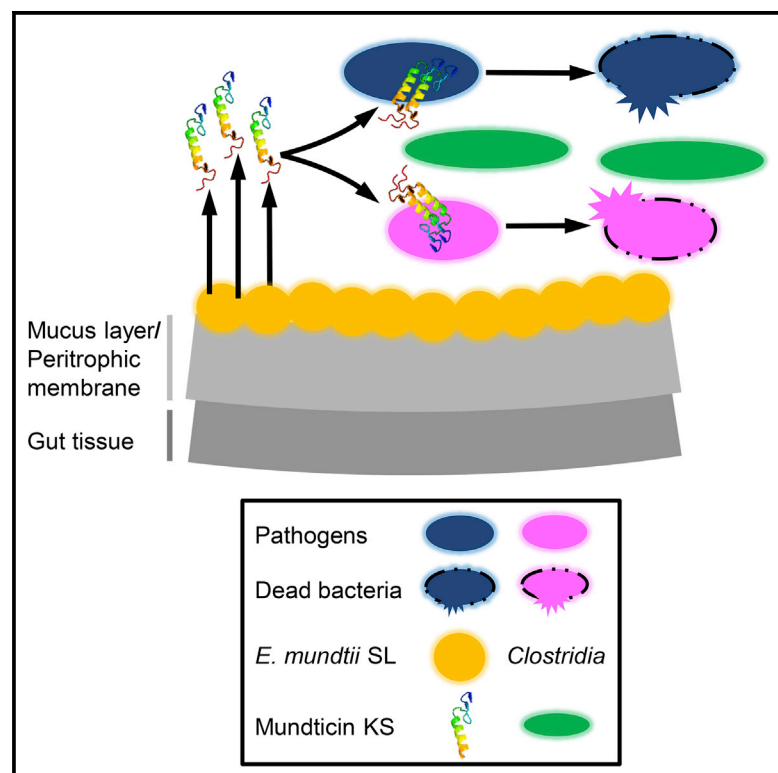


Cell Chemical Biology

Symbiont-Derived Antimicrobials Contribute to the Control of the Lepidopteran Gut Microbiota

Graphical Abstract



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In Brief

Shao et al. show that the extracellular symbiont *E. mundtii* of the cotton leafworm *S. littoralis* selectively clears pathobionts from the host gut lumen by secreting a stable antimicrobial peptide, which facilitates the normal development of gut microbiota and reduces risks of infection via the gut.

Highlights

- The gut bacterium *E. mundtii* of *S. littoralis* produces an antimicrobial peptide
- Mundtacin KS strongly inhibits some of the competing and potentially pathogenic organisms
- Mundtacin KS expresses in vivo and can impair pathogen colonization



Symbiont-Derived Antimicrobials Contribute to the Control of the Lepidopteran Gut Microbiota

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SUMMARY

Insects develop efficient antimicrobial strategies to flourish in a bacterial world. It has long been proposed that native gut microbiota is an important component of host defense; however, the responsible species have rarely been isolated to elucidate the mechanism of action. Here we show that the dominant symbiotic bacterium *Enterococcus mundtii* associated with the generalist herbivore *Spodoptera littoralis* actively secretes a stable class IIa bacteriocin (mundticin KS) against invading bacteria, but not against other gut residents, facilitating the normal development of host gut microbiota. A mundticin-defective strain lost inhibitory activity. Furthermore, purified mundticin cures infected larvae. Thus, the constitutively produced antimicrobials by native extracellular symbionts create a significant chemical barrier inside limiting invader expansion. This unique property also benefits *E. mundtii* itself by providing a competitive advantage, contributing to its dominance within complex microbial settings and its prevalence across Lepidoptera, and probably promotes the long-term cooperative symbiosis between both parties.

INTRODUCTION

Insects, the largest group of animals on Earth, comprise over a million described species. As nearly half of insects feed on plants, they are the most significant herbivores in the world (Mithoefer and Boland, 2012). Herbivorous insects inhabit diverse niches and typically consume large amounts of plant material. Owing to their immoderate foraging behavior, herbivorous insects also ingest a wide variety of potentially harmful microorganisms from either the food resources that contain abundant microbial flora or the surrounding environment associated with microbes (Herzner et al., 2013; Lindow and Brandl, 2003; Ruffner et al., 2013). Such invading microbes compete for limited re-

sources inside the host, as well as increase the risk of infection via the gut, which is probably the main route of insect infection in nature (Vallet-Gely et al., 2008). Although they face repeated challenges from nonnative microorganisms, herbivorous insects are notably resistant to microbial infections and almost always flourish in microbe-rich environments (Zasloff, 2002). It is not surprising that they have evolved effective antimicrobial strategies, including the immune system as well as the physical and chemical barriers created in the gut, such as the strongly alkaline pH in lepidopteran larvae (Funke et al., 2008). In addition, the native gut microbiota (or indigenous microbiota) is increasingly recognized as an important component of host defense against microbial invaders in many animals, from invertebrates to vertebrates (Donia et al., 2014; Iatsenko et al., 2014). For example, the microbial community of bumblebees was shown to protect against a common intestine parasite *Crithidia bombi* (Koch and Schmid-Hempel, 2011). Experiments on mice have demonstrated that a normal microbiota is necessary and sufficient for mediating pathogen clearance from the gut lumen in primary infections with *Salmonella typhimurium* (Endt et al., 2010). Despite these accumulating observations that the native gut microbiota is involved in combating infections, the species which exert this protective effect have rarely been either identified from the microbial consortia or cultured outside the host (Servin, 2004), and the molecular mechanisms of action are poorly understood (Endt et al., 2010; Hudault et al., 2001; Koch and Schmid-Hempel, 2011; Servin, 2004; Stecher et al., 2013; Vasquez et al., 2012).

Here, we experimentally address the antimicrobial activity of a culturable gut symbiotic bacterium in the cotton leafworm *Spodoptera littoralis* (Lepidoptera, Noctuidae), which represents one of the most polyphagous and widespread insect herbivores in the temperate regions, causing substantial economic losses in crop production. Like most lepidopteran larvae, cotton leafworms possess a simple alimentary canal, shaped like a tube without any specialized substructures; however, a large bacterial population, exceeding 10⁷ colony-forming units (CFU)/mL, is evident in the gut (Funke et al., 2008). Recent surveys of the taxonomic composition of this gut microbiota reveal a relatively simple but specific community, dominated by *Enterococcus* and *Clostridium* spp. (Tang et al., 2012). In particular, *E. mundtii* is repeatedly found in normal individuals regardless of diet and

Table 1. General Features of *Enterococcus* Bacteria Isolated from *S. littoralis*

| <i>Enterococcus</i> sp. | Fermentation Pattern | | Pigment | 16s rDNA Identity (%) | Virulence Factor (gelE) | Insect Mortality (%) ^a |
|----------------------------|----------------------|-------------------------------------|---------|-----------------------|-------------------------|-----------------------------------|
| | L-Arabinose | Methyl- α -D-glucopyranoside | | | | |
| <i>E. faecalis</i> SL | + | – | – | 100 | + | 47(a) \pm 10 |
| <i>E. mundtii</i> SL | + | – | + | 99.78 | – | 0(b) \pm 0 |
| <i>E. casseliflavus</i> SL | + | + | + | 100 | – | 40(a) \pm 14 |

SL, strains isolated from the larval gut of *S. littoralis*; +, positive results; –, negative results; gelE, enterococcal gelatinase.

^aAccumulative mortality until adult stage, ANOVA analysis showed significant differences.

persists in the gut across host developmental stages. Our previous study using stable isotope labeling indicates that *E. mundtii* is also the most metabolically active member in the gut microbiota (Shao et al., 2014), which suggests that this symbiotic species plays important roles.

We found that, although *E. mundtii* is a major constituent of the gut microbiota across generations, suggesting the vertical transmission mode of this symbiont, other ubiquitous environmental enterococci, such as *E. faecalis* and *E. casseliflavus*, often started out at high density in early instar when *E. mundtii* had not yet established abundant populations inside the gut (Tang et al., 2012). *Enterococcus* bacteria not only occur in a wide variety of habitats including plants, soil, and water, but also are well known for their ability to adapt to the gastrointestinal tracts of animals and could cause serious infections under certain conditions (Holt et al., 2015). Especially, *E. faecalis* and *E. casseliflavus* have developed many mechanisms to invade the host that allow them to immediately fill empty niches early in life and are generally considered opportunistic pathogens in many animals, whereas *E. mundtii* is rarely documented as a pathogen. It is known that *E. faecalis* poorly supported larval development in the housefly (Ghosh et al., 2014). Certain strains of *E. faecalis* have also been shown to cause lethal infection in Lepidoptera (Doane and Redys, 1970). Since enterococci strains carrying diverse virulence factors have emerged in many habitats and are isolated with alarming regularity (Palmer et al., 2012), these possible pathogens that are often orally acquired by *S. littoralis* nymphs from their diet (first contaminated eggshell, then plants) and the surrounding environment, represent a critical challenge to the host. But, notably, they are quickly depleted from the microbiota concomitant with an increase of the *E. mundtii* population, which subsequently stabilizes in the gut together with clostridia as core components of the flora. It seems likely that the indigenous gut-resident *E. mundtii* has an inherent advantage over those closely related but potentially pathogenic bacteria, although they have already blocked the gut niche (highly adherent to gut epithelium) at an early stage, and thus potentially confers invasion resistance benefit on its host. These observations prompted us to isolate and identify the enterococci in *S. littoralis*, ask whether *E. mundtii* could specifically inhibit nonindigenous invading microbes, and investigate the mechanism of action.

RESULTS

Survey and Characteristics of *Enterococcus* Isolated from *S. littoralis*

To study the antagonism between enterococci, we isolated different species from *S. littoralis* larvae and eggs using a selec-

tive differential medium on which enterococci grow well and produce a pink pigmentation that distinguishes species of this genus from others. Using three-step phenotyping (Table 1), we were able to discriminate among purified bacterial strains (Manero and Blanch, 1999). Carbohydrate fermentation tests with L-arabinose or methyl- α -D-glucopyranoside give a yellow color for a positive reaction and a red color for a negative reaction. The production of pigment is considered positive when the strain presents a strong yellow color. Based on those phenotypic characteristics, three *Enterococcus* species, *E. mundtii*, *E. casseliflavus*, and *E. faecalis*, were identified from both the larval gut and eggs. We focused on representative strains of the three species, designated strain SL, for further analysis. Their DNA was extracted and the nearly full-length 16S rRNA genes were amplified and sequenced. The obtained 16S rRNA sequence of each of the three representative strains showed >99% similarity with a published sequence in GenBank, and the closest relatives were selected to construct a phylogenetic tree (Figure 1A), which revealed that the isolates were phylogenetically placed within three clades and enterococci from the larval gut were closely related to that from eggs, suggesting eggs as a source for *Enterococcus* to larvae.

The gut enterococcal population analysis revealed a high proportion of *E. faecalis* and *E. casseliflavus* in the first instar (Figure 1B). *E. faecalis* was on average ten times more prevalent than *E. mundtii* in the gut, but these pathogenic enterococci were completely eradicated in the second instar. In contrast, although *E. mundtii* was the minority member at a very early stage, making up only 6% of the consortium, it accounted for a large percentage of the second-instar population and subsequently colonized the gut until pupation without any significant changes. The level of *E. mundtii* ranged from 5.7×10^6 to 2.1×10^8 CFU per larva, and it was consistently recovered from different batches of samples ($n = 9$). Culture-independent pyrosequencing further confirmed that *E. mundtii* was the major constituent of the gut microbiota in mature larvae and also revealed that *Clostridium* species was another dominant gut bacterium (Figure S1). Symbiotic clostridia resist cultivation outside the host.

Because *E. faecalis* and *E. casseliflavus* are commonly described as opportunistic pathogens, we screened related virulence factors in isolates. A major virulence gene, *gelE*, which encodes a lethal protein to lepidopterans (Gaspar et al., 2009), was detected in *E. faecalis* isolates, but not in *E. mundtii* SL (Table 1). Infection assays of cured (*E. mundtii*-free) larvae with *E. faecalis* SL and *E. casseliflavus* SL resulted in similar disease symptoms (Figure 1C) and high mortality (Table 1) as described in other lepidopterans (Doane and Redys, 1970).

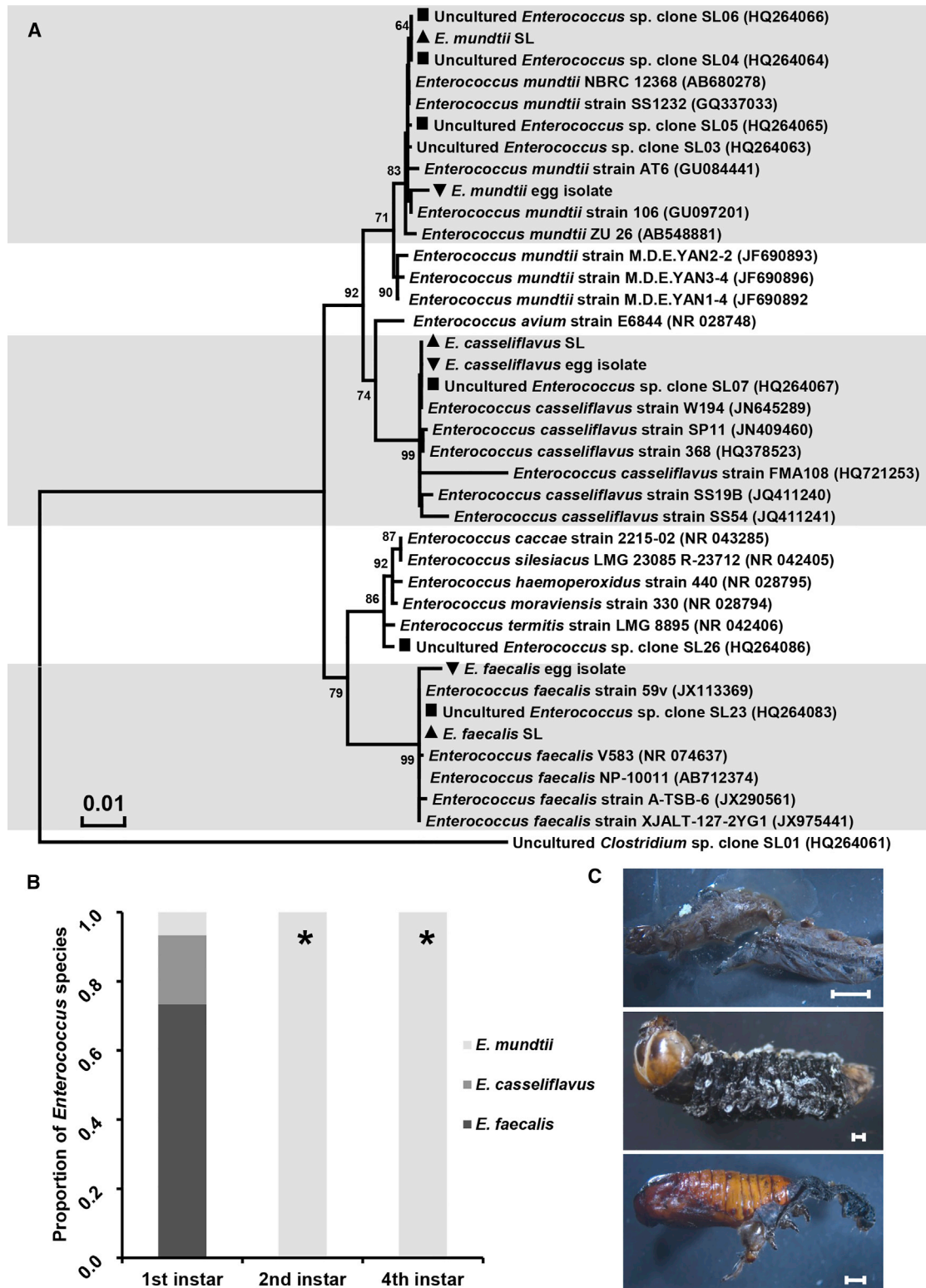


Figure 1. Survey and Characteristics of *Enterococcus* Bacteria from *S. littoralis*

(A) Phylogenetic position of enterococci identified from *S. littoralis*. Phylogeny was estimated by using the maximum likelihood method based on the Kimura two-parameter model. The scale 0.01 is the genetic distance. Bootstrap values (in percent) are based on 1,000 replications. The near full-length 16S rDNA sequences retrieved from previous clone-library-based studies of gut microbiota of *S. littoralis* are indicated by squares (■); the 16S rDNA sequences of *Enterococcus*

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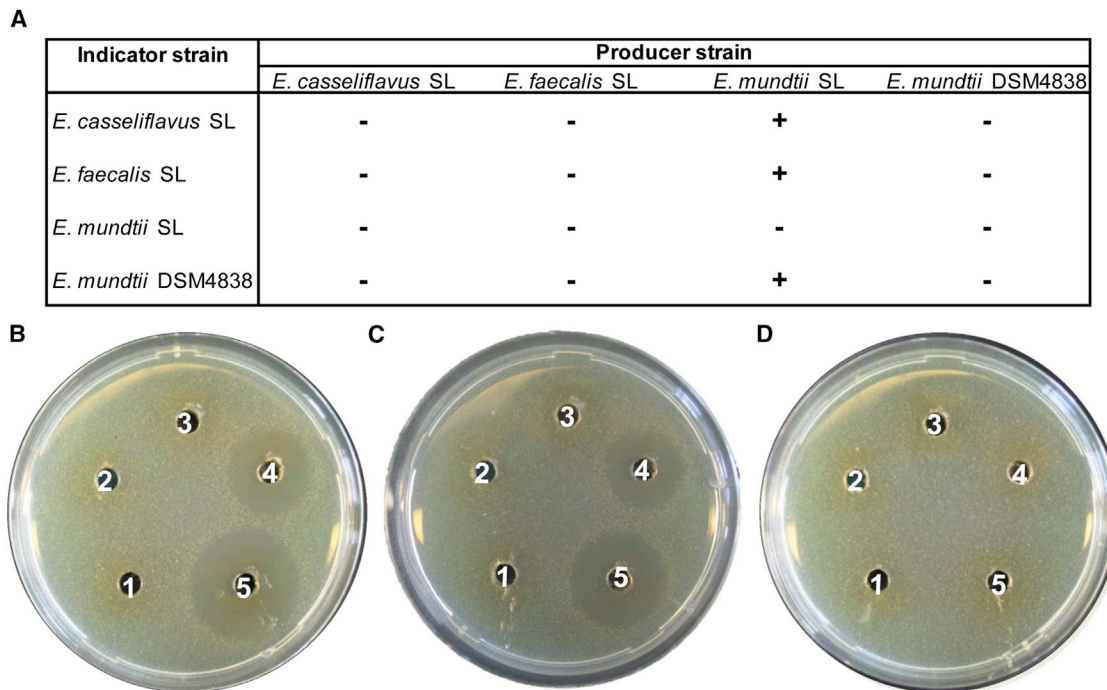


Figure 2. The Native Gut Symbiont *E. mundtii* SL Efficiently Antagonizes Invading Enterococci

(A) Bacteriocin activity and immunity. +, growth inhibition of indicators; −, no inhibition of indicators; SL, enterococcal strains isolated from *S. littoralis*. *E. mundtii* DSM4838 is a type strain.

(B–D) (B) Typical results of agar diffusion assays using *E. faecalis* SL, (C) *E. casseliflavus* SL, and (D) *E. mundtii* SL as the indicator. 1, cell-free culture supernatant of *E. casseliflavus* SL; 2, cell-free culture supernatant of *E. faecalis* SL; 3, cell-free culture supernatant of *E. mundtii* DSM4838; 4, cell-free culture supernatant of *E. mundtii* SL; and 5, purified mundticin KS (10 ng/μL). Shown are representative results of at least ten independent experiments.

Symbiotic *E. mundtii* Directly Antagonizes Other Invading Enterococci through Its Antimicrobial Properties

The antagonistic effects of the identified enterococci were investigated by using inhibition assays on agar plates. The three representative strains showed different inhibition properties relative to each other (Figure 2A). *E. mundtii* SL exhibited specific antimicrobial activity against *E. faecalis* and *E. casseliflavus*. The cell-free *E. mundtii* SL culture supernatant inhibited the growth of indicator bacteria and resulted in a clear inhibition zone (Figures 2B and 2C), suggesting bactericidal activity that resulted from extracellular and diffusible substances. The mode of action was also confirmed in the liquid culture. A rapid decrease in the optical density at 600 nm, due to the lysis of cells, was observed after adding a purified fraction into the broth culture of indicators. Notably, there was no inhibitory effect from *E. mundtii* type strain DSM4838 on *E. faecalis* or *E. casseliflavus*, which served as a negative control. The growth of *E. mundtii* SL itself was not affected (Figure 2D), indicating that it is immune (self-protective) to the antimicrobials it produces. In

contrast, *E. faecalis* and *E. casseliflavus* isolates were inactive against *E. mundtii*. Next, we focused on characterizing the antimicrobials produced by *E. mundtii* SL both in vitro and in vivo.

Antimicrobial Production, Inhibitory Spectrum and Identification

In contrast to the *E. mundtii* type strain, in which the best growth rate was observed at pH 7.0, *E. mundtii* SL grows well in high pH media. The highest cell density of strain SL can be reached within several hours at pH 9.0. Under these conditions, more bacterial biomass is produced than is produced in bacteria growing at neutral pH (Figure S2A), indicating that this strain is well adapted to the alkaline gut environment of *S. littoralis* larvae. The growth curve and antimicrobial production in a broth culture are shown in Figure 3A. An agar diffusion test indicated that *E. mundtii* cells already began secreting antimicrobial substances during the early exponential growth phase, and production increased as the bacteria grew. Maximum production was recorded at the stationary phase and remained stable up to 20 hr. Thus, antimicrobial production is associated with growth. *Enterococci*, which

isolates are indicated by triangles (▲, enterococci isolated from eggs of *S. littoralis*; ▼, enterococci isolated from the larval gut of *S. littoralis*). The numbers in parentheses are accession numbers extracted from GenBank.

(B) Change of *Enterococcus* population in the gut microbiota of *S. littoralis* larvae across different instars. Proportional data are modeled using the mean number of CFUs counted from five to nine larvae. Statistically significant changes (* $p < 0.05$: second instar versus first instar, fourth instar versus first instar) were determined with ANOVA and a post hoc Tukey honestly significant difference (HSD) test.

(C) *S. littoralis* larvae and prepupae infected with the *E. faecalis* SL strain. Upper panel, dead young larva; middle panel, shrunken cadaver with a characteristic short, dry condition; lower panel, some of the infected larvae attempted to spin a pupa and died as prepupae. Scale bar represents 1 mm.

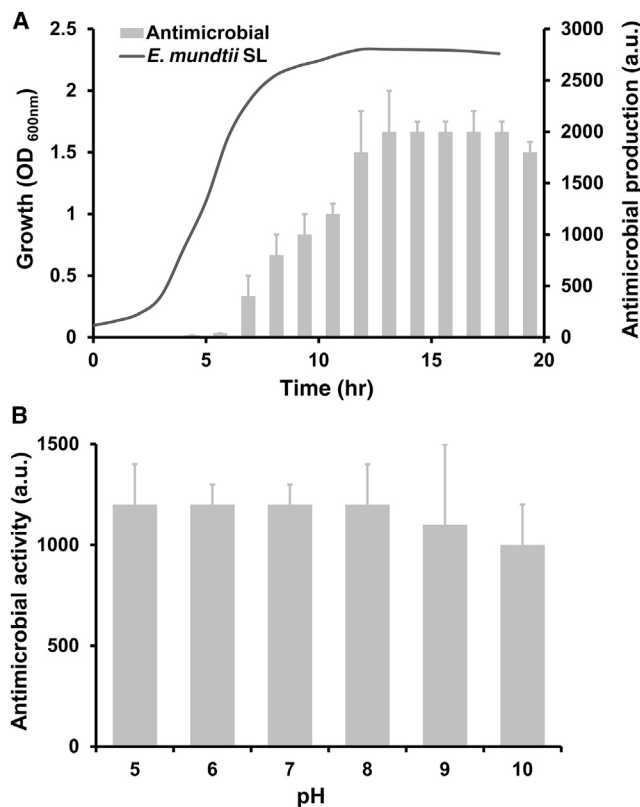


Figure 3. Characterizing Antimicrobial Production

(A) Growth curve and antimicrobial production of *E. mundtii* SL strain in liquid MRS medium at 30°C.

(B) Effect of pH on the activity of mundticin KS. Scale bars represent means from three independent experiments; error bars, SDs.

belong to lactic acid bacteria and produce organic acids during growth, decrease the medium pH (Figure S2B). However, we still observed a clear inhibition zone after the *E. mundtii* culture was neutralized; thus, the major bactericidal activity is not due to the production of acids. Notably, the bactericidal substance retains its activity after being incubated at a wide pH range (Figure 3B) and tolerates heat treatments, even at 100°C for 1 hr (Figure S3). These characteristics reveal the high stability of this antimicrobial compound, suggesting that it can function under the harsh gut conditions.

We further assessed the antimicrobial spectrum of *E. mundtii* SL (Table S1) and found it was particularly effective against some common Gram-positive pathogens, namely *Listeria monocytogenes* and *Listeria innocua*, which opportunistically infect a wide range of hosts including Lepidoptera and usually pose severe threats to larvae (Mukherjee et al., 2010). The test clostridia were insensitive to *E. mundtii* SL. *E. mundtii* SL displayed no significant activity against Gram-negative bacteria used in our assay, including *Escherichia* and *Pseudomonas* spp.

To elucidate the chemical composition of the antimicrobial substance, we performed a bioassay-guided isolation. After being purified by chromatography, a well-separated peak at a retention time of 8.20 min (the only active fraction against test enterococci) was finally collected from high-performance

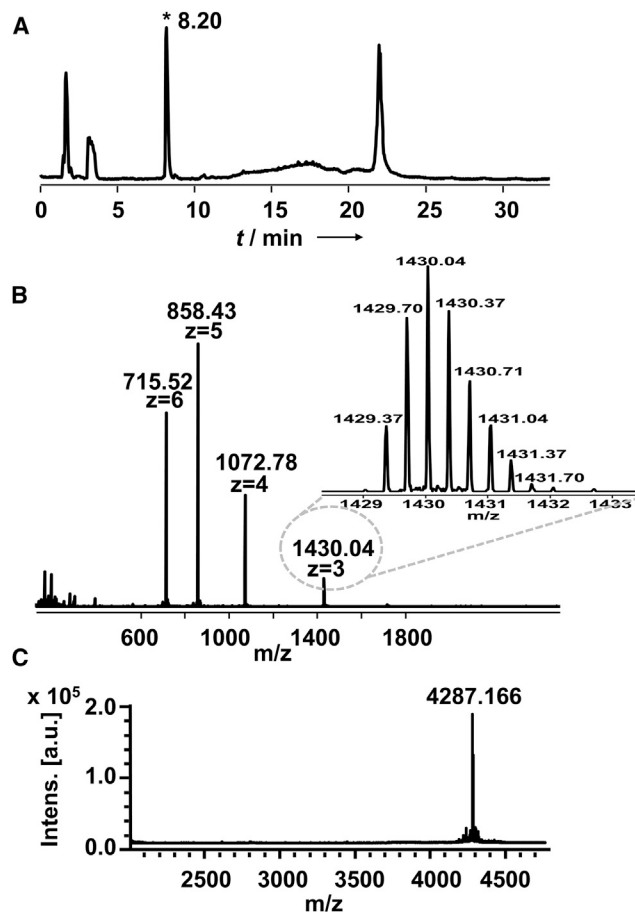


Figure 4. Identification of Inhibitory Molecule

(A) The reverse-phase high-performance liquid chromatography (RP-HPLC) chromatogram profile of the extract from the culture of *E. mundtii* SL. Elution of peptides was monitored by a UV detector at 280 nm. * Indicates the active fraction and retention time.

(B) Electrospray mass spectrum of the purified antimicrobial peptide with multiple charged molecular ions.

(C) MALDI-TOF/MS analysis of mundticin KS obtained from the second reversed-phase chromatography. Intens., intensity.

liquid chromatography (HPLC) and applied to mass spectrometry (MS) (Figure 4A). In liquid chromatography-electrospray ionization time-of-flight mass spectrometry (LC-ESI-TOF/MS), the bioactive compound displayed multiple charged molecular ions ranging from $[M+3H]^{3+}$ to $[M+6H]^{6+}$ (Figure 4B), which is very similar to the molecular mass of the class IIa bacteriocin mundticin KS by comparison with those of other known compounds held in protein databases. MALDI-TOF/MS of the purified substance further confirmed the molecular mass of 4,287.166 Da (Figure 4C). The fragmentation pattern obtained from the post-source decay (PSD) analysis revealed its amino acid sequence. The peptide is composed of 43 amino acid residues, and the sequence is similar to a peptide produced by *E. mundtii* strain NFRI 7393 (Kawamoto et al., 2002) (Figure S4). Taken together, these results suggest that *E. mundtii* SL, originated from *S. littoralis*, produces the bacteriocin mundticin KS as a weapon directly against other bacteria.

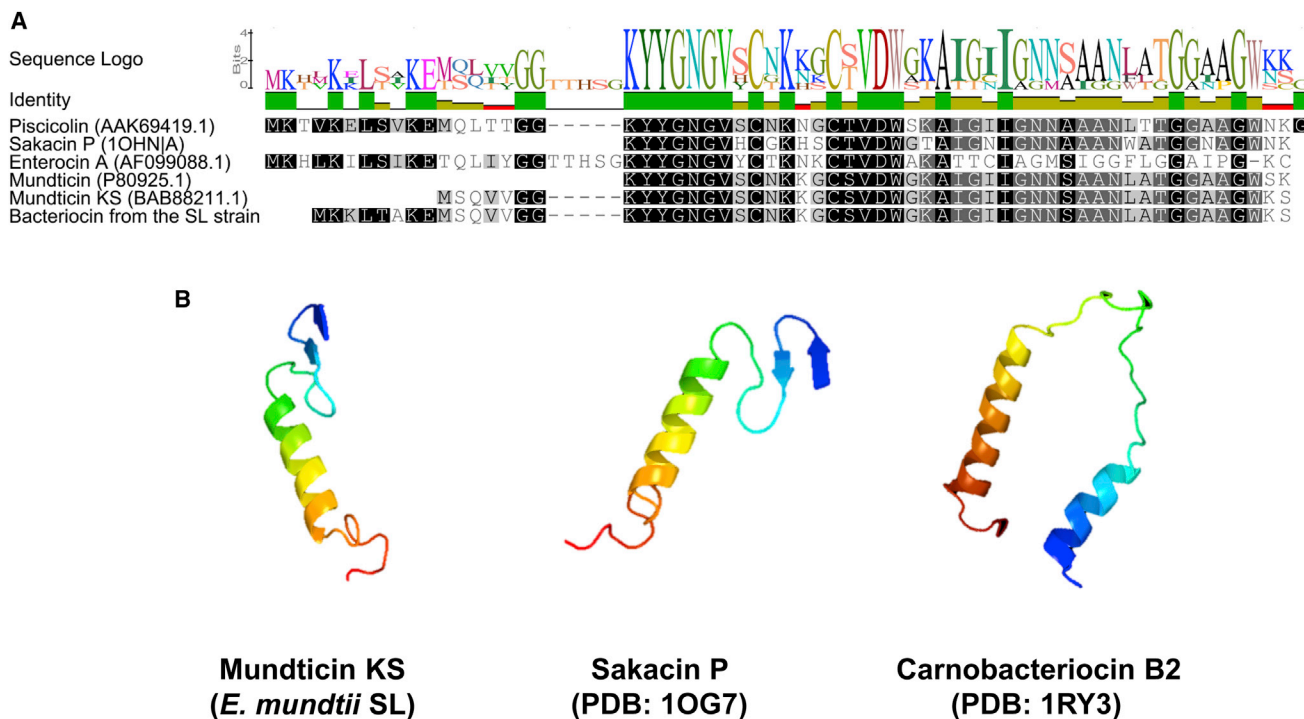


Figure 5. The Primary Sequence and Stereostructure of Mundtacin KS

(A) Amino acid sequence alignment of the mundtacin secreted by *E. mundtii* SL strain with other class IIa bacteriocins (access number extracted from NCBI is displayed). Color key for identity: green means that the residue at the position is the same across all sequences; yellow is for less than complete identity; red refers to a very low identity for the given position.

(B) Tertiary structure prediction for mundtacin KS by Phyre2 in comparison with structures of Sakacin P (PDB: 1OG7) of *Lactobacillus sakei* and Carnobacteriocin B2 (PDB: 1RY3) of *Carnobacterium maltaromaticum* determined by nuclear magnetic resonance spectroscopy. Forty-three residues (100% coverage of mature mundtacin KS) have been modeled with 99.9% confidence by the single highest-scoring template. Image colored by the rainbow N → C terminus.

Functional Gene Determination, Expression of Mundtacin KS In Situ in the Gut, and Its Causal Role in the Clearance of Invading Enterococci

On the basis of the chemical identification, several primers (Table S2) were designed to amplify the responsible gene from the genomic DNA of *E. mundtii* SL. One positive amplicon with the expected size was sequenced, and the nucleic acid sequence revealed that *E. mundtii* SL possesses the mundtacin KS biosynthesis gene, which matches perfectly with that in the strain NERI 7393 (Kawamoto et al., 2002). The sequence involves a region of *munA* and *munB*, which encodes the mundtacin KS precursor peptide and a transport protein. In contrast, *E. mundtii* DSM4838 lacks the mundtacin gene (*munA*) in agreement with its phenotypic characteristic (no antimicrobial activity, Figure 2). The deduced amino acid sequence confirms that the mature antimicrobial peptide consists of 43 amino acids with an estimated molecular mass of 4,289.73 Da, and the sequence is identical with the prediction made from PSD data (Figure 5A). A comparison of the primary structure of mundtacin KS with that of other published class IIa bacteriocins provides insight into some important features of this kind of peptide antimicrobial. These bacteriocins all contain a highly conserved NH₂-terminal YGNGV motif and are largely unmodified except for a disulfide bridge formed by two cysteine residues between positions 32 and 37, which explained the difference between the observed mass (4,287 Da) and that calculated from the deduced

amino acid sequence (4,289 Da). The mode of killing action of mundtacin KS (the bactericidal mechanism) is likely to induce permeabilization of the cell membrane of sensitive bacteria (Cotter et al., 2013). After binding to target bacteria, these peptide molecules perturb the cytoplasmic membrane and induce pore formation and rapid dissipation of intracellular components, which eventually leads to cell death (Diep et al., 2007). Neither the detailed stereostructure of mundtacin KS nor the exact nature of the pores formed on cell membrane has yet been described. By using computer modeling, we illustrated a three-dimensional structure of mundtacin KS (Figure 5B). The N-terminal residues were predicted to form β strands to result in a β sheet domain, which was structurally stabilized by the disulfide bridge, while the C-terminal region harbored a α helices domain that ended with a structurally extended tail. The presence of helical peptides, confirmed by circular dichroism (CD) measurement, facilitates its penetration into the phospholipid bilayer of the cytoplasmic membrane of target bacteria (Figure S5).

During genome sequencing of *E. mundtii* SL (Chen et al., 2016a), we found the mundtacin gene was plasmid encoded. After treatment with a plasmid-curing procedure, some *E. mundtii* SL colonies (*E. mundtii* SL *Mun*⁻) lost their plasmids and the bacteriocin biosynthesis gene, which consequently failed to induce clear zones of inhibition of lawns of *E. faecalis* SL (Figure S6).

To test our hypothesis that mundtacin KS contributes to the demise of *E. faecalis* and *E. casseliflavus* in the gut flora, we further assessed bacteriocin gene expression in vivo. We detected mundtacin KS at the mRNA level and could thus infer that it is produced in the gut. We found that, although mundtacin KS was not detected in the first instar, probably due to the low titer of *E. mundtii* cells at this very early time point, mundtacin KS was highly expressed from the second instar in parallel with the increase of *E. mundtii* population inside the gut (Figure 6A). More importantly, mundtacin KS was continually synthesized by indigenous symbionts across different instars, which reduces the risk of infection. We attempted to infect larvae with a high dose of *E. faecalis*; larvae reassociated with the *E. mundtii* SL strain effectively cleared *E. faecalis* from the gut. However, larvae reassociated with an *E. mundtii* strain lacking the mundtacin biosynthesis gene could no longer inhibit *E. faecalis* (Figure 6B). Since other differences probably occur beyond bacteriocin production between these strains, the purified mundtacin was further orally administered to *E. faecalis*-infected larvae to assess its effect directly. The mean *E. faecalis* SL count recovered from the control larvae (PBS used as placebo) was 4.26×10^4 (mg larva gut)⁻¹, whereas in the mundtacin-treated larvae there was a >60% reduction of *E. faecalis* SL 24 hr following the administration of the bacteriocin (Figure 6C). The curing effect of mundtacin is significant ($p < 0.05$, ANOVA, a post hoc Tukey honestly significant difference test, $df = 5$) compared with the untreated control at each concentration (Figure 6C). By measuring the expression of mundtacin KS and the direct curing experiment, we confirmed that mundtacin KS exerts an antagonistic effect in situ against invading bacteria.

DISCUSSION

Most insects harbor abundant symbiotic bacteria in the gut. It is broadly accepted that the components of the native microbiota can serve beneficial functions, which range from enhancing host energy metabolism to shaping the immune system (Schneider and Chambers, 2008). A simple but specific bacterial community, composed of approximately 40% *E. mundtii*, was found within the larval gut of a generalist herbivore, *S. littoralis* (Tang et al., 2012). The association of *E. mundtii* with lepidopterans has been widely recorded in both laboratory-reared and wild-captured hosts (Chen et al., 2016b; Johnston and Rolff, 2015; Pilon et al., 2013; Tang et al., 2012; Teh et al., 2016; van Frankenhuyzen et al., 2010). For example, *E. mundtii* was found to be the major species in the gut microflora of spruce budworm, *Choristoneura fumiferana* (van Frankenhuyzen et al., 2010). However, to date the ecological role of these *Enterococcus* relatives to the herbivores remains obscure.

In this study, a representative of the predominant gut *E. mundtii* bacteria isolated from *S. littoralis* shows an antimicrobial activity that directly inhibits competitors and potent pathogens from developing in the same ecological niche. Even though the gut niche is blocked early, *E. mundtii* efficiently overcomes invading bacteria and proliferates rapidly to colonize the digestive tract. Subsequently, a mature population of *E. mundtii* is stably maintained throughout larval life. The level of *E. mundtii* is as high as 10^8 CFU in a normal healthy larva. A recent study using fluorescent in situ hybridization revealed that the high density

of *E. mundtii* cells assemble on the gut epithelium and form a biofilm-like structure contributing to its persistence and colonization (Shao et al., 2014). Unlike native *E. mundtii*, other detected enterococcal bacteria are toxic, especially the *E. faecalis* SL strain, which carries a highly virulent factor, enterococcal gelatinase, which decomposes the host's extracellular matrix (Gaspar et al., 2009). Recent studies showed even commensal *E. faecalis* induces rapid death in Lepidoptera following translocation from the gut (Holt et al., 2015). Our cultivation-based observation indicated that *E. faecalis* bacteria are alive inside the host's gut. Once these bacteria successfully establish in large numbers, they damage host tissues and increase the risk of infection (Figure 1C and Table 1) (Doane and Redys, 1970). Not only actively against related *Enterococcus* species, *E. mundtii* SL is also able to inhibit a range of Gram-positive bacteria, in particular some well-known food-borne pathogens and entomopathogens (Table S1). Therefore, the dominant *E. mundtii* is most likely a defensive symbiont that facilitates the survival of its host in a changing environment during development. This phenomenon was also observed in other lepidopterans and insects, such as locusts, ants, and beetles (Dillon et al., 2000; Oh et al., 2009; Scott et al., 2008), a fact that may be common in insects. It has been demonstrated that removing the dominant *E. mundtii* from the larval gut can cause increased susceptibility of the spruce budworm to pathogens, suggesting that *E. mundtii* plays a protective role (van Frankenhuyzen et al., 2010). Similarly, certain *Lactobacillus* strains isolated from the honeybee intestine were able to inhibit growth of the honeybee pathogen *Paenibacillus larvae*, and to some extent reduced the development of disease symptoms in bee colonies (Forsgren et al., 2010).

Although this increasing evidence suggests that native gut bacteria participate in host defense, until now the molecular mechanism has not been clearly demonstrated. We found that *E. mundtii* SL actively secretes a stable class IIa bacteriocin, mundtacin KS, which kills particular kinds of microbial invaders. Bacteriocins, which are ribosomally synthesized antimicrobial peptides, are evolutionarily conserved weapons directly against others (Riley and Wertz, 2002). They have attracted great interest because of their potential use as alternatives to conventional antibiotics and food preservatives (Cotter et al., 2013). For example, nisin, a lantibiotic bacteriocin from *Lactococcus lactis*, has been shown to be more effective than vancomycin against *Streptococcus pneumoniae* in a mouse infection model (Goldstein et al., 1998). *Ruminococcus flavefaciens*, an important member of the rumen microbiota, produces nine different lanthipeptides (Zhao and van der Donk, 2016). However, functional studies of these compounds in their ecological context are limited. We further showed that the symbiotic antibiotic-producing *E. mundtii* efficiently expresses this antimicrobial in situ in the gut, permitting direct control over the gut microbiota. Mundtacin KS was constitutively synthesized across larval stages. Considering the demonstrated strong inhibitory effect, it is not surprising that sensitive *E. faecalis* and *E. casseliflavus* were quickly cleared from the host. Therefore, symbiont-derived bacteriocin participates in the selection process stabilizing the host gut microbiota. Mundtacin KS could also inhibit other bacteria from outside, thus limiting pathogenic microbes in the gut lumen. And its constitutive expression, as an additional chemical barrier, further increases the threshold for disease establishment.

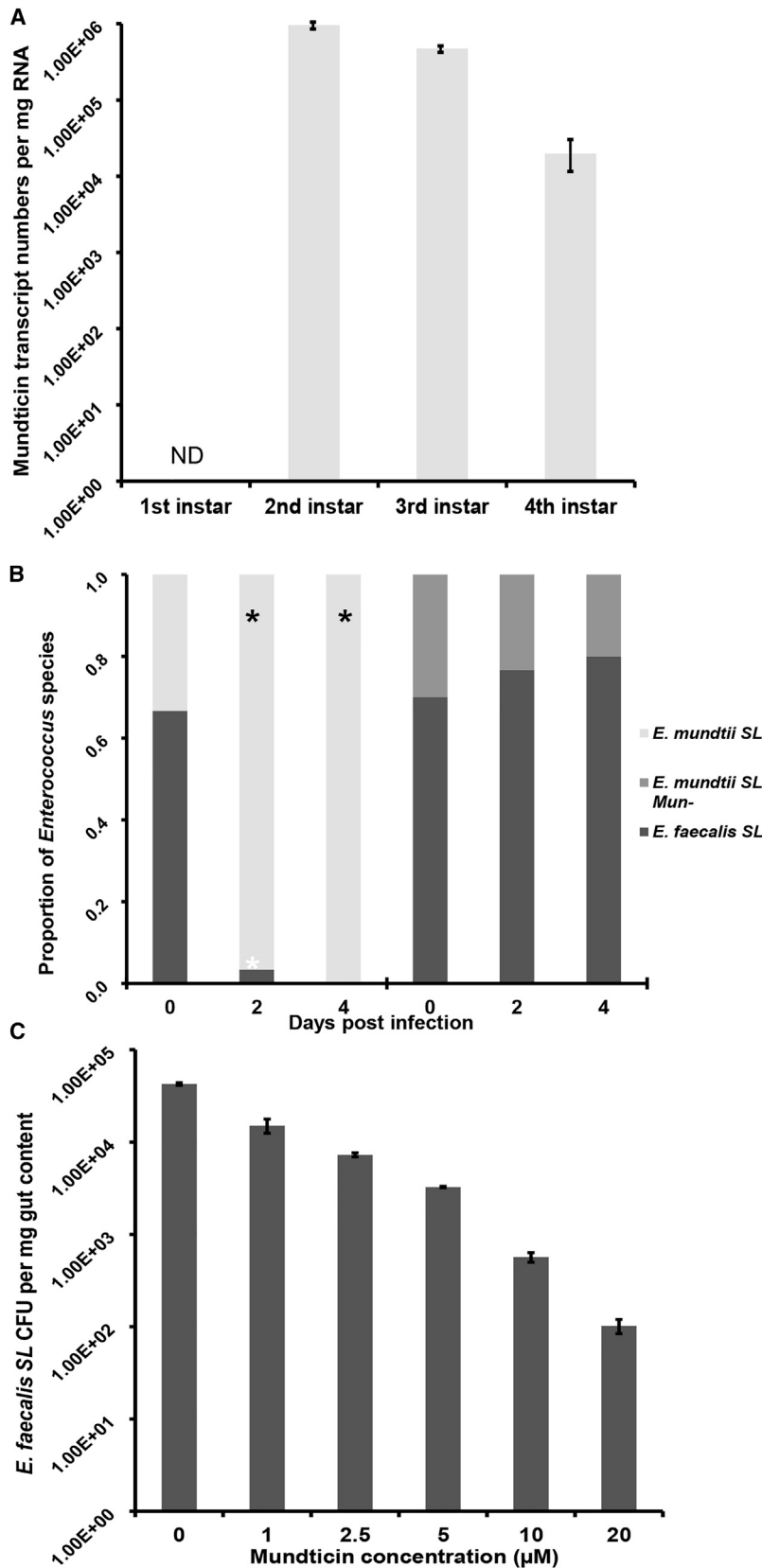


Figure 6. Production of Mundtacin KS In Situ in the Gut and Suppression of Ingested Bacteria

(A) *E. mundtii munaA* transcript numbers in the gut of *S. littoralis* larvae across different instars. Results are average values calculated from biological triplicates. ND, not detected. Error bars, SDs.

(B) Infection assays with *E. faecalis* SL. The same batch of larvae was firstly cured of their native *E. mundtii*, then was randomly divided into two groups and re-introduced of different *E. mundtii* strains. The larvae in both groups were intensively fed with *E. faecalis* SL once and the gut enterococcal population was analyzed. The larval gut re-associated with the *E. mundtii* SL strain could not be infected by ingested *E. faecalis* (left panel), whereas the larval gut re-associated with an *E. mundtii* strain lacking the mundtacin biosynthesis gene does not inhibit *E. faecalis* (right panel). Proportional data are modeled using the mean number of CFUs counted from five to nine larvae. Statistically significant changes (* $p < 0.05$: 2 days post-infection [DPI] versus 0 DPI, 4 DPI versus 0 DPI) were determined with ANOVA and a post hoc Tukey HSD test.

(C) Purified mundtacin cures *E. faecalis* SL-infected larvae. The curing effect of mundtacin is significant ($p < 0.05$, ANOVA, a post hoc Tukey HSD test, $df = 5$) compared with the untreated control at each concentration. Bars represent the mean number of *E. faecalis* CFUs from three to five larvae and the experiment was repeated three times. Error bars, SDs.

Despite the energy costs associated with protein synthesis, the production of peptide antimicrobials by *E. mundtii* symbionts also benefits themselves by providing a competitive advantage in the gut for a safe living space and rich nutrient sources supplied by the host, thus contributing to their dominance within complex microbial settings and promoting long-term cooperative symbiosis between both parties. It is also noteworthy that not all *E. mundtii* bacteria exhibit antimicrobial activity, as the reference strain did not show any inhibition on the indicators. To our knowledge, the strain SL is the first bacteriocinogenic *E. mundtii* isolated from an insect herbivore. These data suggest that this unique feature of *E. mundtii* SL, namely, its ability to effectively produce a peptide antimicrobial, facilitates its prevalence and establishment into the gut niche, and contributes to the relatively simple microbiota inside the herbivore gut.

Because mundticin KS displays a selective antimicrobial spectrum, mainly toxic to the phylogenetically closely related bacteria including *E. faecalis*, *Streptococcus*, and *Lactobacillus*, as well as to the common food-borne pathogen *L. monocytogenes*, it does not influence other indigenous gut residents, resulting in the normal development of gut microbiota. This targeted approach to eliminating undesired bacteria while the normal flora remains relatively unaffected is safe to retain regular functions of the microbiota in energy metabolism and others. Recent reports have revealed that the normal gut microbiota not only confer “colonization resistance” (pathogen blockage by the intestinal microbiota) to protect the host, but also mediate novel pathogen clearance from the highly complex gut environment, which sufficiently complements host immune defenses (Endt et al., 2010). Similarly, Koch and Schmid-Hempel (2011) suggested that the host microbiota be considered an “extended immune phenotype”. Our discovery, that the dominant gut symbiont effectively produces a narrow-spectrum bacteriocin in vivo, represents a molecular mechanism for suppressing invaders primarily mediated by the host native gut microbiota. Understanding the role of indigenous gut residents will contribute to the development of novel biocontrol strategies against herbivorous insect pests. Our observation that insects successfully rely on peptide antibiotic against enterococcal infections also has implications for the struggle against rapidly emerging multi-drug-resistant enterococcal pathogens in humans.

SIGNIFICANCE

Symbiotic bacteria as prevalent as *E. mundtii*, which are found worldwide in Lepidoptera, must have played important roles in the holobiont. Here we reveal that the extracellular symbiont *E. mundtii* of the cotton leafworm *Spodoptera littoralis* selectively clears pathobionts from the host gut lumen by secreting a stable bacteriocin, which facilitates the normal development of gut microbiota. The constitutively produced antimicrobials create an additional chemical barrier inside limiting pathobiont expansion, thus reducing risks of infection via the gut and enhancing host health. Our study together with other investigations into the microbial symbionts of leaf-cutting ants and locusts suggests that protective associations with antibiotic-producing bacteria is a common strategy of insects against microbial invaders; however, their antimicrobials are chemically diverse.

EXPERIMENTAL PROCEDURES

Bacteria were isolated from the normal *S. littoralis* insects and were identified based on both phenotypic characteristics and molecular techniques. For bacteria enumeration, freshly prepared gut bacterial suspensions (five to nine replicates for each sample) were serially diluted in brain heart infusion (BHI) broth (CM1135, Oxoid) and plated on *Enterococcus* Selective Agar (45183, Fluka) plates. Plates were grown at 30°C overnight and the resulting *Enterococcus* colonies were counted and identified. Virulence genes were screened by diagnostic PCR. The infection assay was performed by feeding larvae with each *Enterococcus* strain as described by the original publication (Doane and Redys, 1970). The second- and third-instar larvae were fed with a low amount of antibiotic (10 µg erythromycin/g artificial diet) to eliminate native *E. mundtii* and starved 1 or 2 days depending on age before the tests. Bacterial broth culture was adjusted to 10⁸ CFU/mL with PBS and 100 µL was sprinkled directly on the artificial diet to provoke infection. The artificial diet was changed every day and the mortality was recorded until adult stage. The whole infection assay was performed in triplicate under the same rearing conditions. For further antimicrobial property study, the representative *Enterococcus* strains, including the isolated *E. faecalis* SL, *E. casseliflavus* SL, *E. mundtii* SL strain, and *E. mundtii* type strain (DSM4838), were cultivated in de Man-Rogosa-Sharpe (MRS) broth (X925.1, Carl Roth) at 30°C without shaking. The overnight culture supernatant was adjusted to pH 7.0 with 1 M NaOH and filtered through a 0.22 µm polyvinylidene fluoride membrane for testing. The related inhibitory spectrum was determined by using a well diffusion assay. The indicator strains were grown in the media (BHI or MRS) recommended by culture collections. The growth of gut symbiont *E. mundtii* was monitored by measuring biomass, and antimicrobial production was calculated from the result of an agar diffusion test. The antimicrobial substance produced by *E. mundtii* SL was purified to homogeneity by a three-step procedure consisting of cation-exchange chromatography, absorbent interaction, and reverse-phase HPLC equipped with a C18 column. MS techniques were used to determine the molecular mass of the purified antimicrobial peptide, followed by PSD analysis of its amino acid sequence. The corresponding bacteriocin-coding gene was amplified with a degenerated PCR approach from the *E. mundtii* SL genomic DNA template and confirmed by cloning and sequencing. The peptide secondary and tertiary structures were predicted by Phyre2 using default parameters and structure templates extracted from the PDB. The α helix domain was determined by performing the CD spectroscopy. The abundance of mundticin KS transcripts in the larval gut was calculated by quantitative real-time PCR using the absolute quantification method. Plasmid curing was carried out by a procedure combining three conditions known to lead to plasmid loss: the use of novobiocin and growth at 42°C in the medium lacking its buffering component. The infection assay with *E. mundtii* *Mun*⁻ was performed according to the same procedure described above. For details on reagents, insect rearing, dissection, bacteria characterization, antimicrobial activity screening, peptide purification/sequencing, protein structure analyses, qPCR, infection assay, and other methods used in this study, see [Supplemental Experimental Procedures](#).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, six figures, and two tables and can be found with this article online at <http://dx.doi.org/10.1016/j.chembiol.2016.11.015>.

AUTHOR CONTRIBUTIONS

Y.S. and W.B. conceived the experiments. Y.S., K.I., C.S., and B.C. carried out the experiments, interpreted data, and assisted in the preparation of the manuscript. Y.S. and W.B. wrote the manuscript with contributions from C.H.

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