(E) Schematic alignment of the cytolysin operon and flanking genes of mEF and the *E. faecalis* pathogenicity island. Blue, cytolysin and aggregation substance; black, other pathogenicity island-associated genes; triangles, transposase and IS elements. *rpiR*, family transcriptional regulator; *PTS*, phosphotransferase system; *CylI*, cytolysin immunity protein; *CylA*, cytolysin activator; *CylB*, cytolysin B ABC-type transporter; *CylM*, cytolysin subunit modifier; *CylL+S*, cytolysin subunits L–S; *CylR1+R2*, cytolysin regulators R1 and R2.

(F) MLST-based phylogenetic relationship between mEF and nosocomial (red), community/probiotic (green), and animal (blue) *E. faecalis* strains. See also Figure S4 and Table S3.

DSS-induced colitis, we observed a much greater expansion of *Enterococcus* spp. in the lumen and mucosa of *Il22ra1*$^{-/-}$ mice (~2 log-fold relative to WT littermates) (Figure S5E).

Intestinal dysbiosis can generally enhance the growth of facultative anaerobes such as the Gram-negative Enterobacteriaceae (Lupp et al., 2007; Winter et al., 2013). Therefore, among the facultative anaerobic *Enterococcus* spp., we next sought to determine the expansion dynamics of *E. faecalis* specifically. By analyzing the 16S rRNA sequences of single enterococcal colonies, we identified a predominant expansion of *E. faecalis* among various enterococci in the microbiota of *Il22ra1*$^{-/-}$ mice (from 16% to 70% in the feces and 82% in the cecal mucosa) (Figure 3E). The vast majority of other enterococcal species in the mouse microbiota were identified as *E. gallinarum*, a common animal commensal rarely known to causes disease (Arias and Murray, 2012). By contrast, no significant change in the proportion of *E. faecalis* was detected in WT mice infected with *C. rodentium* (WT versus *Il22ra1*$^{-/-}$ mice: $p < 0.01$). Similarly, during DSS-induced colitis, *Il22ra1*$^{-/-}$ mice had a significantly greater expansion of *E. faecalis* than WT littermates (Figure S5F). Thus, the absence of IL-22RA1 appears to allow overcolonization by *E. faecalis* in relation to other *Enterococcus* spp. during intestinal inflammation.

To further exclude the possibility that the expansion of *E. faecalis* observed may be influenced by the presence of multiple subspecies, which cannot be detected by 16S rRNA gene sequencing, we isolated 30 independent *E. faecalis* isolates from different WT and *Il22ra1*$^{-/-}$ mice colonies over time (Table S4B). Whole-genome sequencing and in vitro hemolytic assays indicated that the microbiota of WT and *Il22ra1*$^{-/-}$ mice

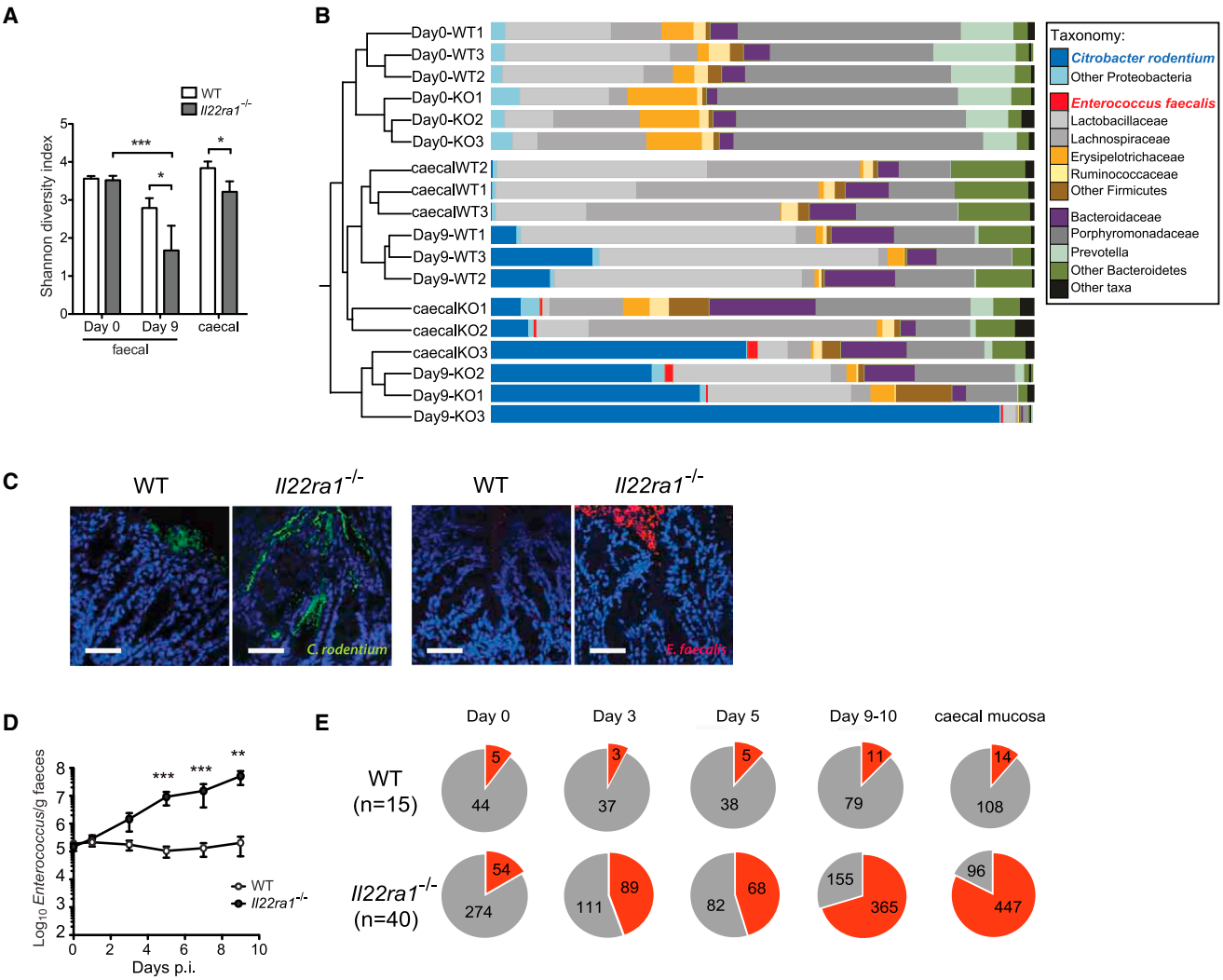

Figure 3. IL-22RA1 Signaling Restricts Intestinal Dysbiosis and *Enterococcus faecalis* Overcolonization during *C. rodentium* Infection

(A) Shannon diversity index of the fecal microbiota of WT and *Il22ra1*$^{-/-}$ mice (mean ± SEM) before *C. rodentium* infection (day 0) and at day 9 p.i. *$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$.

(B) Cluster dendogram of the microbial community structures of WT and *Il22ra1*$^{-/-}$ (KO) fecal and cecal microbiota (n = 3) before infection (day 0) and at day 9 p.i. at the operational taxonomic unit (OTU) level. Bar graphs represent proportional abundance.

(C) Immunofluorescence of *C. rodentium* and *Enterococcus* spp. in the cecum of infected WT and *Il22ra1*$^{-/-}$ mice. DAPI, cell nuclei. Scale bar, 50 μm.

(D) Fecal *Enterococcus* shedding of WT and *Il22ra1*$^{-/-}$ mice (n = 6–15) by selective plating, showing significant overgrowth during *C. rodentium* infection.

(E) Selective plating and 16S rRNA sequencing profile of enterococci in the feces and cecal mucosa of infected WT (n = 15) and *Il22ra1*$^{-/-}$ mice (n = 40) in five independent experiments, showing preferential expansion of *E. faecalis* (red) relative to other intestinal enterococci (*E. gallinarum*, gray). Shown are numbers of *Enterococcus* sequences matched to a species ID.

See also Figure S5.

contained the same *E. faecalis* isolate and that this isolate was recovered repeatedly in the feces, livers, and spleens of *Il22ra1*$^{-/-}$ mice (Table S4B). Taken together, our results demonstrate that IL-22RA1 plays a role in limiting intestinal colonization and dissemination of a particular virulent *E. faecalis* isolate.

Epithelial IL-22RA1 Signaling Mediates Diverse Antimicrobial and Glycosylation Factors

The cytokine IL-22 has been shown to enhance pathogen defense by inducing the bactericidal lectin RegIIIγ (Zheng et al., 2008), which can inhibit various Gram-positive microbes in vitro (Cash et al., 2006), and limit colonization of vancomycin-resistant *Enterococcus faecium* (VRE) in the small intestine (Kinnebrew et al., 2010). During *C. rodentium* infection, *Il22ra1*$^{-/-}$ mice indeed showed diminished expression levels of RegIIIγ (Figure S6A). To directly determine whether RegIIIγ mediates resistance to opportunistic *E. faecalis* in vivo, we infected *RegIIIγ*$^{-/-}$ mice orally with *C. rodentium*. RegIIIγ deficiency does not appear to influence the colonization of *C. rodentium* or *E. faecalis* in the feces or cecal lumen (Figures 4A–4D). However, mucosal colonization of *E. faecalis* at day 9 p.i. increased among *RegIIIγ*$^{-/-}$ mice (Figure 4D). This is consistent with an inhibitory

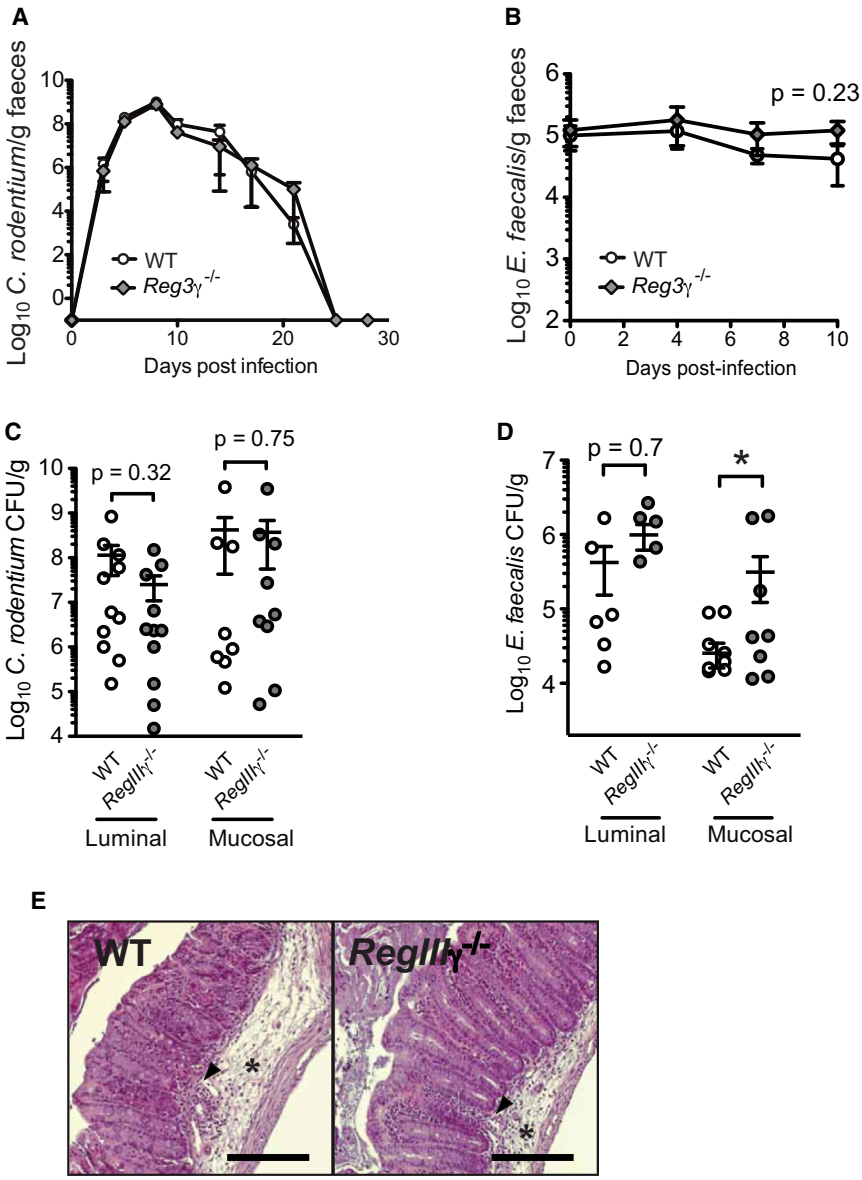

Figure 4. *RegIIIγ*$^{-/-}$ Mice Did Not Show Enhanced Susceptibility to *C. rodentium*

(A–E) WT and *RegIIIγ*$^{-/-}$ mice were infected orally with 10^9 *C. rodentium*. (A) Shedding of *C. rodentium* and (B) *E. faecalis* over the course of infection. Bacterial load of (C) *C. rodentium* and (D) *E. faecalis* in the cecal lumen and mucosal tissues, determined by selective plating. (E) Cecal histopathology of *C. rodentium*-infected WT and *RegIIIγ*$^{-/-}$ mice at day 9 p.i., showing similar submucosal edema (asterisks) and inflammatory infiltrates (arrows). Scale bar, 100 μm. Results are from three independent experiments (n = 6–8 each).

activity of RegIIIγ against adherence by Gram-positive microbes (Vaishnava et al., 2011). However, all *RegIIIγ*$^{-/-}$ mice (n = 14) survived the infection, showing a level of intestinal histopathology similar to that of WT and no evidence of systemic bacterial dissemination (Figures 4E and S6B). Thus, additional factors downstream of IL-22RA1 may be necessary for the maintenance of intestinal colonization resistance.

To interrogate IL-22RA1 signaling specifically at the intestinal epithelium, we derived primary colonic organoids from WT and *Il22ra1*$^{-/-}$ mice (Sato et al., 2011) and evaluated their global transcriptomes by deep RNA sequencing. *Il22ra1*$^{-/-}$ organoids displayed a normal morphology, characterized by columnar enterocytes with microvilli, tight junctions, and mucus-producing goblet cells (Figures 5A and S6C). Consistent with the epithelial expression of IL-22RA1, in vitro stimulation with IL-22 led to an induction of the known antimicrobials RegIIIβ, RegIIIγ, S100a8, and S100a9 in WT and *Il22*$^{-/-}$ organoids, but not *Il22ra1*$^{-/-}$ organoids (Figure S6D). Next, hierarchical clustering of RNA-seq data from WT and *Il22ra1*$^{-/-}$ organoids upon IL-22 stimulation demonstrated their distinct genome-wide expression profiles (Figure 5B). Factors involved in inflammatory response, antimicrobial processes, and wound healing were significantly upregulated, consistent with the previously established functions of the IL-22/IL-22RA1 pathway (Figures 5C and S6E and Tables S5A–S5C). Conversely, genes encoding cellular metabolic processes and DNA replication were downregulated (Figure S6E and Table S5D). Notably, a variety of proteases (e.g., *Prss22*, *Prss27*, *Slpi*) and peroxidases (e.g., *Gpx2*, *Duox1*, *Duox2*, *Duoxa2*) with antimicrobial activity, together with innate recognition molecules (*Tmem173*, *Zbp1*), were also induced by IL-22RA1 signaling, highlighting the unexpectedly diverse antimicrobial function of this pathway (Figure S6F).

Among genes upregulated by IL-22RA1 signaling, we further identified a range of susceptibility genes to inflammatory bowel disease (*Sec1*, *Fut2*, *Muc1*, *Prdm1*, *Xbp1*, *Nupr1*, *Erbb3*, *Efemp2*, *Chac1*, *Ppbp*, *Cxcl5*, *Stat3*, *Slc9a3*, *Bcl2l15*) (Jostins et al., 2012), psoriasis (*Rel*, *Nos2*), and rheumatoid arthritis (*Stom*, *Ptpn22*) (Eyre et al., 2012) represented by a Stat3-focused cluster (Figure 5D). This finding underlines the complex involvement of the IL-22/IL-22RA1 axis in human inflammatory diseases linked to pathological host-microbial interactions and supports the utility of intestinal organoids for dissecting the functional consequences of IL-22RA1 signaling. Finally, we observed a high enrichment of protein glycosylation genes induced by IL-22RA1 ($p < 10^{-4}$; Figures 5C and S6E and Tables S5A–S5C), which suggests a role for epithelial glycosylation in intestinal pathogen resistance.

Fucosylated Glycan Expression Enhances Colonization Resistance in *Il22ra1*$^{-/-}$ Mice

Glycoconjugates produced by the intestinal epithelium provide a rich source of host-derived complex carbohydrates that can

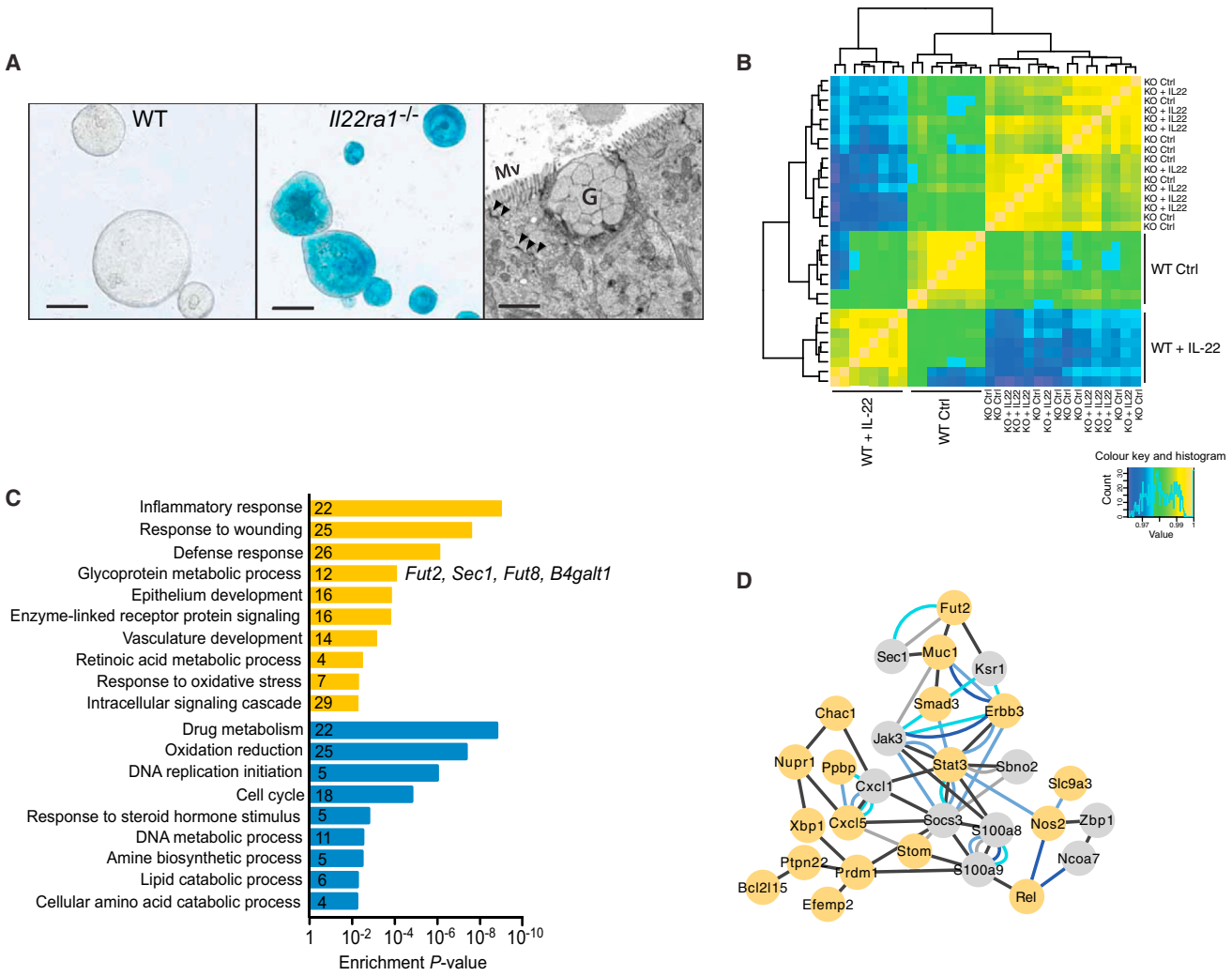

Figure 5. RNA-Seq of IL-22RA1 Signaling in Colonic Epithelial Organoids Identifies Factors Associated with Host-Microbe Interactions

(A) LacZ staining of WT and *Il22ra1*$^{-/-}$ organoids (scale bar, 100 μm) (left) and transmission electron microscopy of an *Il22ra1*$^{-/-}$ organoid with microvilli (Mv), tight junctions (arrowheads), and goblet cell (G) (right; scale bar, 1 μm).

(B) RNA-seq transcriptomes of colonic organoids from wild-type (WT) and *Il22ra1*$^{-/-}$ (KO) mice (n = 4) treated with IL-22 or untreated (Ctrl) in technical replicates, showing hierarchical clustering. Colors indicate levels of correlation (low, light green-yellow; high, dark green-blue).

(C) Enriched biological processes among upregulated (orange) and downregulated (blue) genes downstream of IL-22RA1 signaling, with selected genes associated with glycosylation.

(D) Stat3-focused interaction network of genes induced by IL-22RA1 signaling ($p < 10^{-12}$), 18 of which are associated with human susceptibility to chronic inflammatory diseases (gold). Lines indicate protein-protein interactions (dark/light blue), coexpression (black), colocalization (gray), and shared protein domains (cyan). See also Figure S6.

impact the microbiota structure and function (Becker and Lowe, 2003). Among IL-22RA1-induced genes linked to epithelial glycosylation, *Fut2* encodes an α1,2-fucosyltransferase highly expressed in the distal gut (Bry et al., 1996). The FUT2 enzyme is responsible for the expression of ABO histo-blood group antigens at the intestinal mucosa (Kelly et al., 1995). Importantly, *Fut2* expression may influence the microbiota (Kashyap et al., 2013) and host susceptibilities to various bacterial pathogens (e.g., *Campylobacter jejuni* and *Helicobacter pylori*) and autoimmune disorders (e.g., Crohn's disease) (Ilver et al., 1998; McGovern et al., 2010; Ruiz-Palacios et al., 2003). We found that *Fut2* is highly upregulated by IL-22RA1 signaling both in cultured organoids ($p = 2.21 \times 10^{-188}$) and during *C. rodentium* infection in vivo (Figure 6A and Table S5). Accordingly, the intestinal mucosa of *C. rodentium*-infected *Il22ra1*$^{-/-}$ mice showed reduced staining with the fucose-binding Lotus tetragonolobus lectin compared to WT equivalents (Figure 6B), indicating a decreased fucosylation level in the absence of IL-22RA1 signaling.

To test if induction of epithelial fucosylation mediated by IL-22RA1 may contribute to intestinal host defense, we administered physiologically relevant doses of 2′-fucosyllactose (2′FL), an α1,2-fucosylated oligosaccharide, orally to *Il22ra1*$^{-/-}$ mice during *C. rodentium* infection. Treatment with 2′FL from

day 4 p.i. significantly attenuated the early morbidity and mortality of infected *Il22ra1*$^{-/-}$ mice (Figure 6C). Administration of blood group H disaccharide (another α1,2-fucosylated molecule similar to the mucosal antigen mediated by the FUT2 enzyme) similarly led to attenuation of the infection outcome in *Il22ra1*$^{-/-}$ mice, while administration of lactose alone did not (Figure 6C).

We next investigated the beneficial effect of fucosylated glycans in *Il22ra1*$^{-/-}$ mice by assessing their histopathology, inflammatory responses, and bacterial colonization. 2′FL treatment did not appear to significantly reduce intestinal pathology or the Th1/Th17 inflammation in *Il22ra1*$^{-/-}$ mice at day 9 p.i. (Figures 6D–6G). We also observed similar *C. rodentium* colonization between 2′FL-treated and PBS controls (Figure 7A). However, luminal and mucosal colonization of *E. faecalis* significantly decreased among the 2′FL-treated group (Figure 7B). This correlates with lower levels of *E. faecalis* dissemination and of systemic IL-6 and TNF-α (Figures 7C and 7D), indicating that intestinal abundance of fucosylated molecules may play a role in the restriction of *E. faecalis* in the microbiota.

Within the intestinal microbiota, some obligate anaerobes such as the Ruminococcaceae and *Bacteroides* spp. may benefit from host-derived fucosylated glycans (Marcobal et al., 2011; Martens et al., 2008). We reasoned that fucosylated glycans in the intestine could restrict colonization by opportunistic pathogens (e.g., *E. faecalis*) in part by enhancing the growth of some commensals. By 16S rRNA sequencing, we compared the microbiota diversity and composition of *Il22ra1*$^{-/-}$ mice treated with 2′FL to PBS-treated littermate controls during *C. rodentium* infection. While no significant difference was observed in the baseline microbiota between both groups, 2′FL treatment resulted in an increase of bacterial species richness and diversity (shown by the Shannon diversity index) by day 9 p.i. (Figure 7E). Furthermore, analyses based on the community structure revealed a remarkable shift in the microbial composition upon 2′FL treatment (weighted UniFrac; $p < 0.001$) (Figure 7F). In particular, we detected a significantly greater abundance of *Bacteroides* spp. (2.4-fold; $p < 0.0001$) and Ruminococcaceae (7.9-fold; $p < 0.0001$) in the 2′FL-treated group. Thus, our results suggest that IL-22RA1-mediated production of intestinal fucosylated conjugates plays a role in colonization resistance by restoring the anaerobic commensal diversity.

DISCUSSION

Given that IL-22 dysregulations are implicated in many human diseases linked to pathological host-microbiota interactions (Sabat et al., 2014), and *Il22* deficiency can lead to different infection susceptibility phenotypes (Feinen and Russell, 2012; Graham et al., 2011), we reasoned that the indigenous microbiota could be a critical factor for disease induction. Although absence of IL-22 has been reported to associate with a colitogenic microbiota (Behnsen et al., 2014; Zenewicz et al., 2013), we found that our *Il22ra1*$^{-/-}$ mice harbor a microbiota highly similar (in both diversity and community structure) to that of WT controls. This difference underscores the potential impact of animal husbandry conditions in different facilities and suggests that factors besides a healthy SPF microbiota might be necessary for disease induction in *Il22ra1*$^{-/-}$ mice.

We observed that *Il22ra1* deficiency predisposes mice to a severe disseminated bacterial infection upon disruption of the microbiota by *C. rodentium* infection or DSS colitis. Although extraintestinal spread of *C. rodentium* has been reported in *Il22*$^{-/-}$ mice (Zheng et al., 2008), the contribution of invasion by *C. rodentium* or other intestinal bacteria to host susceptibility has not been fully explored. We identified that an *E. faecalis* isolate was the predominant intestinal bacterium that disseminates among susceptible *Il22ra1*$^{-/-}$ mice and that this *E. faecalis* isolate possesses a range of important virulence factors relevant for nosocomial disease in humans. Notably, *E. faecalis* possesses a functional cytolysin associated with bacterial invasion and virulence (Huycke et al., 1991) and is capable of invading the intestinal epithelium in a manner similar to that of the human pathogen *E. faecalis* V583 (Benjamin et al., 2013). Furthermore, mice are highly susceptible to systemic *E. faecalis* infection, and *C. rodentium*-infected *Il22ra1*$^{-/-}$ mice may be rescued through antibiotic inhibition of *E. faecalis*. Thus, *E. faecalis* represents an opportunistic pathogen capable of disease induction in the *Il22ra1*$^{-/-}$ host.

Intestinal dysbiosis often acts as a predisposing factor to infectious diseases, as diverse bacterial pathogens such as *S.* Typhimurium and *Clostridium difficile* may exploit disruptions of the microbiota to establish replicative niches (Lawley et al., 2012; Stecher et al., 2007). Here we found that while WT mice were able to maintain a low level of colonization by *E. faecalis* (~10^4–10^5 colony-forming units [cfu]/g of feces), *Il22ra1*$^{-/-}$ mice were predisposed to unrestricted *E. faecalis* proliferation (10^7–10^9 cfu/g) during both *C. rodentium*- and DSS-induced dysbiosis. Moreover, we noted a preferential expansion of a pathogenic *E. faecalis* variant in comparison to other commensal enterococci (i.e., *E. gallinarum*). Thus, although intestinal inflammation is thought to generally promote the growth of facultative anaerobes, our result highlights the importance of assessing intestinal dysbiosis at the species level to potentially distinguish between disease-causing and commensal microbes belonging to the same bacterial taxa.

The antimicrobial response of IL-22 to bacterial pathogens has been shown to involve RegIIIγ, which is highly expressed in the small intestine and bactericidal against Gram-positive bacteria (Cash et al., 2006; Kinnebrew et al., 2010). Interestingly, while we found that *RegIIIγ*$^{-/-}$ mice had increased levels of mucosa-associated *E. faecalis*, their expansion in the luminal microbiota and translocation to systemic organs were unaffected. Thus, together with the diverse network of antimicrobial factors uncovered by our RNA-seq analysis of epithelial organoids, this finding underlines the multifactorial nature of IL-22RA1 signaling in host-microbiota interactions.

By our organoid transcriptomic approach, we further identified the α1,2-fucosyltransferase gene *Fut2* as a candidate factor regulated by IL-22RA1 signaling. *Fut2* is critically involved in regulating the fucose nutrient environment of the microbiota (Kashyap et al., 2013) as well as host susceptibility to bacterial and viral pathogens (Ilver et al., 1998; Lindesmith et al., 2003; Ruiz-Palacios et al., 2003). Interestingly, fucosylated glycans typically increase during gut maturation (Bry et al., 1996) and infection (Deatherage Kaiser et al., 2013), a process that is thought to beneficially enhance bacterial colonization. Here we found that epithelial expression of *Fut2* is directly induced by IL-22RA1

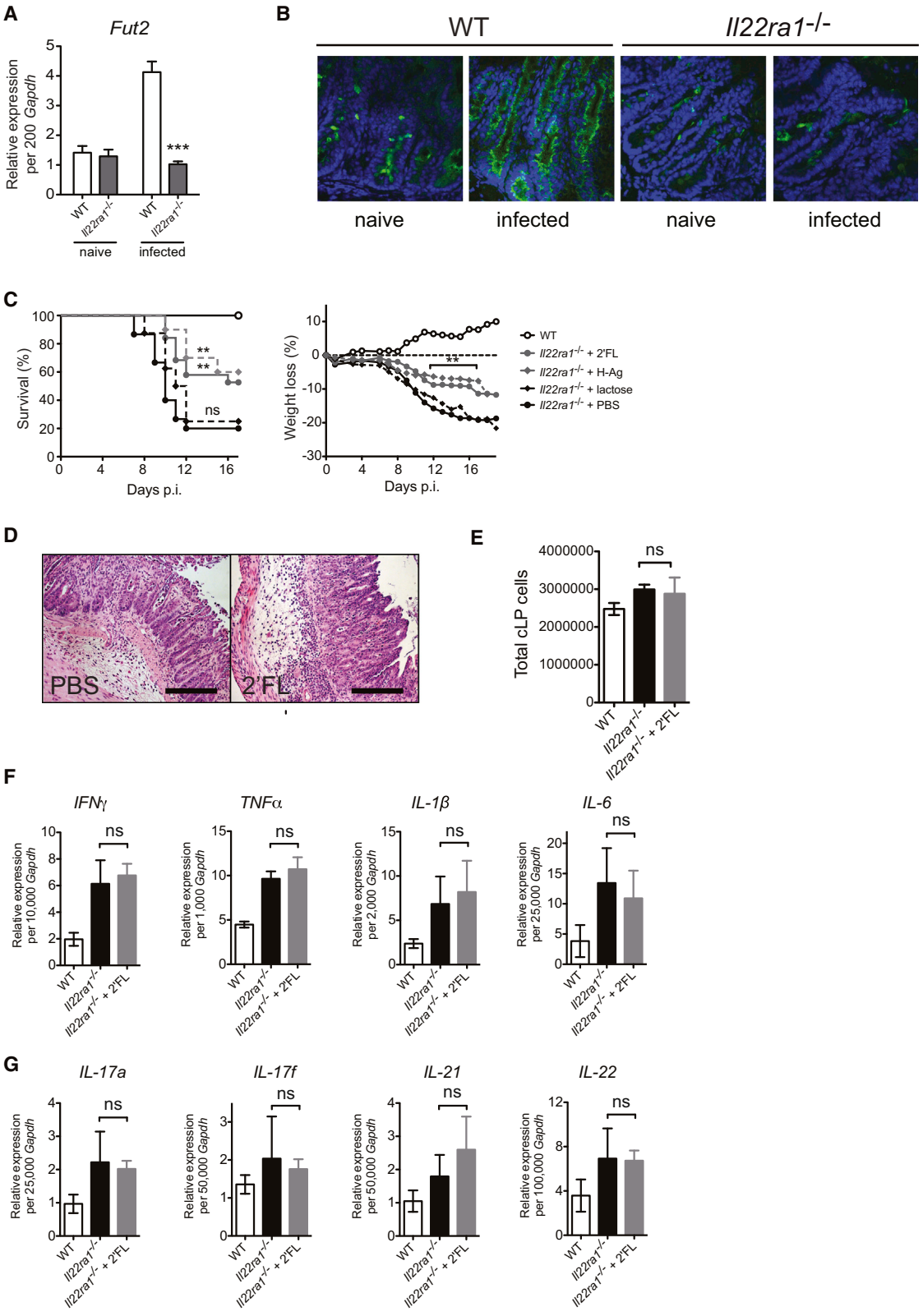

Figure 6. IL-22RA1-Mediated Fucosylated Glycan Expression Contributes to Host Defense

(A) Cecal *Fut2* transcripts of WT and *Il22ra1*$^{-/-}$ mice (n = 9–12) at day 9 p.i. with *C. rodentium*.

(B) Immunofluorescence staining of WT and *Il22ra1*$^{-/-}$ cecal tissues with Lotus tetragonolobus lectin (green).

(legend continued on next page)

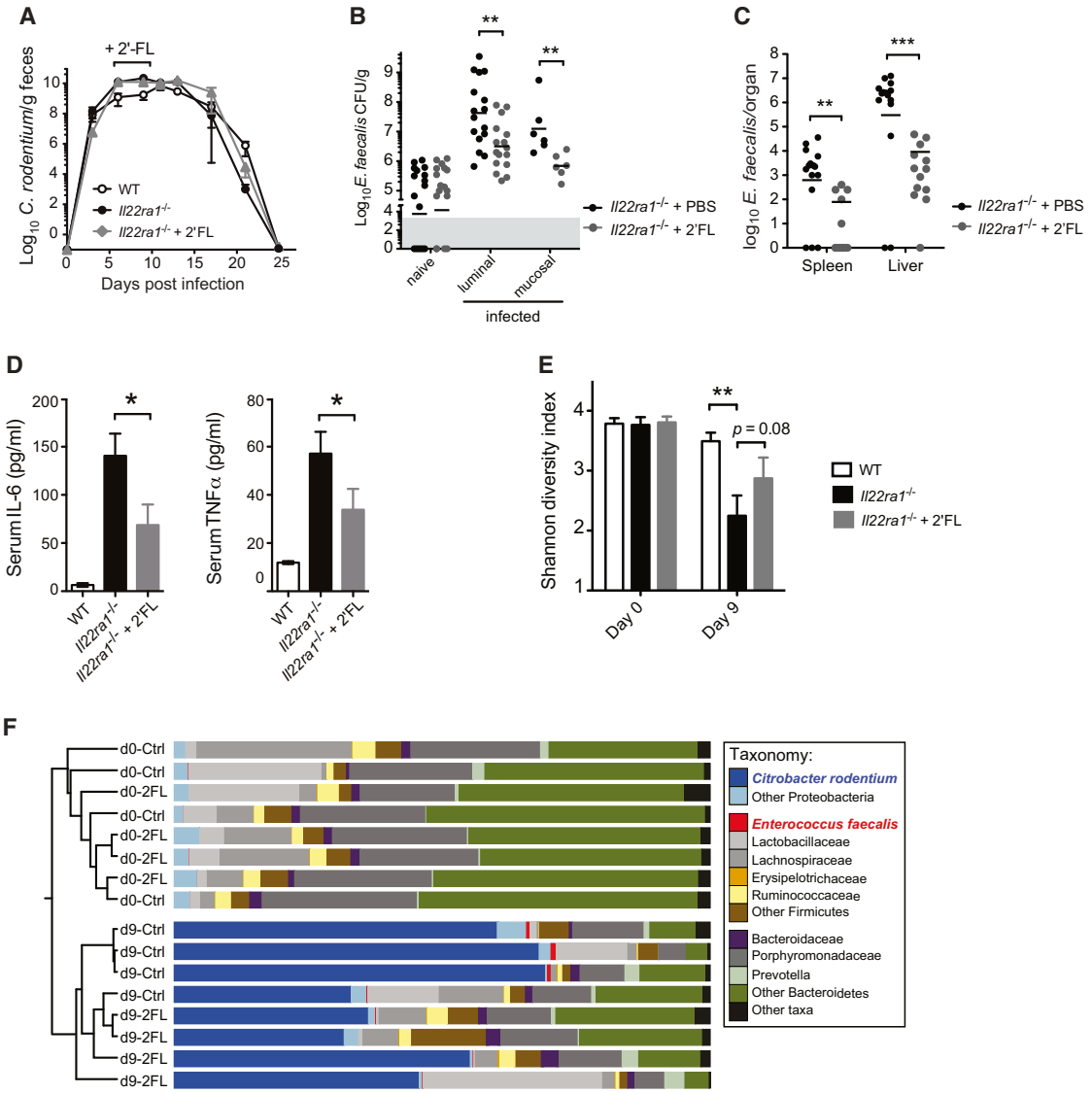

Figure 7. Fucosylated Glycan Expression Enhances Colonization Resistance to *E. faecalis*

(A–D) *C. rodentium*-infected WT mice and groups of *Il22ra1*$^{-/-}$ littermates were treated orally with 2′-fucosyllactose (2′FL) or PBS. Shown are (A) *C. rodentium* shedding, (B) *E. faecalis* cfus in the colonic lumen and mucosa (gray area, detection limit), (C) *E. faecalis* cfus in the liver and spleen, and (D) serum IL-6 and TNF-α levels at day 9 p.i. Mean ± SEM of data from three independent experiments (n = 3–5 each); *p < 0.05, **p < 0.01, ***p < 0.0001.

(E) Shannon diversity index of the fecal microbiota before infection (day 0) and at day 9 p.i.

(F) Cluster dendogram of the fecal microbiota community structures of 2′FL-treated *Il22ra1*$^{-/-}$ mice and PBS-treated controls (Ctrl). Bar graphs represent proportional abundance.

signaling, which is consistent with a lower level of fucosylated glycans at the colonic mucosa of *Il22ra1*$^{-/-}$ mice during infection.

We further observed that restoration of fucosylated molecules in the intestine can attenuate the susceptibility of *Il22ra1*$^{-/-}$ mice and decrease *E. faecalis* colonization at the intestinal lumen and mucosa. Although 2′FL administration did not appear to have an anti-inflammatory effect, we detected a clear impact on the microbiota diversity and composition, characterized by an increased abundance of some anaerobic commensal populations. Interestingly, colonization resistance to VRE has been shown to involve *Barnesiella* spp., which are Gram-negative saccharolytic commensals belonging to the family Porphyromonadaceae (Ubeda et al.,

(C–E) WT mice and groups of *Il22ra1*$^{-/-}$ littermates were treated orally with 2′-fucosyllactose (2′FL), blood group H disaccharide (or H antigen; H-Ag), lactose, or PBS between days 5 and 8 p.i. during *C. rodentium* infection. Shown are (C) survival (left) and weight loss (right), (D) histopathology (scale bar, 200 μm), and (E) total number of cLP leukocytes at day 9 p.i.

(F and G) qPCR of (F) Th1 cytokines (IFNγ, TNF-α, IL-1β, IL-6) and (G) Th17-associated cytokines (IL-17a, IL-17f, IL-21, IL-22) in the cecal tissues at day 9 p.i.

2013). In agreement with this study, we observed a higher colonization of Porphyromonadaceae in the 2′FL-treated group (1.5-fold). Moreover, the fucose-utilizing, anaerobic Ruminococcaceae and *Bacteroides* spp. were remarkably enriched (7.9-fold and 2.4-fold, respectively), which further supports the notion that intestinal fucosylation provides a nutrient foundation for symbiotic commensals (Hooper and Gordon, 2001).

The intestinal microbiota represents a complex and highly controlled nutrient network that is crucial for colonization resistance to diverse bacterial pathogens. For example, upon depletion of commensal microbes, *S*. Typhimurium can upregulate its fucose utilization operon (Deatherage Kaiser et al., 2013) and exploit the increased abundance of fucose and sialic acids to colonize the intestine (Ng et al., 2013). Therapeutic modulation of the microbiota, by dietary intake of prebiotic substances or administration of health-associated bacteria, therefore provides an attractive opportunity for the management of intestinal infections (Lawley et al., 2012). Here, our data support a mechanistic model whereby the IL-22RA1/Fut2 axis is involved in the maintenance of a diverse healthy microbiota, which in turn promotes intestinal colonization against the opportunistic pathogen *E. faecalis*. Further insights into the molecular interactions between IL-22RA1-mediated glycosylation pathways and symbiotic members of the intestinal microbiota could potentially yield opportunities for the treatment of intestinal dysbiosis in infectious and autoimmune diseases.

EXPERIMENTAL PROCEDURES

Mouse Infection

Il22ra1$^{tm1a/tm1a}$, *RegIIIγ*$^{tm1a/tm1a}$, and WT C57BL/6N mice were maintained and phenotyped by the Sanger Mouse Genetics Programme (White et al., 2013). Endogenous *Il22ra1* expression was detected by overnight incubation with 0.1% (w/v) X-gal (Invitrogen). All animals were kept under specific pathogen-free conditions, and colony sentinels tested negative for *Helicobacter* spp. Mice were infected orally with 10^9 cfu of Kanamycin (Kan)- and nalidixic acid (Nal)-resistant luminescent *Citrobacter rodentium* ICC180 (Wiles et al., 2006) or intravenously (i.v.) with 10^5 cfu *Salmonella enterica* serovar Typhimurium M525 TETc (Clare et al., 2003). Where indicated, *C. rodentium*-infected mice were administered intraperitoneal (i.p.) ampicillin (50 mg/kg; Sigma-Aldrich), oral 2′-fucosyllactose, H disaccharide, lactose (2 mg/day; Carbosynth), or PBS controls. DSS colitis was induced by administering 2% (w/v) DSS (MP Biomedicals) in drinking water for 7 days, followed by normal autoclaved water. The care and use of mice were in accordance with the UK Home Office regulations (UK Animals Scientific Procedures Act 1986) and were approved by the Sanger Institute's Animal Welfare and Ethical Review Body.

C. rodentium Immunoglobulin ELISA

NUNC Maxisorp ELISA plates were coated overnight with 100 μg/well *Citrobacter* EspA protein and blocked with 3% BSA (Sigma-Aldrich). Serum and fecal supernatant samples were added in 1:5 serial dilutions for 1 hr at 37°C. We detected immunoglobulin levels with HRP-conjugated rabbit α-mouse total IgG or IgA antibodies (1/1,000; Invitrogen).

Quantitative Real-Time PCR

Total RNA was extracted from mouse cecal tissues using the RNeasy Mini Kit (QIAGEN). Quantitative real-time PCR was conducted with a 7900HT RealTime PCR system (Applied Biosystems), using ABsolute Blue Rox Mix (Thermo Scientific), TaqMan primers, and probes as detailed in the Supplemental Experimental Procedures.

16S rRNA-Based Identification of Bacterial Species

To identify microbial species from the livers and spleens of mice, we homogenized mouse tissues aseptically under laminar flow. Organ lysates were immediately cultured in nonselective Luria-Bertani (LB), BHI (Brain Heart Infusion), and FAA (Fastidious Anaerobe Agar) media under aerobic and anaerobic conditions for 36–48 hr. All colonies from each plate, or within a defined section, were picked in an unbiased manner for DNA extraction and 16S rRNA gene sequencing using the universal primers: 7F, 5′-AGAGTTTGA TYMTGGCTCAG-3′; 926R, 5′-ACTCCTACGGGAGGCAGCAG-3′. Bacterial identifications were performed using the 16S rRNA NCBI Database for Bacteria and Archeae.

Bacterial Genomic and Phylogenetic Analysis

Genomic DNA from *Enterococcus faecalis* isolates were prepared from overnight cultures using a standard phenol-chloroform extraction procedure and sequenced by Illumina MiSeq 2000. We performed genome assembly and comparative genomics with the previously sequenced *E. faecalis* V583 genome (Paulsen et al., 2003) and the *E. faecalis* MMH594 pathogenicity island (GenBank accession number AF454824) (Shankar et al., 2002). To profile the diversity of enterococci in the mouse microbiota, we sequenced the 16S rRNA gene of single enterococcal colonies on selective Bile Esculin Azide agar (Sigma-Aldrich) in an unbiased manner. Representative *Enterococcus faecalis* isolates were selected for whole-genome sequencing, generating ~50× genome coverage/isolate. Phylogenetic analysis was performed using the previously published full and draft genomes of 43 *E. faecalis* strains collected from diverse human and animal sources (McBride et al., 2007). See also Supplemental Experimental Procedures.

Microbiota Analyses

Bacterial DNA was obtained using the FastDNA Spin Kit for Soil (MBio) and FastPrep Instrument (MPBiomedicals). V5-V3 regions of bacterial 16S rRNA genes were PCR amplified with high-fidelity AccuPrime Taq Polymerase (Invitrogen) and primers: 338F, 5′-CCGTCAATTCMTTTRAGT-3′; 926R, 5′-ACTCCTACGGGAGGCAGCAG-3′. Libraries were sequenced by 454 (Roche) or MiSeq (Illumina). Analyses were performed with the mothur software (Schloss et al., 2009), using quality filtering and analysis parameters as described in the Supplemental Experimental Procedures. For quantification of group-specific bacteria, we used the following primers: F, 5′-ATGGC TGTCGTCAGCTCGT-3′; R, 5′-CCTACTTCTTTTGCAACCCACTC-3′ (for Enterobacteriaceae) and F, 5′-CCCTTATTGTTAGTTGCCATCATT-3′; R, 5′-ACTCGTTGTACTTCCCATTGT-3′ (for *Enterococcus* spp.).

Intestinal Organoid Transcriptomics

Organoids from mouse colons were isolated and cultured in vitro as described (Sato et al., 2011). For RNA-seq, 1-week-old organoids of WT and *Il22ra1*$^{-/-}$ mice (n = 4) were treated with IL-22 (50 ng/ml) or controls in complete growth medium. mRNA libraries were prepared using the Illumina TruSeq protocol and sequenced by Illumina HiSeq to yield ~6.4 ± 0.25 giga-base pairs (Gbp)/sample. Normalized read counts (aligned to the reference mm10/NCBIM37 genome) were used to assess the statistical significance of the interaction between IL-22 treatment and *Il22ra1* genotype, as described in the Supplemental Experimental Procedures. At $p < 10^{-12}$, 364 genes were identified as upregulated and 197 genes were identified as downregulated by IL-22/IL-22RA1 signaling.

Statistical Analysis

We applied nonparametric Mann-Whitney U tests for pairwise statistical comparisons and two-way ANOVA for comparisons of grouped data using the Prism 5.0 software (GraphPad).

ACCESSION NUMBERS

The European Nucleotide Archive (ENA) accession numbers for the *E. faecalis* genome data, microbiota sequencing data, and RNA-seq data reported in this paper are ERR225616, ERP002393, and ERR247358–ERR247389, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.chom.2014.08.017.

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