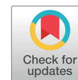

Enterococcus faecalis bacteriocin EntV inhibits hyphal morphogenesis, biofilm formation, and virulence of *Candida albicans*

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***Enterococcus faecalis*, a Gram-positive bacterium, and *Candida albicans*, a fungus, occupy overlapping niches as ubiquitous constituents of the gastrointestinal and oral microbiome. Both species also are among the most important and problematic, opportunistic nosocomial pathogens. Surprisingly, these two species antagonize each other's virulence in both nematode infection and in vitro biofilm models. We report here the identification of the *E. faecalis* bacteriocin, EntV, produced from the *entV* (*ef1097*) locus, as both necessary and sufficient for the reduction of *C. albicans* virulence and biofilm formation through the inhibition of hyphal formation, a critical virulence trait. A synthetic version of the mature 68-aa peptide potently blocks biofilm development on solid substrates in multiple media conditions and disrupts preformed biofilms, which are resistant to current antifungal agents. EntV^{68} is protective in three fungal infection models at nanomolar or lower concentrations. First, nematodes treated with the peptide at 0.1 nM are completely resistant to killing by *C. albicans*. The peptide also protects macrophages and augments their antifungal activity. Finally, EntV^{68} reduces epithelial invasion, inflammation, and fungal burden in a murine model of oropharyngeal candidiasis. In all three models, the peptide greatly reduces the number of fungal cells present in the hyphal form. Despite these profound effects, EntV^{68} has no effect on *C. albicans* viability, even in the presence of significant host-mimicking stresses. These findings demonstrate that EntV has potential as an antifungal agent that targets virulence rather than viability.**

Candida albicans | *Enterococcus faecalis* | biofilms | bacteriocins

Opportunistic pathogens present unique clinical challenges. Although rare or niche pathogens of immunocompetent individuals, they take advantage of breakdowns of immunological or physiological barriers to impose significant morbidity and mortality. Two such organisms are the fungus *Candida albicans* and the Gram-positive bacterium *Enterococcus faecalis* (1–3), which occupy overlapping niches in the normal mammalian microbiome, including in the gastrointestinal tract, oral cavity, and urogenital tract (2, 4). Both species are among the most common causes of sepsis in hospitalized patients (5), and they are frequently coisolated in a variety of human infections (6).

C. albicans and *E. faecalis* are robust biofilm-forming organisms, presenting another therapeutic complication (7, 8). *C. albicans* biofilms are polymorphic structures that can exceed 200 μm in depth and contain hyphal and yeast cells encased in a carbohydrate-based extracellular matrix that increases resistance to antimicrobial drugs and immune surveillance (8). Biofilms are a key factor in device-associated infections on catheters, denture materials, and other medical implants, as well as on mucosal surfaces in the oral cavity and urogenital track, and pose a significant clinical problem (7–9). Agents that specifically target biofilms would potentially be highly synergistic with existing antifungal drugs, but are not yet available.

Biofilms in nonsterile mucosal sites are usually polymicrobial, and the interaction between the resident microbes can be either antagonistic or synergistic (reviewed in refs. 10–12). *Pseudomonas aeruginosa* and *C. albicans* have an intricate relationship in which one bacterial product (phenazines) kills hyphal cells, whereas another (homoserine lactones) induces a switch to the resistant yeast form (13, 14). However, a synergistic interaction between *C. albicans* and either *Staphylococcus aureus* or *Streptococcus gordonii* increases biofilm biomass (15, 16). We previously reported antagonistic interactions between *C. albicans* and *E. faecalis* by using both a *Caenorhabditis elegans* model, in which a coinfection was significantly less virulent than either monomicrobial infection, and a biofilm model in which a factor secreted from *E. faecalis* inhibited hyphal differentiation (17).

In this work, we identity this factor as EntV, a secreted bacteriocin. A synthetic version of this peptide inhibited *C. albicans* hyphal morphogenesis and biofilm formation at subnanomolar concentrations. Furthermore, EntV was active against mature biofilms, shrinking their depth and biomass. Interestingly, EntV did not kill *C. albicans*, nor inhibit its growth, even at high concentrations. Nevertheless, EntV completely protected *C. elegans* from *C. albicans* infection, and increased fungal clearance by murine macrophages. Additionally, EntV dramatically reduced invasion and inflammation in a murine model of oropharyngeal candidiasis (OPC), a biofilm-related infection that is common in immunocompromised patients (18). Because the rise of drug-resistant fungal infections threatens to undermine the current small arsenal of available treatments, we propose that EntV, or similar compounds, may offer a viable therapeutic alternative, either alone or in combination with existing agents.

Significance

Hospitalized patients are susceptible to serious infections that are rarely encountered by healthy people. Two of the most problematic causes are *Enterococcus faecalis* and *Candida albicans*, a bacterium and fungus, respectively. Both are members of our normal microbial communities, but cause significant mortality in immunocompromised individuals. The formation of biofilms—microbial communities that grow on biotic or artificial surfaces—is a common feature of these infections and presents a formidable barrier to their treatment. We show here that *E. faecalis* produces a small protein that is a potent inhibitor of the ability of *C. albicans* to form biofilms and reduces fungal virulence in several models, raising the possibility that it might be developed as an antifungal agent.

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Results

E. faecalis Supernatant Inhibits C. albicans Hyphal Morphogenesis and Biofilm Formation. To facilitate identification of the *E. faecalis* inhibitor, we developed a *C. albicans* in vitro biofilm model in which cells are grown on a polystyrene substrate in an artificial saliva medium (YNBAS; adapted from ref. 19). This media supported robust biofilm growth, whereas supplementation with supernatant from *E. faecalis* cultures decreased both biofilm biomass and hyphal morphogenesis compared with biofilms grown in medium alone, as observed microscopically in biofilms stained with calcofluor white (Fig. 1 *A* and *B*) or by quantitation of biomass using the redox-reactive dye resazurin (20) (Fig. 1*C*). The reduction in hyphal formation was assayed by using a GFP reporter strain under the control of the hyphal-specific *HWP1* gene (*$HWP1_p$::GFP*) (Fig. 1 *D–F*) (21). A direct correlation between hyphal abundance and biofilm biomass was observed, in agreement with previous studies (22, 23).

EntV Inhibits Hyphal Morphogenesis and Biofilm Formation in C. albicans. Our previous studies in the *C. elegans* infection model suggested that the secreted inhibitor is a heat-stable peptide, of 3–10 kDa, regulated by the Fsr two-component quorum sensing system (17). We confirmed that biofilm inhibition was mediated by a factor with similar characteristics (Fig. S1). The Fsr system regulates multiple virulence-related traits, including the secreted proteases GelE and SprE and a bacteriocin produced from the *ef1097* gene (24–26). *ef1097* encodes a 170-aa prepeptide, and cleavage of the secretion leader sequences results in the export of a 136-residue propeptide ($EntV^{136}$) that has bactericidal activity against Gram-positive bacteria and has been referred to as enterococcin V583 and enterocin O16 (25, 27). The *ef1097* gene is present in all *E. faecalis* strains sequenced to date (25), and we propose to name the gene product EntV, for Enterocin originally found in V583.

We tested the hypothesis that EntV is the secreted inhibitor by generating a deletion mutant. Indeed, the biofilm inhibitory activity was lost in supernatants derived from the *entV* mutant strain relative to those from the WT *E. faecalis* strain (Fig. 2*A*), confirming that EntV is necessary for the inhibition of biofilm formation. Inhibitory activity was restored in a complemented strain in which *entV* was restored to the endogenous locus (Fig. 2*A*). To test whether $EntV^{136}$ was sufficient for the inhibitory activity, $rEntV^{136}$ was produced in *Escherichia coli* with a hexahistidine tag. The purified protein was functional, with an IC_{50} of ∼1,000 nM (Fig. 2*B*). Thus, EntV is both necessary and sufficient for inhibition of *C. albicans* biofilm formation.

$EntV^{136}$ is larger than the 3–10 kDa indicated by our preliminary characterization. Recent work (25) suggests that $EntV^{136}$ is further processed in a GelE-dependent manner to a 7.2-kDa peptide encompassing the 68 carboxyl-terminal amino acids ($EntV^{68}$). A predicted disulfide bond encompasses nearly the entire length of the active peptide (amino acids 4–65) and is necessary for its antibacterial activity (25, 27). Attempts to express and purify $EntV^{68}$ were unsuccessful because of apparent toxicity in *E. coli*. As an alternative, the peptide was synthetically produced, including the disulfide bond ($sEntV^{68}$). $sEntV^{68}$ was 3,000-fold more effective at inhibiting biofilm formation (IC_{50} = 0.3 nM) than the unprocessed peptide in a variety of media conditions (Fig. 2*B* and Fig. S2). $sEntV^{68}$ was also active against biofilms formed by other pathogenic *Candida* species, including *C. glabrata*, *C. tropicalis*, and *C. parapsilosis* (Fig. S3). In contrast, $EntV^{68}$ had no effect on the growth of planktonic cells at concentrations as high as 10 μM (Fig. 2*C*). Further, $EntV^{68}$ did not alter fungal susceptibility to host-associated stresses, including oxidative, nitrosative, cell wall, and cell membrane stresses (Fig. S4). Thus, EntV is a specific inhibitor of hyphal and biofilm growth.

$sEntV^{68}$ Is Active Against Mature Biofilms. We used confocal microscopy to visualize the effect of $sEntV^{68}$ on the morphology and depth of *C. albicans* biofilms. When grown in our nutrient-poor artificial saliva medium, control biofilms were 25–30 μm thick after 24 h, but only 5–10 μm in the presence of $sEntV^{68}$, roughly the length of a *C. albicans* yeast cell (Fig. 3*A*). To ask whether $sEntV^{68}$ affected preformed biofilms, we allowed them to develop for 24 h before adding the peptide. Over a subsequent 24 h, peptide-treated biofilms were reduced from ∼30 μm to ∼15 μm, whereas untreated controls grew to >50 μm (Fig. 3*A*), indicating that this peptide could dismantle mature biofilms. The *HWP1p::GFP* hyphal-specific reporter confirmed the decrease in hyphal cells and biomass following $EntV^{68}$ treatment of preformed biofilms (Fig. 3*B*).

Protection of C. elegans by $sEntV^{68}$ During C. albicans Infection. We next asked whether $sEntV^{68}$ was protective in several *C. albicans* infection models. We first used a nematode infection system in which pathogenesis partially depends on hyphal morphogenesis, as it is in mammalian infections (28–30). The presence of $sEntV^{68}$ completely abrogated *C. albicans* virulence in the nematodes at subnanomolar concentrations, such that the lifespan was similar to nematodes fed on nonpathogenic *E. coli* (Fig. 4*A*). At these concentrations, no apparent toxicity of $sEntV^{68}$ was observed (Fig. 4*A*). To ask whether the virulence effect was due to morphological differences, we quantified the proportion of *C. elegans* in which filamentous *C. albicans* could be observed, and found that even 1 pM $sEntV^{68}$ dramatically decreased the number of worms showing evidence of invasive fungal hyphae relative to

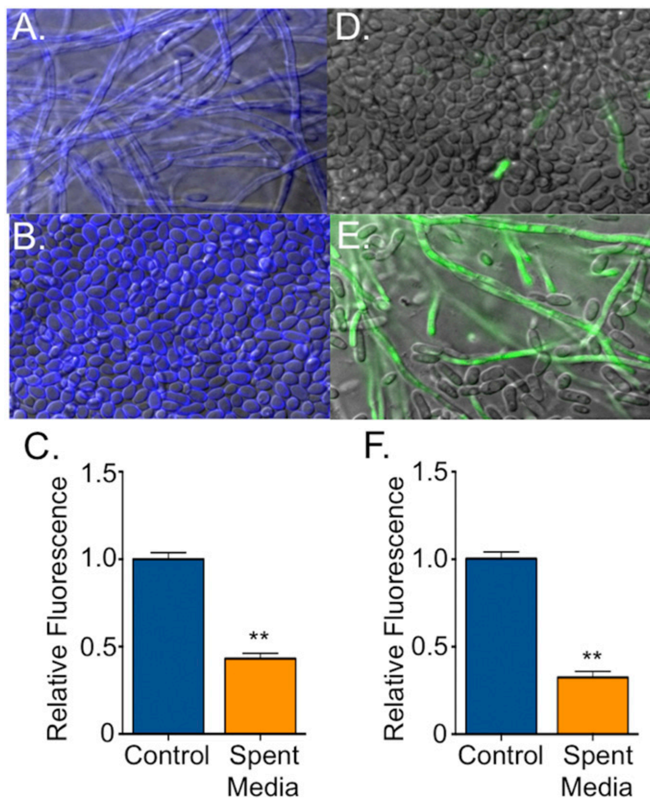

Fig. 1. *E. faecalis* supernatant inhibits *C. albicans* hyphal morphogenesis and biofilm formation. *C. albicans* biofilms grown for 24 h in YNBAS. Representative images of *C. albicans* (strain SC5314) biofilms in the absence (*A*) and presence (*B*) of *E. faecalis* supernatant and stained with calcofluor white. (*C*) Biofilm density was quantified by measuring resazurin fluorescence. Representative images of *C. albicans* hyphal reporter strain (*$HWP1_p$::GFP*) in the absence (*D*) or presence (*E*) of *E. faecalis* supernatant. (*F*) Hyphal morphogenesis was quantified by measuring GFP fluorescence as a ratio to the OD_{600}. Experiments were performed three times and analyzed by using Student's *t* test (**$P < 0.01$).

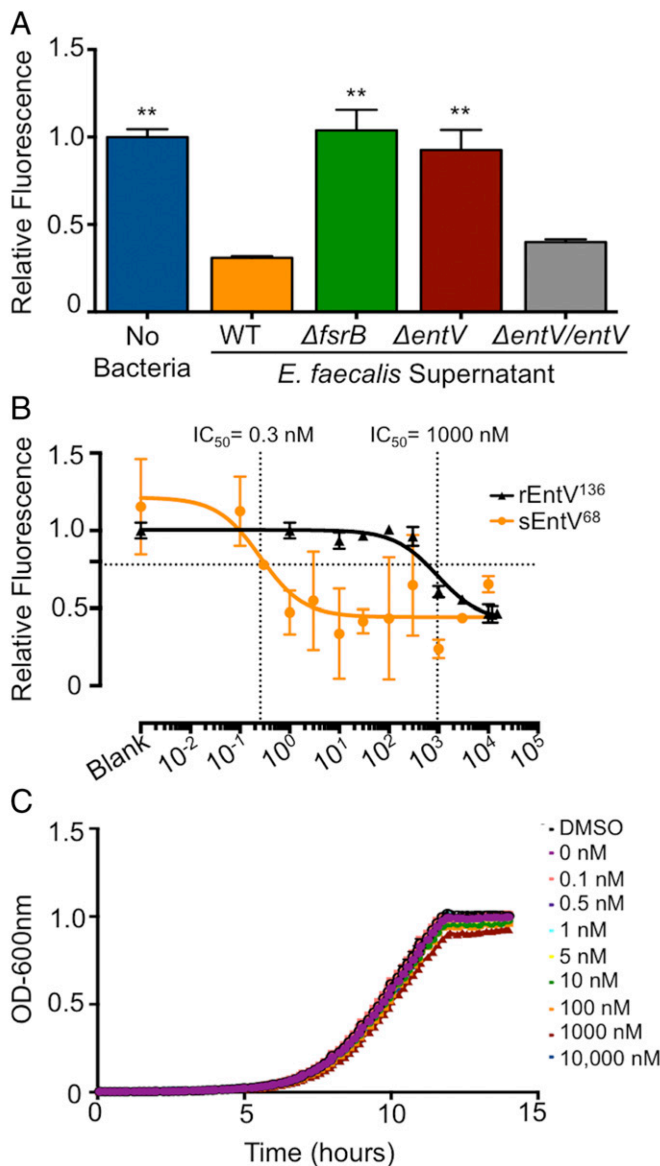

Fig. 2. EntV inhibition of hyphal morphogenesis and biofilm formation in *C. albicans*. (*A*) *C. albicans* SC5314 biofilms grown for 24 h in YNBAS with sterile media (M9HY) or spent supernatant from wild-type and mutant *E. faecalis* strains. Hyphal morphogenesis was quantified by measuring GFP fluorescence as a ratio to the OD_{600}. The experiment was performed three times and analyzed by using one-way ANOVA (**$P < 0.01$). (*B*) Biofilms were grown with increasing concentrations of $rEntV^{136}$ or $sEntV^{68}$ and the IC_{50} calculated at 1,000 nM and 0.3 nM, respectively. (*C*) *C. albicans* was grown at 30 °C in YPD for 15 h in a 96-well plate with increasing concentrations of $sEntV^{68}$, and OD_{600} was measured every 2 min in a microplate reader.

controls (Fig. 4*B*). Consistent with these results, the Δ*entV E. faecalis* mutant was also unable to protect *C. elegans* from *C. albicans* infection (Fig. S5). Taken together, these results suggest $sEntV^{68}$ is effective at protecting *C. elegans* during infection with *C. albicans* via inhibition of hyphal morphogenesis.

Protection of Murine Macrophages by $sEntV^{68}$ During *C. albicans* Infection. C. *albicans* is readily phagocytosed by murine macrophages, whereupon it activates a complex transcriptional and morphogenetic program that promotes fungal survival and results in hyphal-dependent macrophage lysis (31). Using a standard coculture system (32), we observed a significant decrease in lactate dehydrogenase (LDH) release, a measure of macrophage membrane damage, from *C. albicans*-exposed cells in the presence of $sEntV^{68}$ relative to our controls (Fig. 5*A*), indicating that the peptide reduces fungal-induced cytotoxicity. Conversely, $sEntV^{68}$ potentiates the antifungal activity of the macrophage, resulting in fewer surviving fungal cells (Fig. 5*B*). To assess whether $sEntV^{68}$ inhibits hyphal morphogenesis within macrophages, we used the *HWP1p::GFP* reporter strain modified to constitutively express mCherry (*ADH1p::mCherry*). Cells treated with $sEntV^{68}$ had a decrease in hyphal growth and GFP fluorescence compared with control treated cells (Fig. 5*C*). No toxicity of the peptide toward macrophages or HeLa cells was observed. In contrast, some toxicity was observed in the *C. elegans* infection model above the protective concentration of 0.1 nM (Fig. S6). Taken together, these results indicate that $sEntV^{68}$ protects murine macrophages by inhibition of *C. albicans* hyphal morphogenesis.

$sEntV^{68}$ Reduces the Pathology Associated with OPC. *C. albicans* is a normal resident of the oral microflora, but OPC is a common cause of pathology in immunocompromised individuals, notably infants, adults who are HIV^+, and dental implant wearers (33–35). We used an established OPC model (36) in which steroid immunosuppressed mice were given a sublingual inoculation of

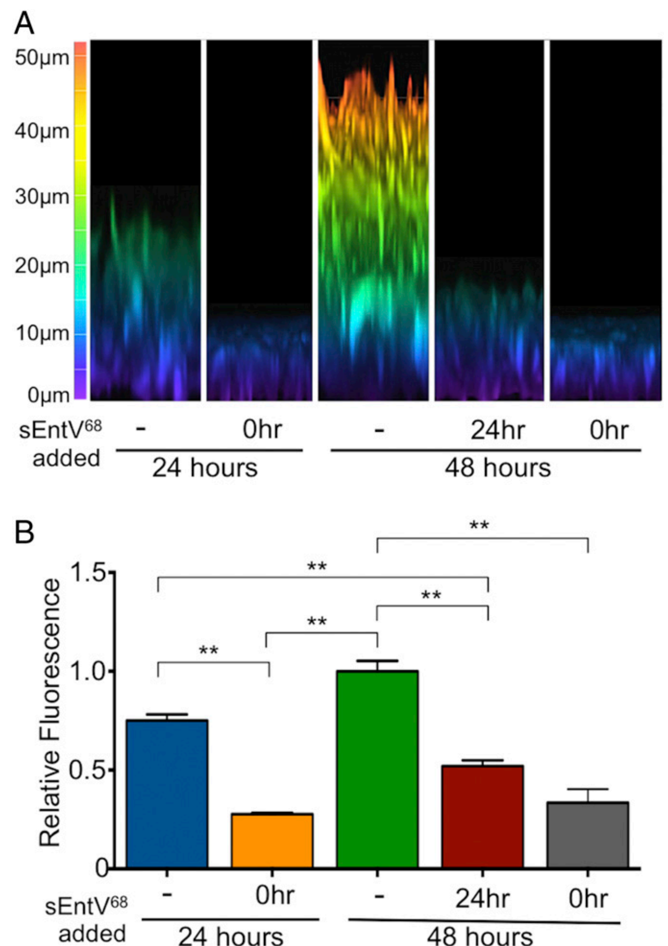

Fig. 3. Characterization of $sEntV^{68}$ inhibitory activity. *C. albicans* SC5314 biofilms were grown with 0.01% DMSO or 100 nM $sEntV^{68}$ added at different time points during biofilm formation (-, not added; 0hr, added at the beginning of the experiment. (*A*) Representative image of 24-h and 48-h biofilms observed by confocal microscopy. (*B*) Hyphal morphogenesis was quantified by measuring GFP fluorescence as a ratio to the OD_{600} for three separate experiments (**$P < 0.01$).

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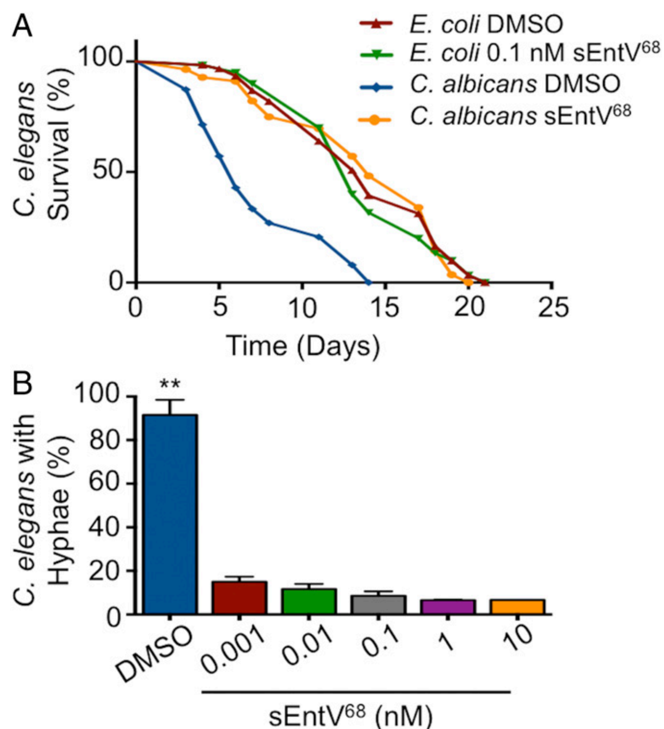

Fig. 4. sEntV^{68} protects *C. elegans* from killing by *C. albicans*. *C. elegans* was exposed to *C. albicans* SC5314 on BHI agar or *E. coli* OP50 on NB agar for 2 h, then washed with sterile PBS and transferred to six-well plates containing increasing concentrations of sEntV^{68} or DMSO at ∼30 nematodes per well. (*A*) Representative data of three independent experiments where nematode viability was scored daily. (*B*) Nematodes were assayed for evidence of visible *C. albicans* filaments penetrating the cuticle after 7 d of infection (**P > 0.01).

C. albicans and then provided drinking water containing sEntV^{68} or DMSO for up to 5 d. The mice were euthanized and tongues were excised at days 3 and 5 to examine the histology and fungal burden. Control mice showed classical signs of OPC, including extensive invasion of the epithelium by fungal hyphae, disruption of the outer layers of epithelial cells, and infiltration of neutrophils (Fig. 6*A*). In contrast, mice that were treated with 100 nM sEntV^{68} had significantly reduced invasion; most fungal cells were in the yeast or pseudohyphal form, indicating that the peptide can inhibit morphological differentiation in vivo as well (Fig. 6*B*). To quantitate epithelial invasion, we scored the proportion of the epithelial surface infected in multiple tongues from control or sEntV^{68}-treated mice, as recently described (37). A clear difference was apparent between treated and control animals (Fig. 6*C*). Hyphal cells were more abundant in control animals, which can lead to an underestimation of colony-forming units (CFUs) because of their multinucleate and adhesive nature. Thus, to assess fungal burden we quantitated fungal DNA by using quantitative PCR (qPCR) as described (38, 39); a significant decrease in tongue fungal burden was evident in peptide-treated relative to control mice (Fig. 6*D*). These results indicate that sEntV^{68} is effective in a complex mammalian infection model that simulates clinically relevant, infection conditions.

Discussion

Hyphal differentiation is crucial for biofilm formation as it is for many aspects of *C. albicans* pathogenicity (23, 29, 30). We demonstrate here that the *E. faecalis* bacteriocin EntV potently inhibits biofilm growth by preventing the switch to the hyphal form. The implications of this inhibitory activity are clearly seen in the decreased virulence of *C. albicans* cells exposed to this peptide in *C. elegans*, in macrophages, and in the mouse OPC model.

EntV is a bacteriocin originally studied for its killing activity against other Gram-positive bacteria, including species of lactococci and streptococci (25, 27). The propeptide is processed into a 68-aa peptide containing a disulfide bridge that cyclizes

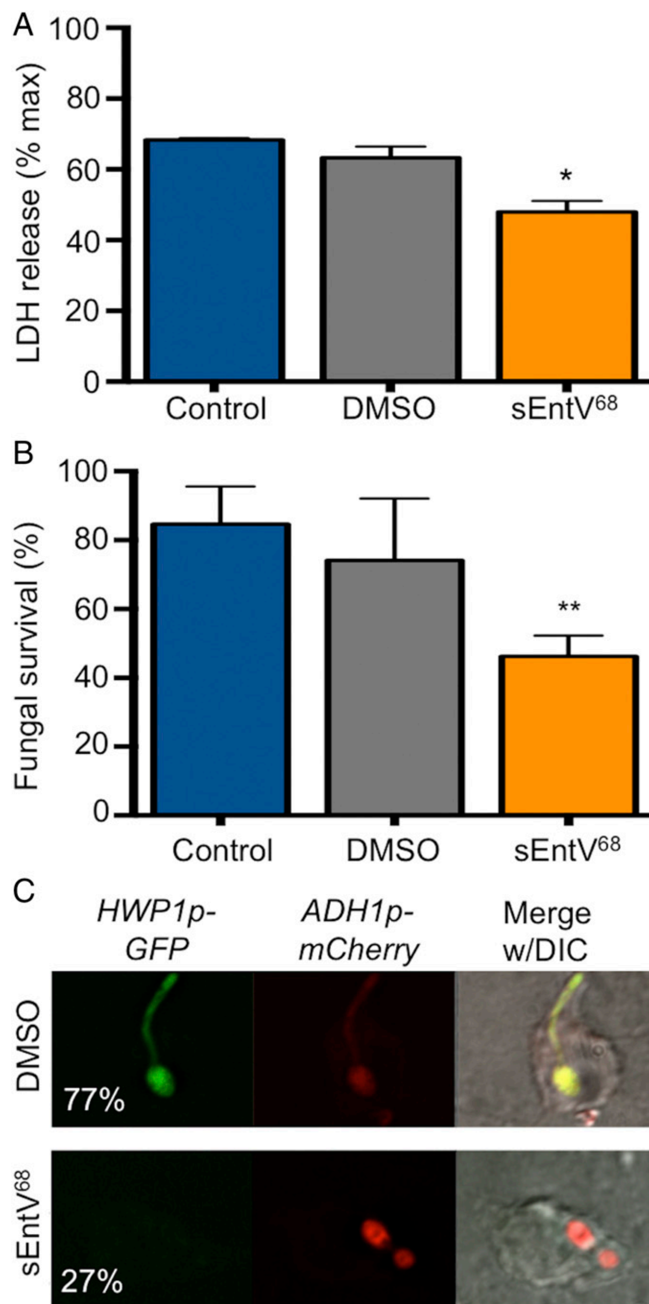

Fig. 5. Protection of murine macrophages by sEntV^{68} during *C. albicans* infection. (*A*) RAW264.7 murine macrophages were incubated with *C. albicans* SC5314 for 4 h with or without 100 nM sEntV^{68}. Macrophage killing was evaluated by using the LDH cell toxicity assay, and the percentage of killed macrophages was calculated. (*B*) *C. albicans* survival in murine macrophages was assessed by using the XTT cell viability assay. (*$P < 0.05$; **$P < 0.01$). (*C*) RAW264.7 cells were infected with *C. albicans* (*HWP1$_p$::GFP, ADH1$_p$::mCherry*) for 1 h ± peptide followed by fixation and visualized by fluorescence microscopy. The percentage of hyphal cells was scored after 2 h of coculture and is given in the left image for both conditions. At least 200 cells were counted per replicate, and the experiment was repeated three times.

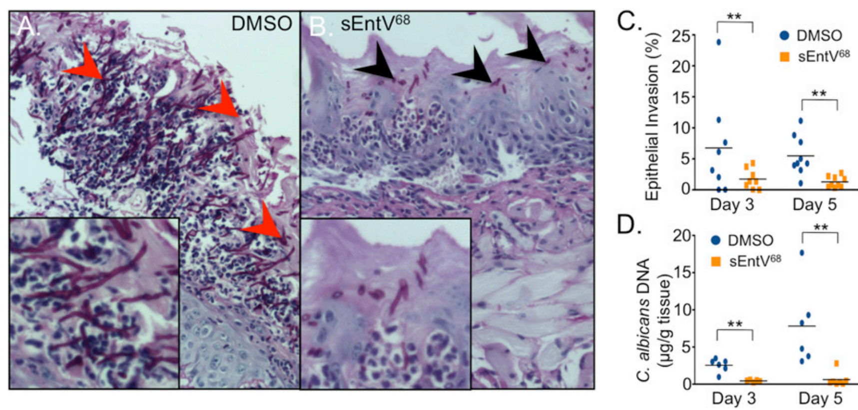

Fig. 6. sEntV^{68} is protective in a murine OPC model. Immunosuppressed mice were inoculated sublingually with *C. albicans* SC5314 with 0.01% DMSO or 100 nM sEntV^{68}. Water containing sEntV^{68} (100 nM) or vehicle (DMSO) alone was provided ad libitum. After 3 or 5 d, mice were euthanized and the tongues were excised for histological examination of DMSO control (*A*) or sEntV^{68}-treated (*B*) mice. Red arrowheads indicate hyphal cells, and black arrowheads indicate yeast cells. (*C*) The percentage of the epithelial surface showing evidence of fungal invasion was calculated for control and treated tongues. (*D*) DNA was extracted from the tongues and the fungal burden was estimated from qPCR amplification of the 5.8S ITS2 region. Statistically significant differences were calculated by using one-way ANOVA (**$P < 0.01$).

nearly the entire protein. Between the cysteines, the structure of the mature form of EntV is predicted in silico to consist of two helical elements, separated by a flexible loop region (25, 27). The bactericidal activity of EntV is not due to lysis and there is no evidence of an immunity protein, so the mechanism remains enigmatic (25, 27). Likewise, the mechanism of the antihyphal activity is unclear, although it does not lyse fungal cells either. In fact, growth and chemical sensitivity of *C. albicans* cells is unaffected, even when exposed to concentrations four logs higher than the IC_{50}. Thus, EntV exerts a true antivirulence effect on this important fungal pathogen. It has been speculated that therapeutics that target virulence might be less likely to select for resistance. In support of this idea, a small molecule also exhibiting *C. albicans* biofilm and hyphal morphogenesis-inhibiting properties did not induce resistance after repeated exposure (40).

In the OPC model, EntV reduced, but did not eliminate, fungal colonization. In contrast, fungal invasion of the epithelium and inflammation, which are responsible for the pathology of OPC, were almost entirely absent. Thus, in the presence of EntV, *C. albicans* reverts to a benign commensal interaction with the host. We have speculated that these two species might find it advantageous to suppress virulence attributes in favor of colonization (10); indeed, *C. albicans* promotes *E. faecalis* colonization in the gastrointestinal tract (41, 42). This proposal is counter to studies that suggest these species are commonly isolated together in many clinical samples (6) but this, too, might represent a coordination of behavior through sensing host weaknesses to switch cocommensal to copathogen. In support of this notion, EntV^{68} does not affect hyphal growth stimulated by mammalian serum, the strongest inducing factor (17) and an obvious host signal during disseminated infection. A precedent for this apparent contradiction is seen in the interaction between *C. albicans* and *P. aeruginosa*, species that are antagonistic in vitro (13, 43), but associated with significantly worse clinical outcomes when found together in several clinical settings (44, 45). It is possible that some of the protective effects of EntV in the OPC model are due to its activity against other bacterial species in the mouth, although we would expect this to enhance fungal colonization rather than suppress it. We are thus only beginning to understand the complex interactions among the microbiota and their varied effects on the human host, but the knowledge is likely to have important impacts on the development of novel antimicrobials.

Materials and Methods

Microbiological and Molecular Biological Methods. *Candida* and *Enterococcus* strains used in this study are listed in Table S1. Standard culture media were used for routine propagation, as described in *SI Materials and Methods*. The *entV* mutant strain was constructed as described to create an in-frame markerless deletion by using a P-*pheS** counterselection system (46) and complemented by using a reported strategy to regenerate the wild-type locus (47). The recombinant hexahistidine-tagged 136-aa EntV propeptide was expressed from pET28a in *Escherichia coli* BL21 and purified on TALON resin, as described (48). Attempts to purify the fully active 68-aa peptide from *E.coli* were unsuccessful; the peptide was synthesized with the disulfide bond (Lifetein). The hyphal-specific *HWP1p-GFP* reporter strain DHC271 (21) expressing a constitutive mCherry was generated via transformation with plasmid pADH1-mCherry (49).

In Vitro Biofilm Assays. Biofilm assays in conditions mimicking the oral cavity were modified from published reports (15, 50, 51) by using an artificial saliva media [0.17% Yeast Nitrogen Base (vol/vol), 0.5% casamino acids (vol/vol), 0.25% mucin (vol/vol), 14 mM potassium chloride, 8 mM sodium chloride, 100 µM choline chloride, 50 µM sodium citrate, 5 µM ascorbate] adapted from ref. 19. This media was supplemented with conditioned bacterial supernatant or recombinant or synthetic peptide, as indicated. Biofilm development was allowed to proceed for 24–48 h and then assessed in three ways: by estimating biomass using the redox dye resazurin, as described (20), by measuring fluorescence from the *HWP1p-GFP* hyphal-specific reporter strain (21), or through confocal microscopy.

Nematode Infection Model. *C. elegans glp-4*(*bn2*);*sek-1*(*km4*) nematodes were propagated by using standard techniques on *E. coli* strain OP50 on nematode growth medium (NGM) agar (52). The liquid infection assay was performed as described (17, 28); briefly, young adults were incubated for 4 h on solid medium with the wild-type *C. albicans* SC5314 strain, then the worms were collected, washed, and transferred to six-well plates containing 20% Brain Heart Infusion (BHI) and 80% M9W, after which viability was assayed daily. Filamentation of *C. albicans* within the worms was scored via microscopic analysis.

Murine Macrophage Infection. To assess whether EntV^{68} protects phagocytes from fungal-induced damage, we used a coculture assay in which C. *albicans* cells were incubated with RAW264.7 murine macrophage-like cells as described (32). Fungal viability was assessed by using a modified end-point dilution assay in which respiratory activity is measured using a tetrazolium dye, XTT (53, 54). Lactate dehydrogenase was assayed by using the Cytotox96 kit (Promega). *C. albicans* morphology was assayed microscopically.

Murine Oropharyngeal Candidiasis Model. Adult BALB/c mice were immunosuppressed with subcutaneous injections of cortisone acetate before sublingual inoculation with *C. albicans* according to Solis and Filler (36). Mice were given EntV or vehicle in drinking water ad libitum. Three and five days after inoculation, mice were euthanized and the tongues excised and halved longitudinally. Part of the tongue was processed for histopathological analysis, whereas the rest was homogenized for assessment of fungal burden via qPCR. DNA was extracted by using the Yeast DNA Extraction Kit (Thermo Scientific), and the Internal Transcribed Spacer (ITS1) of the rDNA genes was amplified as described (38, 39). Experiments were performed in accordance with protocols approved by the Animal Welfare Committee of the University of Texas Health Science Center at Houston.

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