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Lethal Toxin is a Critical Determinant of Rapid Mortality in Rodent Models of *Clostridium sordellii* Endometritis

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Abstract

The toxigenic anaerobe *Clostridium sordellii* is an uncommon but highly lethal cause of human infection and toxic shock syndrome, yet few studies have addressed its pathogenetic mechanisms. To better characterize the microbial determinants of rapid death from infection both *in vitro* and *in vivo* studies were performed to compare a clinical strain of *C. sordellii* strain (DA-108), isolated from a patient who survived a disseminated infection unaccompanied by toxic shock syndrome, to a virulent reference strain (ATCC9714). Rodent models of endometrial and peritoneal infection with *C. sordellii* ATCC9714 were rapidly lethal, while infections with DA-108 were not. Extensive genetic and functional comparisons of virulence factor and toxin expression between these two bacterial strains yielded many similarities, with the noted exception that strain DA-108 lacked the *tcsL* gene, which encodes the large clostridial glucosyltransferase enzyme lethal toxin (TcsL). The targeted removal by immunoprecipitation of TcsL protected animals from death following injection of crude culture supernatants from strain ATCC9714. Injections of a monoclonal anti-TcsL IgG protected animals from death during *C. sordellii* ATCC9714 infection, suggesting that such an approach might improve the treatment of patients with *C. sordellii*-induced toxic shock syndrome.

Keywords

Clostridium infection; endometritis; toxic shock syndrome; reproductive tract infections; anaerobes

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1. Introduction

Clostridium sordellii is a soil-dwelling, anaerobic, spore-forming Gram-positive bacillus that uncommonly causes infections in humans and animals [1]. Diseases caused by *C. sordellii* include bloodstream and necrotizing soft tissue infections associated with black tar heroin use [2–4]; uterine infections following childbirth or spontaneous/medical abortion [5,6]; and soft tissue infections associated with contaminated cadaveric orthopaedic graft material [7]. The mortality of these infections is high, approaching 70% [1] and varies with the clinical presentation. Although rare, *C. sordellii* infections attract attention because of their high mortality and propensity to present with a characteristic “toxic shock syndrome.” This stereotypical syndrome includes the sudden onset of weakness, nausea, and vomiting; progressive and refractory hypotension; local and spreading edema; severe hemoconcentration; and a marked leukemoid reaction [8].

C. sordellii infections are remarkable for their heterogeneity in both clinical presentation and outcome. While some patients present (and die) with refractory toxic shock despite harboring a localized infection of the soft tissues [9,10], other patients have local or disseminated infections in the absence of this stereotypical syndrome [11,12]. The degree of tissue necrosis may also vary among patients with *C. sordellii* disease, despite similar modes of infection [2,13,14]. The clinical manifestations of *C. sordellii* infection are theorized to derive primarily from a diverse array of secreted toxins, including a cholesterol-dependent cytotoxin/hemolysin (*a.k.a.* sordellilysin) [14]; phospholipase C (lecithinase) [15]; neuraminidase (sialidase) [16]; DNase [17]; hyaluronidase [1]; collagenase [17]; and two members of the large clostridial cytotoxin class of toxins, hemorrhagic toxin (TcsH) and lethal toxin (TcsL) [1]. The role each of these individual toxins plays in the pathogenesis of human infection remains incompletely defined. The expression of these toxins by clinical isolates of *C. sordellii* is highly variable [14,18]. Some have postulated that different “virotypes” of *C. sordellii* (expressing distinct virulence factor profiles) might explain the marked variability in clinical presentation during infection [14].

A clinical strain of *C. sordellii* was isolated from a patient who survived postpartum *C. sordellii* endometritis and bacteremia that was not complicated by toxic shock syndrome. We questioned whether the *C. sordellii* strain isolated from this patient was deficient in TcsL production, which might explain the absence of toxic shock and rapid death. We attempted to answer this question using both *in vitro* comparative analyses with a TcsL-positive reference strain of *C. sordellii* (ATCC9714) and *in vivo* experiments. A new mouse model of *C. sordellii* endometritis was developed for these studies. Our results, for the first time, directly correlate the expression of virulence factors by a clinical isolate of *C. sordellii* with the outcome of infection in a human host and in animal models of disease. These data shed new light on the nature of *C. sordellii* pathogenesis and might facilitate the development of preventive or therapeutic strategies against this deadly infection.

2. Materials and Methods

2.1 Animals

125–150 g female Wistar rats were obtained from Charles River Laboratories (Portage, MI), 3–6 week old female C57BL/6J mice from the Jackson Laboratory (Bar Harbor, ME), and 8–10 week old female 129 SvEv mice from Taconic (Hudson, NY). Animals were treated per NIH guidelines for the use of experimental animals with the approval of the University of Michigan Committee for the Use and Care of Animals.

2.2 Reagents

Reinforced clostridial medium (RCM) and brain heart infusion broth were from BD Biosciences (San Jose, CA). A mouse monoclonal anti-TcsL IgG1 (lot CSORN A3B2–3B8) was prepared in a bioreactor as previously described [19]. This IgG protects mice from death from TcsL injection [19]. *C. sordellii* polyclonal antitoxin IRP 501(04) containing 170 antitoxin units per mL (AU/mL), was produced in goats in August 2004 using *C. sordellii* strain 7502–1–11 (obtained originally from Montana State University, Bozeman, MT, on September 16, 1968) and standardized against the World Health Organization gas-gangrene (*C. sordellii*) international antitoxin, equine origin. The *C. sordellii* strain 7502–1–11 is toxic to mice and expresses TcsL but does not express TcsH (data not shown). Goat polyclonal IgG against *C. difficile* toxin A was from EMD Chemicals, Inc (Gibbstown, NJ).

2.3 Bacteria

C. sordellii strain ATCC9714 was from the American Type Culture Collection (Manassas, VA). The clinical strain DA-108 was isolated from the blood of a patient who survived postpartum endometritis. It was verified to be *C. sordellii* by PCR amplification of a *C. sordellii*-specific portion of the 16S rRNA gene (not shown; see Table 1 for primer pairs). Bacteria were grown in broth overnight at 37°C in RCM in an anaerobic chamber (Coy Laboratory Products, Grass Lake, MI).

2.4 Toxin production

For *in vivo* experiments, crude toxin preparations were made using 10 ml of overnight broth cultures of *C. sordellii*. Cultures were subjected to centrifugation (3,000 RPM, 10 min, 22°C). The supernatant was filtered (0.22 µm) into Amicon Ultra-4 centrifugal filters with a 100 kDa molecular weight cut off (Millipore, Billerica, MA). Amicons were centrifuged (5,000 × g, 20 min, 22°C) and the ~100-fold concentrated retentate stored at –20°C. For immunoblot analyses, neuraminidase assays, and TcsH assays (see below), concentrated toxins from ATCC9714 and DA-108 were prepared as follows. Bacteria were grown in anaerobic meat medium for 72 h then inoculated into brain heart infusion dialysis flasks (1L trypsin flasks). After anaerobic incubation (37°C, 72 h), cells were removed by centrifugation (7,000 RPM x 60 min). A protease inhibitor cocktail (Sigma) was added to 0.22 µm-filtered culture supernatant. Toxins were concentrated with acetone. Ice cold supernatants were re-filtered through 0.45 µm pore polycarbonate filters and acetone (–20°C) was added slowly with stirring to a final concentration of 5:1 (vol/vol) acetone: supernatant. 200 ml was concentrated and resuspended in 20 ml of 0.01 M PBS. Toxin was aliquoted and stored at –80°C until further use.

2.5 Phospholipase C and neuraminidase assays

Phospholipase C activity was assayed according to a previously published protocol [20] using toxin samples (100 µl) prepared from overnight cultures as above. Neuraminidase (sialidase) activity was determined using *C. sordellii* toxins, prepared as above, with a commercially available neuraminidase activity kit according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). *C. perfringens* neuraminidase was included as a positive control.

2.6 Pleural effusion volume measurement

C57BL/6J mice were infected i.u. with 1×10^7 CFU of ATCC9714 or DA-108 bacteria. At the immediate time of death (ATCC9714-infected mice) or after 48 hr (DA-108-infected mice) the volume of intrathoracic (bilateral pleural space) fluid was measured, then compared with that of uninfected mice.

2.7 Intrauterine infection, intraperitoneal infection, and intoxication

Intrauterine infection was performed in rats or C57BL/6J mice according to a previously published protocol [21]. Mice were given an intraperitoneal (i.p.) inoculation with live *C. sordellii* in 100 μ l of sterile PBS. In other experiments mice were injected i.p. with 100 μ l of sterile PBS containing a 1:5 dilution of crude *C. sordellii* toxins prepared as above (20 μ l toxin mixed with 80 μ l PBS).

2.8 Administration of anti-toxin or anti-TcsL immunoglobulin in vivo

Rats were infected i.u. with *C. sordellii* ATCC9714 (1×10^{10} CFU) in 100 μ l containing 60 μ l of antitoxin or anti-TcsL IgG. Rats receiving antitoxin were treated daily with i.p. injections of antitoxin (300 μ l) on days 1, 2, and 3 post-infection while animals given anti-TcsL IgG subsequently were treated with i.p. injections of anti-TcsL IgG (300 μ l twice daily) on days 1, 2, and 3 post-infection. C57BL/6 mice were infected i.u. with *C. sordellii* ATCC9714 (1×10^6 CFU) in 25 μ l of anti-TcsL IgG. Mice were subsequently treated with i.p. injections of anti-TcsL IgG (75 μ l twice daily) on days 1, 2, and 3 postinfection.

2.9 Immunoprecipitation of TcsL from *C. sordellii* ATCC9714 toxins

To remove TcsL from *C. sordellii* ATCC9714 filtered culture supernatants, 100 μ l of filtered supernatant was added to 200 μ l of sterile PBS, then 20 μ l of the monoclonal anti-TcsL IgG was added. This was rotated overnight (4°C). Next, 100 μ l of protein A-Sepharose (Amersham, Uppsala, Sweden) was added to each sample and rotated for 5 hr (4°C). Samples were then centrifuged (14,000 RPM x 5 min) and this cycle of anti-TcsL IgG/protein A-Sepharose was repeated. The TcsL-free supernatant was divided into equal volumes for i.p. injection into 5 mice. Control animals (n = 5) received filtered culture supernatant subjected to the identical immunoprecipitation protocol, performed with goat anti-*C. difficile* toxin A IgG instead of anti-TcsL IgG.

2.10 Western immunoblotting

Thirty micrograms of protein from toxin preparations (generated as above) were subjected to gel electrophoresis, then transferred to nitrocellulose membranes that were incubated with primary antibodies to TcsL (mouse monoclonal; 1:250 dilution). Membranes were incubated with peroxidase-conjugated secondary antibodies then visualized by an ECL chemiluminescence kit (Amersham).

2.11 PCR

DNA was isolated from *C. sordellii* cultures using the protocol for bacterial cells in the Easy-DNA™ Kit (Invitrogen). PCR amplifications were performed on each *C. sordellii* strain using the primers for the appropriate encoding gene listed in Table 1.

2.12 Statistical analysis

Comparisons between 2 groups were performed with Student's *t*-test; comparisons among ≥ 3 experimental groups were performed with analysis of variance (ANOVA) with Bonferroni correction as indicated. Differences in survival were compared with a Mantel-Cox log-rank test. Differences were considered significant if $P \leq 0.05$.

3. Results

3.1 *C. sordellii* clinical strain DA-108 causes non-lethal infections in rodents

We have previously established a rat model of *C. sordellii* endometritis using the ATCC9714 strain [21]. The LD₅₀ for that model is $\sim 5 \times 10^8$ CFU and an inoculum of $1 \times$

10^{10} CFU killed all infected rats within 24 hrs of infection [21]. We therefore infected rats i.u. with 1×10^{10} CFU of *C. sordellii* DA-108 or ATCC9714 and recorded animal survival (Fig. 1A). While all animals infected with the latter strain died, none of the DA-108 infected rats died and none exhibited typical signs of illness associated with *C. sordellii* infection (bradykinesia, piloerection, ocular dryness, tachypnea). We followed these animals out beyond 3 weeks of infection without observing death or signs of illness (data not shown).

A relatively large bacterial inoculum was used in the rat model. Mice, however, are more susceptible to death from strain ATCC9714 (see below) and have been used in earlier studies of *C. sordellii* pathogenesis [22]. Therefore, female SvEV129 mice were inoculated with 1×10^7 CFU of *C. sordellii* ATCC9714 through an i.p. route and all of the animals died (Fig. 1B). Conversely, no animals infected similarly with DA-108 died or developed clinical signs, even when infected with 10-fold more bacteria (1×10^8 CFU). A new mouse model of *C. sordellii* endometritis was developed in C57BL/6J mice and the LD₅₀ ($\sim 5.1 \times 10^4$ CFU of ATCC9714) was $\sim 10,000$ -fold lower than observed in rats. We infected C57BL/6J mice i.u. with 1×10^7 CFU of *C. sordellii* ATCC9714 and all died within 4 d of infection ($n = 5$), while animals inoculated i.u. with a similar burden of DA-108 survived (data not shown). These data demonstrate that the clinical *C. sordellii* strain DA-108 is not acutely lethal to rats or mice in models of endometritis or peritonitis.

3.2 The secreted toxins of *C. sordellii* strain DA-108 are non-lethal in mice

To determine whether the lack of virulence observed with DA-108 was due to a lack of expression of secreted toxins, we injected female SvEV129 mice i.p. with a 1:5 dilution of a filtered culture supernatant prepared from this strain or ATCC9714, as described in *Materials and Methods*. We observed that no animals died when injected with filtered culture supernatants from the DA-108 strain while all animals administered supernatants from ATCC9714 died very rapidly, often within 10 hr of injection (Fig. 1C). We confirmed the importance of secreted toxins in the pathogenesis of rapidly lethal *C. sordellii* endometritis using a polyclonal goat anti-toxin raised against crude *C. sordellii* toxins to protect animals from death after infection with the ATCC9714 strain. As observed (Fig. 1D), the anti-toxin prevented the acute lethality of uterine infection from the ATCC9714 reference strain. These data suggest that a secreted toxin (or toxins) is responsible for the acute mortality observed in *C. sordellii* infection and that the DA-108 strain is not producing such a factor(s).

3.3 *C. sordellii* strain DA-108 lacks the *tcsL* gene

As the complete genome sequence of *C. sordellii* is not yet available, we used published DNA sequence information for four *C. sordellii* toxins (sordellilysin, phospholipase C, neuraminidase, and TcsL) to determine which of these were present in DA-108. We discovered that DA-108 lacks the *tcsL* gene, but maintains the presence of the other genes (Figs. 2A and B). Four unique primer pairs were used to identify *tcsL* in these strains. We verified the absence of the ~ 250 kDa TcsL protein by immunoblot using crude toxin preparations from the two *C. sordellii* strains (Fig. 2C). Functional phospholipase C (lecithinase) and neuraminidase enzymes were verified for both strains (Figs. 2D and E).

3.4 Lethal toxin is required for rapid death from *C. sordellii*

The absence of TcsL in toxins produced by DA-108 suggests that this toxin is important for causing the toxic shock syndrome characteristic of many *C. sordellii* infections, a speculation supported by animal studies with purified TcsL [23]. However, a direct correlation between the presence or absence of the *tcsL* gene and human disease has not been published. What is more, the role of TcsL in reproductive tract infections remains undefined. We therefore examined the role of TcsL in the lethality of infections caused by

ATCC9714. As shown in Fig. 3A, i.p. injections of the monoclonal anti-TcsL antibody significantly improved the survival of rats and mice following uterine infection with *C. sordellii*. As a complementary approach, immunoprecipitation with the monoclonal anti-TcsL IgG was used to deplete TcsL protein from crude ATCC9714 toxin preparations. An anti-*C. difficile* toxin A IgG was used as a control for these immunoprecipitation experiments, since it should not cross-react with TcsL. Animals receiving TcsL-depleted toxins all survived while control mice all died (Fig. 3B). These data further demonstrate the importance of TcsL in the pathogenesis of rapid death during *C. sordellii* ATCC9714 infection.

3.5 Lethal toxin contributes to rapid accumulation of pleural effusions during endometritis

Mice injected with purified TcsL develop large pleural effusions soon after intoxication [23], predicting that infection with the TcsL-deficient *C. sordellii* strain (DA-108) would cause smaller pleural effusions than those elicited by the TcsL⁺ ATCC9714. Consistent with this, only infection with the ATCC9714 strain caused significant accumulation of pleural effusions within the first 48 hr of i.u. infection (Fig. 3C).

4. Discussion

The pathogenesis of *C. sordellii* infections is poorly understood and only recently has been the subject of detailed study. Such infections are uncommon, but frequently complicated by a refractory toxic shock syndrome that is rapidly lethal [1]. The lack of specific or effective therapy for *C. sordellii*-associated toxic shock syndrome provides the rationale for investigating the pathogenesis of these infections. The present study attempts to better define the contribution of *C. sordellii* toxins in causing rapid lethality. We compared two unique strains of *C. sordellii* both *in vivo* and *in vitro*; a reference (virulent) strain (ATCC9714) and a clinical strain (DA-108) isolated from a patient with disseminated infection not complicated by toxic shock or death. The results support an important role for TcsL in driving the rapid mortality from this rare pathogen. This is the first time, to our knowledge, that a clinical isolate of *C. sordellii* has been studied extensively in animal models of infection and *in vitro* to correlate the presence of virulence factors with clinical features of infection.

The injection of *C. sordellii* toxins into animals causes rapid mortality [22] and studies in the late 1960s identified a discrete lethal toxin as a major causative factor in this mortality [17]. In addition, the injection of purified TcsL into mice recapitulated severe human disease, with rapid death and major vascular leak caused primarily by damage to vascular endothelial cells [23]. However, certain clinical and environmental *C. sordellii* isolates lack the *tcsL* gene [14,24,25], and *tcsL* deficient strains have been isolated from survivors of *C. sordellii* infection [25]. Thus, we questioned whether strain DA-108 lacked the *tcsL* gene.

Surprisingly, the DA-108 strain did not simply demonstrate reduced virulence compared to ATCC9714, but failed to cause death (or obvious sickness behaviors) in mice or rats infected in the uterus or in the peritoneum. Animals infected with DA-108 did not exhibit any of the signs of illness usually observed with inoculations of the ATCC strain [21]. Genetic comparisons between these strains revealed that DA-108 differed from the ATCC9714 isolate by lacking *tcsL*, confirming our hypothesis. We have not identified previous studies comparing *tcsL*⁺ and *tcsL*⁻ strain in animal models of infection. Although we did not find other genetic or phenotypic differences between these bacteria, our comparisons were incomplete in large part because of a paucity of genetic sequence data and reagents available for *C. sordellii*. There are likely to be numerous differences between these strains, some of which might alter virulence. At the present time isogenic mutants cannot be

generated, so strain comparisons such as this one are important for identifying putative genetic determinants of disease.

Investigations with *C. sordellii* ATCC9714 confirmed the importance of toxins in mediating the rapid death associated with infection. A major role of the toxin TcsL in causing refractory toxic shock was supported by *in vivo* studies that removed TcsL or blocked its function. The protection of infected rats from death with serial injections of a goat polyclonal anti-toxin preparation (Fig. 1D), which was not specific for TcsL, demonstrated that rapid lethality is toxin-mediated. When TcsL was specifically depleted by immunoprecipitation from filtered culture supernatant of ATCC9714, the supernatant lost its lethality (Fig. 3B). When we blocked the function of TcsL *in vivo* using serial i.p. injections of the monoclonal anti-TcsL IgG during uterine infection of rats or mice we also largely prevented animal death (Fig. 3A). Why this approach provided incomplete protection is unclear and might be due to partial removal of TcsL in the face of ongoing bacterial propagation *in vivo*, or due to the effects of other virulence factors. Future studies are needed to address this issue. Although a possible limitation of these studies was that crude toxin preparations were used, the results with the monoclonal anti-TcsL IgG strongly support our conclusions and establish that the presence of TcsL is crucial for a rapidly lethal infection to occur.

The pathogenesis of infection caused by several *Clostridium* species is related to the production of large protein cytotoxins [26]. Almost 40 years ago it was suggested that *C. sordellii* produced two distinct toxins, an edema-producing TcsL and a hemorrhage-inducing TcsH [17]. To date there has been greater characterization of TcsL, including characterization of the *tcsL* gene encoding this toxin. On the other hand, TcsH activity has been described via biochemical and antigenic similarity with *C. difficile* toxin A [27], but as yet the genetic basis for this toxic activity has not been described. Investigations led by other groups suggest that both TcsH and TcsL are important determinants of severe disease, conclusions based largely on *in vivo* animal studies using crude or purified toxins [17,27,28]. TcsL was found to be more potently lethal to animals than TcsH [28]. It is noteworthy that there is a lack of specific reagents for TcsH (and no reported gene sequence), so we were unable to accurately test for TcsH in these two strains.

The clinical strain (DA-108) did not induce large pleural effusions in mice 48 hr following uterine infection (Fig. 3C). These findings support a recent mouse study demonstrating that purified TcsL, when injected i.p. into mice, caused rapid third-spacing in association with impaired endothelial cell barriers [23]. Thus, it seems that TcsL is important for endothelial cell damage during infection.

In summary, our studies provide new information about the relative contribution of TcsL to the clinical features of *C. sordellii* infection and the pathogenesis of the rapidly lethal toxic shock syndrome occasionally associated with this increasingly recognized reproductive tract pathogen. These results also suggest that screening pregnant women (or other “at-risk” populations) for *C. sordellii* colonization should include determination of *tcsL* status. Furthermore, targeted immunotherapy against *C. sordellii* TcsL might prove to be an effective therapy for infections complicated by toxic shock.

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Figure 1.

The clinical *C. sordellii* strain DA-108 does not cause acute death in (A) a rat model of endometritis or (B) mouse peritonitis. (A) Rats were inoculated intrauterine with 1×10^{10} CFU *C. sordellii* clinical strain DA-108 or the reference strain ATCC9714. (B) SvEV129 mice were infected intraperitoneally with 1×10^8 CFU *C. sordellii* DA-108 or 10-fold less (1×10^7 CFU) *C. sordellii* ATCC9714. CFU = colony forming units; n = 5 animals per group. (C) Cell-free toxins prepared as described in *Materials and Methods* from strain ATCC9714 caused rapid death in SvEV129 mice following i.p. injection while toxins similarly prepared from the DA-108 strain did not cause death. n = 5 animals per group. (D) Rats were infected with 1×10^{10} CFU *C. sordellii* ATCC9714 admixed with a goat polyclonal anti-toxin and then received 300 μ l once daily i.p. injections of this IgG on days 1, 2, and 3 post-infection as detailed in *Materials and Methods*. n = 5 rats

Figure 2.

The clinical *C. sordellii* strain DA-108 lacks the gene for lethal toxin (*tcsL*). (**A** and **B**) Genetic comparison of virulence factor genes between the DA-108 and ATCC9714 strains. Genomic DNA was isolated and subjected to PCR for the genes encoding the cholesterol-dependent cytolysin (sordellilysin); lecithinase (phospholipase C); neuraminidase; and lethal toxin. Four different primer pairs were used to amplify the lethal toxin gene. (**C**) Western immunoblot detection of TcsL in crude toxin preparations from ATCC9714 and DA-108 strains. The TcsL appears at ~250 kDa. (**D**) Phospholipase C activity was confirmed for both strains using an egg yolk-phospholipid hydrolysis activity assay. Results are the mean $\pm$ half-range for two independent determinations. A.U. = arbitrary units. (**E**) Neuraminidase activities for each strain and a *C. perfringens* neuraminidase (positive control) were determined as described in *Materials and Methods* and data expressed as the mean $\pm$ half-range for two determinations (absorbance units at 560 nm).

Figure 3.

C. sordellii TcsL is important for causing rapidly lethal death during infection. (A) Rats (n = 5 per group) or C57BL/6J mice (n = 10 per group) were infected i.u. with 1×10^{10} or 1×10^6 CFU *C. sordellii* ATCC9714, respectively, admixed with the mouse monoclonal anti-TcsL IgG. Animals then received twice daily i.p. injections of this IgG on days 0, 1, 2, and 3 post-infection as detailed in *Materials and Methods*. Survival at 72 hr (after the last IgG injection) was determined (* $P < 0.05$, ** $P < 0.01$ compared to untreated animals by Mantel-Cox log-rank test). (B) Mice (n = 5 per group) were injected i.p. with a 1:5 dilution of crude toxins prepared from strain ATCC9714 after immunoprecipitation (IP) was performed with anti-TcsL or anti-*C. difficile* toxin A IgG (control). (C) Mice (n = 5 per group) were infected i.u. with 1×10^7 CFU of *C. sordellii* ATCC9714 or DA-108 (or were left uninfected) and pleural fluid volumes were measured as described in *Materials and Methods*. *** $P < 0.0001$ vs. uninfected animals.

Primers used for PCR.

Table

Gene of Interest	Primer sequence	Reference
16S rRNA encoding gene	Forward (8F) = 5'-AGAG TTGATCCTGGCTCAG-3'	[14]
broad 16S rRNA	Reverse (1492R) = 5'-GGTTACCTTGTTACGACTT-3'	
specific 16S rRNA	Forward (CISOR-F) = 5'-TCGAGGCGACCTTTCGG-3'	[6]
	Reverse (CISOR-R) = 5'-CACCACCTGTCACCAT-3'	
<i>tcsL</i>		[14]
lethal toxin		
Primer pair #1	Forward = 5'-GACTGACATATGATGAACCTTAGTTAA CAAAGGCCAA-3'	
	Reverse = 5'-GACTGAGGATCCTTATACTGTATTTTGAGCAAAATC-3'	
Primer pair #2	Forward = 5'-GACTGACATATGCTTGATAAAGATTATGTTTCTAAA-3'	
	Reverse = 5'-GACTGAGGATCCTTAGTCTATTCTGATAATACCAA-3'	
Primer pair #3	Forward = 5'-GACTGACATATGTTTAAATAATCAATAACTTTA-3'	
	Reverse = 5'-GACTGAGGATCCTTACTCACTATTTGCTATAAGAAAT-3'	
Primer pair #4	Forward = 5'-GACTGACATATGGAAGATAATCAACGACAAGTTAAA-3'	[14]
	Reverse = 5'-GACTGAGGATCCTTATTCATACTAACTAATTTCAGC-3'	
<i>cdc</i>	Forward = 5'-GTACATATCCAGGAGCATTACAAC-3'	[6]
cholesterol-dependent cytolysin (sordellilysin)	Reverse = 5'-CCACCATTCCCAAGCAAGACCTGT-3'	
<i>esp</i>	Forward (CLS-F2) = 5'-TAAAGATGCAGTAGCTAATAAGGATTT-3'	[20]
phospholipase C	Reverse (CLS-R2) = 5'-JTCCTGAAAATTTGATCTTCTGAAACC-3'	
<i>nms</i>	Forward = 5'-CTCGAGAGTAATTTAAACACAACCTAATGAACCTCAAAAAAC-3'	[20]
neuraminidase	Reverse = 5ATTGGATCCCTATTTTAAATTTTATTATTCTCAATAGATAAATAAATCTGT-3'	