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A secreted bacterial peptidoglycan hydrolase enhances tolerance to enteric pathogens

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Abstract

The intestinal microbiome modulates host susceptibility to enteric pathogens, but the specific protective factors and mechanisms of individual bacterial species are not fully characterized. We show that secreted antigen A (SagA) from *Enterococcus faecium* is sufficient to protect *Caenorhabditis elegans* against *Salmonella* pathogenesis by promoting pathogen tolerance. The NlpC/p60 peptidoglycan hydrolase activity of SagA is required and generated muramyl-peptide fragments that are sufficient to protect *C. elegans* against *Salmonella* pathogenesis in a *tol-1*-dependent manner. SagA can also be expressed and secreted in other bacteria and improve the protective activity of probiotics against *Salmonella* pathogenesis in *C. elegans* and mice. Our study highlights how protective intestinal bacteria can modify microbial-associated molecular patterns to enhance pathogen tolerance.

Dysbiosis of the gut microbiota is associated with metabolic disorders, inflammatory bowel disease and increased pathogen susceptibility (1). Nonetheless, individual bacterial species and factors involved in host protection have been difficult to characterize (2). *Enterococci* are lactic acid bacteria associated with the intestinal microbiome of diverse species ranging from humans to flies and can attenuate host susceptibility to enteric pathogens, including *Salmonella* (3, 4). Non-pathogenic strains of *E. faecium* have been used as probiotics, but their protection mechanisms are unclear (5). Since *E. faecium* can colonize the *C. elegans* intestine without causing apparent disease (6), we employed *C. elegans* as a model organism (7) to elucidate the protective mechanism(s) underlying *E. faecium* probiotic activity. To investigate whether *E. faecium* can attenuate enteric bacterial pathogenesis in *C. elegans*, we developed a treatment-infection assay with *Salmonella enterica* serovar Typhimurium, (fig.

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Supplementary Materials

Materials and Methods

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S1A), which causes persistent intestinal infection and death in *C. elegans* (8–10). In our assay, *E. faecium*-treated animals appeared less fragile and more motile than control *Escherichia coli* OP50-treated animals after *S.* Typhimurium infection (fig. S1B). *C. elegans* survival was increased in animals fed *E. faecium* prior to infection as compared to animals fed *E. coli* OP50 or *Bacillus subtilis* 168 (Fig. 1A, fig. S1C). Multiple strains of *E. faecium*, including a pathogenic strain, were able to inhibit *S.* Typhimurium pathogenesis (fig. S1D–E). *E. faecium*-treated animals were also more resistant to the intrinsic pathogenesis of *E. coli* OP50 (fig. S1F) as well as pathogenesis caused by *Enterococcus faecalis* V583 (11) (fig. S1G). These results suggest that the mechanism of protection is conserved amongst *E. faecium* strains and is active against diverse enteric pathogens.

We next analyzed the effect of *E. faecium* on *S.* Typhimurium colonization and persistence. Fluorescence imaging of mCherry-*S.* Typhimurium 3 days post-infection (dpi) showed comparable *S.* Typhimurium colonization with or without *E. faecium* treatment (Fig. 1B, fig. S1H). Viable *S.* Typhimurium (CFUs) recovered from lysed worms revealed a ~2 log decrease in *S.* Typhimurium colonization 1 dpi in *E. faecium*-treated *S.* Typhimurium-infected animals (Fig. 1C). However, by 3 dpi, *S.* Typhimurium titer was similar in OP50- and *E. faecium*-treated *S.* Typhimurium-infected animals (Fig. 1C). To determine if this transient decrease in *S.* Typhimurium colonization represented niche competition early in our assay, we monitored *E. faecium* CFUs throughout the infection assay (fig. S1I). While *E. faecium* initially colonized worms to ~10^5 CFUs/worm, *E. faecium* numbers decreased to ~10–10^2 CFUs/worm 1 dpi, demonstrating that the transient decrease in *S.* Typhimurium colonization was not concomitant with an increase in *E. faecium* load. Electron microscopy of worm transverse sections 4 dpi revealed substantial degradation of the intestinal microvilli in OP50-treated *S.* Typhimurium-infected animals as compared to uninfected or *E. faecium*-treated animals (Fig. 1D). In OP50-treated *S.* Typhimurium-infected animals, bacteria had escaped the intestinal lumen and caused extensive tissue damage (Fig. 1D, middle panel). In contrast, *E. faecium*-treated *S.* Typhimurium-infected animals contained a similar bacterial load to the intestinal lumen and showed no apparent tissue damage (Fig. 1D, right panel), suggesting improved epithelial barrier integrity. These results demonstrate that *E. faecium* does not prevent *S.* Typhimurium colonization or replication, but may enhance host tolerance to pathogens.

We next explored whether specific factors produced by *E. faecium* were sufficient for protection against *S.* Typhimurium pathogenesis. *E. faecium* culture supernatant was as effective as live bacterial cultures in inhibiting *S.* Typhimurium pathogenesis (Fig. 2A). Activity of the supernatant was sensitive to proteinase-K treatment, trichloro-acetic acid precipitation, and 10-kDa size exclusion (fig. S2A–C), leading us to analyze the protein composition of *E. faecium* culture supernatant by mass spectrometry (fig. S2D–E, table S1). This revealed a number of secreted proteins and an enrichment of peptidoglycan remodeling factors (Fig. 2B). We focused on secreted antigen A (SagA), the most abundant protein identified in the supernatant (Fig. 2B), which encodes a putative secreted NlpC/p60 peptidoglycan hydrolase that is essential for *E. faecium* viability (12). Imaging of animals treated with *E. faecium*-expressing mCherry under the *sagA* promoter (*psagA:mcherry*) showed that *E. faecium* expresses SagA *in vivo* (Fig. 2C). Treatment of animals with recombinant SagA-His_6 purified from either *E. coli* BL21-RIL(DE3) or *E. faecium* Com15

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was sufficient to inhibit *S*. Typhimurium pathogenesis (Fig. 2, D–E, fig. S3, table S2). All sequenced *E. faecium* strains encode a *sagA* ortholog in their genomes whereas sequenced *E. faecalis* strains do not. We inserted *sagA-his*$_6$ into the *E. faecalis* OG1RF chromosome to generate *E. faecalis-sagA* (fig. S4, fig. S5). Treatment of *C. elegans* with *E. faecalis-sagA* attenuated *S*. Typhimurium pathogenesis comparably to *E. faecium*, while treatment with wild-type *E. faecalis* was not protective (fig. S6A, Fig. 2F). *S*. Typhimurium load was similar across all infected conditions, demonstrating that *E. faecalis-sagA* does not inhibit *S*. Typhimurium colonization *in vivo* but rather affects pathogen tolerance (fig. S6B). SagA expression also counteracted the intrinsic pathogenesis of *E. faecalis* OG1RF (6) (fig. S6C). These results demonstrate that SagA is sufficient to enhance host tolerance against distinct bacterial pathogens.

The protective activity of *E. faecium* against multiple enteric pathogens suggested that SagA may engage host pathways to limit pathogenesis. A survey of *C. elegans* immunity-associated mutants indicated no major role for the p38 MAPK/Pmk-1 pathway (13, 14), the TGF-β-like/Dbl-1 pathway (15), the insulin-like receptor/Daf-2 pathway (16), or the Npr-1 mediated pathogen avoidance pathway (17, 18) (fig. S7). *C. elegans* encodes one homologue of Toll-like receptor, *tol-1* (19). *C. elegans* lacking the *tol-1* TIR signaling domain [*tol-1(nr2033)*] exhibit defective pathogen avoidance to *S. marsescens* (20) and increased susceptibility to *S*. Typhimurium infection (21). We assessed SagA-mediated protection in *tol-1(nr2033)* animals and found that neither *E. faecium* nor *E. faecalis-sagA* were protective against *S*. Typhimurium infection in this mutant background, suggesting SagA enhances pathogen tolerance through *tol-1* signaling (Fig. 2G).

To evaluate the mechanism of SagA protection (22), we generated an active site mutant as well as a C-terminal domain truncation of the NlpC/p60 hydrolase domain (Fig. 3A, fig. S8A). Neither mutant was able to inhibit *S*. Typhimurium pathogenesis, indicating that the NlpC/p60 hydrolase activity is required (Fig. 3B). SagA did not affect *S*. Typhimurium colonization of *C. elegans* or directly attenuate *S*. Typhimurium growth or virulence mechanisms (fig S8B–E). In culture, recombinant SagA had no effect *E. coli* growth rate (fig. S9A), but induction of SagA expression caused a decrease in culture optical density (OD) (Fig. 3C, S9B–C), indicating cell lysis. In contrast, expression of the active site mutant or cytoplasmically-localized SagA did not induce *E. coli* cell lysis (Fig. 3C, fig. S9B–C). These data suggest that while exogenous addition of SagA is not bacteriolytic, SagA is a functional hydrolase that can cleave peptidoglycan when targeted to the periplasm.

We hypothesized that SagA generates peptidoglycan fragments responsible for enhancing pathogen tolerance. Consistent with this hypothesis, we found that the flow-thru from 5 kDa-MWCO column-filtered culture supernatants of *E. coli* expressing SagA, but not the active site mutant, protected *C. elegans* from *S*. Typhimurium pathogenesis (Fig. 3D, fig. S10A), suggesting that lower molecular weight products of SagA enzymatic activity are sufficient for protection. To test if SagA-generated *E. coli* peptidoglycan fragments can protect *C. elegans* from *S*. Typhimurium, we digested purified *E. coli* peptidoglycan with lysozyme and either SagA or the active site mutant, then filtered the digests to exclude protein. *C. elegans* treated with the SagA peptidoglycan digests survived similarly to SagA-treated animals, whereas active site mutant digests failed to attenuate pathogenesis (Fig. 3E).

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These results suggest that SagA-generated peptidoglycan fragments, and not SagA itself, are responsible for enhancing pathogen tolerance.

To identify the peptidoglycan fragment(s) generated by SagA, we analyzed filtered bacterial culture supernatants by ANTS labeling and gel-based profiling (23, 24). From *E. coli* expressing SagA, we detected a SagA-specific product that migrated similarly to the synthetic peptidoglycan fragments MurNAc-L-Ala and GlcNAc, but not to MurNAc-L-Ala-D-Glu (MDP) or MurNAc (Fig. 3F). ANTS analysis of *E. faecium*, *E. faecalis*, and *E. faecalis-sagA* peptidoglycan extracts revealed that SagA expression alters the muropeptide profile (fig. S11). From *E. faecalis-sagA* culture supernatant, we detected an ANTS-labeled product that co-migrates with MurNAc (Fig. 3G), suggesting that heterologous SagA expression induces muropeptide shedding in both *E. coli* and *E. faecalis*. In contrast, 10 kDa-MWCO filtered *E. faecium* culture supernatant did not yield detectable levels of MurNAc-L-Ala or MurNAc (Fig. 3G) and was not protective when administered to *C. elegans* (fig. S2C). *E. faecium* that expresses SagA endogenously is likely resistant to SagA-induced peptidoglycan shedding. As SagA is abundantly secreted by *E. faecium* (fig. S4, tables S1, S4) and is protective after purification (Fig. 2D–E), soluble SagA may hydrolyze extracellular peptidoglycan fragments derived from digested bacteria *in vivo*. Indeed, incubation of purified *E. coli* peptidoglycan with lysozyme and recombinant SagA, but not the active site mutant, yielded a peptidoglycan cleavage product with similar mobility to MurNAc-L-Ala (fig. S10B). These data suggest that heterologous expression of SagA in bacteria can remodel bacterial peptidoglycan (fig. S11), induce shedding of small muropeptide fragments (Fig. 3F–G), and cleave extracellular peptidoglycan when secreted (fig. S10B). We next assessed the protective activity of SagA-generated peptidoglycan fragments, MurNAc and MurNAc-L-Ala, as well as GlcNAc and MDP. Treatment of *C. elegans* with either MurNAc or MurNAc-L-Ala was sufficient to inhibit *S*. Typhimurium pathogenesis, while MDP or GlcNAc were not (Fig. 3H). MurNAc and MurNAc-L-Ala were not protective in *tol-1(nr2033)* animals (Fig. 3I), suggesting that *tol-1* is required for mediating host protection in response to these peptidoglycan fragments. These data are consistent with the activity of muropeptides in mammals (25, 26), but show MurNAc-L-Ala and MurNAc are the minimal peptidoglycan components that enhance pathogen tolerance in *C. elegans*.

We next evaluated SagA-mediated protection against *Salmonella* pathogenesis in mice. Germ-free mice were mono-colonized with *E. faecium, E. faecalis*, or *E. faecalis-sagA* 7 days prior to infection with *S.* Typhimurium. *Enterococcus* and *Salmonella* load were measured in the feces, and mouse survival was tracked. All *Enterococcus* strains were similarly recovered from the feces after gavage, indicating efficient intestinal colonization (fig. S12). Consistent with our results in *C. elegans, S.* Typhimurium CFUs in the feces were similar across all conditions throughout infection (Fig. 4A), suggesting that *E. faecium* does not inhibit *Salmonella* colonization. Remarkably, mice gavaged with *E. faecium* or *E. faecalis-sagA* prior to infection exhibited reduced weight loss and prolonged survival, with a median survival of 9 days, as compared to *E. faecalis*-treated mice (Fig. 4B–C). Although *Enterococci* are used as probiotics in livestock, their pathogenic potential makes them problematic for use in humans (27). We thus introduced *sagA* into a non-pathogenic probiotic, *Lactobacillus plantarum* (28), and confirmed its expression and secretion (fig.

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S13). *sagA*-expressing *L. plantarum* significantly prevented weight loss and improved survival in an antibiotic-induced *S.* Typhimurium infection model compared to *L. plantarum* (Fig. 4D–F, fig. S14). These results indicate that SagA is sufficient to attenuate *Salmonella* pathogenesis in mammals and is protective even when expressed by other probiotic bacteria.

Here we demonstrate that *C. elegans* is an effective model to explore the protective mechanisms of intestinal bacteria and find that SagA from *E. faecium* is sufficient to protect *C. elegans* and mice from enteric pathogens. Our results suggest that the NlpC/p60 hydrolase activity of SagA generates unique peptidoglycan fragments that may activate host immune pathways to enhance epithelial barrier integrity and confine pathogens to the intestinal lumen, ultimately promoting tolerance to infection (fig. S15). Our analysis of *E. faecium* and engineered SagA-expressing bacterial strains in mice suggests SagA also improves intestinal epithelial barrier integrity to limit bacterial pathogenesis in mammals (29). The protective activity of *E. faecium* and SagA in mice requires the TLR signaling adaptor MyD88, the peptidoglycan pattern recognition receptor NOD2, and the C-type lectin RegIIIγ (29). These results together suggest that *E. faecium* and SagA may function through evolutionarily conserved pathways to enhance epithelial barrier integrity and protect animals from enteric pathogens. Finally, this study suggests that bacterial NlpC/p60-type peptidoglycan hydrolases (30–33) can enhance host tolerance to pathogens and that these enzymes could be used to improve the activity of existing probiotics.

Supplementary Material

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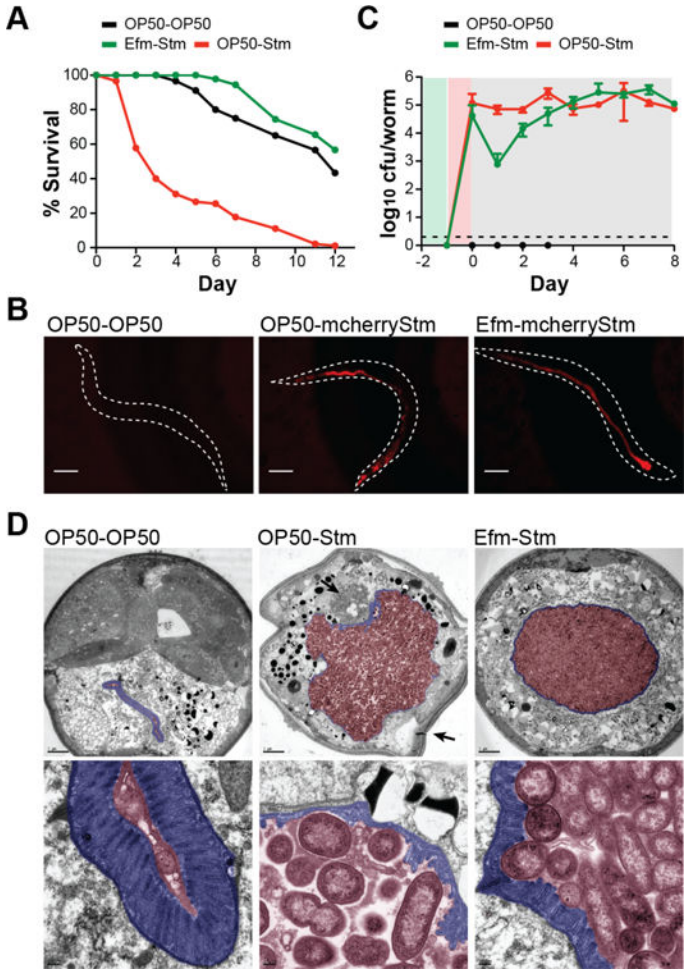

Figure 1. *E. faecium* induces host tolerance to *S.* Typhimurium

(A) Survival curve showing *E. faecium* (Efm, Com15)-mediated inhibition of *S.* Typhimurium (Stm, 14028) pathogenesis ($p<10^{-10}$). The legend indicates treatment-infection. Control worms were fed *E. coli* OP50 for both the treatment and infection stages of the assay. For *C. elegans* survival curves in all figures, significance was calculated by log-rank test with Bonferroni correction for multiple comparisons. Data points represent mean survival from 90 worms from a representative experiment independently replicated at least twice. **(B)** Fluorescence images of *C. elegans* infected with Stm-expressing plasmid-encoded mcherry (mcherryStm) at 3 dpi. The dotted lines indicate an outline of the worm body. Scale bar = 100 μm. **(C)** Stm CFUs measured in *C. elegans* throughout the infection assay. Data points represent average CFUs from 5 worms ± standard deviation of two independent experiments. The dotted line indicates detection limit. The background shading represents stage of the treatment-infection assay. Green indicates treatment, red indicates infection, and grey indicates *E. coli* (OP50) feeding. **(D)** Electron microscopy of transverse sections of *C. elegans* (top), and magnification of intestinal region (bottom) at 4 dpi. The intestinal microvilli are highlighted blue; the intestinal lumen is highlighted red. In the top middle panel, the top arrow indicates bacteria that have breached the epithelial barrier, and the

bottom arrow indicates loss of overall turgidity. Scale bar (top row) = 5 μm. Scale bar (bottom row) = 200 nm.

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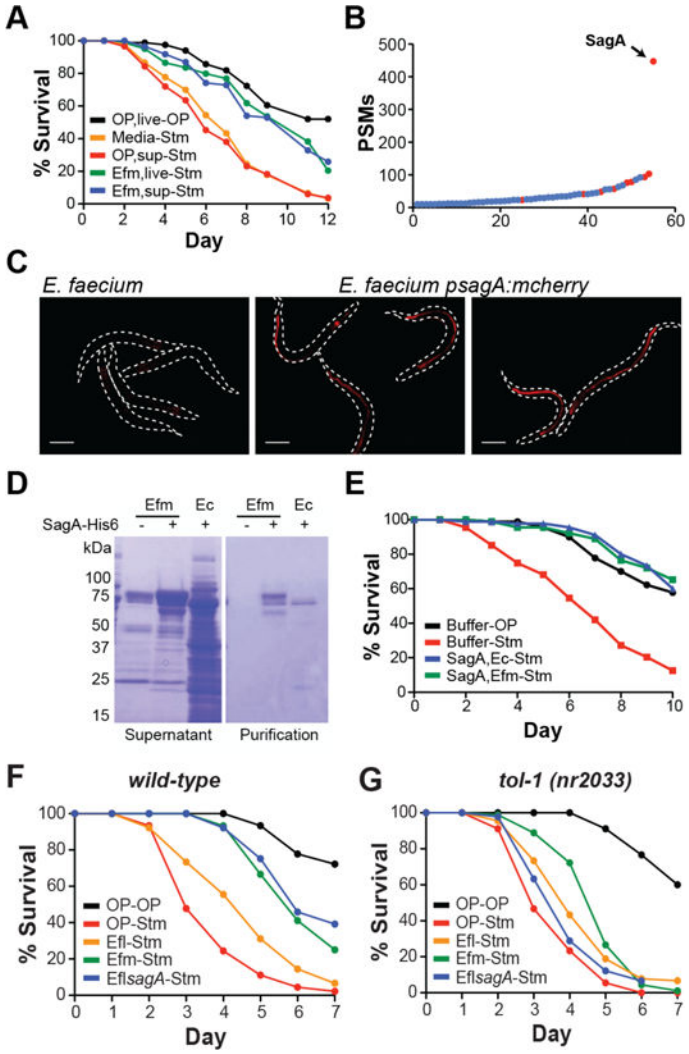

Figure 2. SagA is sufficient for inducing pathogen tolerance in a *tol-1*-dependent manner

(A) Survival curve showing that both *E. faecium* culture supernatant (Efm, sup) ($p<10^{-6}$) and live *E. faecium* culture (Efm, live) ($p<10^{-7}$) inhibit S. Typhimurium (Stm)-induced death. OP50 culture supernatant (OP, sup) is not protective (p=1). **(B)** Summary of proteins identified in Efm culture supernatant by mass spectrometry with at least 10 peptide spectrum matches (PSMs). Proteins involved in peptidoglycan remodeling are in red (See Supplementary Table 1). The x-axis represents arbitrary protein number. **(C)** Fluorescence images of *C. elegans* treated for 1 day with wild-type Efm or Efm-expressing mcherry under the *sagA* promoter (*psagA:mcherry*). The dotted lines indicate an outline of the worm body. Scale bar = 200 μm. **(D)** Coomassie stained SDS-PAGE of culture supernatants and SagA-His_6 purifications from *E. faecium* Com15 (Efm) and *E. coli* BL21-RIL(DE3) (Ec). **(E)** Survival curve showing that SagA-His_6 purified from either *E. coli* BL21-RIL(DE3) (SagA, Ec) ($p<10^{-10}$) or *E. faecium* Com15 (SagA, Efm) ($p<10^{-10}$) inhibits Stm pathogenesis. **(F)** Survival curve from a continuous infection assay (see fig. S6A) showing that *E. faecalis* (Efl, OG1RF)-*sagA* inhibits Stm pathogenesis ($p<10^{-10}$) similarly to Efm (Com15) (p=1) compared to *E. faecalis* (Efl, OG1RF) *and* OP50. **(G)** Survival curve from a continuous

infection assay showing that Efl-*sagA* (p=0.053) does not inhibit Stm pathogenesis in *tol-1(nr2033) C. elegans.*

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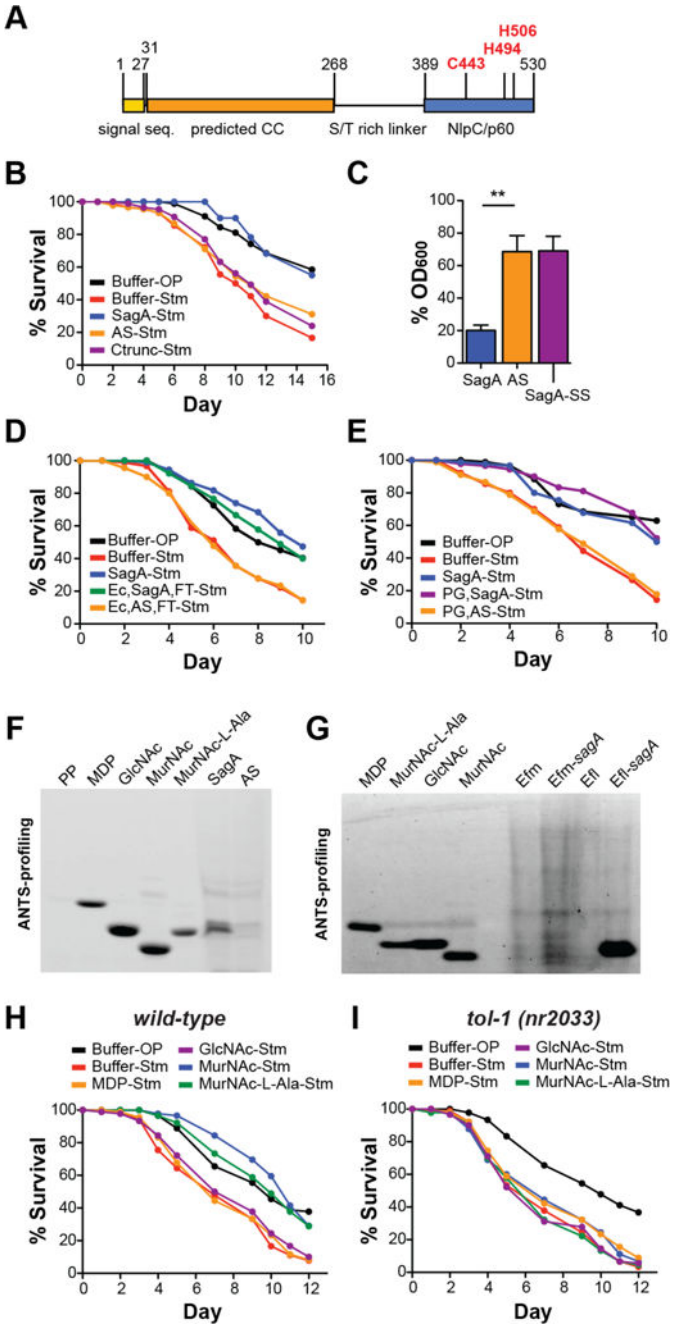

Figure 3. Enzymatic activity of SagA is required for enhancing pathogen tolerance

(A) Schematic of SagA domain organization: the signal sequence is yellow, a predicted coiled-coil (CC) domain is orange, and the NlpC/p60 type hydrolase domain is blue. Active site residues are in red type. **(B)** Survival curve showing that SagA inhibits *S.* Typhimurium (Stm) pathogenesis ($p<10^{-10}$) while an active site mutant (AS) and C-terminal truncation mutant (Ctrunc) do not (p=0.42 and 0.98 respectively). **(C)** OD_{600} of *E. coli* BL21-RIL(DE3) expressing SagA, the active site mutant, or cytoplasmically-localized SagA (SagA-SS) 1 hour post-induction. Bars represent mean ± s.e.m. from three independent experiments. Significance was calculated by unpaired t test. For **, $p < 0.01$. **(D)** Survival

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curve showing that 5-kDa MWCO column filtered *E. coli* culture supernatants expressing SagA-His_6 (Ec, sagA-FT) inhibit Stm pathogenesis ($p<10^{-4}$), while filtered *E. coli* culture supernatants expressing the active site mutant (Ec, AS-FT) do not (p=1). **(E)** Survival curve showing that purified *E. coli* peptidoglycan treated with SagA (PG, SagA) can inhibit Stm pathogenesis ($p<10^{-10}$), while *E. coli* peptidoglycan treated with the active site mutant (PG, AS) cannot (p=1). **(F)** ANTS visualization of *E. coli* culture supernatants expressing SagA-His_6 or the active site mutant. A sugar-less pentapeptide (PP) shows UV signal specificity. **(G)** ANTS visualization of peptidoglycan fragments in Efm, Efm-*sagA*, Efl, *and* Efl-*sagA* culture supernatants. **(H)** Survival curve showing that treatment with MurNAc ($p<10^{-5}$) or MurNAc-L-Ala ($p<10^{-10}$) can inhibit Stm pathogenesis, while MDP (p=1) and GlcNAc (p=1) are not protective. **(I)** Survival curve showing that MurNAc (p=1) and MurNAc-L-Ala (p=0.61) do not inhibit pathogenesis in *tol-1(nr2033) C. elegans*.

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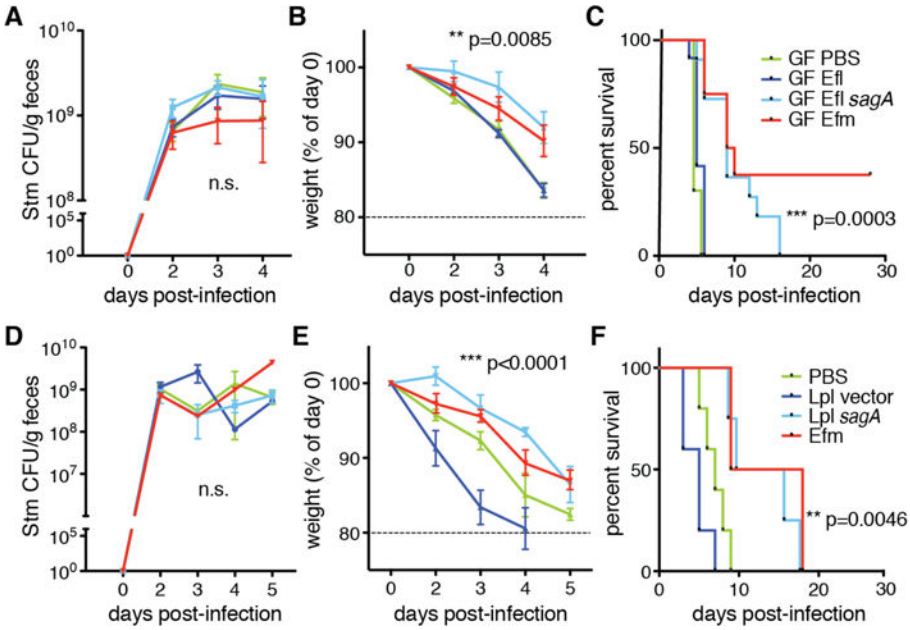

Figure 4. *E. faecium* and SagA enhance pathogen tolerance in mice

(A–C) Germ-free (GF) C57BL/6 mice were orally gavaged with 10^8 CFU *E. faecalis* (Efl), Efl-expressing *sagA* (Efl*sagA*) or *E. faecium* (Efm) 7 days before oral infection with 10^2 CFU *S.* Typhimurium (Stm). (A) Stm CFU in feces, (B) weight loss, and (C) survival are shown. Pooled data from 4 independent experiments, n=10–14 mice/group. **(D–F)** Mice were given AMNV-antibiotic cocktail for 14 days and colonized with 10^8 CFU *L. plantarum* (Lpl) harboring an empty plasmid vector (Lpl-vector) or a *sagA* plasmid (Lpl-*sagA*) or 10^8 CFU Efm prior to oral infection with 10^6 Stm. (D) Stm CFU in feces, (E), Weight loss and (F) survival are shown. Pooled data from 2 independent experiments, n=2–5 mice/group. (A, B, D and E) mean±SEM, 2-way ANOVA, p-value shown comparing *sagA*-expressing Efl or Lpl to WT or vector controls, respectively (A, B, D and E). n.s.=not significant. (C and F) Log-rank analysis, p-value shown comparing Efm, *sagA*-expressing Efl or Lpl to WT or vector controls, respectively (C and F). **p≤0.01, ***p≤0.001 for all analyses. Comparisons with no (*) had p>0.05 and were not considered significant.