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Clostridium difficile has two parallel and essential Sec secretion systems

Robert P. Fagan and Neil F. Fairweather
Division of Cell and Molecular Biology, Centre for Molecular Microbiology and Infection, Imperial College London, London SW7 2AZ, United Kingdom
Running head: Characterization of the *C. difficile* accessory Sec system

Address correspondence to: ***current address*** Robert P. Fagan, Dept. of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield, S10 2TN, United Kingdom; r.fagan@sheffield.ac.uk; Tel +44(0)114 222 4182

Protein translocation across the cytoplasmic membrane is an essential process in all bacteria. The Sec system, comprising at its core an ATPase, SecA, and a membrane channel, SecYEG, is responsible for the majority of this protein transport. Recently, a second parallel Sec system has been described in a number of Gram-positive species. This accessory Sec system is characterized by the presence of a second copy of the energizing ATPase, SecA2, and, where it has been studied, is responsible for the translocation of a subset of Sec substrates. In common with many pathogenic Gram-positive species, *Clostridium difficile* possesses two copies of SecA. Here we describe the first characterization of the *Clostridium difficile* accessory Sec system and the identification of its major substrates.

Using inducible antisense RNA expression and dominant negative alleles of SecA1 and SecA2, we demonstrate that export of the S-layer proteins (SLPs) and an additional cell wall protein (CwpV) is dependent on SecA2. Accumulation of the cytoplasmic precursor of the S-layer protein SlpA and other cell wall proteins, was observed in cells expressing dominant negative SecA1 or SecA2 alleles, concomitant with a decrease in the levels of mature SLP proteins in the cell wall. Furthermore, expression of either dominant–negative allele or antisense RNA knock-down of SecA1 or SecA2 dramatically impaired growth, indicating that both Sec systems are essential in *C. difficile*.

Clostridium difficile is a spore forming, Gram-positive, anaerobic bacterium and is the major cause of nosocomial antibiotic-associated diarrhoea (1). The importance of this pathogen is highlighted by the recent increased incidence of *C. difficile* infection (CDI) in the US and many European countries (2). Research has focused largely on two toxins (3,4), which cause tissue damage, neutrophil recruitment and a severe inflammatory response (5). However, other aspects of *C. difficile* virulence are poorly understood. In particular, little is known about the mechanisms of gastrointestinal colonisation, an undoubtedly essential step in pathogenesis. Bacterial pathogens generally secrete a subset of proteins that mediate essential functions during pathogenesis such as adhesion to host tissues and modulation of the host immune response (6). The identity of such proteins and the mechanisms of secretion employed by *C. difficile* is currently a subject of active research and several candidate proteins have been identified (see below).

Exterior to a typical Gram-positive cell wall is the *C. difficile* S-layer, a paracrystalline proteinaceous array that surrounds the bacterium (7). The S-layer is comprised of two proteins, the high molecular weight (HMW) and low molecular weight (LMW) S-layer proteins (SLPs), derived by post-translational extracellular cleavage of the

precursor SlpA (8). Following cleavage, the HMW and LMW SLPs form a high affinity heterodimer, the basic subunit of the assembled S-layer (9). In addition to the S-layer, *C. difficile* also possesses a large family of non-covalently anchored cell wall proteins (CWPs) which are related to SlpA and are characterised by the presence of three Pfam04122 cell wall binding motifs (10). Functions have been assigned to a few of these proteins, including Cwp84, the cysteine protease responsible for SlpA cleavage (11-13), CwpV, a phase-variable auto-aggregating factor (14,15) and a putative adhesin, Cwp66 (16). However, despite a number of these proteins being implicated in bacterial-host interactions (16-18), their exact roles in pathogenesis have yet to be elucidated. Cell surface localisation of several CWPs has been demonstrated and antibodies to many CWPs have been found in convalescent sera from CDI patients (19,20), implying at least some CWPs are expressed and surface exposed *in vivo*.

The mechanisms of secretion of the *C. difficile* S-layer and CWPs is not understood. Translocation of proteins across the cytoplasmic membrane is an essential process in bacteria and is largely mediated by the multi-subunit Sec system (21). Alternative secretion systems, such as the twin arginine translocation system (22), have been found in some Gram-positive species but to date no additional secretion systems have been described in *C. difficile*. The essential core components of the Sec system are the heteromeric SecYEG membrane channel and the SecA ATPase. Recently a parallel accessory Sec system has been described in a small number of Gram-positive species (23-27), characterised by the presence of a second copy of SecA, termed SecA2. Some of these species also possess a second copy of SecY (28). Bacteria which possess an accessory Sec system employ it for translocation of a defined subset of proteins, many of which appear to be virulence factors (28). *C. difficile* strain 630 (29) encodes the core components of the canonical Sec secretion system, including the SecYEG membrane channel and also has a second copy of the SecA ATPase, indicative of an accessory Sec system. Of the organisms possessing a SecA2-only accessory Sec system, *Mycobaterium smegmatis* is perhaps the most intensively studied (26,30,31). In *M. smegmatis*, SecA1 and SecA2 have non-redundant functions in protein translocation; SecA1 provides essential housekeeping functions, whereas SecA2 is responsible for the secretion of a small number of lipoproteins (30). Furthermore, the *M. smegmatis* accessory pathway also appears to require SecA1 (31).

Molecular characterisation of *C. difficile* has recently been made possible by the development of basic genetic tools for the manipulation of the organism, including shuttle vectors transferable by conjugation and insertional gene inactivation using group II introns (32,33). Here we describe a controlled, inducible expression system in *C. difficile*, which we use to study the functions of *secA1* and *secA2*. Using antisense RNA and protein expression techniques, we demonstrate that *C. difficile* has two essential Sec systems, a housekeeping system and an accessory system, and we identify SlpA and several CWPs as the major substrates of the accessory system.

Experimental procedures

Strains, plasmids and growth conditions- All plasmids used in this study are described in Table 1, oligonucleotides are listed in Table S1. *E. coli* strains were propagated in LB broth and on LB agar supplemented with antibiotics as appropriate; chloramphenicol (15 μg/ml), kanamycin (50 μg/ml) or carbenicillin (50 μg/ml). *E. coli* strain NovaBlue (Merck Biosciences) was used as a recipient for all cloning and the donor strain CA434 (HB101 carrying R702) was used for conjugation of plasmids into *C. difficile*. *C. difficile* strain 630 (29) was cultured in TY broth without thioglycolate (34), *C. difficile* Defined Medium (CDDM) (35) or BHI broth and on BHI agar or Blood agar supplemented with thiamphenicol (15 μg/ml) where appropriate. Anhydrotetracycline (20-500 ng/ml) was used for induction of the P_{tet} promoter in the *C. difficile* expression vectors described below.

An inducible expression system for C. difficile- A highly optimised *Staphylococcus aureus* tetracycline-inducible promoter (P_{tet}) (36) was adapted for use in *C. difficile*. The entire P_{tet} promoter was amplified from pRMC2 using oligonucleiotides NF1320 and NF1321 and ligated as a blunt-ended fragment into pSTBlue-1 yielding pRPF135. Extraneous *Bam*HI, *Xho*I and *Kpn*I sites were removed by sequential rounds of

inverse PCR and ligation using NF1322 and NF1323, yielding pRPF139, followed by NF1334 and NF1335 to generate pRPF141.

pCBR023 containing the constitutive P_{cwp2} promoter driving a *C. difficile* codon-optimised *gusA* gene in a pUC19 backbone (15) was modified by inverse PCR with NF1326 and NF1328 to replace the *Eco*RI site between the promoter and *gusA* gene with a *Sac*I site, yielding pRPF137. The P_{cwp2} promoter was then excised from pRPF137 and replaced with the modified P_{tet} promoter on a *Kpn*I-*Sac*I fragment yielding pRPF143. The P_{cwp2}-*gusA* and P_{tet}-*gusA* cassettes were then subcloned as *Kpn*I-*Bam*HI fragments into the *E. coli-C. difficile* shuttle vector pMTL960 to produce plasmids pRPF144 and pRPF146 respectively. Two transcriptional terminators were added, one after the *gusA* gene and a second after the divergent *tetR* gene. The terminator which follows the *slpA* gene in *C. difficile* strain 630 was amplified using NF1437 and NF1438 and cloned as a *Bam*HI-*Bst*XI fragment downstream of the *gusA* gene in pRPF146, yielding pRPF177. The *fdx* terminator from *C. pasteurianum* was reconstituted as two annealed oligonucleotides, NF1469 and NF1470, and cloned downstream of the *tetR* gene between *Nhe*I and *Kpn*I, yielding plasmid pRPF185. This stabilised inducible expression plasmid was used for all further studies.

SecA1 and SecA2 expression plasmids- secA2 was amplified from *C. difficile* 630 using oligonucleotides NF1234 and NF1235 and cloned *Eco*RI-*Bam*HI in pCBR023. The resulting plasmid was then modified by inverse PCR using NF1260 and NF1261 to introduce an N-terminal Strep-Tag II (WSHPQFEKLE) after the initiation methionine and again using NF1327 and NF1328 to change the *Eco*RI site between P_{cwp2} and *secA2* to *Sac*I. The resulting P_{cwp2}-*secA2* cassette was subcloned into pMTL960 between *Kpn*I and *Bam*HI yielding pRPF150. The K106R mutation was introduced by inverse PCR using NF1256 and NF1257 yielding pRPF151. *secA2*(WT) and *secA2*(K106R) were subsequently subcloned (*Sac*I-*Bam*HI) into pRPF185 generating pRPF186 and pRPF187 respectively. *secA1* was amplified from *C. difficile* 630 using oligonucleotides NF1511 and NF1513 and this product used as a template for a second PCR with NF1512 and NF1513 to add an N-terminal Strep-Tag II. The resulting product was cloned as a blunt fragment into pSTBlue-1. The K106R mutation was introduced by inverse PCR using NF1514 and NF1515. *secA1*(WT) and *secA1*(K106R) were subcloned (*Sac*I-*Bam*HI) into pRPF185 generating pRPF193 and pRPF194 respectively.

Antisense RNA expression plasmids- A 178 bp (NF1582 and NF1583) antisense fragment, spanning from the predicted transcription initiation site of *secA1,* was amplified from *C. difficile* 630 genomic DNA and cloned (*Sac*I-*Bam*HI) into pRPF185 yielding pRPF204. A 600 bp (NF1409 and NF1410) antisense fragment covering the first 300 bp of *secA2* and 300 bp upstream of the gene, was amplified from *C. difficile* 630 genomic DNA and cloned (*Sac*I-*Bam*HI) into pRPF146. The *fdx* transcriptional terminator from *C. pasteurianum* was added downstream of the *tetR* gene as described above yielding plasmid pRPF195.

RT-PCR- C. difficile harbouring pRPF195 or pRPF204 were grown to an optical density at 600 nm ($OD_{600\ nm}$) of 0.3-0.4, induced with 500 ng/ml ATc and harvested 90 min later following stabilisation with RNAprotect (Qiagen). RNA was purified using the FastRNA Pro Blue kit (Bio101 systems), followed by DNase treatment (TURBO DNA-free, Applied Biosystems) and a final clean-up step with the RNeasy MinElute Cleanup kit (Qiagen). RNA concentration and purity was assayed using a NanoDrop ND-1000 spectrophotometer. 260/280 ratios were all > 2.1. To ensure complete removal of DNA a 16S rRNA PCR amplification was carried out with 1 µg RNA. First strand synthesis was carried out using the RETROscript kit (Applied Biosystems) according to the manufacturers instructions with random decamers but without heat denaturation of the RNA template. Expression of the individual antisense RNAs was confirmed by semi-quantitative PCR using antisense-specific oligos to *secA1a* (NF1633 and NF1640) and *secA2a* (NF1636 and NF1639). Quantitative real time PCR was carried out with an Applied Biosystems 7300 Real Time PCR system using the SYBR green PCR Master Mix (Applied Biosystems) and primers specific for *secA1* (NF1634 and NF1635), *secA2* (NF1641 and NF1642) and 16S rRNA (NF1643 and NF1644). A dissociation step was included after each qPCR reaction to confirm the production of a single unique product. The expression of *secA1* and *secA2* relative to 16S

rRNA was determined according to the method of Pfaffl (37).

Cell lysis, fractionation and protein analysis- C. difficile are highly resistant to standard chemical and mechanical lysis methods. However, we have observed that *C. difficile* spontaneously lyse at 37°C following a single freeze-thaw cycle. This propensity was exploited for the preparation of whole cell lysates and fractionation of *C. difficile*. For the preparation of whole cell lysates, cultures of *C. difficile* were harvested by centrifugation at 5,000 x *g* for 10 min at 4°C and the pellets frozen at -20°. Bacteria were thawed, resuspended in PBS to an $OD_{600\ nm}$ of 20 and incubated at 37°C for 40 min. This method resulted in consistent and reproducible lysis. For analysis by SDS-PAGE, an equal volume of 2x SDS sample buffer (38) was added. For cell fractionation, the frozen bacterial pellets were resuspended in PBS containing 1.4 mg/ml lysozyme and 0.12 µg/ml DNaseI to an $OD_{600\ nm}$ of 20 and incubated at 37°C for 1 h. Membranes were harvested by centrifugation at 25,000 x *g* for 10 min at 4°C. The supernatant, containing the soluble cytoplasmic proteins, was removed and mixed with an equal volume of 2 x SDS sample buffer. The harvested membranes were washed twice with 500 µl PBS, resuspended in PBS and solubilised with 1% SDS to a final equivalent $OD_{600\ nm}$ of 20 and mixed with an equal volume of 2 x SDS sample buffer. S-layer and cell wall proteins were isolated from intact *C. difficile* cells using low pH glycine as described previously (9,39). SDS PAGE and Western immunoblotting were carried out using standard methods and as described previously (9,39).

β-glucuronidase assay- C. difficile 630 harbouring pRPF185 were grown to an $OD_{600\ nm}$ of 0.3 in TY broth, induced with ATc (20, 100 or 500 ng/ml) for 210 min, harvested by centrifugation and frozen. Bacteria harbouring the empty vector (pMTL960) or a constitutive *gusA* expression plasmid (pRPF144) were included as controls. Bacteria were lysed as described above and the β-glucuronidase activity in the cell lysates was determined as described previously (34).

^{35}S-methionine metabolic labeling- Overnight TY cultures were sub-cultured to an $OD_{600\ nm}$ of 0.05 in complete CDDM, grown to an $OD_{600\ nm}$ of 0.3 and induced with 20 ng/ml ATc. After a 40 min induction 1 ml of each culture was harvested by centrifugation, resuspended in CDDM without methionine and incubated for a further 30 min to exhaust cellular methionine pools. Cultures were then removed from the anaerobic cabinet to a 37°C heating block. 100 µCi EasyTag ^{35}S-methionine (specific activity 1175 Ci/mmol, Perkin Elmer) was added to each culture and 200 µl samples were removed to fresh tubes containing 50 µl 1 M NaN_3 at defined intervals. 800 µl of unlabelled *C. difficile* was added to each tube and S-layer extracts were prepared as described above.

Phase contrast and immunofluorescence microscopy- Overnight *C. difficile* TY cultures were subcultured into fresh TY supplemented with thiamphenicol, with and without 500 ng/ml ATc and grown overnight. Bacteria were fixed and immunolabelled using a rat anti-LMW SLP antibody as previously described (15). Immunolabelled bacteria were visualised and photographed using a Nikon Eclipse E600 microscope fitted with a Retiga 2000R FAST 1394 camera.

RESULTS

Construction of an inducible promoter system for C. difficile. For many years the genetic intractability of *C. difficile* has hampered molecular analysis of this important pathogen, however recent advances have finally opened the way for fundamental genetic analysis. One crucial tool still missing from the repertoire of *C. difficile* genetic tools is the ability to control the expression of a plasmid-borne gene. A *lac*-based inducible promoter, which has been shown to work well in some Clostridial species, does not appear fully functional in *C. difficile* ((33) and our unpublished observations). Tetracycline-inducible promoters have been successfully employed in many Gram-positive species (40,41). We have taken one such system, optimised for use in *S. aureus* (36), and adapted it for use in *C. difficile*. The inducible system consists of a pair of divergent promoters (P_{tetR} and P_{tet}), each with an overlapping *tet* operator sequence. P_{tetR} drives the expression of TetR, a transcription factor which negatively regulates both promoters, while P_{tet} is used to drive expression of the gene of interest. Addition of tetracycline relieves TetR repression and results in increased expression of both the gene of interest and TetR. The resulting negative feedback

ensures tight regulation and dose-dependent induction.

The Tet promoter system from pRMC2 (36) was modified as described in Experimental procedures and transferred into the *E. coli-C. difficile* shuttle vector pMTL960. The *gusA* gene, encoding the β-glucuronidase enzyme, was cloned downstream of the P_{tet} promoter and β-glucuronidase activity used as a readout of inducible gene expression in *C. difficile*. Although *C. difficile* 630 carries *tetM* and is resistant to tetracycline, there is a lag between addition of the antibiotic and complete expression of resistance. To avoid any effects on growth and to allow use of the inducible system in tetracycline-sensitive strains of *C. difficile*, the non-antibiotic analogue anhydrotetracycline (ATc) was used for induction of the system. In the absence of induction there was no detectable β-glucuronidase activity demonstrating the extremely tight repression of P_{tet} by TetR (Fig. 1A). Induction with ATc was exquisitely dose-dependent with a linear induction profile. Furthermore induction with 500 ng/ml ATc resulted in levels of β-glucuronidase activity similar to those seen with the constitutive P_{cwp2} promoter (15).

C. difficile possesses an accessory Sec system. In species which possess both canonical and accessory Sec systems there are several consistent characteristics which distinguish the respective SecA proteins (reviewed in (28)), for example, the housekeeping SecA1 protein has a higher molecular weight than the accessory protein, SecA2. This size difference is largely due to the absence, in SecA2, of the C-terminal linker (CTL); the CTL has been implicated in interactions with both lipid and SecB (42). SecA1 also shares higher sequence identity with the well-characterised SecA proteins of *E. coli* and *B subtilis*. Sequenced strains of *C. difficile* all possess two copies of SecA, putatively annotated as SecA1 and SecA2 (29,43). SecA1 (CD0143 in strain 630) displays 47% and 51% amino acid sequence identity with the *E. coli* and *B. subtilis* SecA respectively, compared with 41% and 47% for SecA2 (CD2792) (Fig. S1). Furthermore, SecA2 is smaller than SecA1 (781 and 891 residues respectively) and lacks the CTL. Based on these characteristics it is likely that CD0143 is the housekeeping SecA1 protein and that the second protein, SecA2, may form the core of an accessory Sec system.

SecA2 is encoded within a genomic locus containing at least 12 surface localized gene products, including the S-layer precursor SlpA, the S-layer protease Cwp84 and the adhesin Cwp66 (Fig. S2). Given the genomic localisation of *secA2* it is tempting to speculate that SecA2 may play a role in the export of the surface layer precursor, SlpA, and perhaps other surface proteins. This would not be without precedent as in *Listeria monocytogenes* one of the substrates of the accessory Sec system, P60, is encoded by a gene adjacent to, but divergent from, *secA2* (24).

Conservative Walker Box mutations in secA1 and secA2 are dominant-negative and lethal in C. difficile. Despite repeated attempts, we were unable to create insertion inactivated mutants of *secA2*, suggestive of an essential function. However we took advantage of a previous observation that a conservative Lys-Arg substitution in the Walker box of SecA, which negates binding to ATP, confers a dominant-negative phenotype in both *E. coli* and *M. smegmatis* (31,44). In *M. smegmatis,* over-expression of a dominant-negative SecA2K129R gave the same phenotype as a *secA2* knock-out (31). Lysine-129 corresponds to Lysine-106 in both SecA1 and SecA2 of *C. difficile* (Fig. S3). *E. coli-C. difficile* shuttle plasmids were constructed with Strep-tagged wild type (pRPF150) and K106R (pRPF151) alleles of *secA2* under the control of the constitutive *cwp2* promoter (P_{cwp2}) (15). The plasmid carrying the wild-type *secA2* allele was transferred into *C. difficile* 630, which carries wild-type *secA1* and *secA2* in its genome, from an *E. coli* conjugal donor strain with high efficiency and the Strep-tagged SecA2 found to be well expressed (Fig. 1B). However, despite repeated attempts, the *secA2K106R* allele could not be conjugated into *C. difficile*, suggesting that *secA2K106R* was lethal in *C. difficile*. Both the wild-type and K106R alleles of *secA2* were sub-cloned into the tetracycline-inducible expression vector pRPF185, yielding pRPF186 and pRPF187 respectively. Expression of SecA2 from pRPF186 was found to be tightly repressed and induction with 500 ng/ml ATc resulted in high-level protein expression, albeit slightly less than seen with the constitutive P_{cwp2} promoter (Fig. 1B). In contrast with the constitutive *secA2K106R* plasmid,

pRPF187 (P_{tet}-*secA2K106R*) was easily conjugated into *C. difficile*. To test if expression of SecA2K106R was indeed lethal, bacteria were grown on BHI agar plates with and without ATc. Expression of SecA2K106R resulted in a severe growth defect whereas expression of the wild-type protein had no apparent affect on *C. difficile* growth (Fig. 2). Plasmids encoding inducible wild-type SecA1 and a K106R derivative were also constructed (pRPF193 and pRPF194 respectively) and conjugated into *C. difficile* 630. Expression of SecA1K106R resulted in a growth defect similar to that seen with SecA2K106R. However, in contrast with SecA2, expression of wild-type SecA1 also resulted in a partial growth defect. Interestingly this growth defect was far greater on Blood agar (Fig. S4).

Expression of SecA1K106R or SecA2K106R blocks translocation of SlpA. In order to examine the effect of expression of the SecA proteins on the surface protein composition of *C. difficile* cells were grown to early exponential phase, induced with ATc for four hours and surface proteins isolated following standard methods. Bacteria over-expressing either SecA1K106R or SecA2K106R had considerably lower levels of S-layer (SecA1K106R, 68% of control levels; SecA2K106R, 48%) and associated cell wall proteins (Fig. 3A & B). SlpA is cleaved post-translocation by the cell wall cysteine protease Cwp84, yielding the LMW and HMW SLP subunits (11). We reasoned, therefore, that inhibition of SlpA translocation should result in the accumulation of the full-length protein in the cell cytoplasm. To determine if SlpA was indeed accumulating in cells expressing SecA1K106R or SecA2K106R, bacteria carrying the appropriate plasmid were induced with ATc as before and whole-cell lysates were analysed for the presence of the full-length SlpA (Fig. 3C & D). Expression of SecA2K106R or SecA1K106R resulted in significant accumulation of SlpA in the cell cytoplasm, although the affect was greater with SecA2K106R than with SecA1K106R. This observation was in complete agreement with the reduction in the amount of the SLPs observed on the cell surface. Expression of wild-type SecA1 also resulted in a small, but clear, accumulation of SlpA in the cell cytoplasm. In contrast, expression of SecA2 had no effect on the amount of SlpA detected.

Pulse labelling reveals a substantial defect in S-layer translocation upon over-expression of dominant-negative SecA1 or SecA2. In order to determine the degree of the S-layer translocation defect, cells were induced with ATc for 40 min, washed and resuspended in a defined medium lacking methionine, incubated to exhaust cellular methionine pools and pulse labelled with ^{35}S-methionine. The surface protein repertoire synthesized during the ^{35}S-methionine pulse was then analysed by SDS PAGE and autoradiography (Fig 3E). Expression of either dominant-negative SecA protein, SecA1K106R or SecA2K106R, resulted in a substantial blockade of translocation of the SLPs and all detectable CWPs. There was also a clear, but less severe, defect in surface protein translocation upon over-expression of wild-type SecA1 but not with SecA2.

Translocation of other CWPs is affected by SecA1K106R and SecA2K106R expression. As SlpA translocation was dramatically inhibited by expression of SecA1K106R and SecA2K106R we sought to confirm if other members of the cell surface protein were also affected in a similar manner. Unlike SlpA, most *C. difficile* CWPs are not modified post-translocation, making it difficult to distinguish the cytoplasmic and surface forms by size alone. However, there is one additional surface protein, CwpV, which undergoes a cleavage event analogous to SlpA resulting in two fragments localised to the cell wall (15). Expression of CwpV is phase-variable (15) but we have recently described a *C. difficile* strain, 630Δ*recV* ON, with a mutation in the gene encoding the recombinase, RecV, responsible for CwpV phase variation, rendering CwpV expression constitutive (14). Using this constitutive CwpV strain we sought to analyse the effect of SecA1K106R and SecA2K106R expression on the translocation of CwpV. Expression of SecA1K106R or SecA2K106R resulted in a severe defect in the surface localisation of CwpV and, analogous to SlpA, there was a concomitant accumulation of full-length CwpV in the cell cytoplasm (Fig. 4A & B). There were also reductions in the amount of several other CWPs on the cell surface, including Cwp2, Cwp66 and Cwp84 (Fig. 4A & C). However, as these proteins are not modified post-translocation it was impossible to demonstrate

accumulation of these proteins in the cell cytoplasm.

Knock-down of SecA2, but not SecA1, disrupts SlpA and CwpV translocation. The experiments described above used plasmids over-expressing *secA1* or *secA2* alleles in the context of wild-type levels of both proteins, driven from chromosomally encoded genes. To investigate the effects of down-regulation of expression of chromosomally encoded *secA1* and *secA2*, antisense fragments to the *secA1* and *secA2* genes were cloned separately under the control of the inducible P_{tet} promoter. Both antisense fragments were designed such that the 5′ end of the target mRNA, including the RBS, would be sequestered in the resulting RNA duplex. Expression of the antisense RNAs and sequestration of the target mRNA was confirmed using semi-quantitative and quantitative RT-PCR (Fig. 5A - D). RNA samples were not heated prior to first strand cDNA synthesis to preserve the mRNA/antisense RNA duplex. As an RNA duplex is refractory to cDNA synthesis, the cDNA synthesised is representative of the available (and translatable) mRNA. When *secA1a* was expressed there was an almost complete sequestration of the *secA1* mRNA but no affect on the amount of available *secA2* mRNA, demonstrating the target specificity of the antisense RNA (Fig. 5A). Expression of *secA2a* resulted in a greater than 70% decrease in the amount of available *secA2* mRNA but had no affect on the levels of *secA1* mRNA (Fig. 5B).

Expression of the *secA1* antisense RNA (*secA1a*) led to a rapid cessation of growth, after induction growth stopped within 2-3 generations. When examined microscopically, there was evidence of severe stress, including defective septation, increased sporulation and cell lysis (Fig. 5G). Induction of the *secA2* antisense RNA (*secA2a*) also resulted in impaired growth but the effect was far less severe than with *secA1a* (growth stopped 8 generations after induction) and there was no visible change in cell morphology when examined microscopically (data not shown). In order to ascertain what affect the knock-down of SecA1 and SecA2 had on surface protein translocation, we examined cells for the accumulation of full-length SlpA and CwpV in the cell cytoplasm (Fig. 5F). Knock-down of SecA2 expression resulted in significant accumulation of both CwpV and SlpA in the cell, demonstrating an absolute requirement for SecA2 for the translocation of both proteins. However, despite the highly efficient sequestration of *secA1* mRNA and the extreme stress phenotype induced by *secA1a* expression, there was no apparent effect on the translocation of either SlpA or CwpV.

Cellular distribution of SecA1 and SecA2. It has been previously reported that *M. smegmatis* SecA1 and SecA2 differ in their subcellular localisation; SecA1 was found equally in cytoplasmic and membrane fractions whereas SecA2 is predominantly cytoplasmic (31). Furthermore, a K129R SecA2 mutation, analagous to the K106R SecA2 mutation described in this study, relocalised SecA2 predominantly to the membrane. We sought to determine if *C. difficile* SecA1 and SecA2 were localized in a similar manner. Wild-type SecA1 and SecA2 differed dramatically in their subcellular localization (Fig. 6); SecA2 was predominantly found in the soluble fraction with only a small amount of protein detected at the membrane, whereas SecA1 was only detected at the membrane. This is in stark contrast to *M. smegmatis* where SecA1 was found to be localised equally between the membrane and cytoplasm (31). However, as was observed in *M. smegmatis*, the dominant-negative K106R mutation in SecA2 switched the localisation of the protein entirely to the membrane fraction.

DISCUSSION

In synthesising and maintaining a complete cell envelope all living cells face a similar physical problem - how to transport largely polar proteins across the non-polar membrane? Bacteria have evolved many complex proteinaceous systems to facilitate this transport but the majority of translocated proteins in bacteria utilise the multi-subunit Sec translocase (21). The Sec systems of the model organisms *E. coli* and *B. subtilis* have been studied in remarkable detail but there are still many research questions remaining to be answered. In the last decade a second, accessory, Sec system has been described in several Gram-positive species (24-27,45). In this study we have characterised the accessory Sec system of the nosocomial pathogen *Clostridium difficile* and identified its major substrates.

A conservative amino acid substitution in the SecA2 Walker box, which prevents binding of

ATP, has previously been shown to confer a dominant-negative phenotype in *M. smegmatis* (31). Indeed, expression of the dominant-negative SecA2 resulted in the same phenotype as a *secA2* knockout. We show that an equivalent mutation (K106R) in the *C. difficile* SecA2 is also dominant-negative. However, unlike in *Mycobacteria* the dominant-negative SecA2K106R was found to be lethal in *C. difficile*, suggesting an essential role for SecA2. Consistent with this, we were unable to construct a *secA2* knockout despite repeated attempts. Previous to these observations, SecA2 has only been found to be essential in one species where an accessory Sec system has been described, *Corynebacterium glutamicum* (45). In the absence of a *secA2* knockout we resorted to using an inducible antisense RNA to knock-down SecA2 expression. Although the knock-down was only partial, as judged by qPCR, there was a significant growth defect. Taken together this data supports the hypothesis that SecA2 performs an essential function in *C. difficile*. We also confirmed the essentiality of SecA1 in *C. difficile* by constructing an analogous dominant-negative allele (SecA1K106R) and knocking down expression with antisense RNA. This result was as predicted due to a requirement for the canonical Sec system for translocation of many essential proteins into and across the cytoplasmic membrane (46). As SecA2 is also essential in *C. difficile* it is likely that one or more of its translocated substrates are essential. It is clear that SecA2 is necessary for the secretion of a subset of proteins and does not rely on SecA1 for this function.

In order to identify the substrates of the *C. difficile* accessory Sec system we used a novel inducible expression system to express dominant-negative SecA1K106R and SecA2K106R proteins and antisense RNAs. We then examined the surface protein complement to identify those proteins whose surface display was SecA2 dependent. Expression of either dominant-negative SecA resulted in a severe defect in the translocation of the S-layer precursor SlpA. However the defect was considerably more severe when SecA2K106R was expressed. In *M. smegmatis* it was observed that translocation of SecA2 substrates also required SecA1 (31), demonstrating a degree of interdependence between the two systems. In order to determine if a similar interdependence existed between the *C. difficile* canonical and accessory Sec systems, we employed antisense RNA to independently knock-down the expression of either SecA1 or SecA2. SecA2 knock-down resulted in the same SlpA translocation defect seen with SecA2K106R expression. However, despite efficient knock-down of SecA1 and resultant severe growth phenotypes, there was no affect on the translocation of SlpA. These data suggest that, unlike in *M. smegmatis*, the *C. difficile* accessory Sec system is not dependent on the canonical SecA. Furthermore, it is clear that transport of the *C. difficile* S-layer precursor across the membrane is dependent on the accessory Sec system. As *C. difficile* appears to possess only the canonical SecYEG translocase, SecA1- and SecA2-dependent translocation presumably utilise the same membrane channels. As the dominant-negative SecA1 is predicted to non-productively interact with the SecYEG translocase (31), over-expression of this mutant SecA1 would likely block SecA2 from accessing the translocase. This would explain the SlpA translocation defect seen upon SecA1K106R expression.

Interestingly, over-expression of wild-type SecA1 also results in a partial SlpA translocation defect but expression of wild-type SecA2 has no effect. We also observed that, when over-expressed, SecA1 accumulates at the membrane. Therefore it is possible that the artificial excess of SecA1 reduces the access of SecA2 to the SecYEG translocase and thus prevents efficient translocation of SlpA. We examined the amount of SecA2 at the membrane in cells with and without over-expressed SecA1 and, although we did observe a slight but reproducible decrease in the amount of SecA2 at the membrane with a concomitant increase in the cytoplasm, the difference was too small to be conclusive (data not shown). However it is possible that even a small decrease in the amount of SecA2 accessing SecYEG could result in the partial SlpA defect observed upon SecA1 over-expression.

SlpA is a member of a large family of *C. difficile* cell wall proteins (10), related by a common putative cell anchoring mechanism. The *C. difficile* strain used in this study, 630, encodes a further 28 of these proteins in addition to SlpA. We have demonstrated that at least one of these proteins, CwpV, is also a SecA2 substrate. The

translocation of three more CWPs, Cwp2, Cwp66 and Cwp84, also appear to be affected by the expression of dominant-negative SecA1 or SecA2 in a manner similar to SlpA. It is possible that SecA2-dependent translocation is a feature of the entire CWP family but confirmation of this will require additional study.

The experiments described here required the development of a number of new genetic tools and techniques for *C. difficile*. The inducible promoter system described in this study adds another powerful genetic tool to our repertoire and will undoubtedly prove useful to the wider clostridial community. The P_{tet} promoter is very tightly repressed and displays exquisite dose-dependent induction with tetracycline or the non-antibiotic analogue anhydrotetracycline. Here we have used this promoter system for the high level expression of several proteins and also for inducible antisense RNA production. This allowed us to study the function of a pair of essential genes, which would previously have been impossible given the current lack of tools to construct conditional mutants in *C. difficile*. The combination of inducible antisense RNA with the ability to test knock-down specificity and efficiency using qPCR should be widely applicable to the study of essential genes in clostridia.

We have demonstrated that the *C. difficile* accessory Sec system in required for the secretion of at least two substrates, the major *C. difficile* surface proteins SlpA and CwpV. SlpA is the most highly expressed protein in *C. difficile*, accounting for approximately 10-15% of total cellular protein. CwpV is an autoaggregating adhesin and, although expressed in a phase variable manner (15), is the dominant cell wall protein in phase ON cells and accounts for 13% of the S-layer (14). While it is possible to knock-out many of the genes encoding cell wall proteins, including *cwpV* (15), we have found it impossible to generate an *slpA* knock-out strain. The essential requirement for *secA2* likely reflects a requirement for the S-layer in *C. difficile*. S-layers are ubiquitous structures in nature, found on many Gram-positive and Gram-negative bacteria and most archaea (47). The clostridia are an evolutionarily ancient lineage of bacteria (48). It will be fascinating to see if other species possessing S-layers also have a dedicated system for their secretion.

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FOOTNOTES

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The abbreviations used are: ATc, anhydrotetracycline; low molecular weight, LMW; high molecular weight, HMW; S-layer protein, SLP; cell wall protein, CWP

FIGURES

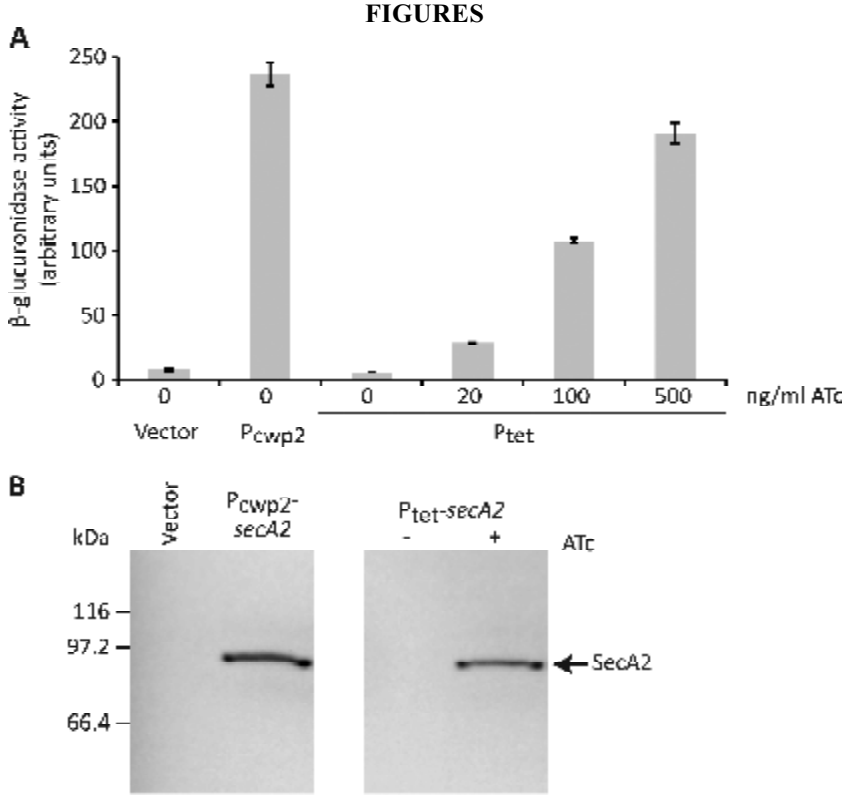

Fig. 1. Inducible protein expression in *C. difficile*. *A*. β-glucuronidase activity of mid-logarithmic phase cultures of *C. difficile* strain 630 harbouring the empty *E. coli-C. difficile* shuttle vector (pMTL960, Vector), a constitutive *gusA* (pRPF144, P_{cwp2}) or the inducible *gusA* (pRPF185, P_{tet}). Cultures of *C. difficile*-pRPF185 were induced with the indicated concentration of ATc for 210 min. Shown are the

averages of triplicate measurements with standard deviations displayed as error bars. *B*. Constitutive and inducible expression of SecA2. Expression of SecA2 in mid-logarithmic phase cultures of *C. difficile* 630 harbouring pMTL960 (Vector), pRPF150 (P_{cwp2}-*secA2*) or pRPF186 (P_{tet}-*secA2*) with (+) and without (-) ATc induction (500 ng/ml, 1 h) was assayed by SDS-PAGE and Western immunoblotting using an anti-Strep Tag II antibody. The position of the Strep-tagged SecA2 is indicated.

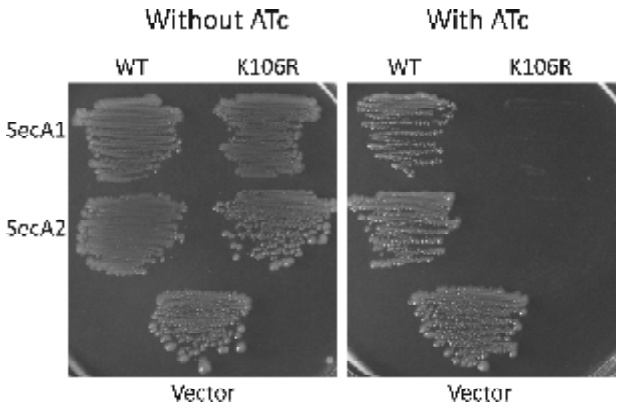

Fig. 2. Growth phenotypes resulting from SecA1 and SecA2 expression in *C. difficile*. *C. difficile* harbouring pRPF193 (SecA1 WT), pRPF194 (SecA1K106R), pRPF186 (SecA2 WT), pRPF187 (SecA2K106R) or pMTL960 (Vector) were streaked on BHI agar plates with and without ATc (500 ng/ml). Plates were incubated anaerobically at 37°C for 48 h and then photographed.

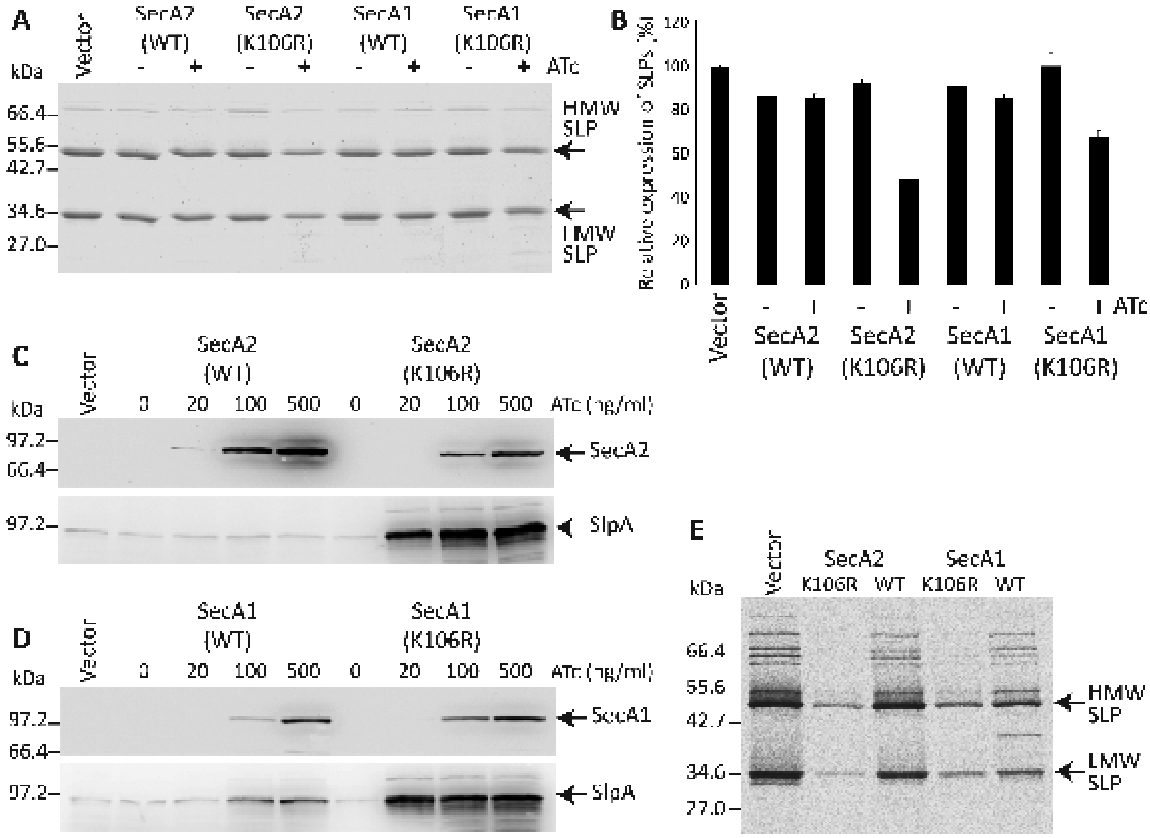

Fig. 3. Expression of SecA1K106R and SecA2K106R causes a severe S-layer translocation defect. *A*. Mid-log cultures of *C. difficile* 630 harbouring pMTL960 (Vector), pRPF186 (SecA2(WT)), pRPF187 (SecA2(K106R)), pRPF193 (SecA1(WT)) or pRPF194 (SecA1(K106R)) were induced with ATc (500 ng/ml) for 4 h and surface proteins were isolated using low pH glycine (39). Surface proteins were separated on 12% SDS polyacrylamide gels and Coomassie stained. *B*. Densitometry analysis of gel shown in panel *A*. Densitometry was performed independently on the LMW and HMW SLPs, shown are the average densitometry values with standard deviations. *C*. and *D*. Western immunoblot analysis of SlpA translocation defect in cultures expressing wild-type and dominant-negative SecA1 and SecA2. Cultures of *C. difficile* 630 harbouring pRPF186 (SecA2(WT)), pRPF187 (SecA2(K106R)), pRPF193 (SecA1(WT)) or pRPF194 (SecA1(K106R)) were induced for 3 h with the indicated concentration of ATc. Whole cell lysates separated on 10% SDS polyacrylamide gels and probed with anti-Strep Tag II or anti-LMW SLP antibodies. *E*. ^{35}S-methionine labelling of *de novo* synthesised *C. difficile* surface proteins. Cultures of *C. difficile* 630 carrying pMTL960 (Vector), pRPF187 (SecA2K106R), pRPF186 (SecA2 WT), pRPF194 (SecA1K106R) and pRPF193 (SecA1 WT) were ^{35}S-labelled as described in Experimental procedures, surface proteins were isolated using low pH glycine and visualised by SDS PAGE followed by autoradiography.

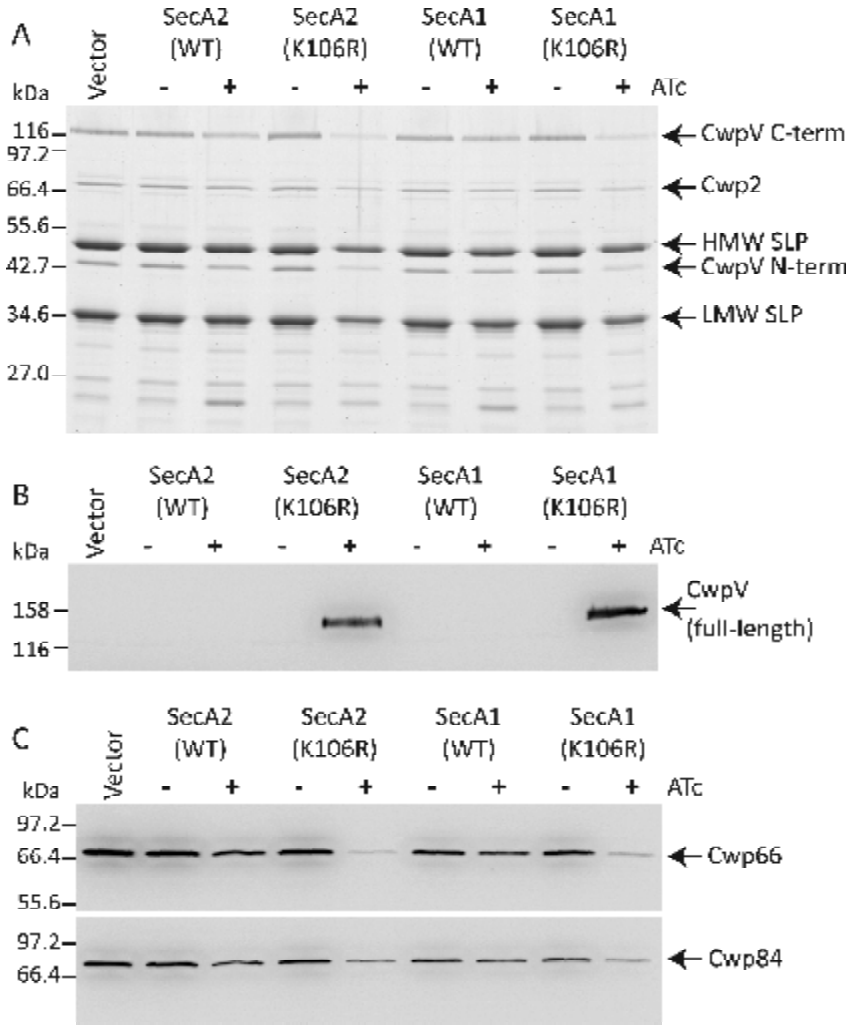

Fig. 4. Expression of SecA1K106R and SecA2K106R impairs the translocation of several CWPs. *C. difficile* strain 630Δ*recV* ON (14) harbouring pMTL960 (Vector), pRPF186 (SecA2(WT)), pRPF187 (SecA2(K106R)), pRPF193 (SecA1(WT)) or pRPF194 (SecA1(K106R)) were induced with ATc (500 ng/ml) for 4 h. Surface proteins were isolated using low pH glycine (39) and analysed by SDS-PAGE (*A*.). Whole cell lysates of the same cultures were also prepared and analysed for the presence of full-length CwpV using an antibody raised against the 41.2 kDa N-terminal fragment of the protein (*B*.). *C*. The surface protein samples described above were further analysed using antibodies recognising two additional cell wall proteins (Cwp66 and Cwp84). The positions of the two CwpV subunits (C-term and N-term), Cwp2, HMW and LWM SLPs, full-length CwpV, Cwp66 and Cwp84 are indicated.

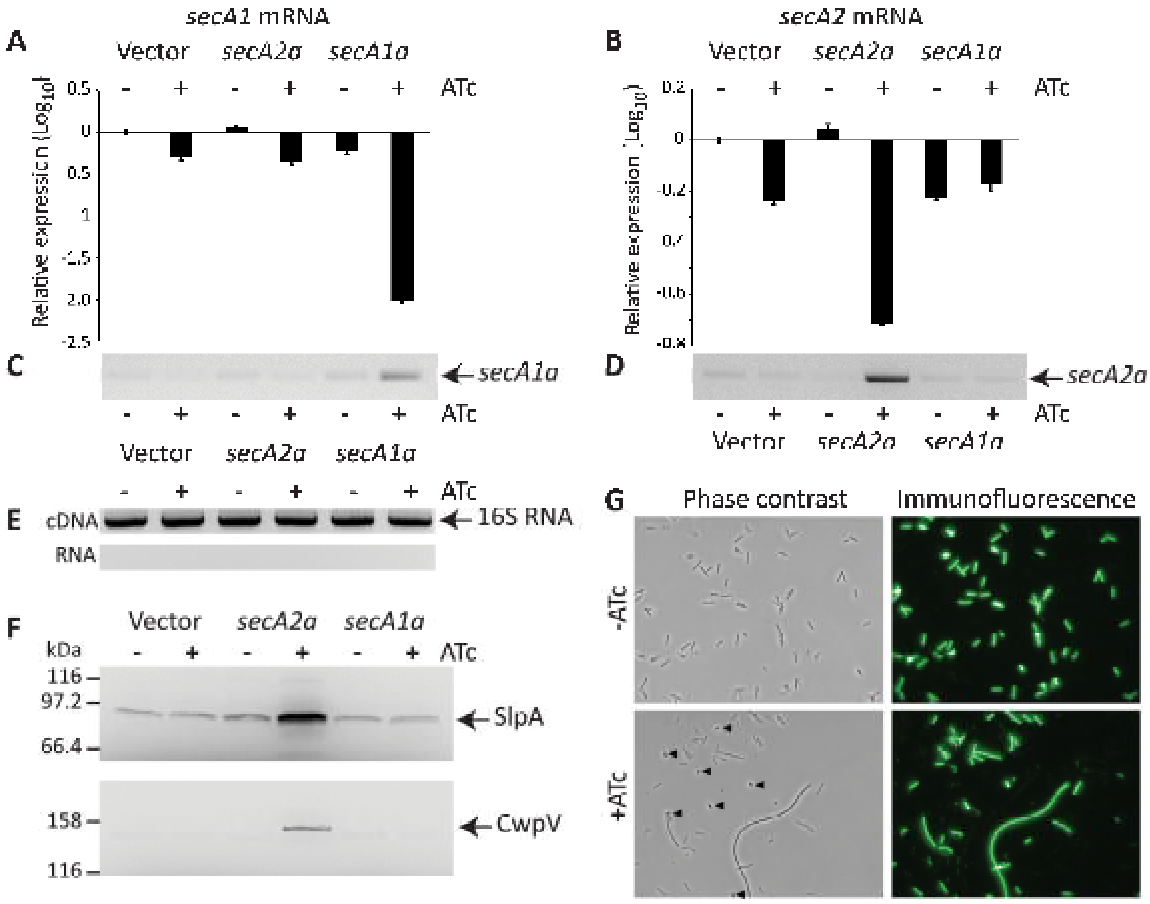

Fig. 5. Inducible antisense RNA knock-down of SecA1 and SecA2 expression. *A.-E.* RNA was isolated from *C. difficile* 630 carrying pMTL960 (Vector), pRPF195 (*secA2a*) or pRPF204 (*secA1a*) grown with (+) and without (-) ATc (500 ng/ml) and the available *secA1* (*A*) and *secA2* (*B*) mRNA was quantified relative to 16S rRNA by qRT-PCR. Displayed are the relative expression values (log_{10}); each is the average of triplicate reactions, error bars indicate standard deviations. RT-PCR using antisense RNA-specific oligonucleotides was used to confirm expression of *secA1a* (*C*) and *secA2a* (*D*). Purified RNA and cDNA samples were subjected to PCR using 16S rRNA specific oligonucleotides to confirm RNA purity and cDNA synthesis (*E*). *F. C. difficile* 630 carrying pMTL960 (Vector), pRPF195 (*secA2a*) or pRPF204 (*secA1a*) were grown overnight with (+) and without (-) ATc (500 ng/ml). Whole cell lysates were prepared and full-length SlpA and CwpV were detected using antibodies raised against the LMW SLP and CwpV N-terminal fragment respectively. *G*. Morphological phenotypes of SecA1 knock-down. Shown are representative phase contrast and immunofluorescence images of *C. difficile* 630 carrying pRPF204 (P_{tet}-*secA1a*) grown overnight with and without ATc (500 ng/ml). Spores present in the induced culture are indicated (◀) and cell debris is clearly visible in the corresponding immunofluorescence image (bottom-right panel).

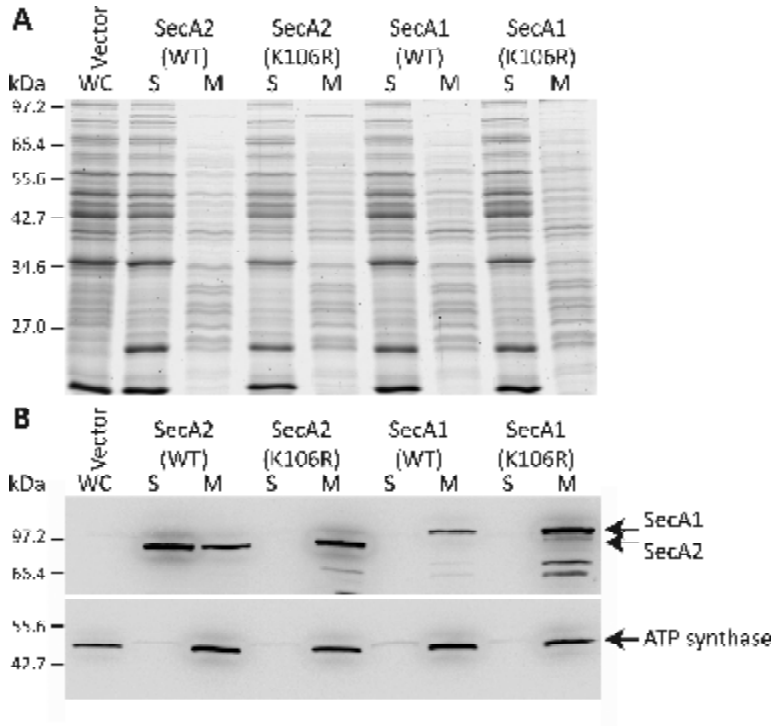

Fig. 6. Subcellular localisation of SecA1 and SecA2. Mid-log cultures of *C. difficile* 630 harbouring pMTL960 (Vector), pRPF186 (SecA2(WT)), pRPF187 (SecA2(K106R)), pRPF193 (SecA1(WT)) or pRPF194 (SecA1(K106R)) were induced with ATc (500 ng/ml) for 90 min. *A*. Coomassie-stained SDS polyacrylamide gel showing whole cell lysate (WC), membrane (M) and soluble (S) protein fractions. *B*. The location of SecA1 and SecA2 was detected using an anti-Strep Tag II antibody. The integral membrane protein ATP synthase was used as a control of cell fractionation.

Table 1. Plasmids used in this study

Plasmid	Relevant details	Reference or source
pCBR023	P_{cwp2} driving a codon optimised *gusA* gene in pUC19	(15)
pRPF137	*Eco*RI site between P_{cwp2} and *gusA* in pCBR023 changed to *Sac*I	This study
pMTL960	*E. coli*-*C. difficile* shuttle vector	Nigel Minton
pRPF144	P_{cwp2}-*gusA* cassette from pRPF137 subcloned into pMTL960	This study
pRPF150	*gusA* gene in pRPF144 replaced with WT *secA2* with an N-terminal Strep-Tag II	This study
pRPF151	*gusA* gene in pRPF144 replaced with K106R *secA2* with an N-terminal Strep-Tag II	This study
pSTBlue-1	*E. coli* cloning vector	Novagen
pRMC2	Tetracycline-inducible vector for *Staphylococcus aureus*	(36)
pRPF135	P_{tet} from pRMC2 cloned into pSTBlue-1	This study
pRPF139	Extraneous *Bam*HI and *Xho*I sites in pRPF135 removed	This study
pRPF141	Extraneous *Kpn*I site in pRPF139 removed	This study
pRPF143	P_{cwp2} in pRPF137 replaced with modified P_{tet} from pRPF141	This study
pRFP146	P_{tet}-*gusA* cassette from pRPF143 cloned in pMTL960	This study
pRPF177	pRPF146 with the *slpA* transcriptional terminator added after *gusA*	This study
pRPF185	pRPF177 with the *fdx* transcriptional terminator added after *tetR*	This study
pRPF186	WT *secA2* with an N-terminal Strep-Tag II cloned into pRPF185	This study
pRPF187	*secA2* K106R with an N-terminal Strep-Tag II cloned into pRPF185	This study
pRPF193	WT *secA1* with an N-terminal Strep-Tag II cloned into pRPF185	This study
pRPF194	*secA1* K106R with an N-terminal Strep-Tag II cloned into pRPF185	This study
pRPF195	Inducible antisense RNA to *secA2*	This study
pRPF204	Inducible antisense RNA to *secA1*	This study