

RESEARCH ARTICLE

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Clostridium difficile has a single sortase, SrtB, that can be inhibited by small-molecule inhibitors

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Abstract

Background: Bacterial sortases are transpeptidases that covalently anchor surface proteins to the peptidoglycan of the Gram-positive cell wall. Sortase protein anchoring is mediated by a conserved cell wall sorting signal on the anchored protein, comprising of a C-terminal recognition sequence containing an "LPXTG-like" motif, followed by a hydrophobic domain and a positively charged tail.

Results: We report that *Clostridium difficile* strain 630 encodes a single sortase (SrtB). A FRET-based assay was used to confirm that recombinant SrtB catalyzes the cleavage of fluorescently labelled peptides containing (S/P)PXTG motifs. Strain 630 encodes seven predicted cell wall proteins with the (S/P)PXTG sorting motif, four of which are conserved across all five *C. difficile* lineages and include potential adhesins and cell wall hydrolases. Replacement of the predicted catalytic cysteine residue at position 209 with alanine abolishes SrtB activity, as does addition of the cysteine protease inhibitor MTSET to the reaction. Mass spectrometry reveals the cleavage site to be between the threonine and glycine residues of the (S/P)PXTG peptide. Small-molecule inhibitors identified through an *in silico* screen inhibit SrtB enzymatic activity to a greater degree than MTSET.

Conclusions: These results demonstrate for the first time that *C. difficile* encodes a single sortase enzyme, which cleaves motifs containing (S/P)PXTG *in-vitro*. The activity of the sortase can be inhibited by mutation of a cysteine residue in the predicted active site and by small-molecule inhibitors.

Keywords: *Clostridium difficile*, Sortase, Cysteine protease, Fluorescence resonance energy transfer (FRET), Enzyme kinetics, Enzyme inhibitors

Background

Sortases are membrane-bound cysteine transpeptidases that anchor surface proteins to the peptidoglycan cell wall in Gram-positive bacteria. Surface proteins anchored via sortases are often essential virulence factors important in colonization and invasion, evasion of the host immune system, and nutrient acquisition. The sorting process is mediated by a conserved C-terminal cell wall sorting signal on the anchored protein, comprised of a C-terminal recognition sequence (often LPXTG, where X is any amino acid), followed closely by a hydrophobic transmembrane domain and a positively charged tail [1]. A conserved catalytic cysteine residue of the sortase cleaves the LPXTG motif of the polypeptide between the threonine

and glycine residues and covalently attaches the protein to the peptidoglycan [2-6].

There are six described sortase families, A-F, that share amino acid similarity [7]. All catalyze similar transpeptidation reactions, but recognize different substrate motifs and serve different functions within the cell. Class A sortases (SrtA), such as the prototypical *Staphylococcus aureus* Sortase A (SaSrtA), are considered housekeeping sortases as they are capable of anchoring many functionally distinct proteins to the cell wall. SaSrtA, which recognizes an LPXTG motif, is responsible for anchoring a variety of surface proteins involved in adherence and immune response evasion, and is essential for virulence in animal models [8,9]. SrtA orthologues have been found in the genomes of almost all Gram-positive bacteria [8,10-16]. Class B sortases are functionally different from class A in their substrate specificity. In *S. aureus* and *B. anthracis*, the sortase B gene (*srtB*) is part of an iron-

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regulated locus *isd* (iron-responsive surface determinant) responsible for heme-iron transport, and anchors the iron transporter protein, IsdC, by recognizing an NPQTN motif [17,18]. Though mutating *srtB* has no effect on establishing infection, SaSrtB is required for persistence of the bacterium in mice [17].

Clostridium difficile, an anaerobic Gram-positive, spore-forming bacillus, is the leading cause of hospital-acquired infectious diarrhea in North America and Europe. Infection with *C. difficile* can result in a range of clinical presentations, from mild self-limiting diarrhea to the life-threatening pseudomembranous colitis (PMC), known collectively as *C. difficile* infection (CDI) [19]. MLST studies have identified that the *C. difficile* population structure forms at least five distinct lineages that are all associated with CDI [20-22]. Complications of severe CDI can lead to toxic megacolon, bowel perforation, sepsis and death in up to 25% of cases [23]. Broad-spectrum antibiotic usage is the greatest risk factor for development of CDI due to the consequent disruption of the intestinal microflora. Treatment of CDI with metronidazole and vancomycin can exacerbate the problem by continuing to disrupt the intestinal microflora. This leaves the patient susceptible to relapse or re-infection. Approximately one third of patients experience CDI relapse following treatment, and those who relapse have a greater risk of succumbing to the infection [23]. A current imperative is the development of therapies that selectively target *C. difficile*, whilst leaving the intestinal microflora intact.

The *C. difficile* reference strain 630 encodes a single predicted sortase, CD630_27180, which has high amino-acid similarity with SrtB of *S. aureus* and *B. anthracis* [24]. A second sortase encoded within the genome is interrupted by a stop codon prior to the catalytic cysteine and is considered a pseudogene. Thus, in contrast to other Gram-positive bacteria, *C. difficile* appears to have only a single functional sortase. As such, a compound that inhibits the activity of *C. difficile* sortase could target the pathogen without disrupting the numerous Gram-negative bacteria that make up the intestinal flora.

In this study, we demonstrate that the predicted sortase encoded by CD630_27180 recognizes and cleaves an (S/P)PXTG motif between the threonine and glycine residues. The cleavage of this motif is dependent on the conserved cysteine residue at position 209 in the predicted active site of the sortase. We have also identified seven putative sortase substrates, all of which contain the (S/P)PXTG motif. These substrates are conserved among the five *C. difficile* lineages and include potential adhesins, a 5' nucleotidase, and cell wall hydrolases. Furthermore, we identified a number of small-molecule inhibitors by means of an *in silico* screen that inhibit the activity of the *C. difficile* SrtB.

Results

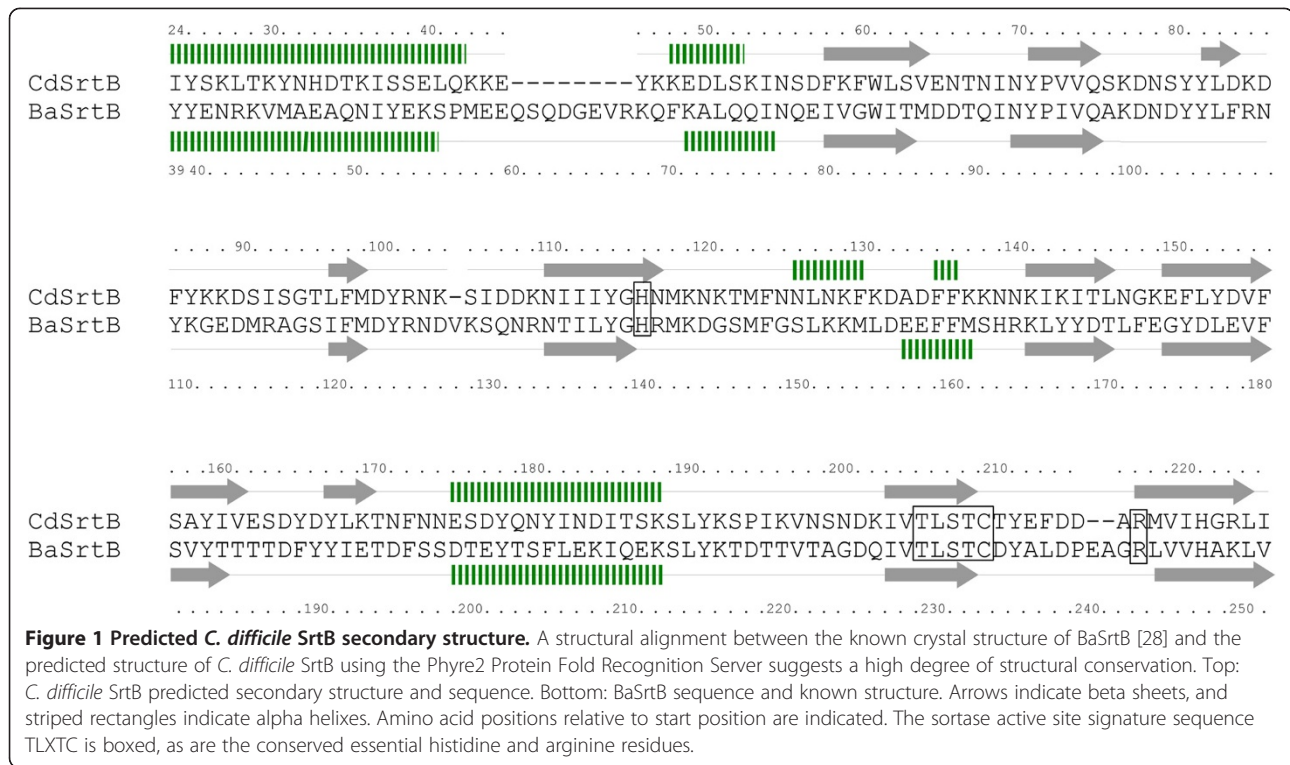
Conservation of the catalytically active residues of sortase

The genome sequence of *C. difficile* strain 630 previously indicated the presence of a single copy of a sortase-like protein, encoded by CD630_27180, based on the presence of the sortase catalytic motif TLXTC [24]. We performed BLAST searches (BlastP) to reveal the protein encoded by CD630_27180 shares 32% and 34% amino acid identity with SrtB from *S. aureus* (SaSrtB) and *B. anthracis* (BaSrtB), respectively. In addition to the TLXTC active site, the catalytically essential histidine (His120 in SaSrtA) and arginine (R197 in SaSrtA) residues [3,25,26] are conserved in the *C. difficile* SrtB. A structural prediction analysis of SrtB was performed using Phyre2 Protein Fold Recognition Server (<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>) [27], and the resulting alignment suggests a high level of conservation between the predicted secondary structure of SrtB and the known crystal structure of the BaSrtB [28] (Figure 1). Expression of *C. difficile* SrtB was analysed *in vitro* using RT-PCR analysis on strain 630, which confirmed that CD2718 is actively transcribed during early exponential, late exponential and stationary phases (Additional file 1: Figure S1).

The *C. difficile* population structure forms at least five distinct clonal lineages that are all associated with human infection [20-22]. To determine whether SrtB is conserved between *C. difficile* strains, representatives for each of the five distinct clades were chosen for analysis based on the availability of a fully annotated sequence: *C. difficile* strains 630 for Clade 1, R20291 and CD196 (RT027) for Clade 2 [29], M68 and CF5 (RT017) for Clade 3 [20], CD305 (RT023) for Clade 4 (unpublished, WTSI), and M120 (RT078) for Clade 5 [20]. BLAST searches of these representative strains show that *srtB* is conserved across all five *C. difficile* lineages. A second sortase-like gene in the 630 genome, classified as a pseudogene because of an in frame stop codon prior to the catalytic cysteine, is absent from the other four *C. difficile* lineages.

Bioinformatic prediction of sortase substrates

A bioinformatics approach was used for the preliminary identification of sortase substrate proteins in *C. difficile* strain 630. The predicted recognition sequence for CD630_27180 has been proposed to be (S/P)PXTG by Pallen *et al.* [11], and recently to also include the sequence NVQTG, found in the surface-associated collagen binding protein CbpA, by Tulli *et al.* [30]. Putative proteins were screened for the patterns (S/P)PXTG and NVQTG at the C-terminus [11,30]. Putative candidates were then assessed for the known features of a sortase substrate: a predicted N-terminal signal peptide sequence, and a cell wall sorting signal comprising of a potential transmembrane domain following the sortase recognition sequence,



and at least two consecutive basic residues (arginine or lysine) at the C-terminus [31-33].

Eight proteins satisfied our definition of a sortase substrate in strain 630 (Table 1). The newly described *C. difficile* collagen binding protein A, CbpA, is the only protein containing the proposed NVQTG motif [30]. The remaining proteins contained one of four observed variations of the (S/P)PXTG motif: SPKTG, PPKTG, and SPSTG and SPQTG. These predicted *C. difficile* sortase substrates are a diverse range of surface proteins that include putative cell wall hydrolases, putative adhesins, a collagen-binding protein, and a 5' nucleotidase/phosphoesterase (Table 1). Transcriptional analysis performed by RT-PCR confirmed that all eight predicted

substrate proteins are transcribed during growth *in vitro* (Additional file 1: Figure S1B-I). The eight predicted substrates are transcribed during all three growth phases examined, with the exception of CD630_25370 and CD630_32460, which do not appear to be transcribed during stationary phase. Four of these putative substrates are conserved across all five *C. difficile* lineages: CD630_01830, CD630_25370, CD630_27680, and CD630_28310.

Purified *C. difficile* SrtB cleaves (S/P)PXTG peptides

To determine whether *C. difficile* SrtB cleaves putative substrates at the predicted motifs, FRET peptides were designed based on the variations observed in the predicted (S/P)PXTG motif (Table 2). Two residues upstream of the

Table 1 Identification of putative *C. difficile* SrtB substrates in strain 630

Protein	Function	C-terminal sorting signal
CD630_01830	Putative cell wall hydrolase	MIH SPSTG KTVSVTSINSSYYTAR FV TAKRIL
CD630_03860	Putative cell surface protein, collagen binding protein	PSD SPKTG DNTNL YGL LALLLTS GAGLAG IFFYKRRKMKKS
CD630_25370	Putative membrane-associated 5'-nucleotidase/phosphoesterase	KEK SPKTG DLGFNSN II FV S STL IC LLNFQKELKDKKSK
CD630_27680	Putative cell-wall hydrolase	FIH SPQTG DVVKVT S MAPGTNY ARR LITATRVLQ
CD630_28310	Putative adhesion, collagen binding protein	PPV PKTG DSTTI IGE ILLV IG AIV L LIVLRNRKNTN
CD630_31450	Collagen binding protein, CbpA	VGQ NVQTG DQSN IM LDL AL MF S LF FL IKNLNTNKYLRRK
CD630_32460	Putative surface protein	IVK SPKTG DETQLMS YV F S VIA IC GLAYQCKIKRN
CD630_33920	Putative cell surface protein, collagen binding protein	PSD SPKTG DSTNL MA FIV M LLVSGGG L AGTYLYKRRKMKKS

Bold = predicted sortase recognition sequence.
 Bold and Italic = hydrophobic residues.
 Italics only = positively charged residues.

Table 2 FRET peptide details

Peptide sequence*	Description
<i>d</i> -IHSPSTGGG- <i>e</i>	Based on CD0183 sequence
<i>d</i> -IHGSSTPGG- <i>e</i>	Control for above peptide
<i>d</i> -SDSPKTGGG- <i>e</i>	Based on CD0386, CD3392 sequence
<i>d</i> -SDGSKTPGG- <i>e</i>	Control for above peptide
<i>d</i> -IHSPQTGGG- <i>e</i>	Based on CD2768 sequence
<i>d</i> -IHGSQTPGG- <i>e</i>	Control for above peptide
<i>d</i> -PVPPKTGGG- <i>e</i>	Based on CD2831 sequence
<i>d</i> -PVGPKTPGG- <i>e</i>	Control for above peptide
<i>d</i> -GQNVQTGGG- <i>e</i>	Based on CbpA sequence
<i>d</i> -QALPETGGG- <i>e</i>	SaSrtA peptide
<i>d</i> -NPQTN- <i>e</i>	SaSrtB peptide
<i>d</i> -IHSPSTGKT- <i>e</i>	Based on CD0183 sequence
<i>d</i> -SDSPKTGDN- <i>e</i>	Based on CD0386 sequence
<i>d</i> -IHSPQTGDV- <i>e</i>	Based on CD2768 sequence
<i>d</i> -PVPPKTGDS- <i>e</i>	Based on CD2831 sequence

*Where *d* is Dabcyl (4-((4-(dimethylamino)phenyl)azo)-benzoyl) and *e* is Edans (5-((2-Aminoethyl)amino)naphthalene-1-sulfonic acid).

motif were included, and two glycine residues were incorporated downstream, as this has been previously shown to improve sortase cleavage efficiency *in vitro* [34]. Fluorescence of the Edans fluorophore within the peptides is blocked when in close proximity to the fluorescent quencher Dabcyl [35]. When the peptide is cleaved, the Edans fluorophore is separated from Dabcyl, and a fluorescent signal is observed.

The N-terminal transmembrane domain of *C. difficile* SrtB (residues 2–25) was replaced with a six-histidine tag (SrtB_{ΔN26}) to improve soluble protein yield. SrtB_{ΔN26} was expressed in *E. coli* NiCo21(DE3) and purified by nickel affinity chromatography from cleared lysates (Figure 2). Purified SrtB_{ΔN26} was then incubated with a

FRET peptide containing the SPKTG sequence. An increase in fluorescence was observed over time, indicating that cleavage of the SPKTG peptide occurred in the presence of SrtB_{ΔN26} over 48 hours (Figure 3). In addition to the SPKTG motif, SrtB_{ΔN26} also cleaved peptides containing the predicted substrate sequences PPKTG, SPSTG, and SPQTG (Figure 4). SrtB_{ΔN26} failed to cleave the scrambled peptide sequences GSKTP, GPKTP, GSSTP and GSQTP (Figure 4). Interestingly, SrtB_{ΔN26} failed to cleave peptides containing the LPETG and NPQTN motifs of SaSrtA and SaSrtB, respectively, and also failed to cleave the proposed sortase recognition motif NVQTG found in the *C. difficile* collagen binding protein, CbpA [30] (Figure 4).

Analysis of FRET reaction

To investigate the importance of the cysteine residue in the proposed active site of *C. difficile* SrtB, site-directed mutagenesis was used to replace the cysteine residue at position 209 with an alanine. When the resulting mutant protein SrtB_{ΔN26,C209A} was incubated with the FRET peptides, the fluorescent signal fell below the limits of detection (Figure 5), indicating that the cysteine residue at position 209 was essential for the activity of the *C. difficile* SrtB. Cleavage in the FRET-based assay was also inhibited by the addition of MTSET (Figure 5), a known cysteine protease inhibitor and inhibitor of sortase function in *S. aureus* [36,37] and *B. anthracis* [15].

The cleavage of the SPKTG, PPKTG, and SPQTG motifs was enhanced at least two-fold by the addition of the two native amino acids immediately downstream of this sequon (data not shown). Analysis of the FRET reaction with these modified peptides revealed that SrtB_{ΔN26} cleaves these peptides between the T and G residues. MALDI analysis of *d*-PVPPKTGDS-*e* peptide incubated with SrtB_{ΔN26} results in a peptide with a mass of 889 Da,

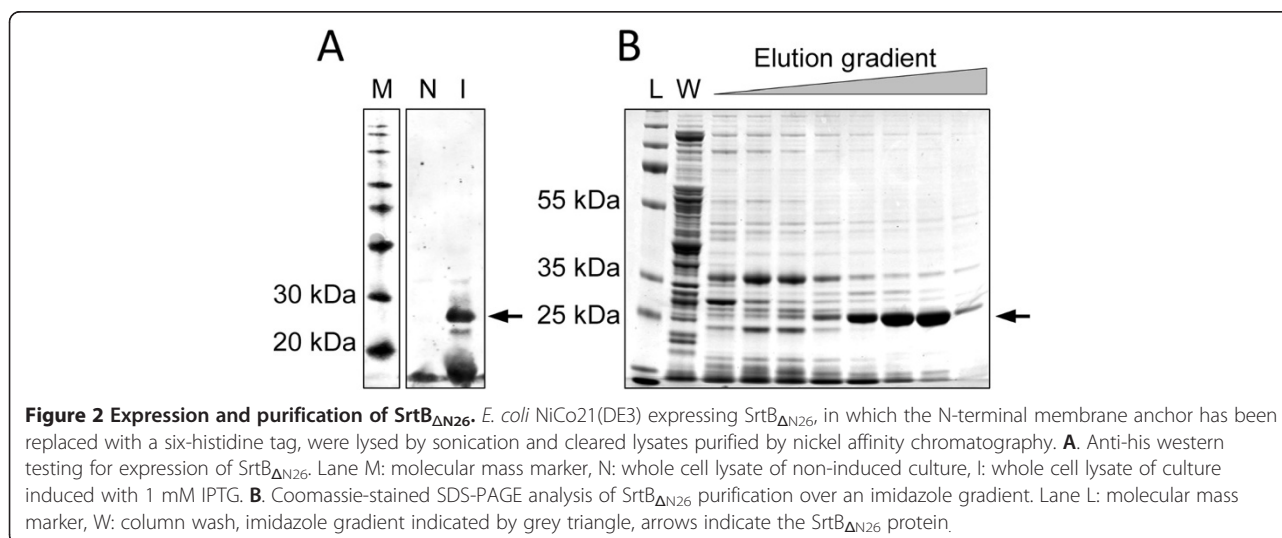


Figure 2 Expression and purification of SrtB_{ΔN26}. *E. coli* NiCo21(DE3) expressing SrtB_{ΔN26}, in which the N-terminal membrane anchor has been replaced with a six-histidine tag, were lysed by sonication and cleared lysates purified by nickel affinity chromatography. **A.** Anti-his western testing for expression of SrtB_{ΔN26}. Lane M: molecular mass marker, N: whole cell lysate of non-induced culture, I: whole cell lysate of culture induced with 1 mM IPTG. **B.** Coomassie-stained SDS-PAGE analysis of SrtB_{ΔN26} purification over an imidazole gradient. Lane L: molecular mass marker, W: column wash, imidazole gradient indicated by grey triangle, arrows indicate the SrtB_{ΔN26} protein.

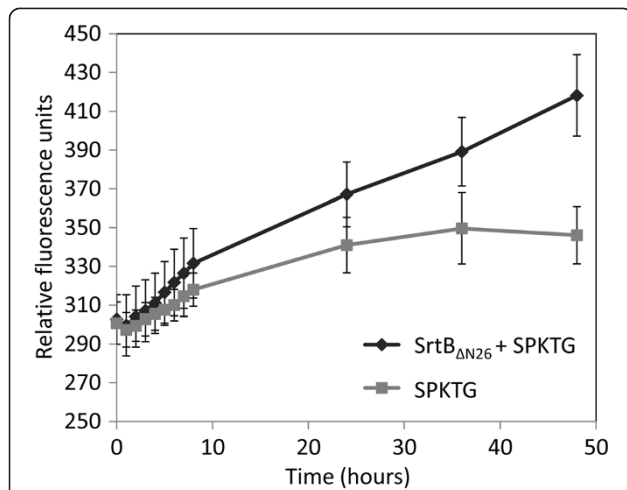


Figure 3 Cleavage of SPKTG peptide by recombinant SrtB_{ΔN26}. Purified recombinant SrtB_{ΔN26} was incubated with a FRET peptide containing the SPKTG motif and fluorescence measured every hour for the first eight hours, and also at 24 h, 36 h, and 48 h. An increase in relative fluorescence units (arbitrary units) was observed, compared with the peptide incubated alone, indicating that SrtB_{ΔN26} mediated cleavage of the SPKTG peptide occurred over a period of 48 hours. Error bars represent the standard error of the mean.

corresponding to the fragment *d*-PVPPKT-OH (Figure 6, top). The peptide control, incubated without SrtB_{ΔN26}, lacked this fragment (Figure 6, bottom). Cleavage between the T and G residues for the *d*-SDSPKTGDN-*e* and *d*-IHSPQTGDV-*e* peptides was also confirmed (data not shown), indicating that *C. difficile* SrtB cleaves the (S/P)PXTG motif between the same residues as other functional sortases [4,15,38,39].

Kinetic measurements of SrtB activity

In order to calculate the *in vitro* kinetic parameters of SrtB_{ΔN26} for the *d*-SDSPKTGDN-*e* and *d*-PVPPKTGDS-*e* peptides, we performed a kinetic analysis of the sortase-

catalyzed hydrolysis reaction. Figure 7A shows the progress curves of the SrtB_{ΔN26} catalyzed hydrolysis reactions at various *d*-SDSPKTGDN-*e* concentrations. For each progress curve, the amount of fluorescent product (after conversion from RFU to concentration) was approximately 5% of the initial substrate concentration. Within the time period analyzed, the progress curves are linear, so the steady state rate (*V*) was determined by fitting the data to a linear function. Figure 7B shows *V* plotted against the concentration of the peptide. Non-linear regression of these data fitted to a modified Michaelis-Menten equation incorporating substrate inhibition (Equation 1):

$$V = \frac{V_{max} \cdot [S]}{K_m + [S] + \frac{[S]^2}{K_i}} \quad (1)$$

Using SciPy 0.11.0 in Python 2.7.3, where V_{max} is the apparent maximal enzymatic velocity, K_m is the apparent Michaelis constant, and K_i is the apparent inhibitor dissociation constant for unproductive substrate binding. This resulted in a K_m of $74.7 \pm 48.2 \mu\text{M}$ and a K_{cat} of $1.1 \times 10^{-3} \pm 6 \times 10^{-4} \text{ min}^{-1}$ for *d*-SDSPKTGDN-*e* (Figure 7B). This analysis was performed for *d*-PVPPKTGDS-*e*, resulting in a K_m of $53.3 \pm 25.6 \mu\text{M}$ and a K_{cat} of $8.3 \times 10^{-4} \pm 3 \times 10^{-4} \text{ min}^{-1}$. SrtB_{ΔN26} is subject to substrate inhibition; at peptide concentrations greater than 30 μM , the rate of SrtB_{ΔN26} activity decreases. Substrate inhibition has previously been observed for other sortase enzymes *in vitro*, and is not expected to be physiologically relevant [40].

Inhibiting SrtB activity

We sought to determine whether *C. difficile* SrtB could be specifically targeted using small-molecule inhibitors. The published crystal structure of the *B. anthracis* SrtB (BaSrtB) [28] was used as a template for the selection of potential *C. difficile* SrtB inhibitors. These orthologous proteins show 70% identity and 90% similarity at the active

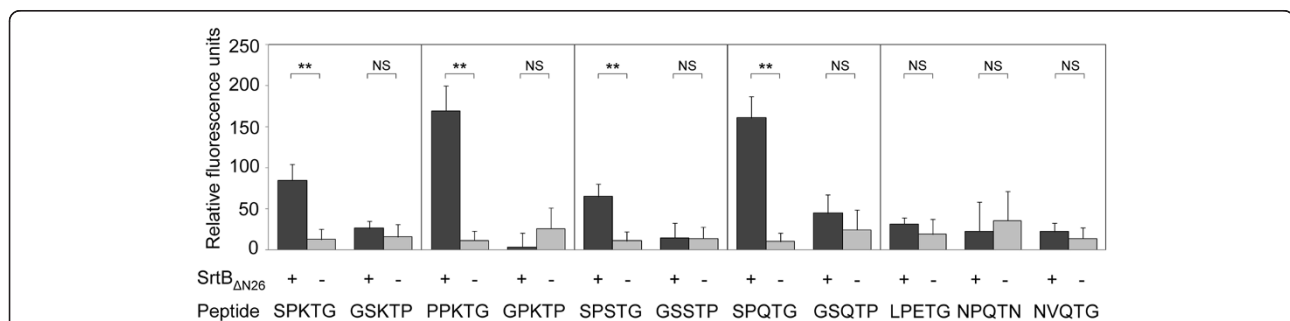
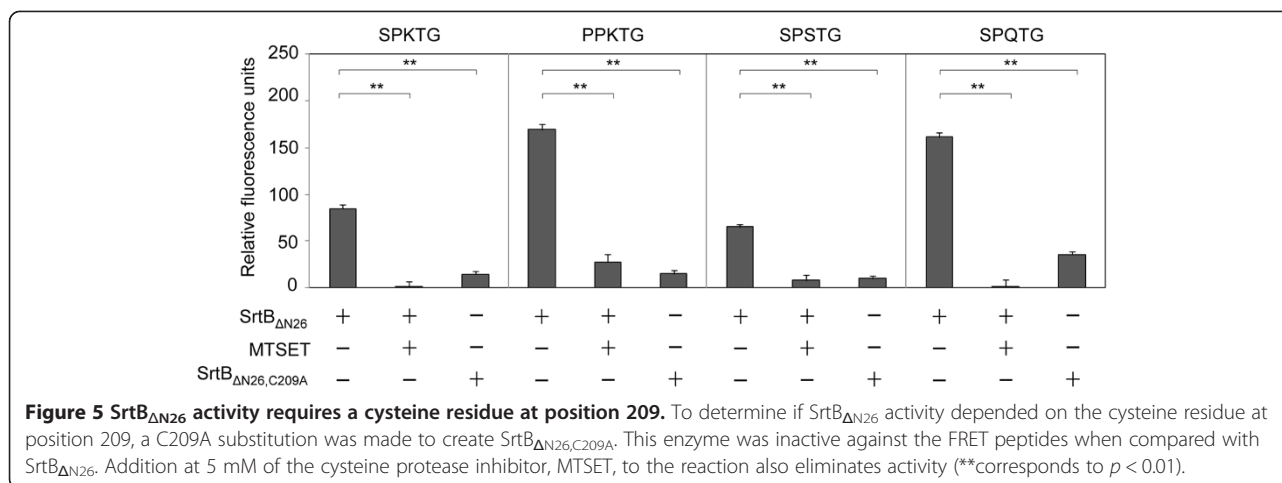


Figure 4 SrtB_{ΔN26} substrate specificity. Purified recombinant SrtB_{ΔN26} protein was incubated with a range of peptide sequences to investigate its substrate specificity. The motifs SPKTG, PPKTG, SPSTG and SPQTG were all recognized and cleaved following incubation with SrtB_{ΔN26}. The scrambled peptide sequences GSKTP, GPKTG, GSSTP, and GSQTP serve as controls for the cleavage specificity of SrtB_{ΔN26}. The sequences LPETG and NPQTN, corresponding to the motifs recognized by *S. aureus* sortase A and B, respectively, do not appear to be substrates for SrtB_{ΔN26}. SrtB_{ΔN26} also failed to cleave the proposed sorting signal NVQTG from recently characterized collagen binding protein, CbpA. Bars indicate the mean, and error bars represent the standard error (**corresponds to $p < 0.01$).



site, and their differences are confined to the periphery of the active site. The proprietary *LeadBuilder* virtual-screening method (Domainex Ltd) was used to interrogate the *PROTOCATS* database of potential protease inhibitors with pharmacophoric and docking filters derived from analysis of the BaSrtB crystal structure. *PROTOCATS* comprises 80,000 commercially-available compounds that may form reversible transition-state-like complexes with protease enzymes. Compounds in *PROTOCATS* contain a carbonyl group which is activated to make a fully reversible complex with the active-site serine/cysteine group by virtue of adjacent moderately electron-withdrawing substituents, which are not leaving groups. Some examples of these functional groups are α -ketoamides and aryl ketones. Figure 8A shows one of the identified compounds docking within the active site structure of BaSrtB.

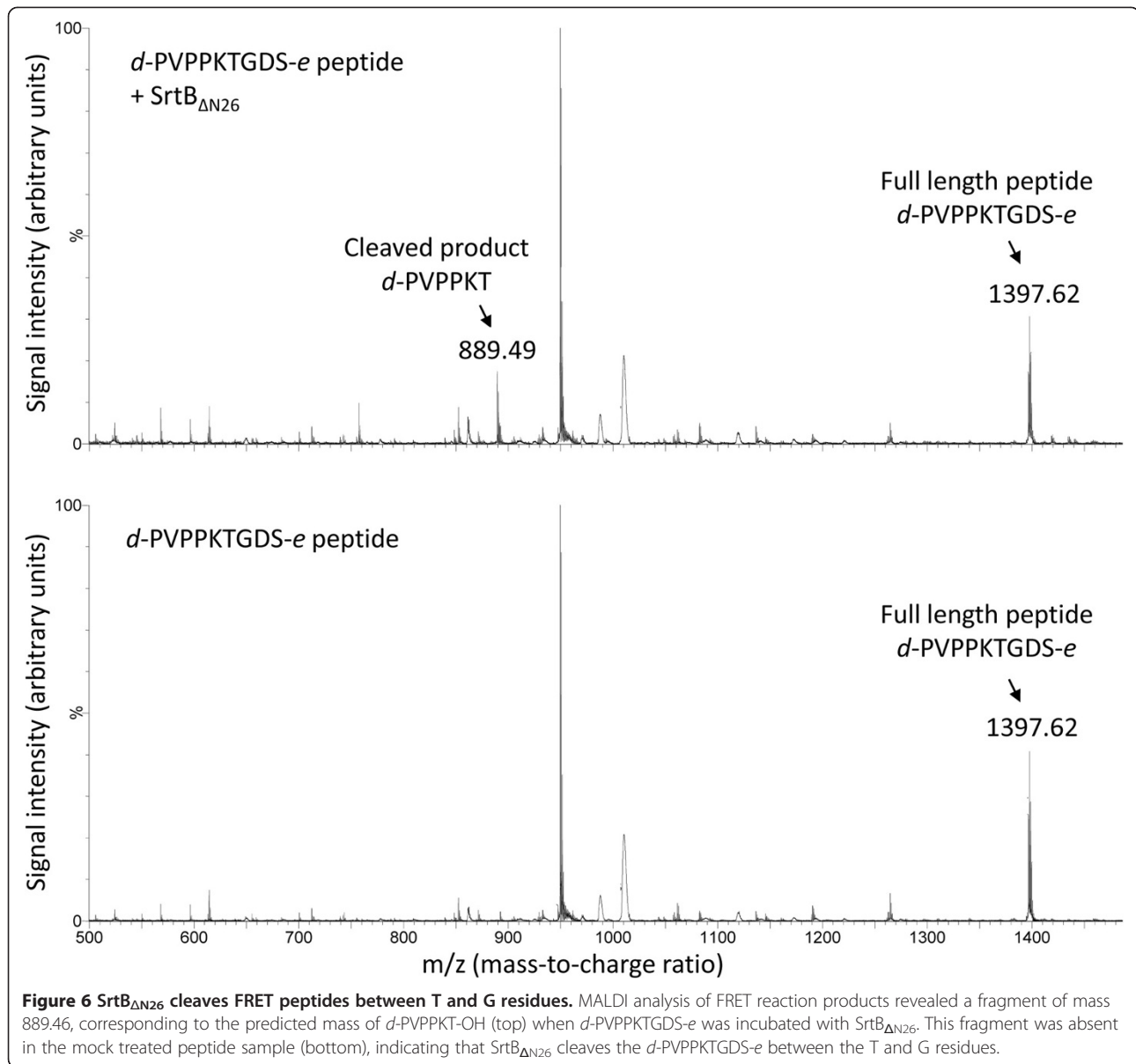
Compounds identified in this screen as potential SrtB inhibitors were tested alongside the cysteine protease inhibitor MTSET at a range of concentrations in the FRET-based assay using the *d*-PVPPKTGDS-*e* peptide to compare IC₅₀ values. Addition of MTSET reduced SrtB_{ΔN26} activity to below the limits of detection at concentrations of 500 μ M and greater. MTSET exhibited an IC₅₀ of $286.7 \pm 16.6 \mu$ M (Figure 8B). A panel of potential *C. difficile* SrtB inhibitors were screened for inhibition of SrtB_{ΔN26} activity. The most effective of the 62 compounds were LSHTM40, LSHTM50, and LSHTM52. They had IC₅₀ values below 100 μ M (Figure 8B, Table 3), at $63.1 \pm 8.8 \mu$ M, $60.1 \pm 4.7 \mu$ M, and $44.1 \pm 6.9 \mu$ M, respectively, showing a good efficacy against *C. difficile* SrtB activity.

Discussion

C. difficile infection is invariably associated with the disruption of the normal intestinal microflora by the administration of broad spectrum antibiotics. Thus there is a pressing need to develop therapies that selectively

target *C. difficile* while leaving the intestinal microflora intact. The *C. difficile* reference strain 630 encodes a single predicted sortase, CD630_27180, which has strong amino acid similarity with SrtB of *S. aureus* and *B. anthracis* [24]. Sortase substrates frequently contribute toward pathogenesis via their involvement in attachment to specific tissues during infection [17,41-44], as well as the bacteria's ability to evade the immune response of the host [32,36]. Sortases, although not essential for growth or viability of the organism, are often essential for virulence in Gram-positive organisms; inactivation of sortases reduces colonization in mice [8,13,44,45], and decreases adhesion and invasion *in vitro* [8,10,14,46,47]. Sortases and their substrates are considered promising targets for the development of new anti-infective compounds [10,14,48]. Unusually for Gram-positive bacteria, *C. difficile* appears to possess a single sortase enzyme that is likely to be important for the viability of the pathogen as we have been unable to construct a *C. difficile* strain 630 SrtB defined mutant (unpublished data). Inhibiting the *C. difficile* sortase could prove to be a strategy to specifically target *C. difficile*.

In this study, we cloned, expressed and characterized the sortase encoded by CD630_27180 of *C. difficile* 630, a predicted class B sortase (SrtB). Sortase nomenclature is based on sequence similarity to the known classes of sortase, A-F [7]. Sortases of class B typically are involved in heme-iron uptake and tend to be expressed in operons with their substrates [17,18]. Genes encoding class A sortases are not found in proximity to their substrates, which consist of a variety of surface proteins with diverse biological functions. Several exceptions to these rules have already been described, notably a class B sortase that polymerizes pilin subunits in *S. pyogenes* [49], and a class E sortase from *C. diphtheriae* that serves a housekeeping function [50]. The potential *C. difficile* sortase substrates identified in this paper comprise a diverse

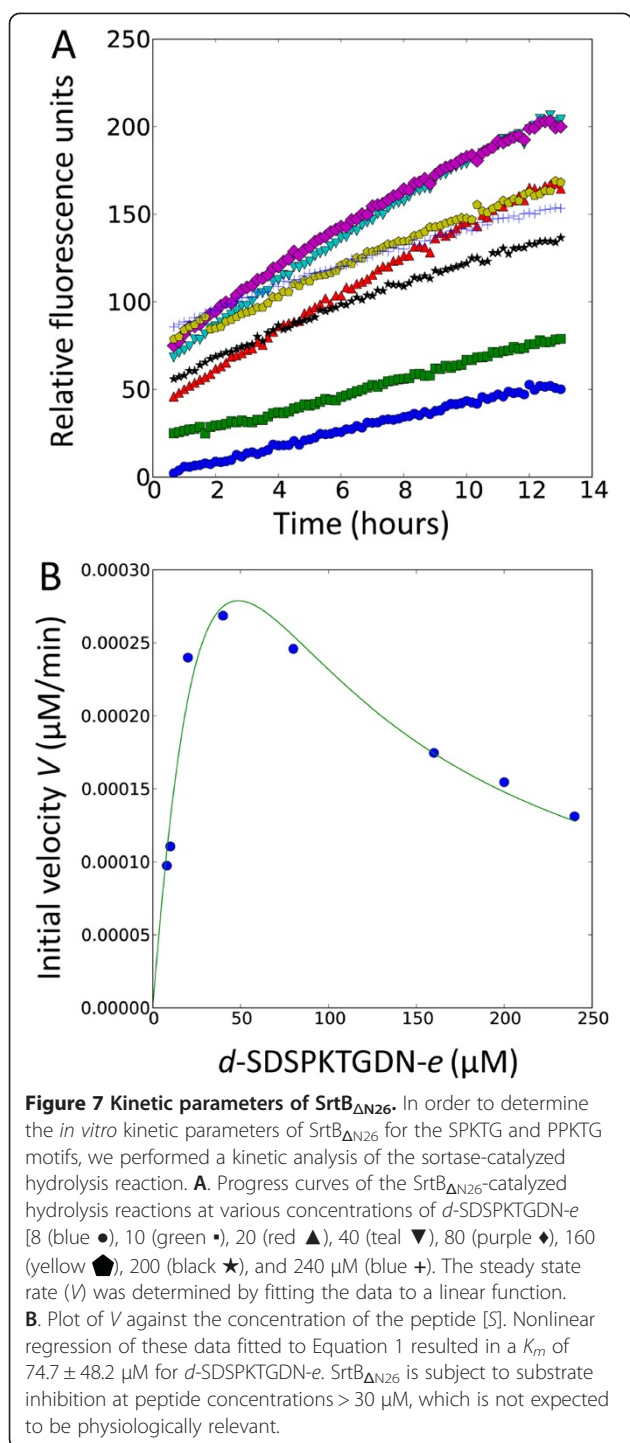


range of surface proteins, suggesting that SrtB may serve as a housekeeping sortase in *C. difficile*, a function usually reserved for class A sortases.

These potential sortase substrates in *C. difficile* strain 630 comprise of seven proteins, all containing an (S/P)PXTG motif, that are predicted to be surface localized and are conserved across *C. difficile* strains. Recently it was proposed that a *C. difficile* collagen binding protein, CbpA, may be sorted to the cell surface by sortase recognizing an NVQTG motif [30]. In this study, we developed a FRET-based assay to demonstrate that SrtB of *C. difficile* recognizes and cleaves the (S/P)PXTG motif between the threonine and glycine residues, and that cleavage is dependent on a single cysteine residue at position 209. SrtB_{ΔN26} does not appear to cleave the *S. aureus*

SrtA and SrtB motifs, LPXTG and NPQTN, respectively, nor the NVQTG motif *in vitro*, suggesting that CbpA from *C. difficile* may be attached to the cell surface by another mechanism.

The FRET-based assay enabled us to determine kinetic parameters for the recombinant *C. difficile* SrtB. Although the catalytic activity appears low, low catalytic efficiency is observed for most sortases *in vitro* [40,51]. The kinetic and cleavage data we report for SrtB_{ΔN26} is consistent with this trend. *In vivo*, the sorting motifs are part of a larger protein, and the transpeptidation substrates are part of a cell wall precursor or mature peptidoglycan [5,6,39]. The transpeptidation reaction has been observed *in vitro* for sortases from bacteria with a Lys-type peptidoglycan, where cross-linking occurs through a peptide



bridge [52,53] such as *S. aureus* and *Streptococcus* species [4,40,54], but not for bacteria with Dap-type peptidoglycan such as *Bacillus* with direct cross-linkages through *m*-diaminopimelic acid [55]. The likely cell wall anchor of the *C. difficile* SrtB substrates is the diaminopimelic acid cross-link [56], similar to *Bacillus*. When transpeptidation is observed *in vitro*, the cleavage efficiency of sortase increases.

This study revealed that recombinant SrtB_{ΔN26} cleaves the (S/P)PXTG motifs with varying levels of efficiency, cleaving the sequences PPKTG and SPQTG with the greatest efficiency. Apparent preferential cleavage efficiency of certain substrate sequences *in vitro* has been observed in other sortases. For example, in *B. anthracis*, BaSrtA cleaves LPXTG peptides more readily than a peptide containing the sequence LPNTA [15]. The biological significance of this peptide sequence preference is unknown.

Small-molecule inhibitors with activity against SrtA and SrtB have been reported that prevent cleavage of fluorescently-labelled peptide compounds by sortase *in vitro* [57]. These compounds inhibit cell adhesion to fibronectin, yet, they have no effect on *in vitro* growth. Inhibitors tested against SrtA, SrtB and SrtC in *B. anthracis* irreversibly modified the active cysteine residue [58]. Several compounds identified in this study had an inhibitory effect on *C. difficile* SrtB activity. However, these lead compounds had no direct effect on *in vitro* *C. difficile* growth (data not shown), which is consistent with observations in *S. aureus* [57]. Inhibition of bacterial growth is not considered vital in the development of sortase-based drug therapies. In both *Staphylococcus* and *Bacillus*, sortase inhibitors show good suitability for further development as therapeutics despite their lack of bactericidal activity. When mice challenged with *S. aureus* were treated with sortase inhibitor compounds, infection rates and mortality were reduced [59], despite these compounds having no effect on staphylococcal growth [57]. The use of *in silico* approaches such as the LeadBuilder method employed by this study to screen databases of putative small-molecule inhibitors for further analysis has been validated. Further analysis of the structural similarities between the hit compounds could lead to a refinement of SrtB inhibitor design and increased potency *in vitro*.

Conclusions

In conclusion, we demonstrate that *C. difficile* encodes a single sortase, SrtB, with *in vitro* activity. We have confirmed the *C. difficile* SrtB recognition sequence as (S/P)PXTG, and show that *C. difficile* SrtB cleaves the (S/P)PXTG motif within peptides between the threonine and glycine residues. The cysteine residue within the predicted active site is essential for activity of the enzyme, and the cleavage of fluorescently-labelled peptides can be inhibited by MTSET, a known cysteine protease inhibitor. SrtB inhibitors identified through our *in silico* screen show a greater level of efficacy than MTSET at inhibiting the protease activity of *C. difficile* SrtB. Such inhibitors provide a significant step in successfully identifying *C. difficile* SrtB inhibitor compounds, which can be further refined to enhance their efficacy, and may

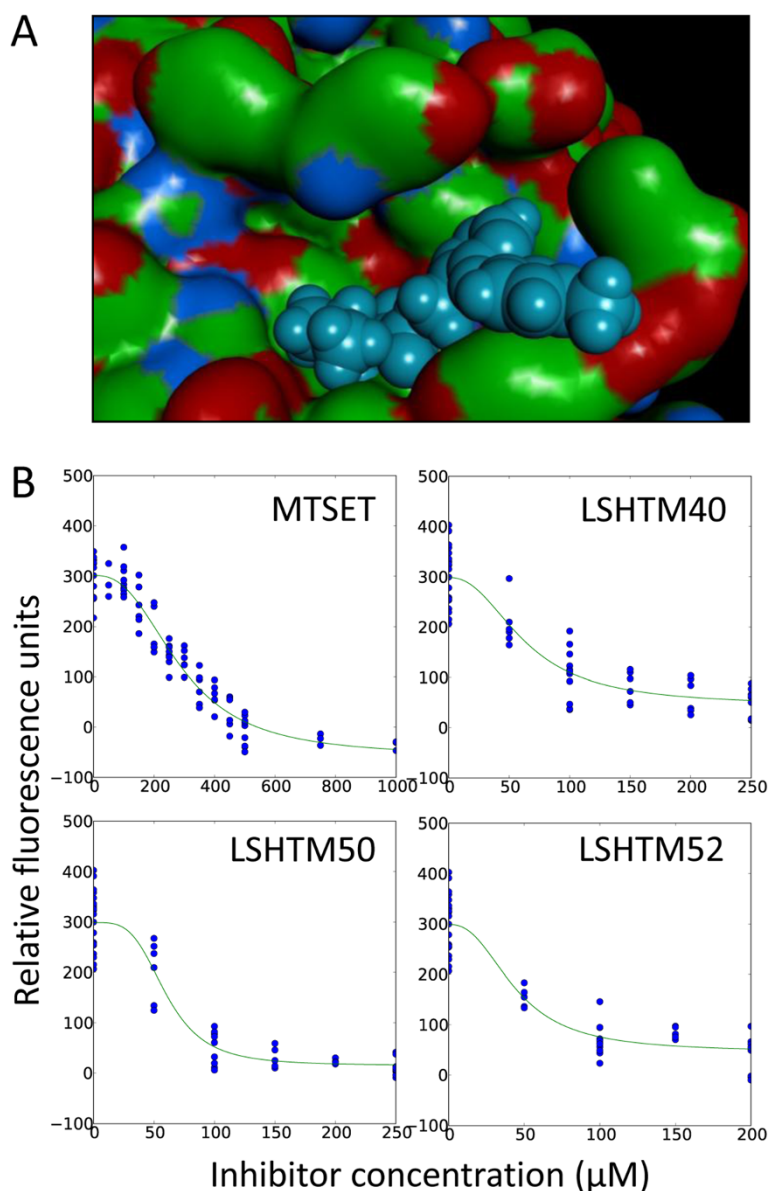


Figure 8 SrtB Δ N₂₆ activity can be inhibited by rationally designed inhibitors. The proprietary *LeadBuilder* virtual-screening method (Domainex Ltd) was used to screen a database of 80,000 potential protease inhibitors, *PROTOCATS*, with pharmacophoric and docking filters derived from analysis of the BaSrtB crystal structure [28]. **A.** Space filling model showing one of the hit compounds fitting into the active site of BaSrtB and interacting with the catalytic cysteine residue. **B.** MTSET and the hits from the virtual screen were tested in the FRET-based assay at varying concentrations to screen for inhibition of SrtB Δ N₂₆ mediated cleavage of *d*-PVPPKTDGDS-*e*. The most effective compounds were LSHTM40, LSHTM50, and LSHTM52, which had IC₅₀ values of 63.1 ± 8.8 , 60.1 ± 4.7 and 44.1 ± 6.9 μ M, respectively. The IC₅₀ for MTSET was 286.7 ± 16.6 μ M, indicating its inhibitory effect on SrtB Δ N₂₆ is less potent than the three identified compounds.

contribute towards the development of novel selective therapeutics against CDI.

Methods

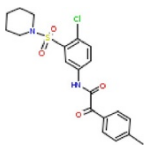
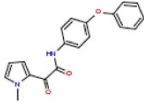
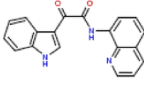
Bacterial culture

C. difficile strain 630 [24] was cultured on Brazier's agar (BioConnections) supplemented with 4% egg yolk (BioConnections) and 1% defibrinated horse blood (TCS Biosciences Ltd.). Liquid cultures were grown in brain

heart infusion broth (Oxoid Ltd.) supplemented with 0.05% L-cysteine (BHIS broth). All media was supplemented with *C. difficile* antibiotic supplement (250 μ g/ml D-cycloserine and 8 μ g/ml ceftioxin, BioConnections). *C. difficile* cultures were incubated at 37°C for 24–48 hours in a Whitley MG500 anaerobic workstation (Don Whitley Scientific Ltd.).

One Shot Top10[®] (Invitrogen) and XL-1 Blue (Agilent) *Escherichia coli* were used for all cloning steps, and

Table 3 Structure of most effective inhibitors of SrtB_{ΔN26}

Compound	Structure	IC50
LSHTM-0040		63.1 ± 8.8 μM
LSHTM-0050		60.1 ± 4.7 μM
LSHTM-0052		44.1 ± 6.9 μM

NiCo21(DE3) *E. coli* (NEB) was used for the expression of recombinant proteins [60]. *E. coli* strains were grown at 37°C on Luria-Bertani (LB) agar (Novagen) or in LB broth (Difco). Media was supplemented with 100 μg/ml ampicillin or 50 μg/ml kanamycin as required.

Genomic DNA isolation

Genomic DNA was isolated from *C. difficile* strain 630 [24,61] by phenol chloroform extraction as previously described [29] and used as a template for cloning. The annotated genome sequences from *C. difficile* strains R20291 and CD196 (RT027) [29], M68 and CF5 (RT017) [20], M120 (RT078) [20], and CD305 (RT023) (unpublished, Wellcome Trust Sanger Institute) were used for analysis.

Identification of sortase substrates

All proteins encoded by *C. difficile* strain 630 [24,61] were searched for the patterns (S/P)PXTG [11] and NVQTG [30] positioned 17–45 amino acid residues from the C-terminus [31]. The resulting candidate protein list was assessed for the known features of a sortase substrate: (i) a suitable N-terminal signal peptide sequence, as predicted by SignalP 4.1 Server (<http://www.cbs.dtu.dk/services/SignalP/>) [62], (ii) a potential transmembrane segment following the C-terminal “LPXTG-like” sequence, as predicted by TMHMM Server v. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>) [63], and (iii) at least two consecutive basic residues (arginine or lysine) at the C-terminus [31-33].

Genetic manipulation

A list of primers and plasmids used in this study can be found in Tables 4 and 5, respectively. The coding

Table 4 Primers used in this study

Primer	Sequence
pET_3	gatattccatggatgaagaactgtaccgtatcggtatc
pET_4	gatgagctcgaggatcagacgaccgtggataacc
pET_17	gatataccatggatgcaccaccaccaccactctaaactgaccaata caaccacgacac
pET_16	gatgagctcgagtagatcagacgaccgtggataac
C209A	tcgttaccctgtctaccgccacctagcaattcgacg
C209A_antisense	cgctcaattcgtagggtggcgtagacagggtaacga
T7F	taatacgactcactataggg
T7R	gctagtattgctcagcgg

sequence for *srtB* (CD630_27180) was codon-optimized for expression in *E. coli* by Celtek Bioscience, LLC (Nashville, TN, USA). The resulting fragment was cloned into the NcoI/XhoI sites of pET28a using primers pET_3 and pET_4 to create pET28a-*srtB*. To improve soluble SrtB yield, the N-terminal transmembrane anchor domain (residues 2–25) was replaced with a six-histidine tag. The truncated gene *srtB*_{ΔN26} was amplified from pET28a-*srtB* using primers pET_17 and pET_16, and cloned into the NcoI/XhoI sites of pET28a to create pET28a-*srtB*_{ΔN26}. The mutant protein SrtB_{ΔN26,C209A} was generated using the QuikChange Site-Directed Mutagenesis kit (Agilent) in accordance with the manufacturer's instructions using pET28a-*srtB*_{ΔN26} as a template and primers C209A and C209A_antisense. Successful construction of recombinant plasmids was confirmed by DNA sequencing using primers T7F and T7R (Source BioScience).

RT-PCR analysis

Total RNA was isolated from *C. difficile* 630 grown in BHIS at early exponential phase (t = 3 hours, OD₆₀₀ = 0.4-0.5), late exponential phase (t = 5 hours, OD₆₀₀ = 0.8-0.9), and stationary phase (t = 24 hours, OD₆₀₀ = 0.6-0.8) using RNAprotect Bacteria Reagent (Qiagen) and the FastRNA Pro Blue Kit (MP Biomedicals LLC., Illkirch,

Table 5 Plasmids used in this study

Plasmid	Description	Reference
pQE30Xa- <i>srtB</i>	Codon optimized <i>srtB</i> , synthesized and cloned in pQE30xa	Obtained from Celtek Bioscience, LLC
pET28a	Commercial protein expression vector	Provided by Neil Fairweather
pET28a- <i>srtB</i>	Codon optimized <i>srtB</i> cloned in pET28a	This work
pET28a- <i>srtB</i> _{ΔN26}	<i>srtB</i> with residues 2–25 replaced with a six-histidine tag	This work
pET28a- <i>srtB</i> _{ΔN26,C209A}	Same as above, with C209A substitution	This work

France) in accordance with the manufacturer's instructions. Genomic DNA was removed from total RNA samples using TURBO DNase (Life Technologies). Equal amounts of RNA were reverse transcribed into complementary DNA (cDNA) for expression analysis. Briefly, one μg random primers (Invitrogen) and 40 units RNasin Plus RNase inhibitor (Promega) was added to one μg RNA in a final volume of 10 μl , and incubated at 65°C for 10 min followed by room temperature for 30 min. The following first-strand mixture was added for cDNA synthesis: four μl of 5x first-strand buffer (Invitrogen), two μl 0.1 M DTT (Invitrogen), two μl 10 mM dNTP mix (New England BioLabs), and 1.5 μl Superscript II (Invitrogen). The reaction mixture was incubated at 25°C for 10 minutes, 42°C for 1 h, and finally 70°C for 15 minutes. RT-PCRs were performed with gene specific primers (Additional file 2: Table S1) using cDNA as a template.

Purification of recombinant protein

Expression constructs were transformed into *E. coli* NiCo21(DE3) (NEB). Cultures grown at 37°C were induced for expression with 1 mM IPTG when the OD₆₀₀ reached 0.6, and harvested after 5 hours. Cell pellets were resuspended in lysis buffer [1x Bugbuster (Novagen), 50 mM NaH₂PO₄, 300 mM NaCl, 40 mM imidazole, 1 mM DTT, 1 mg/ml lysozyme, and 25 U/ml Benzonase nuclease (Novagen) (pH 7.5)]. Lysates were sonicated on ice for 2 min (15 sec on/off) at 50% Vibra Cell™ high intensity ultrasonic processor (Jencon, Leighton Buzzard, Bedfordshire, UK) before centrifugation at 10,000 rpm for 45 min. The supernatant was passed through a 0.22 μm filter before applying to a 1 ml HisTrap HP column (GE Healthcare), pre-equilibrated with buffer (50 mM NaH₂PO₄, 300 mM NaCl, 40 mM imidazole, 1 mM DTT, pH 7.5). SrtB_{ΔN26} was eluted with an imidazole gradient (40 – 500 mM) over 25 column volumes. Fractions containing SrtB_{ΔN26} (as identified by SDS-PAGE) were pooled and injected onto a HiLoad 16/60 Superdex 200 column (GE Healthcare) pre-equilibrated with buffer F (5 mM CaCl₂, 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM DTT). Eluted fractions containing SrtB_{ΔN26} were pooled and concentrated using an Amicon Ultra-15 (10 kDa) centrifuge filter unit (Millipore). Protein samples were quantified using Bradford reagent (Thermo Scientific) and analyzed by SDS-PAGE. The mutant protein SrtB_{ΔN26,C209A} was expressed and purified following the above method. Expression of SrtB_{ΔN26} and SrtB_{ΔN26,C209A} was confirmed by MALDI fingerprinting.

Immunoblotting

Samples were resolved on Novex NuPage 10% Bis-Tris SDS-PAGE gels (Invitrogen) before transferring to Hybond-C Extra nitrocellulose (GE Healthcare). Membranes were

probed with rabbit antiserum directed against 6xHis-tag (1:5000, Abcam), followed by goat anti-rabbit IRDye conjugated secondary antibody (1:7500, LI-COR Biotechnology). Blots were visualized using an Odyssey near-infrared imager (LI-COR Biotechnology).

In vitro analysis of sortase activity

SrtB_{ΔN26} activity was monitored using a fluorescence resonance energy transfer (FRET) assay [58] in buffer F (5 mM CaCl₂, 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 1 mM DTT). Fluorescently self-quenched peptides tagged with 5-((2-Aminoethyl)amino)naphthalene-1-sulfonic acid (Edans) as a fluorophore and 4-([4-(Dimethylamino)phenyl]azo)benzoic acid (Dabcyl) as a quencher [35], and containing the predicted sorting signals of SrtB were purchased from Protein Peptide Research Ltd and solubilized in DMSO (Table 2). The FRET-based assay was performed in a final volume of 100 μl buffer F containing 10 μM SrtB_{ΔN26} and 20 μM fluorogenic peptide in clear-bottomed, black polystyrene 384-well plates (Nunc). Plates were incubated for 48 hours at 37°C, during which fluorescence (excitation = 340 nm, emission = 490 nm) was measured using a Spectra-Max M3 plate reader (Molecular Devices). Five mM 2-(trimethylammonium)ethylmethanesulfonate (MTSET, Affymetrix) was added to the reaction as indicated. Each experiment was performed in triplicate with a minimum of three biological replicates, and the results are presented as the means and the standard error of the data obtained. The two-tailed Student's T-test was used to analyze the data. MALDI analysis of FRET reaction samples was performed by the Protein and Nucleic Acid Chemistry Facility (University of Cambridge) to determine exact cleavage site within each peptide.

Kinetic measurements

Kinetic data for SrtB_{ΔN26} were obtained by incubating varying concentrations of peptide (8, 10, 20, 40, 80, 160, 200 and 240 μM) with 10 μM SrtB_{ΔN26}. All reactions were performed as described above, with fluorescence monitored every ten minutes over a 13 hour period. To correlate fluorescence signal, expressed as arbitrary relative fluorescence units (RFU), with the concentration of product formed, standard curves of the fluorophore Edans were collected. The linear segment of the fluorophore standard curve generated a conversion ratio of 703.9 RFU/ μM Edans. Initial velocities (V) were determined from the progress curves and plotted against substrate concentration [S]. The data were fitted to a modified version of the Michaelis-Menten equation incorporating substrate inhibition using SciPy 0.11.0 in Python 2.7.3, where V_{max} is the maximal enzymatic velocity, K_m is the Michaelis constant, and K_i is the inhibitor dissociation constant for unproductive substrate

binding. All data points were collected in triplicate, and the overall assay was run in duplicate.

Identification of SrtB inhibitors

The proprietary *LeadBuilder* virtual screening method (Domainex, Ltd) was used to interrogate a database (*PROTOCATS*) of 80,000 potential compounds which had been pre-selected as protease inhibitors. The virtual screening protocol used pharmacophoric and docking filters derived from analysis of the BaSrtB crystal structure (with which the *C. difficile* SrtB shows 70% identity and 90% similarity at the active site). Sixty-two compounds identified in this screen as potential SrtB inhibitors were obtained from Enamine, ChemBridge, and Key Organics, and solubilized in DMSO. Selected compounds and MTSET were incubated with 10 μ M SrtB _{Δ N26} at a range of concentrations in the FRET-based assay conditions described above, so that final DMSO concentrations were \leq 3.75%, a concentration shown to have no significant effect on control fluorescence (data not shown). IC50 values were determined by non-linear least squares fit to a four parameter sigmoidal function using SciPy 0.11.0 in Python 2.7.3.

Additional files

Additional file 1: Figure S1. RT-PCR analysis in *C. difficile* strain 630 of CD2718 and its predicted substrates. PCR reactions were performed with 630 cDNA that was prepared from cultures grown to early exponential (E), late exponential (L) and stationary phase (S). M = Hyperladder I (Bioline), G = 630 genomic DNA, W = dH₂O. A "+" indicates cDNA reaction with added reverse transcriptase, "-" indicates cDNA reaction without added reverse transcriptase (control for DNA depletion of RNA sample).

Additional file 2: Table S1. Primers used for RT-PCR analysis.

Abbreviations

SrtA: Sortase A; SrtB: Sortase B; FRET: Fluorescence resonance energy transfer; MTSET: 2-(trimethylammonium)ethylmethanethiosulfonate; Dabcyl or *d*: 4-([4-(dimethylamino)phenyl]azo)-benzoyl; Edans or *e*: 5-((2-Aminoethyl)amino)naphthalene-1-sulfonic acid; CDI: *C. difficile* infection; IPTG: Isopropyl β -D-1-thiogalactopyranoside; RFU: Relative fluorescence units.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Laboratory work: EHD; experimental design: EHD, LFD, EV, SFC, MRM, EL, TRP, BWW; writing of manuscript: EHD, LFD, BWW. All authors read and approved the final manuscript.

Acknowledgments

We thank Jun Wheeler for MALDI mass spectrometry fingerprinting analysis of recombinant proteins; Mark Donahue for assistance with data analysis; Hayley Angove and Wendy Savory for assistance with development of the FRET-based assay and sortase protein expression, respectively. We thank Neil Fairweather, Johann Peltier, Helen A. Shaw and Madeleine Moule for critical reading of the manuscript.

Funding

This research was supported by funding from Wellcome Trust grant number 086418/Z/ and MRC grant number 499 94717.

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Received: 6 June 2014 Accepted: 12 August 2014

Published: 31 August 2014

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doi:10.1186/s12866-014-0219-1

Cite this article as: Donahue *et al.*: *Clostridium difficile* has a single sortase, SrtB, that can be inhibited by small-molecule inhibitors. *BMC Microbiology* 2014 **14**:219.

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