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Toxin-positive *Clostridium difficile* latently infect mouse colonies and protect against highly-pathogenic *C. difficile*

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Abstract

Objective—*Clostridium difficile* is a toxin-producing bacterium and a leading cause of antibiotic-associated disease. The ability of *C. difficile* to form spores and infect antibiotic-treated persons at low MOI underlies its large disease burden. However, *C. difficile*-induced disease might also result from long-harbored *C. difficile* that blooms in individuals administered antibiotics.

Design—Mice purchased from multiple vendors and repeatedly testing negative for this pathogen by qPCR bloomed *C. difficile* following antibiotic treatment. This endogenous *C. difficile* strain, herein termed LEM1, which formed spores and produced toxin, was compared to highly pathogenic *C. difficile* strain VPI10463.

Results—Whole-genome sequencing revealed that LEM1 and VPI10463 shared 95% of their genes, including all known virulence genes. In contrast to VPI10463, LEM1 did not induce overt disease when administered to antibiotic-treated or germ-free mice, even at high doses. Rather, blooms of LEM1 correlated with survival following VPI10463 inoculation, and exogenous

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SUPPLEMENTARY MATERIALS

Supplementary files include methods, tables and figures.

COMPETING INTERESTS

None

ETHICS STATEMENT

All animals were housed at Georgia State University (Atlanta, Georgia, USA). All procedures were performed under institutionally approved animal use (Georgia State University Institutional Animal Care and Use Committee, IACUC protocol number A14033) whose responsibilities are mandated by the Animal Welfare Act (Public Law 98–198), Guide for Care and Use of Laboratory Animals and Public Health Service Policy on Humane Care and Use of Laboratory Animals.

CONTRIBUTORS

L.E.M., B.C., and A.T.G. designed the experiments. L.E.M., B.C., O.A. performed the experiments, L.E.M., B.C., and A.T.G. analyzed and interpreted data. L.M.M. performed whole genome sequencing and bioinformatics analysis. L.E.M. and A.T.G. wrote the manuscript with input from co-authors. L.E.M., B.C., O.A., L.M.M., F.D.B. and A.T.G. critically revised the manuscript.

administration of LEM1 before or shortly following VPI10463 inoculation prevented *C. difficile*-induced death. Accordingly, despite similar growth properties *in vitro* LEM1 strongly outcompeted VPI10463 in mice even at 100-fold lower inocula.

Conclusions—These results highlight the difficulty of determining whether individual cases of *C. difficile* infection resulted from a bloom of endogenous *C. difficile* or a new exposure to this pathogen. In addition to impacting the design of studies utilizing mouse models of *C. difficile*-induced disease, this study identified, isolated and characterized an endogenous murine spore forming *C. difficile* strain able to decrease colonization, associated disease and death induced by a pathogenic *C. difficile* strain.

Keywords

C. difficile; antibiotics; mouse; germ-free; whole-genome sequencing

INTRODUCTION

The spore-forming obligate anaerobic Gram-positive toxin-producing bacterium *Clostridium difficile* (*C. difficile*) is a leading cause of nosocomial disease [1, 2, 3]. *C. difficile*-induced disease includes diarrhea, severe colitis, and, in about 30,000 cases per year in the U.S., death [2, 4]. That room locations of patients manifesting *C. difficile* infections (CDI) have been observed to cluster within hospitals [5], and that its spores are highly stable and shed in abundance by infected hosts, indicates that many cases of *C. difficile* result from susceptible patients encountering spores that had been shed by nearby *C. difficile*-infected patients [6]. However, about 50% of CDI are from strains that not directly related to any previous case of *C. difficile* indicating the origin of many cases remains unclear [7].

Susceptibility to CDI is primarily conferred by use of antibiotics that ablate the microbiota, which normally serves as a strong barrier to *C. difficile* colonization [8, 9, 10, 11, 12]. Concomitantly, rodents are normally impervious to *C. difficile* but become prone CDI upon gut microbiota depletion, achieved by antibiotics or germ-free approaches [1, 8, 13, 14, 15]. However, when setting up a CDI model, we observed that mice from multiple sources that had repeatedly tested negative for *C. difficile* by qPCR would frequently display readily detectable *C. difficile* following treatment with antibiotics but prior to being exposed to *C. difficile*. Herein, we isolated endogenous *C. difficile* that "bloomed" in this manner, termed LEM1, and characterized its genome and phenotypic impact in mice. We demonstrate that *C. difficile* LEM1 is a common albeit very low-abundance member of the microbiota of mice from major suppliers, is not highly virulent, and rather, can protect against highly pathogenic isolate *C. difficile* VPI10463.

MATERIALS and METHODS (see supplement for details)

Bacterial strains: Table S1.

CDI model

Eight-week-old male C57BL/6 mice, bred at GSU or purchased from indicated suppliers, received antibiotics and *C. difficile* spores as previously described [14].

DNA isolation and quantification of *C. difficile* *tcdA* gene in feces

Fecal DNA was phenol-chloroform extracted and subjected to qPCR using primers specific to *C. difficile* toxin A (*tcdA*) (table S2) to measure *C. difficile* loads (quantification limit: 1708 genomic copy number of *C. difficile* *tcdA* per g of feces ($C_T=33.41$)).

DNA extraction and quantification of *C. difficile* VPI10463 and LEM1 loads using specific primers

Whole-genome sequencing identified unique sequences that enabled primers specific to LEM1 and VPI10463 strains (table S2) (limit of quantification 1220 genomic copy number of LEM1 per g of feces ($C_T=31.2$) and 1708 genomic copy number of VPI10463 per g of feces ($C_T=30.99$)).

Statistical analysis

Significance was determined using Student's or Mann-Whitney t-test (GraphPad Prism software, La Jolla, CA). Differences were noted as significant * $P<0.05$.

RESULTS

C. difficile positivity in antibiotic-treated non-inoculated mice

Our initial goal was to set up a model of *C. difficile*-induced disease wherein mice were inoculated with VPI10463 spores and *C. difficile* levels in feces sensitively quantitated by nucleic acid-based measurements, allowing efficient parallel quantitation of microbial genomes and/or gene expression. First, we verified that a Taqman qPCR analysis of *C. difficile* toxin A gene, *tcdA*, could be reliably quantitate purified *C. difficile* VPI10463 spores. While VPI10463 spores were not reliably extracted by commercially DNA isolation kits, bead-beating followed by phenol/chloroform extraction afforded quantitation of purified spores that could reliably detect as few as two copies of *tcdA* per mg of feces (figure S1). While mice are relatively resistant to *C. difficile* colonization, a model of *C. difficile*-induced disease has been established by Kelly wherein mice are orally administered multiple antibiotics via drinking water for 3 days, followed 2 days later by intraperitoneal injection of clindamycin [14]. This treatment renders mice susceptible to *C. difficile* 24h post-clindamycin, as assayed by levels in feces and clinical indicators of disease, such as colitis, weight-loss and death [14, 16]. In accord, we observed that treatment of C57BL/6 mice with the oral antibiotic cocktail only, followed by inoculation with *C. difficile* VPI10463 spores, did not result in observable disease (figure S2) or detectable levels of *tcdA* in feces at any time point assayed (figure S2). In further accord, adding a single dose of clindamycin to this regimen one day prior to inoculation with VPI10463 resulted in some mortality (figure 1A) and weight loss (figure 1B) and, moreover shedding of *C. difficile* at multiple time points, as indicated by levels of fecal *tcdA* (figure 1C–G). Yet, disease penetrance was less than previously reported. More surprisingly, many mice had detectable levels of *tcdA* 1 day following clindamycin treatment regardless of whether or not they had been inoculated with VPI10463. Levels of fecal *tcdA* were at least as high in the sham-inoculated (i.e. PBS) group as that observed in the VPI-inoculated group.

Our initial presumption was that our non-inoculated control mice exhibiting *tcdA* positivity reflected an unintended exposure of these mice to VPI10463 or another *C. difficile* strain present in our vivarium or in the food or bedding it utilizes. To address this possibility, we maintained multiple groups of non-inoculated control mice in distinct rooms, performed similar experimentation on mice from distinct breeding colonies, and utilized mice newly obtained from Jackson Laboratories that were placed in our ABSL2 facility upon arrival and, thereafter, maintained in autoclaved cage with sterile food and water. While the extent of disease penetrance induced by VPI10463 varied in these experiments, we consistently observed that most antibiotic-treated mice displayed *tcdA* positivity 1–3 days following clindamycin treatment, even if never inoculated with VPI10463. In contrast, we did not observe *tcdA* positivity in germ-free animals removed from their sterile isolators and then transferred and maintained in our ASBL2 facility (figure 2A). Moreover, challenge of such formerly germ-free with even a modest number of VPI10463 spores resulted in *tcdA* positivity (figure 2B) and rapid mortality (figure 2C), in accord with previous work [8]. Together, these results suggested that *tcdA* positivity observed in our non-inoculated mice did not result from antibiotic-treated mice being exposed to *C. difficile*, but rather was a consequence of endogenous *C. difficile* that had been harbored at a low (i.e. undetectable) level that "bloomed" upon antibiotic treatment.

We next investigated why *C. difficile* colonization that resulted from such blooms lacked any evidence of associated disease. We performed fecal transplant from conventional mice that had been inoculated with VPI10463 or mice that had bloomed *C. difficile* to germ-free mice (figure 2D). Germ-free mice receiving fecal transplants from VPI-inoculated mice exhibited *tcdA* positivity and mortality, whereas those receiving feces from mice that had bloomed *C. difficile* displayed relatively high levels of fecal *tcdA* but lacked evidence of disease (figure 2E–F). Levels of *tcdA* correlated with fecal toxin levels and persisted for at least 4 weeks post-transplant (figure 2G–J). These results indicated that mice obtained from multiple sources had, upon antibiotherapy, bloomed endogenous *C. difficile* that, although toxin positive, lacked virulence in mice.

Isolation of murine *Clostridium difficile* strain LEM1

We next sought to isolate and characterize the endogenous *C. difficile* that seemed to be latent and widespread, albeit at very low levels, in the murine intestine. Fecal samples that had bloomed *C. difficile* upon antibiotic treatment were grown on TCCFA medium under anaerobic conditions. This yielded an isolate that was positive for *tcdA* and *tcdB* genes and that sporulated on BHIS and 70:30 media (figure S3A). PCR ribotyping, in order to amplify the variable-length intergenic spacer region between the 16S and 23S rRNA genes, did not reveal a match between any of the reference *C. difficile* strains tested (figure S3B), suggesting it was a strain that had not been previously described. To gain insight into why it lacked VPI10463's virulence and enable design of PCR primers to distinguish it from VPI10463, we performed whole-genome-sequencing of this endogenous strain, termed *C. difficile* LEM1, in parallel with *C. difficile* VPI10463 (figure 3). GenBank accession numbers for LEM1 and VPI are pending.

Complete genomes of LEM1 and VPI10463 were aligned using Circos software [17]. The alignment reveals numerous genomic translocations and rearrangements but, nonetheless, a high overall degree of similarity between the two *C. difficile* strains was observed, with some strain-specific regions (figure 3C). Genome Subtractor, a high-throughput *in silico* subtractive hybridization analytical tool [18], was used to compare the two *C. difficile* genomes and demonstrated 90–95% identity between the two strains at the DNA level. In accordance, the LEM1 and VPI10463 genomes had very similar metabolic profiles as analyzed by MG-RAST, which assigns sequences to metabolic categories based on their best Blast hits against the SEED database (figure S4) [19, 20]. Nor did LEM1 and VPI10463 differ appreciably in any known virulence genes. *De novo* genome analysis of LEM1 pathogenicity locus (PaLoc) based on established standards within the *C. difficile* field [21, 22], revealed that LEM1 is toxinotype 0, like VPI10463, with no differences in TcdA and TcdB sequences themselves neither in their respective promoters. However, we observed that LEM1's strain produces lower amounts of toxin A than VPI10463 strain both *in vitro* and *in vivo*.

Genome Subtractor revealed a specific pool of 206 coding DNA sequences (CDS) that were present in LEM1 but not in VPI10463, among which 38 CDS are unique to LEM1 with no identity with any other CDS contained in NCBI. We also identified 371 CDS that were present in VPI10463 but not in LEM1 (92 of these CDS were unique to VPI10463). Such unique CDS in LEM1 and VPI10463 provided potential PCR targets that could differentiate these strains. Testing of over 20 sets of primers resulted in two primer sets that reliably quantitated levels of LEM1 and VPI10463 individually and when mixed together (table S2, figure S5).

Presence of LEM1 correlates with differential susceptibility to virulent strain VPI10463

We next examined the extent to which the heterogeneity in susceptibility to VPI10463-induced disease could be explained by endogenous blooms of LEM1. We retrospectively assayed fecal levels of VPI10463 and LEM1 in mice ($n=10$) purchased from Jackson, administered antibiotics, and inoculated with VPI10463 spores. One cage of such mice ($n=5$) had appeared ill, lost weight and succumbed to death and was termed succumbers while the other cage ($n=5$) lacked evidence of disease and was termed resisters (figure 4A–B). qPCR for LEM1 and VPI10463 indicated that all of the resistant mice had bloomed LEM1 prior to VPI10463 inoculation, whereas none of the mice that succumbed to VPI10463 exhibited detectable levels of LEM1 at any time point assayed (figure 4C). Blooming of LEM1 in the resistant mice correlated with lower levels of fecal VPI10463 (figure 4D). Assay of *C. difficile tcdA*, detecting both LEM1 and VPI10463, corroborated these results (figure 4E). To investigate our hypothesis that LEM1 detected in this cage of mice reflected an expansion of LEM1 that had been harbored at a very low level, we assayed fecal samples for LEM1 prior to antibiotic administration by a highly sensitive, albeit only semi-quantitative, nested-PCR assay. This assay revealed that in the “resisting” cage, 2 of the 5 mice had detectable levels of LEM1 prior to antibiotic administration, while all mice in the “succumbing” cage were negative for this bacterium (figure 4F). These results suggest that while some mice probably became colonized by LEM1 as a result of being exposed to it

from cage mates following antibiotherapy, others, i.e. those positive for LEM1 by nested PCR at day -6, had truly bloomed LEM1 that they had been harboring at very low levels.

Next, we examined the extent to which LEM1 might bloom and correlate with susceptibility to VPI10463 in mice obtained from other vendors. Like mice purchased from Jackson Labs, mice from Charles River Research bloomed LEM1 following antibiotic cocktail treatment, as revealed by specific LEM1 primers (figure 5, S3C). Here also, such blooms correlated with lack of disease in response to inoculation with VPI10463 (figure 5A–B). In contrast, mice from Taconic did not bloom LEM1 and exhibited high mortality following inoculation with VPI10463 (figure 5C–E). Thus, although there is heterogeneity in mice from different sources and also within mice from a single source, colonization with LEM1 appears to be a correlate of susceptibility to VPI10463-induced disease.

LEM1 lacks high virulence in mice

We next examined the consequences of exogenous inoculation of mice with purified spores of LEM1. First, we utilized the antibiotic-induced susceptibility model, in which administration of 1×10^5 spores of VPI10463 resulted in 60% lethality (figure S6). Infection with the same dose of LEM1 spores resulted in a similar level of *C. difficile* colonization (figure S6), but did not cause the weight loss or death induced by VPI10463. When the LEM1 inoculum was increased 100-fold (the highest dose achieved), we observed a modest increase in *C. difficile* fecal loads that was associated with transient weight loss but not death (figure S6). Next, we tested germ-free mice, which are highly sensitive to *C. difficile* infection and lack the confounding variable of potential endogenous bacterial competitors. While inoculation with as little as 1000 spores of VPI10463 resulted in an acute disease and rapid death by 48h post-infection (figure 2A, 6A–B), inoculation with up to 100-fold higher levels of LEM1 spores (highest dose tested) did not result in clinical evidence of disease (figure 6A–B). In both antibiotic and germ-free models, LEM1 persisted at detectable levels (without additional treatment) for at least 4 weeks post LEM1 inoculation (figure 6C–G). Moreover, euthanizing mice at 48h post-inoculation indicated the presence of colitis, based on levels of inflammatory markers (myeloperoxidase and lipocalin-2), cytokine levels, and histopathology in VPI10463-treated mice that was less evident in mice administered LEM1 (figure S7). These results suggest that LEM1 colonized the murine intestine as efficiently as VPI10463 but lacked high virulence even in mice prone to *C. difficile*-induced disease.

LEM1 protects against VPI10463-induced disease *via* outcompeting it

We next directly considered the possibility that LEM1 might protect against the pathogenic strain VPI10463. The frequent but unpredictable blooms of endogenous LEM1 complicate its use in the antibiotic model. Nonetheless, we observed that administration of LEM1 3 days prior to VPI10463 inoculation eliminated VPI-induced mortality (figure 7A–B). In the absence of LEM1 treatment, some mice exhibited delayed blooms of endogenous LEM1 and survived VPI10463 challenge, while some mice did not bloom LEM1 and succumbed to the pathogenic strain (figure 7A, D). Moreover, prophylactic administration of LEM1 resulted in reduced levels of VPI10463 at days 5–8 post clindamycin treatment (figure 7E). These results suggest that LEM1, when blooming endogenously or when administered

exogenously, protects against VPI-induced mortality at least in part by preventing colonization of virulent *C. difficile* strain.

Next, we examined how LEM1 and VPI10463 would impact each other in the absence of microbiota via the use of germ-free mice. Strikingly, the high rate of mortality induced by VPI10463 in germ-free animals was completely prevented by administration of LEM1 prior to, or concurrent with, VPI10463 inoculation (figure 8A). Moreover, curative administration of LEM1 as late as 30 hours after VPI10463 inoculation was able to reduce mortality induced by VPI10463 (figure 8A). Preventive administration of LEM1 fully protected against mortality-induced by VPI10463 even in response to a 10-fold higher dose of VPI10463 spores, that otherwise resulted in 100 percent lethality (figure S8). Lastly, to better understand how LEM1 protects against *C. difficile*-induced disease, we directly measured the ability of these strains to compete against each other *in vivo* and *in vitro*. While germ-free mice on both C57Bl/6 and Swiss-Webster backgrounds are highly prone to VPI-induced disease, the latter are somewhat more resistant thus enabling formal demonstration that either VPI10463 or LEM1 can persist a few days post-inoculation (figure 8C–D). However, when administered together, at equal ratios or 100-fold higher levels of VPI10463 (highest tested), LEM1 dramatically out-competed VPI10463 thus eliminating it by 3 days post-challenge (figure 8E–I). Such ability of LEM1 to outcompete VPI10463, thus nearly eliminating it even the absence of commensal microbes, correlated with complete protection against VPI-induced disease. LEM1 and VPI10463 did not impact each other's growth *in vitro*. Rather, these strains grew in broth medium at similar rates when cultured individually or together (figure 8JS9A–B), thus suggesting that LEM1's ability to outcompete VPI10463 does not reflect a direct interaction between these *C. difficile* strains but, rather, is a consequence of the murine colonic environment.

DISCUSSION

The recent development of mouse models of CDI, which are genetically and immunologically tractable, promised to allow mechanistic study of disease determinants [13, 14]. However, a paucity of expertise in anaerobic culture in many research groups has remained a barrier. Hence, our initial goal was to setup a model of *C. difficile*-induced disease in mice using spores that would permit high-throughput and culture-independent quantification of pathogen loads using frozen fecal samples [23, 24]. This would enable a broad range of researchers using mouse-based approaches to test *C. difficile* strains and query host determinants. Real-time qPCR-based measure of fecal levels of *C. difficile* toxin A, following infection of mice with stable *C. difficile* spores, proved to be a relatively simple and sensitive means to quantitate *C. difficile* levels. However, we made an unanticipated observation, namely that many colonies of mice, including those from some commercial suppliers, harbored endogenous *C. difficile*, a strain of which we herein named LEM1. While, in the absence of antibiotics, LEM1 levels were below the detection limit of our qPCR assay, use of semi-quantitative nested-PCR indicated it was nonetheless present, at least in some mice that later bloomed it following antibiotherapy. The notion that *C. difficile* can be present but yet undetectable by culture was demonstrated by Lawley and colleagues, whom observed that, even in stringently hygienic conditions, mice that had seemingly fully resolved a CDI would bloom the bacteria upon antibiotherapy [25]. While such blooms

observed by Lawley were associated with clinical-type indicators of disease, LEM1 lacked virulence and rather protected mice against pathogenic *C. difficile* VPI10463. These observations have important implications for mouse modeling of CDI and understanding the pathophysiology of *C. difficile*-induced disease in humans.

The frequent, albeit sporadic, blooms of LEM1 observed in our antibiotic-treated mice illustrate the need for animal models of *C. difficile* infection to be carefully controlled. Specifically, control animals subjected to all treatments except *C. difficile* inoculation are needed and should be sampled throughout the experiment. Further, assays should utilize a specific means to be sure that the *C. difficile* strain of interest is the one being quantitated. Regarding this point, hamsters from commercial suppliers also harbored endogenous *C. difficile*, different from LEM1 based on its ribotype [26]. In many cases, it may prove difficult to eliminate blooms of endogenous *C. difficile*, but the presence of such organisms can be taken into account during data interpretation.

LEM1 is clearly distinct from human isolates in that it contains about 100 CDS not found in any previously sequenced human isolate. Based on ribotyping and the PCR protocol developed herein using one of LEM1's novel CDS, we find "LEM1-like" *C. difficile* is a frequent albeit low-abundance resident of the murine intestine but note that sequencing of additional isolates is needed to discern how closely related to such isolates are to LEM1. The extent to which LEM1's low virulence in mice even when administered at high doses might be explained by its modest toxin production relative to VPI10463 (figure S9C). In support of this possibility, we have observed that LEM1 is virulent in hamsters (data not shown), which are far more sensitive to *C. difficile* toxins and highly susceptible to *C. difficile*-induced mortality [27]. Concomitantly, LEM1's ability to protect against a virulent strain is analogous to observations that non-toxigenic *C. difficile* strains can prevent *C. difficile*-induced mortality in hamsters [28, 29, 30] and reduce the rate of recurrence in humans [31]. While such non-toxigenic *C. difficile* strains may soon be utilized to treat *C. difficile*-associated disease, their failure to eliminate recurrence and associated adverse events highlights the importance of mechanistically understanding how such strains confer protection, thus allowing more effective and specific strategies to prevent recurrence [30, 32, 33]. We are hopeful that mouse modeling of protection against virulent *C. difficile* by an avirulent strain, as shown herein, will prove a useful platform for achieving this goal.

The mechanism by which LEM1 protects against VPI10463 remains unclear. We initially favored the hypothesis that LEM1 might activate an immune response that affects VPI10463 more than LEM1. However, deletion of several host genes that mediate innate immunity (MyD88, Tlr5, and Nlrc4) and adaptive immunity (Rag1) had minimal effects on ability of LEM1 to protect against VPI10463-induced disease (data not shown) arguing against this possibility. Another possible mechanism for LEM1's ability to outcompete VPI10463 is that bacteriophages and/or anti-microbial compounds may be produced by LEM1 *in vivo* that may directly impede VPI10463; analogous to hypotheses from others [34, 35], although that LEM1 did not impede growth of VPI10463 fails to support this scenario. Hence, we view the ability of LEM1 to outcompete VPI10463 in germ-free mice to reflect that LEM1 outcompetes VPI10463 for a limiting niche in the mouse gastro-intestinal tract [8, 25]. Such ability of LEM1 to protect against VPI10463 was not shared by known probiotic strains or

non-pathogenic *E. coli* strains, both of which, in our hands, had only modest ability to protect against VPI10463-induced disease even when administered prophylactically (data not shown). Nor did administration of fecal transplants to antibiotic-treated mice offer strong protection against VPI10463 (data not shown), thus highlighting the specificity, strength, and perhaps ultimate potential usefulness of the protection conferred by LEM1.

Extrapolating the results from this study in mice to *C. difficile*-induced disease in humans questions the reliability of the widely held assumption that newly diagnosed CDI result from exposure of the antibiotic-treated host to spores of *C. difficile*. Indeed, while Gerding *et al.* demonstrated that this is one major route of transmission at least in the 1980s, Eyre and colleagues sequence-based study of hospital-manifesting CDI in Oxfordshire, U.K from 2007–11 2013 found that the majority of such cases were not transmitted from another symptomatic patient [5, 7]. This study revealed that almost half (45%) of all *C. difficile* cases in the Oxfordshire area were genetically distinct from all other previous cases of *C. difficile* [7]. In this context, we view our results to suggest that some CDI that manifest in hospitals are, in fact, the result of *C. difficile* that was harbored at very low, perhaps undetectable levels. While studies analyzing the microbiome of healthy humans have not frequently reported the presence of *C. difficile* [36], such studies typically 1) lack the depth to detect low-abundance bacteria, 2) use DNA extraction methods that work poorly on *C. difficile* spores, and 3) target regions of the 16S RNA genes that do not resolve *C. difficile* from other Clostridia (Tor Savidge, Baylor College of Medicine, personal communication). Indeed, our use of 16S sequencing, using relatively standard methodology and a depth of over 20,000 sequences per sample failed to detect any sequences classified as *C. difficile* even in mice that had clearly bloomed it by qPCR. Hence, we submit that the true rate of carriage of *C. difficile* in the general population is not easily discerned from existing studies. While culture-based studies indicate it is quite high (up to 70%) in infancy and relatively low in healthy persons over age 2 [37], we speculate that use of highly sensitive methodologies such as nested-PCR might ultimately reveal that low-level carriage of *C. difficile* is relatively high.

Our observation that colonization of mice by a *C. difficile* that did not induce overt disease protected against a highly virulent strain is analogous to observations that hospital patients who were positive for *C. difficile*, upon initial hospital culture, had less clinical manifestations of disease [5]. However, this does not exclude the possibility that some strains of *C. difficile* that are harbored at low levels may bloom and become highly virulent. Indeed, as demonstrated by Lawley, the ready-ability of other administered *C. difficile* strains to establish latency in mice argues that the lack of virulence is not prerequisite for long-term maintenance of latency [38, 39]. We view our results to suggest that many arising instances of *C. difficile* positivity, including strains that induce symptomatic disease and/or protect against more virulent strains, may have resulted from blooms of *C. difficile* long harbored by affected individuals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CDS	coding DNA sequences
<i>C. difficile</i>	<i>Clostridium difficile</i>

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SUMMARY BOX**What is already known about this subject?**

Clostridium difficile is a leading cause of antibiotic-associated disease. Its disease burden presumably results from its ability to form spores and infect antibiotic-treated persons at low doses. However, *C. difficile* infections might also result from long-harbored *C. difficile* blooming upon antibiotic therapy.

What are the new findings?

Mice from various sources bloomed an endogenous *C. difficile* strain, herein termed LEM1, which shared 95% of its genome with VPI10463. LEM1 was avirulent in mice. Rather, blooms of LEM1 correlated with lack of disease following VPI10463 challenge. Moreover, exogenous administration of LEM1 strongly outcompeted and protected against VPI10463-induced disease in antibiotic-treated and germ-free mice.

How might it impact on clinical practice in the foreseeable future?

These results demonstrate the difficulty of determining whether individual cases of *C. difficile* resulted from a bloom or new exposure to the pathogen. Furthermore, we have identified, isolated and characterized an endogenous murine spore forming *C. difficile* strain that prevents colonization and disease induced by pathogenic *C. difficile*.

Figure 1. C57BL6 WT mice bred at GSU exhibited unanticipated *tcdA* positivity and moderate disease upon VPI10463 challenge

(A). Kaplan-Meier survival curve of mice uninfected (PBS, $n=7$) or infected with 1×10^5 spores of *C. difficile* VPI10463 ($n=10$) at day 0. (B). Average body weight measured daily starting from the day of infection (day 0). (C–G). *C. difficile* load in feces was quantified by q-PCR using Taqman probe and primers specific to the gene encoding *C. difficile* toxin A at day -6 (C), day 0 (D), day 1 (E), day 2 (F) and day 7 (G).

Each point represents an individual animal from 2 colonies of wild-type mice bred at Georgia State University. Results are the means $\pm$ SEM ($n=7-10$).

Figure 2. Germ-free mice exhibit disease in response to VPI10463 spores and VPI10463-containing feces but not feces exhibiting *C. difficile* blooms

(A–B) *C. difficile* load in feces was quantified by q-PCR using Taqman probe and primers specific to the gene encoding *C. difficile* toxin A at day 0 (A) and day 1 (B). (C). Kaplan-Meier survival curve of C57Bl6 germ-free mice uninfected (PBS, $n=3$) or infected with 1×10^3 spores of *C. difficile* VPI10463 ($n=4$) at day 0. (D). Schematic representation of the experimental design. (E). Kaplan-Meier survival curve of Swiss Webster germ-free mice receiving fecal microbiota transplant from conventional antibiotic-treated mice without a

bloom (fecal sample previously *tcdA* negative by qPCR, $n=2$), with a bloom (fecal sample previously *tcdA* positive by qPCR, $n=4$) or *C. difficile* VPI-infected mice (fecal sample previously *tcdA* positive by qPCR, $n=4$). **(F)**. Average body weight measured daily starting from the day of infection (Day 0). **(G and I)**. *C. difficile* load in feces was quantified by q-PCR using Taqman probe and primers specific to the gene encoding *C. difficile* toxin A at day 1 **(G)** and day 28 post-infection **(I)**. **(H and J)**. Vero cell cytotoxic assay was used to determine the $\log_{10}$ reciprocal cytotoxin dilution per gram of fecal sample at day 1 **(H)** and day 28 post-infection **(J)**.

Each point represents an individual animal. Results represent the means $\pm$ SEM ($n=3-4$ for panels B and C); results represent the means ($n=2-4$ for panels G to J).

Figure 3. Genomic comparison of *C. difficile* strains LEM1 and VPI10463
(A). Comparison of *C. difficile* LEM1 and *C. difficile* VPI10463 genomes. (B). Venn diagram representing the number of sequences unique or shared between the two strains, where 206 sequences specific to *C. difficile* LEM1 genome and 371 sequences specific to *C. difficile* VPI10463 genome were identified. (C). Comparative analysis of the genome sequences of *C. difficile* VPI10463 and *C. difficile* LEM1 using Circos software tool where blue lines represent high homology between the two genomes, orange lines visualize the

differences (modest homology) and the darker blue lines represent overlapping areas of homology.

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Figure 4. Variation in susceptibility to *C. difficile* VPI10463-induced disease amongst Jackson laboratories mice correlates with LEM1 bloom

(A). Kaplan-Meier survival curve of infected with 1×10^5 spores of *C. difficile* VPI10463 ($n=5$ mice for resisting cage, $n=5$ for succumbing cage). (B). Average body weight measured daily starting from the day of infection (Day 0). (C–E). *C. difficile* loads in feces were quantified by q-PCR using SYBR Green technology and primers specific to *C. difficile* LEM1 (C) or primers specific to *C. difficile* VPI10463 (D) or using Taqman technology with probe and primers specific to the gene encoding *C. difficile* toxin A (E) at day -6, day 0

and day 1 post-infection. **(F)**. Agarose gel electrophoresis of nested PCR products in fecal DNA samples of succumbing and resisting animals on fecal samples collected upon receipt from Jackson laboratories (day-6). M: molecular size DNA marker (1kb Plus DNA ladder), lanes 1–5: succumbing animals, lanes 6–10: resisting animals, lanes 11–12: *C. difficile* LEM1 (positive control), lanes 13–14: negative control. Lanes 1, 2 and 3 have non-specific bands.

In C–E, each point represents an individual animal. Results are the means $\pm$ SEM ($n=5$). Significance was determined by Mann-Whitney test, * indicates $p<0.05$.

Figure 5. Variation of *C. difficile* susceptibility in mice from Charles River Laboratories and Taconic farms

(A). Kaplan-Meier survival curve of infected Charles River ($n=5$) or Taconic ($n=5$) mice with 1×10^5 spores of *C. difficile* VPI10463. (B). Average body weight measured daily starting from the day of infection (Day 0). (C–E). *C. difficile* loads in feces were quantified by q-PCR using Taqman probe and primers specific to the gene encoding *C. difficile* toxin A (C) or using SYBR Green technology and primers specific to *C. difficile* LEM1 (D) or primers specific to *C. difficile* VPI10463 (E) at day 0 and day 1 post-infection. Each point represents an individual animal. Results are the means $\pm$ SEM ($n=5$). Significance was determined by Mann-Whitney test, * indicates $p < 0.05$.

Figure 6. *C. difficile* LEM1 strain lacks virulence in germ-free mice

(A). Kaplan-Meier survival curve of Swiss Webster germ-free mice infected with *C. difficile* LEM1 (1×10^5 , $n=4$; 5×10^4 , $n=3$; 1×10^4 , $n=3$; 1×10^3 spores $n=3$) or *C. difficile* VPI10463 (1×10^3 spores, $n=4$). (B). Average body weight measured daily starting from the day of infection (Day 0). (C–G). *C. difficile* loads in feces were quantified by q-PCR using Taqman technology with probe and primers specific to the gene encoding *C. difficile* toxin A at day 1 (C) and day 28 post-infection (F) or using SYBR Green technology and primers

specific to *C. difficile* LEM1 at day 1 (**D**) and day 28 post-infection (**G**) or primers specific to *C. difficile* VPI10463 at day 1 post-infection (**E**).

Each point represents an individual animal. Results are the means $\pm$ SEM ($n=3-4$).

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Figure 7. *C. difficile* LEM1 protects mice against *C. difficile* VPI10463-induced disease and death in conventional antibiotic-treated mice

(A). Kaplan-Meier survival curve of Jackson mice uninfected ($n=5$) or infected with *C. difficile* LEM1 (1×10^5 spores, $n=5$, the dotted line represents the mice that did not show an endogenous bloom for *C. difficile* LEM1) and challenged 72 hours later with *C. difficile* VPI10463 (1×10^5 spores). (B). Average body weight measured daily starting from the day of intra-peritoneal injection of clindamycin. (C–E). *C. difficile* loads in feces were quantified by q-PCR using Taqman technology with probe and primers specific to the gene

encoding *C. difficile* toxin A (**C**) or using SYBR Green technology and primers specific to *C. difficile* LEM1 (**D**) or primers specific to *C. difficile* VPI10463 (**E**) at day 2, 3, 4 5, 6 and 8 post-clindamycin respectively.

Each point represents an individual animal. Results are the means $\pm$ SEM ($n=5$).

Significance was determined by Student's t-test, * indicates $p<0.05$.

Figure 8. *C. difficile* LEM1 outcompetes *C. difficile* VPI10463 and protects against VPI-induced disease in germ-free mice

(A). Kaplan-Meier survival curve of germ-free C57Bl6 mice infected with 1×10^3 spores of *C. difficile* VPI10463 ($n=15$) or given 1×10^5 spores of *C. difficile* LEM1 48 hours ($n=2$), 36 hours ($n=6$) prior to or concomitant to ($n=2$) VPI10463 challenge. In another experiment, germ-free mice were challenged with *C. difficile* VPI10463 (1×10^3 spores) and treated 30 hours later with *C. difficile* LEM1 (1×10^5 spores). (B). Average body weight measured daily starting from the day of infection (Day 0). (C–J) Competition experiments between *C.*

difficile VPI10463 (black line) and LEM1 (pink line) were performed *in vivo* in germ-free Swiss Webster mice (**C–I**) and *in vitro* (**J**); the number of bacteria recovered in feces or TY medium was quantified by quantitative PCR of *C. difficile* VPI10463 and *C. difficile* LEM1 (with *C. difficile* LEM1 being defined as 1) and then expressed as a competitive index (CI). The CI was defined as: (LEM1 / VPI10463) output / (LEM1 / VPI10463) input and then expressed as a percentage of each strain. (**C–I**). Mixed spores suspensions were administered to germ-free mice at different ratio (**J**) *C. difficile* VPI10463 and LEM1 were grown in equal amount of each bacteria in TY medium. Results are the means $\pm$ SD ($n=2-6$).