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Antimicrobial peptide resistance mediates resilience of prominent gut commensals during inflammation

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Abstract

Resilience to host inflammation and other perturbations is a fundamental property of gut microbial communities, yet the underlying mechanisms are not well understood. We have found that human gut microbes from all dominant phyla are resistant to high levels of inflammation-associated antimicrobial peptides (AMPs) and have identified a mechanism for lipopolysaccharide (LPS) modification in the phylum Bacteroidetes that increases AMP resistance by four orders of magnitude. *Bacteroides thetaiotaomicron* mutants that fail to remove a single phosphate group from their LPS were displaced from the microbiota during inflammation triggered by pathogen infection. These findings establish a mechanism that determines the stability of prominent members of a healthy microbiota during perturbation.

Human gut microbial communities reside in an open ecosystem subject to disruptions ranging from dietary change to toxin exposure and pathogen invasion. Many of these perturbations, and functional disorders such as irritable bowel syndrome, are accompanied by nonspecific immune responses that impart a disruptive influence on community structure and function (1, 2). Host inflammatory mechanisms to remove harmful organisms and restrict bacteria to the lumen commonly target conserved molecular patterns found on pathogens and commensals alike, yet healthy gut microbial communities can remain stable for years in humans (3).

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Supplementary Materials www.sciencemag.org Materials and Methods

Because cationic antimicrobial peptides (AMPs) represent one of the most prominent and nonspecific components of the host response to pathogens, we determined the minimum inhibitory concentration (MIC) of polymyxin B (PMB) for a common human gut commensal, *Bacteroides thetaiotaomicron*. PMB is a bacterial cationic AMP with a similar mode of action to mammalian AMPs (4). Strikingly, this commensal exhibits 680- to 2400-fold increased resistance to PMB by comparison with mammalian enteropathogens or *Escherichia coli* (Fig. 1A, fig. S1, and table S1). A survey of 17 prominent human commensals, representing the three major bacterial phyla in the gut (5), revealed that resistance against multiple inflammation-associated human and murine AMPs is a general feature of the human gut microbiota (Fig. 1B, fig. S1, and table S1).

We screened transposon mutant populations of five human gut commensal species for genes required for fitness in the presence of PMB. This approach identified a single gene (encoded by *BT1854* in *B. thetaiotaomicron*) that mediated PMB resistance in all species tested (Fig. 2A, table S2-4). Targeted deletion and complementation of *BT1854* confirmed this phenotype for multiple AMPs (Fig. 2B and table S1).

BT1854 has limited homology to the phospholipid biosynthetic enzyme PgpB, a phosphatidylglycerol phosphatase (6) that is not implicated in AMP resistance. However, an unusual PgpB homolog, LpxF, catalyzes the removal of the negatively charged 4'-phosphate group from the lipopolysaccharide (LPS) lipid A anchor in a small, phylogenetically heterogeneous group of AMP-resistant pathogens (7-9). Notably, *B. thetaiotaomicron* produces an under-phosphorylated lipid A structure when compared to most gram-negative organisms (10) (fig. S2A-B).

Mass spectrometry (MS) analysis of lipid A extracted from *B. thetaiotaomicron* revealed a predominant peak at m/z 843.6, consistent with the published (10) doubly-deprotonated ($[M-2H]^{2-}$) ion structure of penta-acylated 4'-dephosphorylated lipid A (predicted exact mass: 844.7 m/z) (Fig. 2C). In contrast, MS analysis of lipid A extracted from the *B. thetaiotaomicron* *BT1854* deletion mutant revealed a peak consistent with the $(M-2H)^{2-}$ ion of penta-acylated, bis-phosphorylated lipid A (predicted exact mass: 884.6 m/z) (Fig. 2C). An additional minor peak consistent with a tetra-acylated structure is observed for all strains (Fig. 2C and fig. S2C-D). Complementation restores the wildtype lipid A profile (fig. S2E). Based on these results, we designated this protein as LpxF.

Characterization of wildtype and *lpxF* mutant strains showed that LpxF increased resistance to inflammation-associated AMPs by neutralizing the negative charge of the cell (Fig. 2D), decreasing AMP binding at the bacterial surface (Fig. 2E and fig. S3A), and reducing AMP-dependent membrane disruption (Fig. 2F and fig. S3B).

If resistance to inflammation-associated AMPs determines Bacteroidetes fitness in the gut, then a strain with reduced AMP resistance should be outcompeted by an isogenic wildtype strain in a mammalian host during inflammation. We established a gnotobiotic model of gut inflammation by colonizing germfree mice with a mixture of wildtype, *lpxF* deletion mutant, and complemented (*lpxF*, *lpxF*⁺) *B. thetaiotaomicron* strains 7d prior to infection with

Citrobacter rodentium, a murine enteropathogen that mimics human gastrointestinal infection (11), or an avirulent *tir* mutant incapable of inducing colitis (fig. S4A-C) (12).

We next monitored the relative abundance of each strain over time in these animals. The *B. thetaiotaomicron lpxF* deletion mutant was rapidly displaced by wildtype and *lpxF, lpxF*⁺ complemented strains in mice infected with *C. rodentium* (Fig. 3A and table S5). Displacement occurred at the onset of inflammation (fig. S5A and S5B) and concurrent with increased AMP secretion measured from colonic tissue explants (fig. S5C). By contrast, the *lpxF* deletion mutant was able to persist in mice that had not been infected with *C. rodentium* (Fig. 3B) and in mice infected with the non-inflammatory *C. rodentium tir* mutant, which colonizes the gut to similar levels as the wildtype pathogen (Fig. 3C). The *lpxF* mutant was also outcompeted in gnotobiotic mice exposed to dextran sulfate sodium (DSS), a chemical inducer of intestinal inflammation, at the onset of inflammation (Fig. 3D, fig. S4A-C, and fig. S5). Exposure *in vitro* to numerous AMPs, but not *C. rodentium* or DSS, recapitulates the *in vivo* results (fig. S4D-E). LpxF thus appears to be a general determinant of resilience during inflammation and not a specific requirement for response to *C. rodentium*.

If resistance to AMPs is also important for resilience in the context of a multi-phylum human gut microbiota residing in the mammalian gut environment, then a mutant sensitive to AMPs should exhibit a loss of resilience in this community. We colonized germfree mice with 14 bacterial species, including wildtype *B. thetaiotaomicron*, that represent the three dominant phyla observed in the human gut (tables S6-S8) (13). Strikingly, this community remained largely stable through the course of *C. rodentium* infection (Fig. 4A) and associated inflammatory events. To determine whether LpxF is required for this stability during perturbation, we replaced *B. thetaiotaomicron* with the *lpxF* deletion mutant. Unlike wildtype *B. thetaiotaomicron*, the *lpxF* mutant was rapidly and specifically displaced from the defined human gut microbiome upon *C. rodentium* infection and consequent host inflammation (Fig. 4B and fig. S6A). By contrast, in the absence of *C. rodentium* infection both the *B. thetaiotaomicron* wildtype and *lpxF* mutant-containing communities remained stable for the duration of the experiment (fig. S6B).

Germfree mice have an immature innate immune system (14) that is largely rescued by colonization with mouse, but not human, microbiota (15). We tested whether *B. thetaiotaomicron* requires LpxF in animals with a complete mouse microbiota and a mature innate immune system. Because human gut *Bacteroides spp.* are rapidly outcompeted in specific pathogen-free (SPF) wildtype mice (16), we screened multiple strains of mice and found that *B. thetaiotaomicron*, like *B. fragilis* (16), stably colonized SPF *Rag*^{-/-} animals (Fig. 4C). In these mice, the *B. thetaiotaomicron lpxF* deletion mutant colonized to equivalent levels as the wild type (Fig. 4C). *B. thetaiotaomicron* resilience during *C. rodentium* infection of these SPF mice remained dependent on LpxF, indicating that the inflammation caused by pathogen infection, rather than other members of the commensal mouse microbiota, mediated the requirement for AMP resistance (Fig. 4D). Many innate immune defects observed in germfree mice are also abrogated by segmented filamentous bacteria (SFB), a mouse commensal that interacts directly with the gut epithelium (15). The *lpxF* deletion mutant remained stable over time in gnotobiotic mice monoassociated with

SFB, indicating that even a pure population of immunostimulatory commensal members of the host's native microbiota do not induce a requirement for AMP resistance (fig. S6C).

LpxF orthologs are readily identified in all sequenced human-associated Bacteroidetes and all characterized LPS structures in this phylum reveal an under-phosphorylated lipid A structure (fig. S7), suggesting that all human gut Bacteroidetes use this mechanism to resist inflammatory perturbations. We conducted a human study to determine whether our observations from sequenced type strains extend to gut commensals captured directly from humans. To this end, we cultured 733 species-level phylotypes from 12 unrelated, healthy donors (table S9). These cultured strains represent ~98% of the donors' original, uncultured communities at the phylum level, ~95% at the class and order levels, ~80% at the family level, and ~50% at the genus level (fig. S8A). Quantification of PMB resistance across these culture collections established that the AMP resistance profiles of fecal microbial communities isolated directly from humans mirror the phylum-level patterns observed in sequenced type strains (Fig. 4E, fig. S8B). Understanding mechanisms of microbiota stability is important for efforts to manipulate these communities for therapeutic purposes. We found that the ability of prominent human gut commensal bacteria to resist killing by AMPs also mediated stability during infection. This resilience hinges on a protein that removes a single phosphate group from the bacterial LPS to determine whether the host maintains or removes commensal bacteria in response to inflammation. In this way, commensal-encoded mechanisms for persistence in the host during inflammation complement host-encoded mechanisms for immune tolerance of the microbiota (17). As observed in certain pathogens, lipid A modification may provide additional benefits to commensal microbes beyond AMP resistance, including reduced activation of the host Toll-like receptor 4-myeloid differentiation factor 2 (TLR4-MD2) complex that recognizes common forms of bacterial LPS (7, 8, 10, 18-23). Our studies indicate that LpxF is dispensable in the absence of inflammation, suggesting that the broad conservation of this enzyme across commensal Bacteroidetes reflects selective pressures imposed by periodic inflammatory events. A delicate balance between microbial resilience and host tolerance thus allows for commensal persistence throughout a diverse range of perturbations while preventing commensal overgrowth or depletion, either of which could have deleterious effects on the host.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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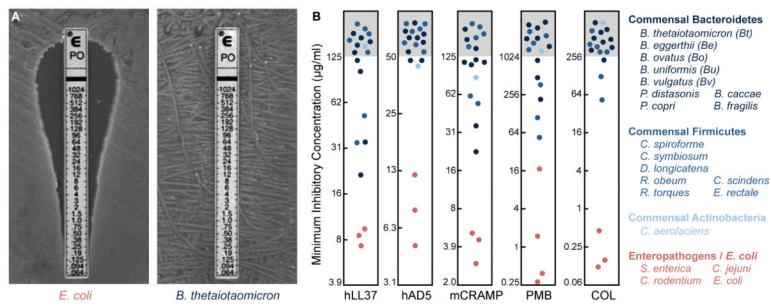


Fig. 1. Human gut commensals are highly resistant to cationic AMPs
 (A) MIC of PMB against *Escherichia coli* and *Bacteroides thetaiotaomicron*. (B) MICs of human and mouse inflammation-induced AMPs and bacterial surrogates against prominent human gut commensal bacteria (blue) and enteropathogens (red). MICs of PMB and colistin were determined using E-test strips; others were determined using the microtiter broth dilution method. See also fig. S1 and table S1.

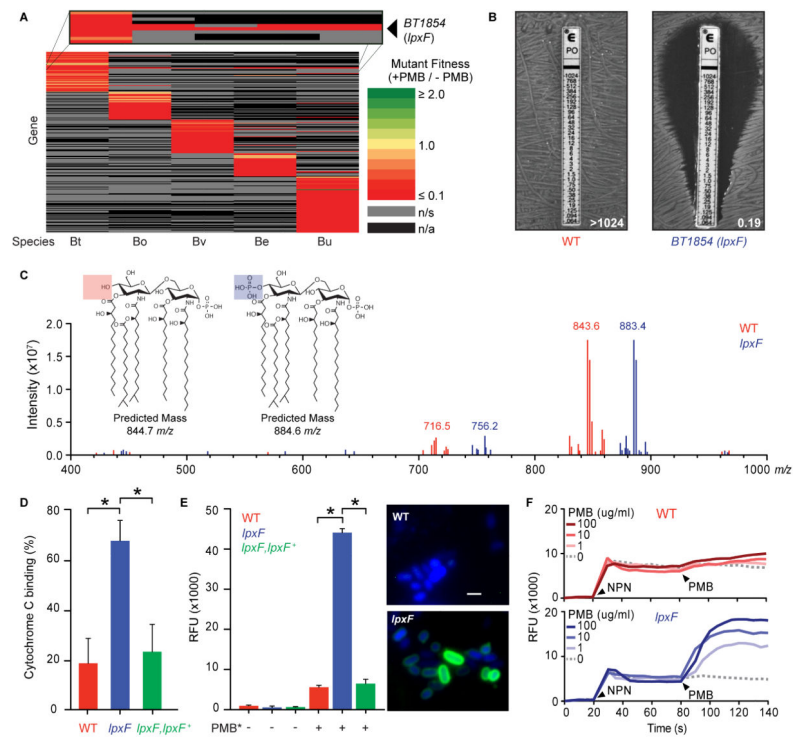


Fig. 2. Lipid A dephosphorylation mediates AMP resistance in prominent human gut bacteria (A) Heat map of the relative fitness of transposon mutant strains in the presence and absence of PMB. Columns indicate human gut bacterial species tested (abbreviations from Fig. 1B); rows indicate gene orthologs with insertions displaying a significantly altered fitness ($q < 0.05$) in the presence of PMB in at least one species. n/s, not significantly altered; n/a, no ortholog. (B) BT1854 (*LpxF*) is necessary for PMB resistance in *B. thetaiotaomicron*. Representative MIC assessed using the E-test method is shown. (C) *LpxF* is required for dephosphorylation of lipid A. FT-ICR MS analysis of lipid A isolated from wildtype (red) and *lpxF* deletion mutant (blue) *B. thetaiotaomicron* revealed a shift of the predominant peak by ~ 40 m/z , consistent with the gain of a single phosphate group. Inset shows predicted structures and m/z values of the doubly deprotonated ($[M-2H]^{2-}$) ions for monophosphorylated (red) and bis-phosphorylated (blue) penta-acylated lipid A. Minor peaks are consistent with tetra-acylated lipid A structures (fig. S2C-D). (D) Deletion of *lpxF* increases cationic cytochrome C binding to *B. thetaiotaomicron*, indicating altered surface charge. (E) Increased PMB-Oregon Green (PMB*) binding to *lpxF* deletion mutant cells measured by fluorescence quantification (left panel) and microscopy (right panels). Error bars represent standard deviation and asterisks indicate significance ($p < 0.01$). Representative fluorescence microscopy images of bacterial cells incubated with PMB* (green) are shown as an overlay with 4', 6' diamino-2-phenylindole (DAPI) (blue). Scale bar indicates 2 μm . (F) *LpxF* protects the outer membrane from PMB perturbation. 1-*N*-phenyl naphthylamine (NPN) uptake profiles of select strains were measured followed by challenge with the indicated concentration of PMB. Arrowheads indicate the addition of NPN (20s) and PMB (80s). Readings were taken in 5s intervals. Each experiment was

performed in triplicate with representative results shown. See figure S3 for complementation.

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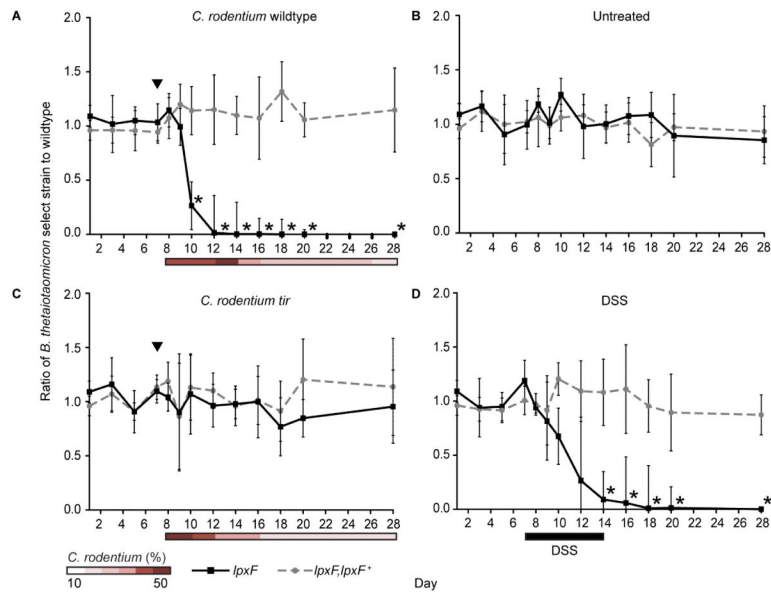


Fig. 3. AMP resistance determines the resilience of a prominent human gut symbiont in gnotobiotic mice with colitis

Germfree mice ($n=5$ /group) were colonized with wildtype, the *lpxF* deletion mutant (solid lines), and complemented (*lpxF*,*lpxF*⁺; dashed lines) *B. thetaiotaomicron* strains 7 days prior to initiation of inflammation by *C. rodentium* infection (A), no further treatment (B), infection with a non-inflammatory *C. rodentium tir* mutant (C), or exposure to 3% DSS *ad libitum* for 7 days (black bar) (D). Infection with *C. rodentium* strains is indicated by black arrowheads and relative *C. rodentium* abundances are reported as percent of total fecal DNA (shaded bars); error bars represent standard deviation and asterisks indicate significant ($p<0.01$) differences.

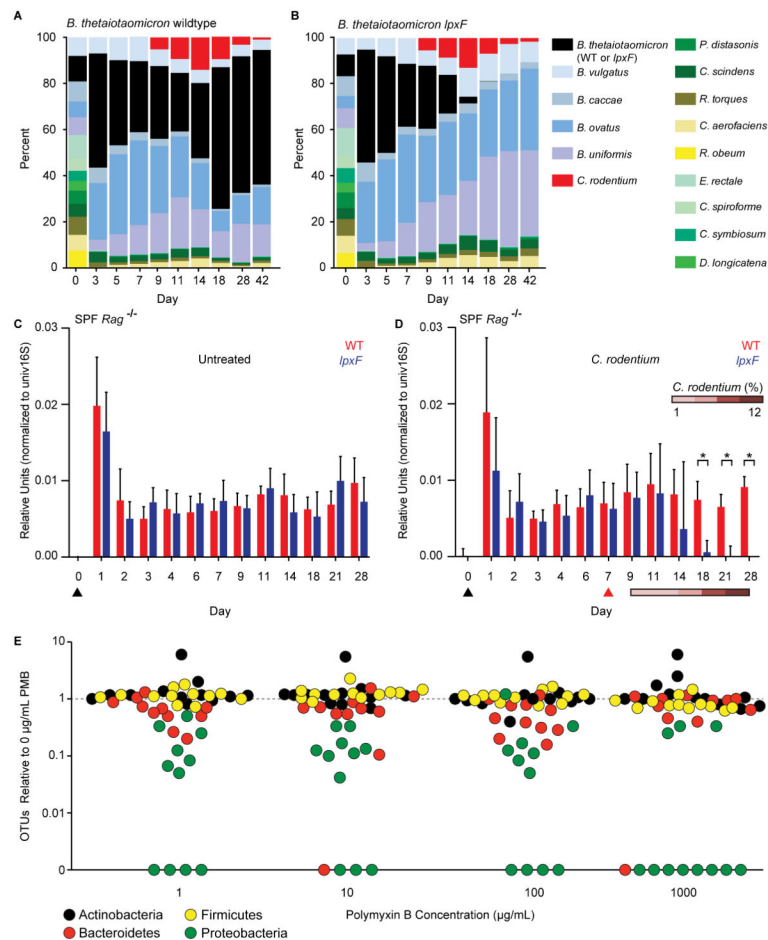


Fig. 4. AMP resistance determines commensal resilience in the context of a human or murine gut microbiota

(A, B) Germfree mice ($n=5/\text{group}$) were colonized with 14 prominent human gut microbes, including *B. thetaiotaomicron* wildtype or *lpxF* deletion mutant, 7 days prior to infection with *C. rodentium*; community composition was monitored by species-specific quantitative polymerase chain reaction (qPCR) and reported as median percent of total. Uninfected controls are shown in fig. S6B. (C, D) Specific pathogen free *Rag*^{-/-} mice ($n=5/\text{group}$) were gavaged (black arrowhead) with either *B. thetaiotaomicron* wildtype or *lpxF* deletion mutant; in (D), mice were infected with *C. rodentium* 7 days later (red arrowhead). Colonization levels were assessed by qPCR from fecal DNA. *C. rodentium* levels are reported percentage of total fecal DNA. Error bars indicate standard deviation and asterisks indicate significance ($p < 0.05$). (E) AMP resistance is a general feature of human gut Bacteroidetes, Firmicutes, and Actinobacteria. Fecal samples from 12 unrelated, healthy human donors were cultured on varying PMB concentrations (x-axis) and the number of species-level phylotypes (OTUs) belonging to each phylum observed from each donor (points, colored by phylum) was normalized to the number observed in culture in the absence of PMB. A weighted (abundance) analysis provides similar results (fig. S8B).