New tools and insights in physiology and chromosome dynamics of Clostridioides difficile

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CHAPTER 2

The signal sequence of the abundant extracellular metalloprotease PPEP-1 can be used to secrete synthetic reporter proteins in *Clostridioides difficile*

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Abstract

Clostridioides difficile is an opportunistic pathogen and the main cause of antibiotic-associated diarrhoea. Adherence of *C. difficile* to host cells is modulated by proteins present on the bacterial cell surface or secreted into the environment. Cleavage of collagen-binding proteins is mediated by the zinc metalloprotease PPEP-1, which was identified as one of the most abundant secreted proteins of *C. difficile*. Here, we exploit the PPEP-1 signal sequence to produce novel secreted enzymes. We have constructed two functional secreted reporters, AmyE^{opt} and sLuc^{opt} for gene expression analysis in *C. difficile.* AmyE^{opt} extracellular activity results in starch degradation and can be exploited to demonstrate promoter activity in liquid or plate-based assays. sLuc^{opt} activity could reliably be detected in culture supernatant when produced from an inducible or native promoter. The secreted reporters can be easily assessed under aerobic conditions, without the need for complex sample processing.

Introduction

Clostridioides difficile is a gram-positive anaerobic bacterium and is the leading cause of antibiotic-associated diarrhoea in the healthcare environment. Symptoms of *Clostridioides difficile* infection (CDI) can range from mild diarrhoea to pseudomembranous colitis and even death [1,2]. The incidence and severity of CDI have increased worldwide in the past decades due to the appearance of epidemic strains. Recently, an increase of CDI cases in the community has been noted [2]. Consequently, the interest in the physiology of the bacterium has increased.

The ability of *C. difficile* to adhere to intestinal epithelial cells plays a crucial role in the development of the disease. Adherence is modulated by proteins present on the cell surface or secreted into the environment, such as the S-layer proteins that cover the *C. difficile* cell surface [3] or the components of the flagella that confer motility to the cells [4]. The secreted toxins TcdA and TcdB compromise the intestinal barrier by disrupting the actin cytoskeleton of the epithelial cells, leading to morphological alterations and eventually cell death [2,5]. Recently, the metalloprotease PPEP-1 (CD2830; EC 3.4.24.89) has been identified amongst the most highly secreted proteins in both the laboratory strain 630Δ*erm* as well as the epidemic strain R20291 (a representative of the PCR ribotype 027, BI, NAP01) [6]. PPEP-1 has been suggested to regulate the switch between adhesion and motility phases through the cleavage of Pro-Pro peptide bonds in the collagen-binding protein CD2381 and other proteins [6-8].

Gene expression analysis of *C. difficile* can be challenging. Many common reporters used to study gene expression in bacteria are not suitable to use in *C. difficile* studies, as they require oxygen for maturation or are produced at insufficient levels due to different codon usage. A number of genetic tools are available to study gene expression in *C. difficile,* including β-glucuronidase (*gusA*), alkaline phosphatase (*phoZ*) and various fluorescent proteins [9-14]. However, no secreted reporters have been described for *C. difficile* to date.

In the present study, we show that the signal sequence of PPEP-1 can be fused to synthetic constructs to yield secreted proteins in *C. difficile*. We exemplify this strategy by generating two novel secreted reporters, AmyE^{opt} and sLuc^{opt}, that allow screening of gene expression activity on plates as well as in liquid medium, without the need for complex processing of samples.

Results and Discussion

A synthetic amylase, AmyE^{opt}, is functional in *C. difficile*

Our lab strain of *C. difficile*, 630Δ*erm* [15,16] is not capable of breaking down starch, suggesting that no functional amylase is produced under laboratory conditions (Fig. 1A).

Bacillus subtilis does produce a functional α-amylase, encoded by the *amyE* gene, and this feature has been exploited to ascertain double crossover integration of DNA into this locus [17]. We reasoned that it might be possible to use the properties of this enzyme to engineer a synthetic amylase that could function as a plate-based reporter for promoter activity in *C. difficile.* We, therefore, fused a codon-optimized AmyE from *B. subtilis* to the signal sequence of PPEP-1, resulting in the secreted reporter AmyE^{opt}. To ensure high level induction the previously published anhydrotetracycline (ATc) inducible promoter was used [9].

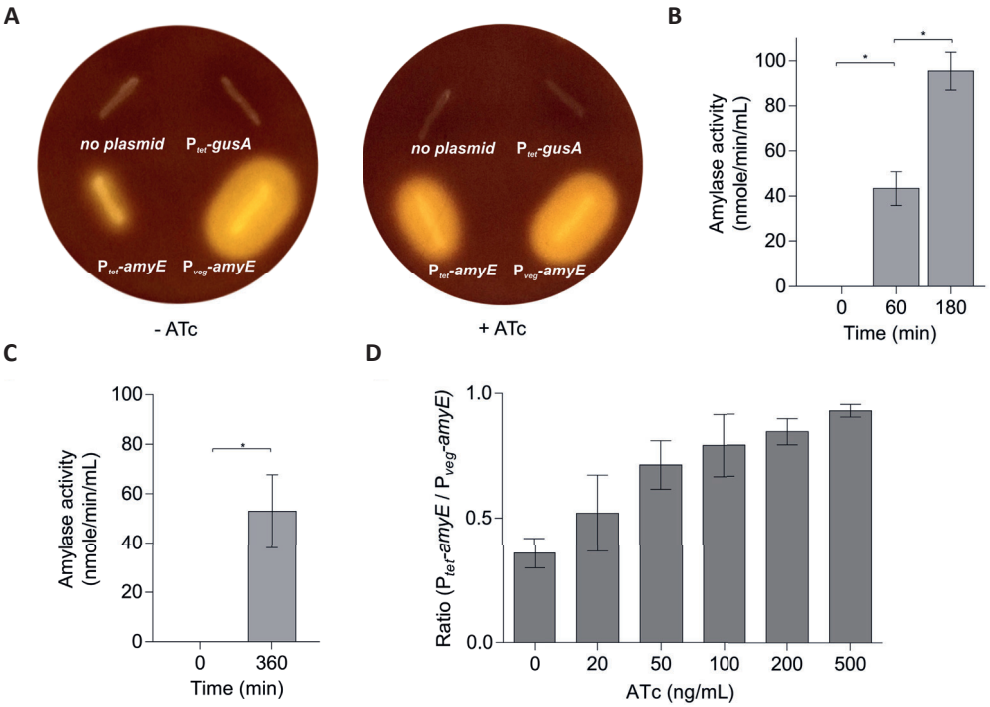

Fig. 1 - Visualization of secreted amylase activity. A) Staining with 0.2% iodine solution of BHI agar plates supplemented with 0,1% soluble starch. Growth of the *C. difficile* strains 630Δ*erm* (no plasmid), WKS1588 (P$_{tet}$-*gusA*), WKS1594 (P$_{tet}$-*amyE*^{opt}) and SJ113 (P$_{veg}$-*amyE*^{opt}) growth after 24h in the presence or absence of 500 ng/mL ATc. Clear halos (yellow) indicate starch degradation by the α-amylase. **B)** Quantification of amylase activity of the strain WKS1594 (P$_{tet}$-*amyE*^{opt}) at 0, 60 or 180 min after induction with 200 ng/mL ATc. **C)** Quantification of amylase activity of the strain SJ113 (P$_{veg}$-*amyE*^{opt}) assayed at 0 and 360 min after inoculation. **D)** Quantification of the relative halo size (halo size of P$_{tet}$-*amyE*^{opt} /halo

size of P_{veg}-*amyE*^{opt}) on BHI agar plates supplemented with 0,1% soluble starch in the presence of increasing amounts of ATc (0, 20, 50, 100, 200 and 500 ng/mL). Error bars represent the +/- standard deviation of triplicate samples. Using a Student's t-test for statistical analysis, *p<0.05, the following combinations were found to be significantly different from each other: 0 vs 50, 100, 200 and 500; 20 vs 200 and 500; 50 vs 500 ng/mL ATc.

We found that the *C. difficile* strain expressing AmyE^{opt} was able to hydrolyse the starch, as visible by halo formation after staining starch-containing plates with an iodine solution (Fig. 1A). As expected, the halo was larger when the strain was induced with 500 ng/mL ATc compared to the non-induced condition. It has previously been noted that the P_{tet} promoter is leaky and prolonged incubation in combination (in our case 24h) with the efficient enzymatic activity of the synthetic amylase could explain the observed breakdown of starch under non-inducing conditions (Fig. 1A). Nevertheless, halo formation was specific for the amylase containing plasmid, as it did not occur with cells containing the control plasmid pRPF185 [9], harbouring P_{tet}-*gusA* (Fig. 1A). Amylase-producing colonies could easily be distinguished from amylase-negative colonies; in a 5:1 mixed culture of wild type and P_{tet}-*amyE* harbouring *C. difficile*, 21% of the colonies were found to hydrolyse the starch when plated (Fig. S1).

Several methods exist to quantify amylase activity in liquid samples. We used a colourimetric assay (see Methods) to determine amylase activity in liquid cultures. A *C. difficile* strain capable of expressing AmyE^{opt} was induced with 200 ng/mL ATc in BHI and supernatants collected for quantification of the amylase activity (Fig. 1B). At the time of induction (0 min), no amylase activity was detected, suggesting the leakiness of the P_{tet} promoter is less pronounced in the timeframe of this assay. At 60 min amylase activity was detected (43,27±7,5 nmol/min/mL) with a further 2-fold increase at 180 min (95,38 ± 8,4 nmol/min/mL).

In liquid medium, the expression of AmyE^{opt} does not allow the growth on starch as sole carbon source (data not shown), likely due to an inability to further break down the disaccharides that result from the degradation of starch [18].

We conclude that the signal sequence of PPEP-1 is able to drive the secretion of the functional AmyE^{opt} and the predicted extracellular activity is correlated with the starch degradation observed in the solid medium and amylase activity in liquid cultures. Therefore, AmyE^{opt} can be used as a reporter for gene activity in *C. difficile*.

AmyE^{opt} can be used for promoter trap experiments

Next, we wanted to demonstrate whether the observed effect could be extended beyond the inducible promoter P_{tet}. Different organisms demonstrate different preferences for promoter sequences. As a result, it is frequently necessary to optimize promoter sequences for inducible gene expression systems, or screen a library of promoter fragments to determine desired characteristics. A plate-based screening method could facilitate these promoter-trap experiments.

We placed the well-characterized *B. subtilis* promoter P_{veg} [19,20] upstream of the *amyE*^{opt} sequence to determine whether it can be used to drive gene expression in *C. difficile*. The incubation of the *C. difficile* strain carrying the P_{veg}-*amyE*^{opt} construct produced large halos on starch plates (Fig. 1A), which appeared comparable to the strain carrying the inducible P_{tet}-*amyE*^{opt} construct. However, when amylase activity of the *C. difficile* strain carrying the P_{veg}-*amyE*^{opt} was quantified after 6 hours of inoculation (Fig. 1C), values were approximately 2-fold lower (52,8 nmol/min/mL) than those measured for the strain with the induced P_{tet}-*amyE*^{opt} (Fig. 1B).

The results above suggest that a plate-based readout of amylase activity has its limitations. P_{veg} is believed to be a constitutively expressed promoter [19,20]. We reasoned that the use of a P_{veg}-*amyE*^{opt} control might allow for a semi-quantitative measure of amylase activity that can overcome some of the inherent variability of a plate-based assay. We determined the relative halo size at varying amounts of ATc (0, 20, 50, 100, 200 and 500 ng/mL) after 24h of incubation. An increase in halo size was evident with increasing amounts of ATc (Fig. 1D), although they only reached statistical significance when the highest and lowest concentrations of inducer were compared (Fig. 1D).

Together, these data show that P_{veg} from *B. subtilis* is a functional and highly expressed promoter in *C. difficile* and that AmyE^{opt} can be used to verify the presence and relative strength of promoter sequences in plate-based or liquid assays.

Construction and validation of a secreted luciferase reporter, sLuc^{opt}

To extend the use of the signal sequence of PPEP-1 for the secretion of synthetic proteins beyond the amylase, we generated a novel secreted luciferase reporter. Luciferase-based bioluminescence assays are widely used for gene expression studies and promoter activity. However, due to the requirement for oxidation in the reaction with the substrate the application to anaerobic organisms is limited. We anticipated that efficient secretion of the luciferase would allow assaying of culture supernatant in an aerobic environment without

removing the original culture from the anaerobic environment, which could cause stress that affects gene expression levels.

We fused the PPEP-1 signal sequence to a codon-optimized luciferase based on NanoLuc [21], yielding the sLuc^{opt} reporter. We analyzed P$_{tet}$ dependent expression of sLuc^{opt} by assaying luciferase activity in diluted culture supernatants at different concentrations of ATc (0, 20, 50, 100, 200 and 500 ng/mL) at 180 min after induction (Fig. 2A).

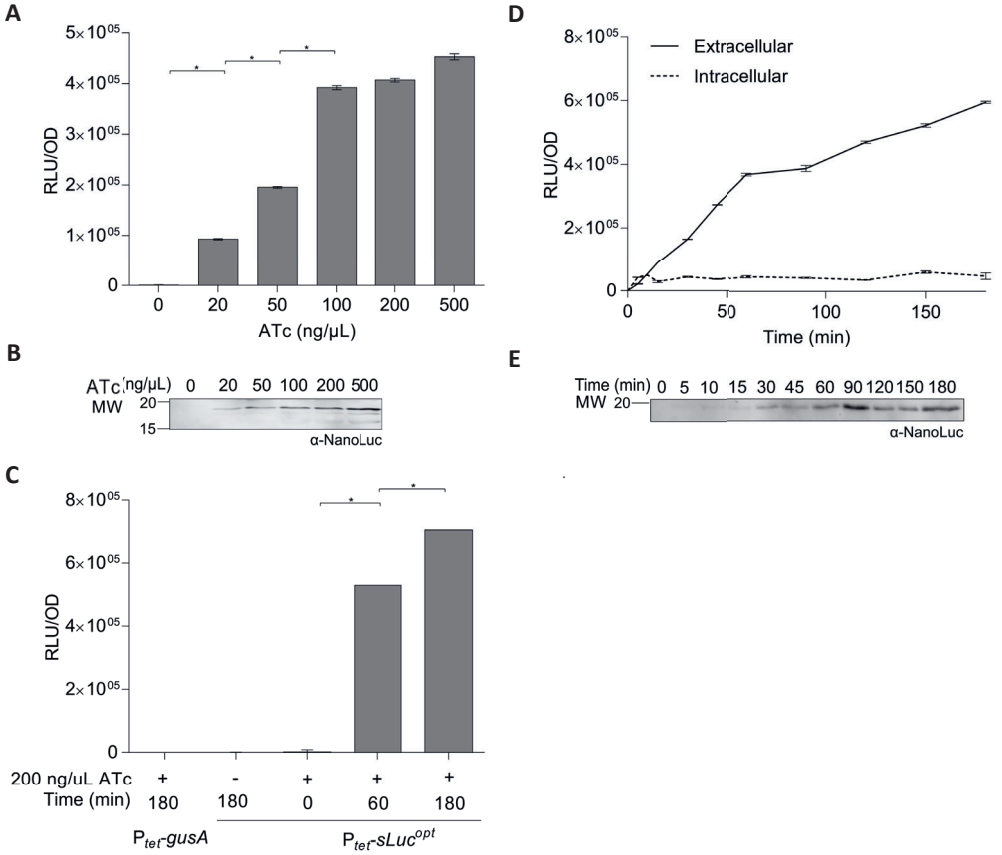

Fig. 2 - Luciferase reporter assays with the inducible P$_{tet}$ promoter. A) Luciferase activity of 1:100 diluted culture supernatant of *C. difficile* strain harbouring P$_{tet}$-*sLuc*^{opt} induced with different amounts of anhydrotetracycline (ATc) at 180 min. **B)** Western blot analysis of the same samples with anti-NanoLuc antibody. **C)** Luciferase activity of *C. difficile* strains harbouring P$_{tet}$-*sLuc*^{opt}, assayed at 0, 60 and 180 min after induction. P$_{tet}$-*gusA* and non-induced samples were used as controls and for these, luciferase activity was measured at 180 min. **D)** Luciferase activity of the *C. difficile* strain harbouring P$_{tet}$-*sLuc*^{opt}. Extracellular (line) and intracellular (broken line) luciferase activity following induction at 5, 15, 30, 60, 90, 120, 150 and 180 min. **E**) Western blot analysis of the culture supernatant of the same samples. Error bars represent the +/- standard deviation of triplicate samples. Student's t-test was used for statistical analysis, *p<0,000001.

In the absence of inducer, a low background signal was detected (830 ± 30 RLU/OD). Induction with ATc increased the signal up to ~500-fold (452713 ± 6022 RLU/OD, p<0,000001) in extracellular luciferase activity, in a dose-dependent manner. No significant increase of luciferase activity was observed when cells were induced with >100 ng/mL of ATc, suggesting maximal expression from this promoter.

The luciferase activity largely mirrored the detection of sLuc^{opt} in culture supernatant by immunoblotting using anti-NanoLuc antibodies (kindly provided by Promega)(Fig. 2B). Much greater sample volumes were required for the immunoblot detection, suggesting less sensitivity than the luciferase assay. The signal decay in our hands was identical to that described for NanoLuc activity [21] and after storing cell-free supernatants for 1 month at -20°C identical signals were obtained (data not shown). Thus, samples can be harvested throughout a growth experiment and assayed for luciferase activity in a microtiter plate after all samples have been collected.

As a result of the stability of sLuc^{opt}, continuous expression of the reporter is expected to result in increased signals over time. To determine if this was the case, we assayed the extracellular luciferase activity of *C. difficile* strains expressing *sLuc^{opt}* and *gusA* under the control of P$_{tet}$, induced with 200 ng/mL ATc over time (Fig. 2C). No signal was detected for the P$_{tet}$-*gusA* containing strain. Upon induction of sLuc^{opt}, luciferase activity increased significantly between 60 (529815 ± 771 RLU/OD) and 180 min (704365 ± 7320 RLU/OD) (P<0,0000001). Consistent with our earlier observations, the luciferase activity increased ~500-fold at 180 min compared to the moment of induction (0 min).

Reducing agents, such as glutathione and thioglycolate, are commonly used in the growth of anaerobic bacteria [22,23]. To determine the influence of these compounds on luciferase activity, *C. difficile* expressing *sLuc^{opt}* was grown in the presence of 0,1% thioglycolate or 1 mg/mL glutathione. After 1 hour induction, no significant differences in sLuc^{opt} luciferase activity were detected between the different media (Fig. S2). Thus, the presence of thioglycolate or glutathione does not negatively affect *sLuc^{opt}* expression and detection.

We wanted to determine if sLuc^{opt} accumulates intracellularly before detectable luciferase activity in the medium, due to a lag between the production and secretion. Therefore, we harvested cells expressing the sLuc^{opt} reporter under the control of the P$_{tet}$ promoter, and determined the presence of intracellular luciferase activity (using whole cell lysates) in relation to the luciferase activity in culture supernatant in time after induction with 200 ng/mL of ATc (Fig. 2D). Low level intracellular luciferase activity was detected but remained constant

throughout time. In contrast, extracellular luciferase activity strongly increased in time (Fig. 2D) and the presence of extracellular sLuc^{opt} was confirmed by immunoblotting (Fig. 2E).

We conclude that secretion of sLuc^{opt} does not pose a significant bottleneck for gene expression analysis and that extracellular luciferase activity, therefore, is a good representation of intracellular promoter activity in *C. difficile*.

The sLuc^{opt} reporter can be used to monitor gene expression

We established that the sLuc^{opt} reporter could reliably be detected in culture supernatant when produced by an inducible promoter. Next, we wanted to confirm its application for gene expression profiling. Toxin production is influenced by nutrient availability and growth conditions, and a change in conditions can result in different expression profiles [22,24]. We placed the promoter of the toxin A gene (*tcdA*) [22] upstream of sLuc^{opt} and assayed luciferase activity in time. We chose the *tcdA* promoter as *tcdA* is expressed at higher levels than the toxin B gene (*tcdB*) [22].

We found that toxin A gene expression in BHI medium occurs in the exponential growth phase, with the maximum luciferase activity at 12h (158840 ± 2411 RLU/OD) (Fig. 3A), consistent with previous quantitative real-time PCR experiments [25-27]. The extracellular and intracellular luciferase activity remained stable in stationary growth phase, suggesting that P$_{tcdA}$ expression is switched off upon entering the stationary phase. In contrast, the toxicity of bacterial supernatants towards Vero cells is generally only detectable from stationary growth phase [28]. Consistent with our findings, we did not detect significant extracellular levels of toxins in the exponential phase (T5), but readily detected toxins from the transition into stationary phase (T8, Fig. 3B). Intracellularly, toxin A was detectable by immunoblotting during exponential growth (Fig. 3C). Our results suggest that toxin synthesis occurs well before the toxins are detectable in the medium. The presence of glucose results in repression of *C. difficile* toxin gene expression but this effect is influenced by the medium composition [22,24]. As BHI contains 0,5% glucose, we evaluated the effect of glucose on the expression of the P$_{tcdA}$-*sLuc^{opt}* in TY medium [22]. Overall, the toxin A gene expression profile in TY medium was similar to the profile observed in BHI medium (data not shown). In the presence of 1% glucose luciferase activity was detected at 3 hours (26198 ± 20 RLU/OD) and 8 hours post-inoculation (52078 ± 561 RLU/OD). No significant increase of P$_{tcdA}$-*sLuc^{opt}* signal was detected after 8 hours, in contrast to TY medium without glucose (Fig. 3D), confirming the catabolite repression of the toxin A gene expression.

In total, sLuc^{opt} has been used to investigate the transcriptional dynamics of the *tcdA* gene under different growth conditions. This construct is valuable for those that wish to further characterize factors that influence the transcription of the *C. difficile* toxin genes.

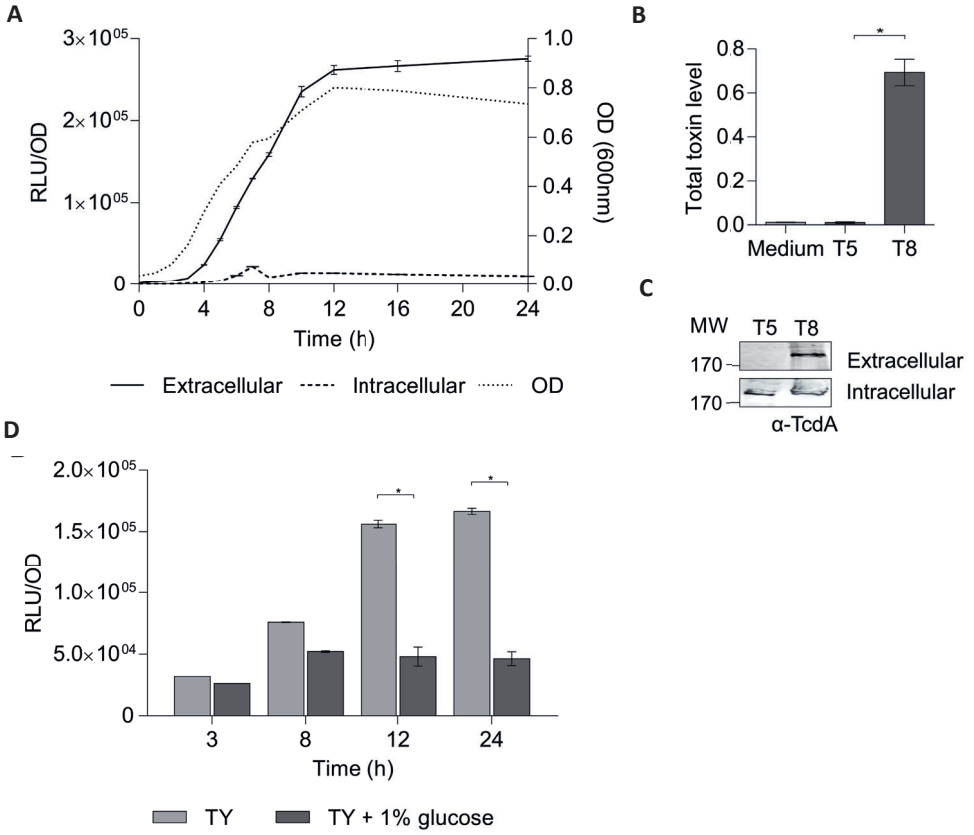

Fig. 3 - Dynamics of P_{tcdA}-dependent luciferase activity. A) Luciferase activity of a *C. difficile* strain harbouring P_{tcdA}-*sLuc^{opt}*. Extracellular (solid line) and intracellular (broken line) luciferase activity at the indicated time points of 1:100 diluted samples. Growth curve is represented as a dotted line. **B)** Immunoassay of 1:10 diluted samples at 5h (T5) and 8h (T8) post-inoculation. **C)** Western blot analysis of TCA-precipitated samples with anti-TcdA antibody, 5h (T5) and 8h (T8) post-inoculation intracellularly and extracellularly. **D)** Luciferase activity of a *C. difficile* strain harbouring P_{tcdA}-*sLuc^{opt}* in TY medium with or without 1% glucose, at 0, 8, 12 and 24 hours after inoculation. Error bars represent the mean value of triplicate samples +/- standard deviation. Student's t-test was used for statistical analysis, *p<0,000001.

Conclusion

The signal sequence of the PPEP-1 is useful to generate efficiently secreted proteins in *C. difficile*. The signal sequence was fused to codon-optimized heterologous proteins to produce strains of *C. difficile* capable of degrading starch or secreting a luciferase. AmyE^{opt} is reliable

and easy for plate-based gene expression assays, but can also be used in liquid cultures. Using this reporter, we determined that P_{veg} from *B. subtilis* is highly expressed and can be used for constitutive expression in *C. difficile*. The $sLuc^{opt}$ reporter is a particularly valuable tool for highly sensitive gene expression studies, as exemplified by our study of the dynamics of *tcdA* expression. Both reporters can be easily assessed for cells grown under anaerobic conditions and do not require complex processing of samples.

Methods

Strains and growth conditions

Escherichia coli strains were cultured in Luria Bertani (LB) broth supplemented with chloramphenicol at 10 µg/mL or 20 µg/mL kanamycin when appropriate. For agar plates, 20 µg/mL chloramphenicol was used.

C. difficile strains were cultured in Brain Heart Infusion broth (BHI, Oxoid), with 0,5 % w/v yeast extract (Sigma-Aldrich), supplemented with 20 µg/mL thiamphenicol and *Clostridioides difficile* Selective Supplement (CDSS; Oxoid), when necessary. 0,1% thioglycolate (pH 7,4) and 1 mg/mL glutathione (pH 7,5) were added from the beginning of growth for the assay represented in Fig. S2. Tryptone Yeast medium (TY, 3% Bacto Tryptose (BD Difco), 2% Yeast Extract (Sigma-Aldrich), 0,1% thioglycolate, pH7,4) [22], supplemented with 1% glucose when necessary, was used for the assay represented in Fig. 3D. *C. difficile* strains were grown anaerobically in a Don Whitley VA-1000 workstation with an atmosphere of 10% H_2, 10% CO_2 and 80% N_2. The growth was followed by optical density reading at 600 nm.

Plasmids (Table 1) were maintained in *E. coli* strains DH5α or MC1061, grown aerobically at 37°C. Plasmids were transformed into *E. coli* CA434 [29] by standard procedures [30]. Introduction of plasmids into *C. difficile* strain 630Δ*erm* by conjugation was performed as previously described [29]. Briefly, pellets from 1 mL of an overnight culture of *E. coli* CA434 harbouring the conjugative plasmids were mixed with 100 µL of the recipient *C. difficile* strain, spotted onto pre-reduced BHI agar plates and incubated overnight at 37°C. Growth was harvested and serial dilutions were plated onto BHI plates with CDSS and thiamphenicol. Single colonies were re-streaked 2 more times onto fresh selective plates before strains were confirmed by PCR and growth on CLO plates (Biomerieux). All the strains are described in Table 2.

Anhydrotetracycline (ATc; 20-500 ng/mL final concentration) was used for induction of the strains containing the P_{tet} promoter in the *C. difficile* expression vectors described below. *C.*

difficile strain 630Δ*erm* with or without pRPF185[9] was used as a control for the reported experiments.

Table 1 - Plasmids used in this study.

Name	Relevant features	Source/Reference
pRPF185	*tetR* P_{tet}-*gusA*; *catP*	[9]
pWKS1583	*tetR* P_{tet}-*amyE*^{opt}; *catP*	This study
pSJ111	P_{veg}-*amyE*^{opt}; *catP*	This study
pJ201	*kan*	DNA2.0
pAP18	*sluc*^{opt}; *kan*	This study
pAP24	*tetR* P_{tet}-*sluc*^{opt}; *catP*	This study
pAP43	*tetR* P_{tcdA}-*sluc*^{opt}; *catP*	This study

* *km* – kanamycin resistance cassette, *catP* – chloramphenicol resistance cassette

Table 2 - *C. difficile* strains used in this study.

Name	Relevant Genotype/Phenotype	Origin /Reference
C. difficile 630Δ*erm*	Erm^S	[15,16]
WKS1588	630Δ*erm* pRPF185; $Thia^R$	This study
WKS1594	630Δ*erm* pWKS1583; $Thia^R$	This study
SJ113	630Δ*erm* pSJ111; $Thia^R$	This study
AP34	630Δ*erm* pAP24; $Thia^R$	This study
AP48	630Δ*erm* pAP43; $Thia^R$	This study

* Erm^S – Erythromycin sensitive, $Thia^R$ – Thiamphenicol resistant

Construction of a synthetic amylase

To construct a synthetic amylase for expression in *C. difficile*, the DNA sequence encoding the predicted signal sequence (amino acids 1-28) of PPEP-1 was fused to a codon-optimized amylase gene, based on the amylase of *B. subtilis* subsp. *subtilis amyE* (Genbank CAB12098.2) lacking its signal sequence. The resulting hybrid aminoacid sequence (AmyE^{opt}; for codon-optimized α-amylase) is available from Genbank (accession number KT895263). The gene was synthesized and cloned into pRPF185 [9] by DNA2.0 (Menlo Park, CA, USA), placing it under control of the ATc-inducible promoter P_{tet}, yielding pWKS1583 (Addgene 70188) (Fig. S3A). *In silico* analysis of the hybrid gene using SignalP4.1 [31] indicates that cleavage of the signal sequence is expected between the aminoacids 26 and 27 (Fig. S4C).

The P_{veg} promoter of *B. subtilis* subsp. *Subtilis* [19,20] was reconstituted by annealing the oligonucleotides oWKS-1529 (5'-

CCTTATTAACGTTGATATAATTTAAATTTTATTTGACAAAAATGGGCTCGTGTTGTACAATAAATGTA GAGCTC - 3') and oWKS-1530 (5' - CTACATTTATTGTACAACACGAGCCCATTTTTGTCAAATAAAATTTAAATTATATCAACGTTAATAAGG GTACC - 3'). The resulting dsDNA fragment was ligated into *KpnI-SacI* digested pWKS1583, yielding pSJ111 (Addgene 72985).

All plasmids were sequence verified using primers NF1323, NF793 and/or NF794 [9].

Amylase activity assays

To visualize amylase activity of *C. difficile* strains on solid media, plates were prepared with 0.1% soluble starch (Sigma-Aldrich), supplemented with 20, 50, 100, 200 or 500 ng/mL ATc when required. Single colonies were replicated onto the starch plates and incubated for 24h anaerobically. To visualize amylase activity of mixed cultures of *C. difficile* strains the cultures were mixed 5:1 (non-producing versus producing) when reached OD_{600} of 0,3, serially diluted, plated onto starch plates and incubated for 24h. To assess amylase activity, plates were removed from the anaerobic cabinet and stained with a 0,2% iodine solution for 20 minutes. Excess iodine was removed and plates were imaged against a white background. The diameter of the halo formed was measured perpendicular to the streak.

To measure the amylase activity in liquid media, 1 mL culture samples were taken at the indicated time points. The amylase activity was measured with the Amylase Activity Assay Kit (Sigma-Aldrich MAK009), according to the manufacturer's instructions.

Construction of a luciferase reporter

To construct a secreted luciferase reporter, we fused the DNA sequence encoding the predicted signal sequence (aa 1-28) of PPEP-1 to a codon-optimized luciferase gene, based on Nanoluc (Promega; Genbank AFI79295.1) [21]. The resulting hybrid aminoacid sequence ($sLuc^{opt}$; for secreted codon-optimized luciferase) is available from Genbank (accession number KT895264). The gene was synthesized by DNA2.0 (Menlo Park, CA, USA) in their proprietary vector pJS201, yielding pAP18. *In silico* analysis of $sLuc^{opt}$ using SignalP4.1 [31] indicates that cleavage of the signal sequence is expected between aminoacids 26 and 27 (Fig. S4D). To generate an anhydrotetracycline-inducible luciferase, the *SacI-BamHI* fragment of pAP18 carrying the $sluc^{opt}$ gene was cloned into similarly digested pRPF185 [9], yielding plasmid pAP24 (Addgene 70190) (Fig. S3B).

To construct pAP48, the toxin A promoter region (P_{tcdA}) was amplified using primers oAP17 (5' – CATGGTACCGGTCAGTTGGTAAAATCTATTAAGC - 3') and oAP18 (5' -

CAGGAGCTCTTATTTTTGATAATAAATCCAC - 3'). The resulting fragment was *SacI-BamHI* digested and cloned into similarly digested pAP24, yielding vector pAP48.

All plasmids were sequence verified using primers NF1323, NF793 and/or NF794 [9].

Luciferase activity assay

To measure luciferase activity, 1 mL culture sample was taken at the indicated time points. The cells were pelleted (5 min, 4ºC, 14000 rpm) and culture supernatants were filtered (0.22 µm) to remove any remaining cells. Bacterial pellets were suspended in 1 mL lysis buffer (10mM Tris, 100 mM EDTA, 1X AESBF, 0.5 mg/mL Lysozyme) and incubated for 30 min at 37ºC. All the measurements were performed with 100 µL 1:100 sample (either whole cell lysate or culture supernatant) in triplicate in a 96-well plate with 20 µL NanoGlo Luciferase System (Promega N1110), according to manufacturer's instructions. Luciferase activity was determined using a Mithras LB940 Luminometer (Berthold) for 0.1 s. Data were normalized to culture optical density measured at 600 nm (OD_{600}).

Western blot analysis

For western blotting 2 mL culture was used. The cell pellet was collected by centrifugation (5 min, 4ºC, 14000 rpm) and suspended in lysis buffer. For $sLuc^{opt}$ detection, the supernatant samples were concentrated in Amicon Ultra Centrifugal Filters (Millipore), to a final volume of 100 µL. All the samples were normalized for OD_{600}, analysed by SDS-PAGE (12.5% polyacrylamide) and transferred onto a nitrocellulose membrane. The membrane was probed with a rabbit polyclonal anti-NanoLuc antibody (Promega) 1:2500 in TBSTB (20 mM Tris-HCl pH 7,5, 150 mM NaCl, 0,05% Tween-20, 5% BSA).

For TcdA detection, culture supernatants were precipitated with trichloroacetic acid (TCA). Briefly, 400 µL TCA was added to the culture supernatant and incubated for 10 min at 4ºC. The samples were centrifuged (5 min, 4ºC, 14000 rpm) and the pellet suspended in 200 µL cold acetone. This step was repeated two more times. The precipitated protein was eluted in 1x Laemmli loading buffer. The samples were normalized for OD_{600} and analysed by SDS-PAGE (6% polyacrylamide), transferred onto nitrocellulose membrane and the membrane was probed with a rabbit monoclonal anti-TcdA antibody (TCC8; tgcBiomics) 1:3000 in TBSTB.

The probed membranes were analysed using a secondary anti-rabbit HRP antibody 1:3000 (Dako), and Pierce ECL2 Western blotting substrate (Thermo Scientific). A Typhoon 9410 scanner (GE Healthcare) was used to measure the chemiluminescent signal.

Immunoassay

For total toxin detection, 1 mL culture sample was taken at the indicated timepoints. The cells were pelleted (5 min, 4ºC, 14000 rpm) and culture supernatants were filtered (0.22 µm). Toxins A and B relative amount was quantified using the Wampole *C. difficile* TOX A/B II (TechLab), according to the manufacturer instructions with 1:10 diluted supernatant.

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Supplemental Figures

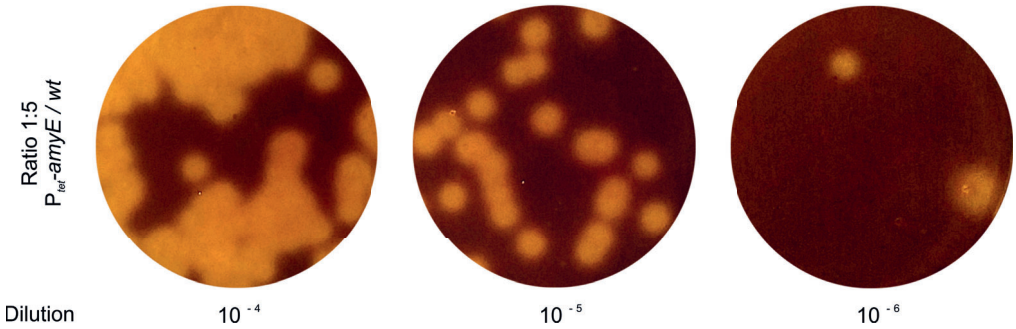

Fig. S1 - Visualization of secreted amylase activity in mixed cultures. *C. difficile* strains 630Δ*erm* (wt) and WKS1594 (P$_{tet}$-*amyE)* were mixed in 5:1 ratio, serially diluted and plated onto BHI agar plates supplemented with 0.1% soluble starch and 500 ng/mL ATc. Staining with a solution of 0.2% iodine was performed after 24h. Halos (yellow) indicate starch degradation by the α-amylase. Note that non-producing colonies are not visible in this picture due to the dark colour as a result of the iodine stain and that a proper dilution is important to reliably identify halo-producing colonies.

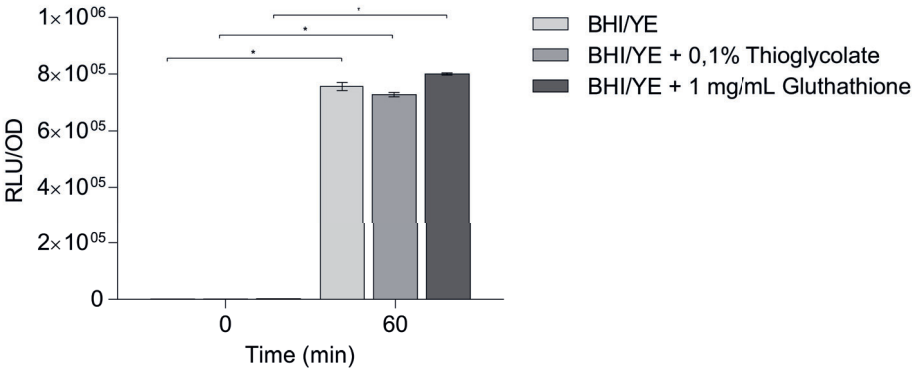

Fig. S2 - Luciferase activity of *C. difficile* in the presence of reducing agents. Luciferase activity of diluted 1:100 culture supernatant of *C. difficile* strain harbouring P$_{tet}$-*sLuc^{opt}* induced with 200 ng/mL anhydrotetracycline in the presence 0,1% thioglycolate or 1 mg/mL glutathione, following induction at 0 and 60 min. Error bars represent the +/- standard deviation of triplicate samples. Student's t-test was used for statistical analysis, *p<0,0001.

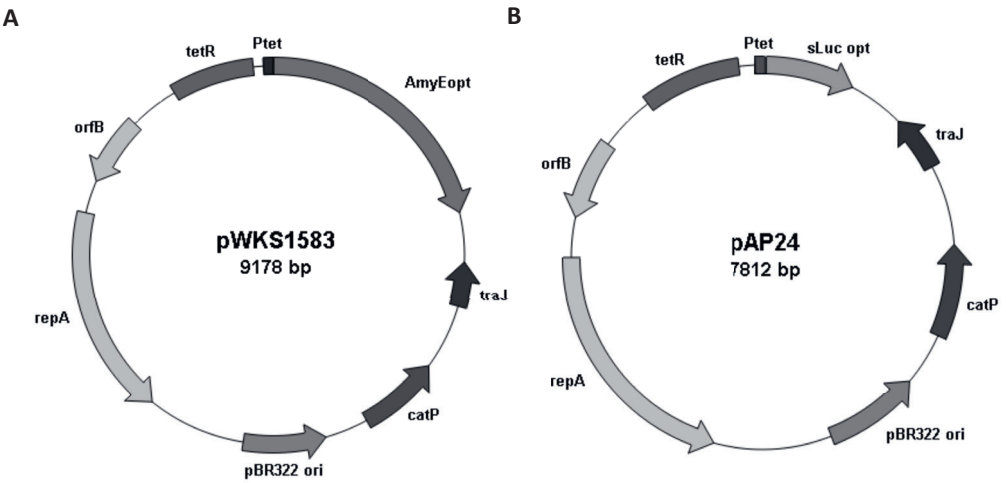

Fig. S3 - Schematic representation of pWKS1583 and pAP24. A) Schematic representation of pWKS1583 vector, encoding AmyE^{opt}, under the control of anhydrotetracycline inducible promoter P_{tet}. **B)** Schematic representation of pAP24 vector, sLuc^{opt}, under the control of anhydrotetracycline inducible promoter P_{tet}.

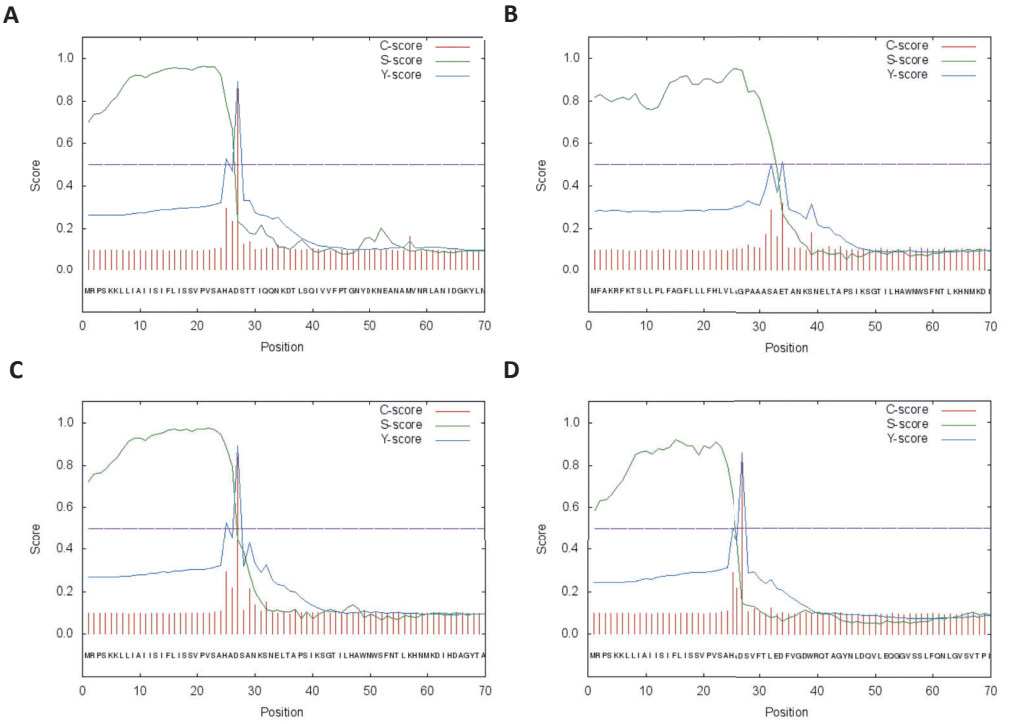

Fig. S4 - *In silico* analysis of the signal sequences. Analysis of the hybrid proteins **A)** CD2380, **B)** AmyE, **C)** AmyE^{opt} and **D)** sLuc^{opt} using SignalP4.1 server [31]. The X-axis shows the residue positions of the proteins. The Y-axis indicates the raw cleavage score (C-score), signal peptide score (S-score), and combined cleavage site score (Y-score) generated by SignalP4.1.

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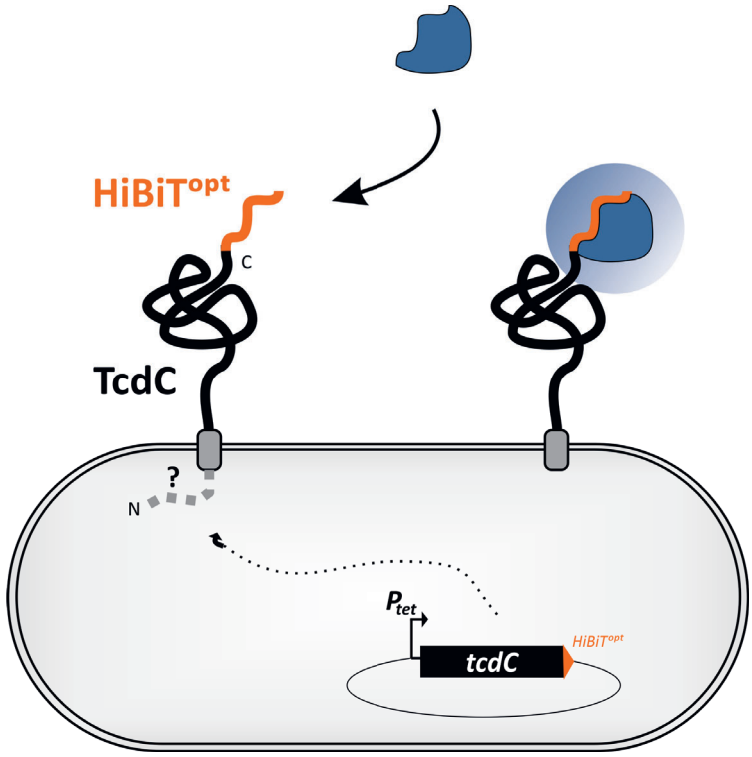
HiBiTopt
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