

Expression and delivery of an endolysin to combat *Clostridium perfringens*

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Abstract *Clostridium perfringens* is a cause for increasing concern due to its responsibility for severe infections both in humans and animals, especially poultry. To find new control strategies to treat *C. perfringens* infection, we investigated the activity and delivery of a bacteriophage endolysin. We identified a new endolysin, designated CP25L, which shows similarity to an *N*-acetylmuramoyl-L-alanine amidase domain and is distinct from other *C. perfringens* endolysins whose activity has been demonstrated in vitro. The *cp25l* gene was cloned and expressed in *Escherichia coli*, and the gene product demonstrated lytic activity against all 25 *C. perfringens* strains tested. The probiotic strain *Lactobacillus johnsonii* FI9785 was engineered to deliver the endolysin to the gastrointestinal tract. The integration of the *nisRK* two-component regulatory system from the *Lactococcus lactis* nisin A biosynthesis operon into the chromosome of *L. johnsonii* allowed constitutive expression of the endolysin under the control of the *nisA* promoter (P_{nisA}), while the use of a signal peptide (SLPmod) led to successful secretion of the active endolysin to the surrounding media. The high specificity and activity of the endolysin suggest that it may be developed as an effective tool to enhance the control of *C. perfringens* by *L. johnsonii* in the gastrointestinal tract.

Keywords *Clostridium perfringens* · *Lactobacillus johnsonii* · Endolysin · Delivery · Probiotic

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Introduction

Clostridium perfringens is a rod-shaped, Gram-positive, anaerobic spore-forming bacterium with the ability to produce potent toxins. According to the pattern of toxin expression, *C. perfringens* strains are classified into five toxinotypes: A, B, C, D, and E (Miyamoto et al. 2012; Petit et al. 1999). The interest in this microorganism is increasing because it is responsible for a range of debilitating conditions, including food poisoning, gas gangrene, necrotic enteritis (NE) both in humans and animals, and non-food-borne gastrointestinal infections (Keyburn et al. 2008; Lindstrom et al. 2011). *C. perfringens* associated food poisoning is classed among the most common gastrointestinal diseases in industrialized countries (Lindstrom et al. 2011; Scallan et al. 2011). One of the most frequently occurring infections caused by *C. perfringens* type A is NE in poultry; in countries where the use of antimicrobial growth promoters in feed has been abolished, the incidence of NE has increased, and the productivity of the poultry industry has been reduced (Lee et al. 2011; Van Immerseel et al. 2009). A range of different alternatives to the conventional antimicrobial growth promoter approach are being investigated to reduce the numbers of these pathogens in the food chain and improve poultry health. In addition to vaccination (Lee et al. 2011) or the use of natural antimicrobials (Timbermont et al. 2010), there are a number of examples of using probiotic microbes (Gil de los Santos et al. 2012; Kizerwetter-Swida and Binek 2009; La Ragione et al. 2004) or lytic enzymes (Liu et al. 2010; Zhang et al. 2010) to control NE in poultry. Bacteriophages and their endolysins present a further opportunity to reduce *C. perfringens* without disturbing the balance of the microbiota in the gastrointestinal (GI) tract.

Bacteriophage therapy is a rapidly growing area of research (Lu et al. 2011; O'Flaherty et al. 2009), and tests in poultry have demonstrated the potential of bacteriophages to control levels of *C. perfringens*, *Salmonella*, and *Campylobacter*

(Atterbury et al. 2007; El-Shibiny et al. 2009; Miller et al. 2010). However, the narrow host range of bacteriophages and the rapid development of resistance can limit their application as control agents. An alternative approach is to develop bacteriophage proteins as novel antimicrobial agents. At the end of their lytic cycle, most dsDNA bacteriophages release their progeny by lysis of the host cell via the activity of two proteins—the holin and the endolysin. The holins are small hydrophobic proteins which disrupt the cell membrane to produce holes, allowing the endolysin to access the cell wall (Catalao et al. 2012). The endolysins target bonds in the peptidoglycan, resulting in the destruction of the cell and the release of newly produced virions. Although evolved to act from inside the cell, endolysins which act on Gram-positive bacteria are also effective when applied exogenously, giving them the potential to be used as targeted antibacterial agents (Fischetti 2010).

Although there are several variations in endolysin architecture, the majority of endolysins from bacteriophages infecting Gram-positive bacteria consist of two functional regions: the enzymatically active region contains one or more domains providing the catalytic activity of the enzyme (e.g., muramidase, glucosaminidase, endopeptidase, or amidase), attacking specific bonds in the peptidoglycan, while the cell wall binding region allows the endolysin to bind to specific ligands found in the cell wall of the host bacterium and so provides specificity (Schmelcher et al. 2012). Properties such as this specificity for their host pathogen, leading to minimal disturbance of the normal flora, the low risk of bacterial resistance, and the ability to kill colonizing pathogens on mucosal surfaces make them ideal anti-infective agents (Fischetti 2010; O'Flaherty et al. 2009). Recent studies demonstrated the efficiency of endolysins in killing or controlling pathogenic bacteria in animal models (Fenton et al. 2010; Grandgirard et al. 2008; Gupta et al. 2011; Yoong et al. 2006); they have also shown efficacy against biofilms (Meng et al. 2011), as disinfectants (Hoopes et al. 2009) and as the basis of detection systems for bacteria (Kretzer et al. 2007). Several bacteriophage endolysins which produce lytic activity against *C. perfringens* cells have been described (Nariya et al. 2011; Schmitz et al. 2011; Simmons et al. 2010; Zimmer et al. 2002). A number have also been identified by sequencing and genome mining, producing a rich resource of information for such enzymes (Morales et al. 2012; Oakley et al. 2011; Schmitz et al. 2011; Volozhantsev et al. 2012; Volozhantsev et al. 2011).

Lactobacillus johnsonii FI9785 is a poultry-isolated strain which has been shown to act as a competitive exclusion agent against *C. perfringens* in chickens (La Ragione et al. 2004). As well as acting as probiotics in their own right, lactic acid bacteria have also shown promise as delivery systems for the secretion of biologically active interleukins or peptidoglycan hydrolases (Braat et al. 2006; Fernandez et al. 2009; Turner

et al. 2007). The promoter of the structural gene encoding the lantibiotic nisin A (P_{nisA}) is induced by nisin A via signal transduction using the two component regulatory system NisRK (Kuipers et al. 1995). This system has been exploited to develop gene expression systems in *Lactococcus lactis*, *Leuconostoc lactis*, and *Lactobacillus helveticus* (de Ruyter et al. 1996; Kleerebezem et al. 1997), and in this study, we have used the NisRK system to express the endolysin CP25L, originating from a prophage of *C. perfringens* (Gervasi et al. 2013), in a probiotic strain of *L. johnsonii* which was engineered for delivery to the GI tract.

Materials and methods

Bacterial strains and growth conditions

C. perfringens strains (listed in Table 1) were obtained from the NCTC (HPA, London, UK), or from in-house culture collections (IFR, Norwich, UK). *C. perfringens* 5416–97 is a type A strain (Grant et al. 2008). Strains were maintained in Robertson's cooked-meat medium (SGL) at room temperature and were grown anaerobically at 37 °C in brain heart infusion broth (BHI, Oxoid) supplemented with vitamin K [50 µg/l], hemin [5 mg/l], resazurin [1 mg/l], and L-cysteine [0.5 g/l], termed BHI+C. *Escherichia coli* strains were grown in L broth with shaking at 37 °C, and *L. johnsonii* strain FI9785 (NCIMB 41621) (La Ragione et al. 2004) and derivatives were grown in MRS broth (Oxoid) at 37 °C. Commensal, environmental, and clostridial strains (listed in Tables 1 and 2) were obtained from IFR culture collections, the DSMZ (Braunschweig, Germany), or the NCIMB (Aberdeen, UK) and were grown as recommended by DSMZ or in BHI+C.

Endolysin CP25L analysis and subcloning

The endolysin sequence is available in the vB_CpeS-CP51 genome nucleotide sequence, accession number KC237729. Endolysin amino acid similarities and conserved domains were determined using Blastp (Altschul et al. 1997) and the NCBI non-redundant protein sequences database. Amino acid alignments were performed using the ClustalW algorithm in Vector NTI (Invitrogen) and visualized with GeneDoc (Nicholas et al. 1997).

Genomic DNA was extracted from cells of *C. perfringens* 5416–97 (grown to mid-exponential phase) using the Genomic DNA extraction kit with a Genomic Tip 20/G column (Qiagen) as described by the manufacturer, with the addition of 50 U mutanolysin (Sigma) to aid cell lysis. The putative endolysin gene *cp25l* was amplified from genomic DNA using Phusion DNA polymerase (Finnzymes). To facilitate cloning into the NdeI and XhoI sites of expression vector pET15b (Novagen), primers were designed to incorporate an

Table 1 Lytic activity of CP25L against sensitive strains

Bacterial strains	Lytic activity ^a		
	CP25L	Lag (min)	Lysozyme
<i>C. perfringens</i> NCTC 3110	79.5±1.0	1	–
<i>C. perfringens</i> NCTC 8238	59.2±0.9	6	21.8±1.5
<i>C. perfringens</i> NCTC 8239	28.6±0.4	6	10.6±0.6
<i>C. perfringens</i> 5146-97 ^b	58.8±4.4	4	8.5±0.5
<i>C. perfringens</i> 5810-97 ^b	17.1±2.5	10	5.6±0.9
<i>C. perfringens</i> 2144-98 ^b	39.8±3.1	10	22.5±0.0
<i>C. perfringens</i> 2118-98 ^b	23.0±0.2	–	16.7±0.4
<i>C. perfringens</i> 2536-01 ^b	44.6±0.7	2	30.2±0.6
<i>C. perfringens</i> 2551-01 ^b	50.7±1.7	4	25.4±1.0
<i>C. perfringens</i> 4519-98 ^b	70.5±1.7	8	15.6±0.1
<i>C. perfringens</i> 2151-88 ^b	34.2±0.4	–	17.5±0.8
<i>C. perfringens</i> 6081-97 ^b	38.0±0.7	–	16.7±0.0
<i>C. perfringens</i> 3011-98 ^b	38.8±0.3	–	22.0±0.0
<i>C. perfringens</i> 2540598 ^b	37.9±0.6	8	–
<i>C. perfringens</i> F3278	39.4±1.2	15	20.2±2.7
<i>C. perfringens</i> N151/151	49.4±2.0	–	9.8±0.3
<i>C. perfringens</i> N147/155A	46.4±2.3	4	7.3±0.0
<i>C. perfringens</i> DP2	39.4±2.1	6	–
<i>C. perfringens</i> DP3	35.7±0.0	–	24.3±0.0
<i>C. perfringens</i> DP5	46.2±1.8	–	15.7±0.4
<i>C. perfringens</i> CH1	37.5±5.9	4	–
<i>C. perfringens</i> CH2	35.4±0.2	6	–
<i>C. perfringens</i> FD00389 ^c	26.5±9.7	22	–
<i>C. perfringens</i> FD00413 ^c	37.8±0.5	–	–
<i>C. perfringens</i> FD00412 ^c	5.5±0.2	6	–
<i>B. cereus</i> NCIMB 11796	17.3±1.3	6	46.7±1.5
<i>B. subtilis</i> ATCC 6633	5.5±2.5	6	–
<i>C. acetobutylicum</i> BL75141	9.5±0.3	18	33.2±1.3
<i>C. bif fermentans</i> NCTC 13019	56.6±0.5	4	–
<i>C. beijerinckii</i> NCIMB 8052	13.5±2.3	6	46.6±1.0

^a Percent drop in OD₆₀₀ in 4 min linear lysis (mean±standard deviation), dash no lysis/lag

^b Strains kindly provided by Dr. M. Brett, (HPA, UK)

^c Strains kindly provided by Prof. R La Ragione (AHVLA, UK)

NdeI site at the initiating methionine codon CP25L_NDE (5'-GAATGTCATATGTATATAAATCAATCA-3', altered nucleotides underlined throughout) and a XhoI site downstream of the stop codon CP25L_XHO (5'-ACTCGAGGTGGGATAA TTCCTACC-3'). Due to the presence of an internal NdeI site, internal primers were designed to create a 1 bp substitution (T771C) without changing the resulting amino acid sequence. Primer CP25L_GTG (5'-TATTGCATGTGGTAGTCAA AAGAT-3') was used in conjunction with CP25L_NDE, and primer CP25L_CAC (5'-TTGACTACCAATGCAATATAA AAATG-3') was paired with CP25L_XHO, then the two products were spliced and amplified using overlap extension

Table 2 Strains resistant to lysis by CP25L

Bacterial strains
<i>Anaerococcus hydrogenalis</i> DSMZ 7454
<i>Bacillus amyloliquefaciens</i> 0880
<i>Bifidobacterium adolescentis</i> DSMZ 20083
<i>Bifidobacterium angulatum</i> DSMZ 20098
<i>Bifidobacterium bifidum</i> DSMZ 20082
<i>Bifidobacterium longum</i> DSMZ 20219
<i>Bifidobacterium pseudocatenulatum</i> DSMZ 20438
<i>Clostridium cellobioparum</i> DSMZ 1351
<i>Clostridium coccoides</i> NCTC 11035
<i>Clostridium colinum</i> DSMZ 6011
<i>Clostridium difficile</i> NCTC 11204
<i>Clostridium innocuum</i> DSMZ 1286
<i>Clostridium leptum</i> DSMZ 753
<i>Clostridium nexile</i> DSMZ 1787
<i>Clostridium ramosum</i> DSMZ 1402
<i>Clostridium sordellii</i> NCTC 13356
<i>Clostridium sporogenes</i> ATCC 17886
<i>Clostridium tyrobutyricum</i> NCIMB 9582
<i>Enterococcus faecalis</i> FI10734
<i>Enterococcus hirae</i> FI10477
<i>Eubacterium barkeri</i> DSMZ 1223
<i>Lactobacillus casei</i> FI10736
<i>L. johnsonii</i> FI9785
<i>Lactobacillus plantarum</i> FI08595
<i>Lactobacillus rhamnosus</i> FI10737
<i>L. lactis</i> MG1363
<i>Leuconostoc mesenteroides</i> subsp. <i>mesenteroides</i> ATCC 8293
<i>Listeria innocua</i> NCTC 11288
<i>Listeria ivanovii</i> NCTC 11007
<i>Micrococcus luteus</i> FI10640
<i>Pediococcus pentosaceus</i> FI10642
<i>Pediococcus acidilactici</i> FI10738
<i>Salmonella enterica</i> serovar Typhimurium FI10739
<i>Salmonella enterica</i> serovar Enteritidis FI10113
<i>S. aureus</i> FI10139

polymerase chain reaction (Horton et al. 1989) with primers CP25L_NDE and CP25L_XHO. The spliced product was restricted with NdeI and XhoI (New England Biolabs) and cloned into restricted pET15b that had been dephosphorylated with Antarctic Phosphatase (New England Biolabs), using Fastlink ligase (Epicentre). Ligation products were transformed into chemically competent *E. coli* TOP10 cells (Invitrogen), and transformants were selected with ampicillin (100 µg/ml). After confirmation by sequencing, construct pET15b-*cp25l* and the empty control vector pET15b were transformed for expression into chemically competent *E. coli* BL21(DE3) cells (Invitrogen).

An endolysin expression and secretion construct was produced in pUK200 (Wegmann et al. 1999) placing the endolysin coding sequence downstream of the signal peptide SLPmod (Fernandez et al. 2009), and a 6xHis tag, all under the control of the promoter P_{nisA} (pUK200-*slpmod-6xHis-cp25l*). Ligation products were transformed into electrocompetent *E. coli* MC1022, selected with chloramphenicol (15 µg/ml) and confirmed by sequencing. To provide the two-component regulatory system required for P_{nisA} -controlled expression, the *nisRK* genes from *L. lactis* FI5876 (Dodd et al. 1992) were inserted into the chromosome of *L. johnsonii* FI9785 using the thermosensitive pG+host9 vector (Maguin et al. 1996). An integration region was amplified from *L. johnsonii* FI9785 genomic DNA, and the resulting 1,033-bp product was cloned into the blunt-ended *SpeI* site of pG+host9 (creating plasmid pFI2657). A 2.4-kb region encoding *nisRK* was amplified from the genome of *L. lactis* FI5876 using the primer pair *nisRK_F* (5'-CCCGGGAGAATCTTAAAGAGTCTAGGG-3') and *nisRK_R* (5'-AAAAAGTAATCCTTAGAGATTAC-3') and cloned into a blunt-ended *BstEII* site located within the integration region of pFI2657, creating pFI2652. Subsequently, *L. johnsonii* FI9785 was transformed with pFI2652, and the *nisRK* sequences were integrated into the chromosome by gene replacement (Maguin et al. 1996) using 30 °C as the permissive temperature and 42 °C as the non-permissive temperature, to create the *nisRK*-carrying strain FI10744. The lysin expression construct and the control vector pUK200 were transformed into electrocompetent *L. johnsonii* FI10744 as described (Horn et al. 2005), and positive transformants were selected using chloramphenicol (7.5 µg/ml) to give the endolysin delivery strain FI10744-L and the vector control FI10744-V.

Protein expression, analysis, and partial purification

Crude protein extracts were produced from IPTG-induced *E. coli* BL21(DE3) cells containing pET15b-*cp25l* or pET15b in NP buffer (20 mM sodium phosphate buffer pH 6.5) as previously described (Mayer et al. 2008). His-CP25L was purified using the nickel-nitrilotriacetic acid (Ni-NTA) Fast Start kit (Qiagen). For expression in *L. johnsonii*, cells were grown to mid-exponential phase then incubated for 2 h with or without nisin (10 ng/ml). Cells were harvested by centrifugation for 10 min at 2,500×g and 4 °C, and the cell pellet was frozen while the supernatant was concentrated 20-fold using Amicon columns (Ultra-4 ultracell-30 k, Millipore) or 166-fold by shaking for 5 min after the addition of an equal volume of 100 % ethanol pre-cooled to -80 °C, followed by centrifugation for 30 min at 10,000×g and 4 °C. The resulting pellet was freeze-dried prior to resuspension in Ni-NTA elution buffer (EB, 50 mM sodium phosphate, 300 mM NaCl, 250 mM imidazole pH 8). Crude cell extracts were produced in EB by bead beating as with *E. coli*. Protein quantification, electrophoresis, and Western blot analysis with a His Tag®

monoclonal antibody (Novagen) were all performed as described previously (Mayer et al. 2008).

Lysis assays

The lysis of *C. perfringens* NCTC 3110 cells resuspended in phosphate-buffered saline (PBS) was assessed by turbidity reduction assay as previously described (Mayer et al. 2008) in duplicate 300-µl assays comprising 270 µl resuspended cells and 30 µl crude or Ni-NTA purified endolysin extract or the appropriate buffer controls. Cells for lysis assays were harvested by centrifugation, flash-frozen in liquid nitrogen, and stored at -20 °C until use. Hen egg white lysozyme (Sigma) was used as a positive control at 500 U per 300 µl assay. The activity in different media was assessed by replacing the PBS with BHI+C or chemostat medium, a complex medium used for in vitro fermentations (CM: peptone water 2 g/l (Oxoid), yeast extract 2 g/l (Oxoid), NaCl 0.1 g/l, K₂HPO₄ 0.04 g/l, KH₂PO₄ 0.04 g/l, MgSO₄·7H₂O 0.01 g/l, CaCl₂·6H₂O 0.01 g/l, NaHCO₃ 2 g/l, Tween 80 2 ml, hemin 0.02 g/l dissolved in 400 µL 1 M NaOH, vitamin K1 10 µl of 5 %v/v aqueous solution, cysteine HCl 0.5 g/l, bile salts 0.5 g/l, and 1 % glucose all from Sigma (Mandalari et al. 2007)), which was adjusted to pH 6 to approximate colon conditions. Activity against different species was assessed using 1 µg of Ni-NTA purified protein during a 1-h incubation. The endolysin activity of crude protein extracts and concentrated culture supernatants from the engineered *L. johnsonii* strains was measured by plate assay as described previously (Stentz et al. 2010). Briefly, *C. perfringens* cells were grown to stationary phase in 100 ml BHI broth, autoclaved, pelleted by centrifugation then resuspended in 1 ml of PBS, and added to 100 ml of PBS with 1 % agar at 55 °C. After pouring into plates, small holes were punched into the seeded agar, and 10 µl of protein extract (equivalent to 30 µg total crude protein) or 20 µl of concentrated supernatants was loaded into the wells, and the plates were then incubated for 24 h at 37 °C. To demonstrate lytic activity from growing colonies, *C. perfringens* cells were processed in the same way then incorporated into plates containing MRS agar with 7.5 µg/ml chloramphenicol. Overnight cultures of FI10744-L and FI10744-V were subsequently streaked onto these plates, and the plates were incubated for 1–3 days at 37 °C.

C. perfringens NCTC 3110 viability assays were performed by adding 166 µg of Ni-NTA purified CP25L protein to 5 ml of pre-reduced BHI+C under anaerobic conditions. The protein was added to the media either immediately before *C. perfringens* inoculation (20 µl of a fresh overnight culture) or during the early exponential phase of growth (optical density [OD₆₀₀] ca. 0.3). To determine *C. perfringens* viability, samples were taken every 2 h for 8 h and serially diluted in PBS before being plated onto BHI agar and incubated for 24 to 48 h at 37 °C. Results were calculated as the total number of bacteria from triplicate counts.

Results

Bioinformatic analysis of the CP25L sequence

The *cp25l* gene was identified previously within the genome of a mitomycin C-inducible prophage of *C. perfringens* 5416–97, vB_CpeS-CP51 (Gervasi et al. 2013). Blastp analysis of CP25L identified two regions with similarity to conserved domains separated by a central section (Fig. 1). In the N-terminal part of the protein, there is an area with similarity to domains associated with cell wall binding and lysis, including the peptidoglycan recognition protein (PGRP, cd06583, amino acids 19–135, *E* value 1.24e-23), lysozyme (PHA00447, amino acids 22–134, *E* value 3.62e-25) and amidase_2 (*N*-acetylmuramoyl-L-alanine amidase, pfam01510, amino acids 20–128, *E* value 2.37e-22). An area with similarity to a bacterial SH3 domain (SH3_3, pfam08239, amino acids 322–375, *E* value 4.14e-09) is located at the C terminus. Alignments of this protein with other *C. perfringens* endolysins whose activity has been demonstrated experimentally revealed that there are only very low levels of amino acid conservation with the amidase_3 domain-containing lysins ply3626 (Zimmer et al. 2002) (28 % consensus/14.7 % identity), plyCP26F, or plyCP390 (Simmons et al. 2010) (14.1 %/7.4 % and 13.5 %/7.7 %, respectively), or with the glycosyl hydrolase_25 domain-containing Pms (Nariya et al. 2011) (26.3 %/16.3 %) or PlyCM (Schmitz et al. 2011) (25.5 %/15.5 %). The conservation with Pms and PlyCM is highest in the region of the SH3_3 hypothetical domain near the C terminus, with 44.8 % consensus/29.3 % identity between this 54 amino acid region in CP25L and the corresponding regions in Pms and PlyCM. The region of the amidase_2 domain also showed some homology to predicted endolysins from podoviruses of *C. perfringens* (Volozhantsev et al. 2012); for example, the CP25L sequence shows 66.9 % consensus/57.4 % identity over the first 135 amino acids covering the amidase_2 domain with the predicted lysin from ΦZP2. Interestingly, when CP25L was analyzed by Blastp, the sequences showing the highest similarity had the amino acid conservation concentrated in the center of the sequence, between the enzymatic and SH3 domains in an area without homology to a functional domain (Fig. 1). These sequences were all annotated as putative amidases from *C. perfringens* sequenced genomes in the NCBI databases, and it is possible that conservation of this area is relevant to the target cell wall.

Expression and activity of CP25L

CP25L was expressed in *E. coli* as a His-tagged protein, giving a product of c. 45 kD which was visible in crude extracts and bound a His-tag antibody (data not shown). Crude protein extracts showed lytic activity when incubated with *C. perfringens* cells (58.5±1.8 % drop in OD₆₀₀ over

4 min when using 33 µg/ml protein), while lytic activity of the crude protein extracts from empty vector control samples was minimal over this period and similar to buffer controls; lysis of cells which had not been frozen after harvest was slower (12.4±0.1 %) but still resulted in complete lysis within 10 min (data not shown).

Ni-NTA columns were used to purify His-tagged CP25L from *E. coli* cells to assess lytic activity and host range of the endolysin (Fig. 2a). Strong lytic activity was shown by an effective drop in the OD₆₀₀ reading within 6 min of addition of 3, 33, or 100 µg/ml of Ni-NTA-purified protein to frozen cells of *C. perfringens* (Fig. 2b). The speed of cell lysis obtained with 3 µg/ml of protein was similar to that obtained with 33 µg/ml, the OD₆₀₀ dropping from c. 0.65 to c. 0.13 in less than 5 min. Lower concentrations gave slower lysis—0.3 µg/ml gave a 9.1±0.1 % drop in OD₆₀₀ over 4 min compared with 79.5±1.4 % using 3 µg/ml, while 0.03 µg/ml failed to produce appreciable lysis. However, lysis from 100 µg/ml was slightly slower than that from 33 µg/ml; this phenomenon of reduced activity with higher concentrations has been observed previously (Mayer et al. 2010) and may be associated with precipitation of the protein at high concentrations. As noted with other clostridial species (Mayer et al. 2008; Mayer et al. 2012), slow gradual lysis of cells was also commonly observed in buffer controls.

The Ni-NTA-purified CP25L was stable at 4 °C for several months, and its activity was not affected by a 30-min incubation at 37 °C or 45 °C; however, treatment at 65 °C for 30 min, or 42 °C overnight, or a pasteurization treatment at 72 °C for 20 s all abolished lytic activity (data not shown). Stability at room temperature was monitored over 3 weeks, the activity remaining largely unchanged after 8 days compared with a sample stored at 4 °C, but, after 15 and 22 days storage at room temperature, the endolysin activity had reduced to c. 83 % and c. 62 % respectively, compared with the protein samples stored at 4 °C (Fig. 3).

Lysis assays were performed to examine the activity of CP25L when cells were suspended in broth media such as BHI+C or CM (Fig. 2c). Lytic activity was observed when cells were resuspended in BHI+C but was reduced in comparison to PBS; however, the lysis in CM, a complex medium designed to simulate GI tract conditions, was more effective. When tested under anaerobic conditions in BHI+C growth medium, which allows rapid growth of *C. perfringens*, the addition of endolysin to *C. perfringens* culture at the point of cell inoculation resulted in a dramatic 5-log reduction in viable cell counts compared with the culture not exposed to endolysin (Fig. 4a). However, on continued culturing, the pathogen was able to recover its population density. When the lysin was added to the culture during the log phase, it failed to reduce the bacterial count (Fig. 4b).

The host range of CP25L was tested using frozen cells of *C. perfringens* strains and a variety of clostridial and non-

CP25L	-----MYINQSNIKFNGRLRYCNDPNKTIHNAATSCSVYDIDRW	40
EIA17471	-----MYINQSNIKFNGRLNYCNPNNKTIHNAHPNCSVYDVDRW	40
ZP_02633232	-----MNIKTLTSYNYRNGRNCNSIDYVCHFTGNQNDKASGNANY	42
ZP_02640305	-----MKINKRLSTTNVT--LNANNPKHIIHETNTSKGAGAETHC	40
ZP_02636955	-----MKIAVRGGHNFQAKGASALIDETIEDRKVKDSVILNLRKECHEVLDVTPGNCVNTDLRYGVNKA	65
ZP_02630819	MSKNIKKIAVRGGHNFQATGAVALIGETSED RKVKDSVIYVLRQECYQVLDVTPGNCQITDLRYGVNKA	70
ZP_02953092	-----MKIAVRGGHNFQATGAALIGETSED RKVKDSVIECLRQECHQVLDVTPGNCQITDLRYGVNKA	65
CP25L	HKNG--WSGIGYDYFIRKEGS-----VW-TGRPENAI GAHTIGQNSSSIGTCLEGAFMREKPTRA	98
EIA17471	HKNG--WSGIGYHYFIRKDG-----VW-TGRPENAKGAHTIGQNSSSIGTCLEGALMREKPTRA	98
ZP_02633232	FRCVN--RQASAHYFVDDNEIVQVVRREGDTSVHCDDGNRGYGITNSNS-----IGIEMCATNGDISEK	103
ZP_02640305	KAQANGNIGKASVHYVDDTGVYQAVEHKHATVNCDDGNRNYGINNKNT-----ISIEICVNSDSDYNK	104
ZP_02636955	EEWGA--DLFISIHFDKAYDSYNGALGTGTWICGAGQAEVYARRIVNSIANGTGLNRNGVKTNPPLYEL	133
ZP_02630819	EEWGA--DLFISIHFDKAYDSYNGALGTGTWIVGTGGKAEVYARRIVNSIASGTGLKNRNGVKTNSKLYEL	138
ZP_02953092	EEWGA--DLFISIHFDKAYDSYNGALGTGTWIVGTGGKAEVYARRIVNSIANGTGLKNRNGVKTNSKLYEL	133
CP25L	QLNSLYELIADIRARR--GNLPVYGHKDFNN-----TDCPGINFPLEQFKNNSYRPTGG-EIVSDNG	157
EIA17471	QLNSLYYLIADIRARR--GNLPVYGHKDFNN-----TDCPGANFPLEQFKNNSYRPTGGTEIVSDNG	158
ZP_02633232	TIENLWLVKSLMNKYGIDIDHVVRRHYDASRKCPCSPFSPNNWSRWWEFKERLKGTVEN-IEVTTQSTNG	172
ZP_02640305	AVDNTVELVRYLKNKY-YSNCOVVRHYDASRKNCPRRILAN--GYWNTFLERVNSKSDSSQTPANTSYKQ	171
ZP_02636955	RKTSMPAVIIEVCFCE--ATTDVSIYKAKGSN-----LIGELIAEGICNKDIKTDNIPSQT-QSSVSLDG	195
ZP_02630819	RNTSMPAVIVVCFCE--ATTDVAIYKAKGPK-----LIGELIAEGICNKDIHTDNTPSLTPQDSVSLDG	201
ZP_02953092	RNTSMPAVIVVCFCE--ATTDVAIYKSKGPK-----LIGELIAEGICNKDISSDNTTNTQTEQPSVSLDG	196
CP25L	FYRSDEERINATIVGEGNIEVLDKNCKVITENRYSISLDRVFLGIYPASKYIEIYYPAGNEKYHAYISIE	227
EIA17471	FYISNEERENATIVGEGNIEVLDEKGNVITQGRHYSISLDRVFLGIYPSRNHIEIYYPGKDEKYHAYISIE	228
ZP_02633232	FYESDIEKINATIVGLGDIIEVLNDKCEVIKDRVYSISLDRIVYVGIYPSRNIEVIYQKDKKYHAYIDIK	242
ZP_02640305	FYESSETRINATIVGEGSISIKVLDEEONHVSGRWIDSLDRLFVIGIYPSRKFIEVVYPAGDKKYHAYIGIE	241
ZP_02636955	FYESSETRINATIVGAGRISVNLKNCPEIPNRYIDSLDRIFVIGIYPSLKFIEIVYPGSEKMYHAYIDIE	265
ZP_02630819	FYESSETRINATIVGEGRIEVLNKNCPPIPNRYIDSLDRIFVIGIYPSLKFIEVVYPASGKMYHAYIDIE	271
ZP_02953092	FYESSETRINATIVGKRIAVLNKNCPIDPRYIDSLDRIFVIGIYPSLKFIEVVYPASGKMYHAYIDIE	266
CP25L	NYSRLSFDYHMQYKNDNGVTVVWVDSQVNVKHEHEELQANQKASPMYRVGKWLRTFYRTDGTSDGFV	297
EIA17471	NYSRLSFDYHMQYKNDNGVTVVWVDSKVNVDKDHDEELQPHQKASPMYRTNGWLRTFYREDGTSDGYV	298
ZP_02633232	YYSRLSFDYHMQYQNDNGDITYVWVSSKDVNKTEPNEILSPNKKASPMYRENGWLRTFYRDNGVATDGFV	312
ZP_02640305	YYNRILFDYHKEYIINDGVTYVWVNASDVNVKDHNEELQPNQKASPMYRTGEWLRTFYRENGTSDGYV	311
ZP_02636955	NYNRLSFDYHFGYHNDNGDITYVWVNSDDVNEKEPEDEILLPNYKASPMYRTNGWLRTFYRADGNP	335
ZP_02630819	NYNRLSFDYHFGYHNDNGDITYVWVNSDDVNEKEPEDEILLPNYKASPMYRTNGWLRTFYRADGNP	341
ZP_02953092	NYNRVSFDYHFGYHNDNGDITYVWVNSDDVSEKEPEDEILLPNYKASPMYRTNGWLRTFYRADGNP	336
CP25L	RYEGEQAQVYVEEKEIKGIVVNTYLNVRDSINGNIICKVFNGEBSIITWKDGWYYIDYNTNHGKCRG	367
EIA17471	RYEGEQKERFYR--KGKVVNVRTSLTVRAGAGTNYSAGISLEPNENVDILGKAEGWYYVEYNTKNERKRG	366
ZP_02633232	RYEGEQSVKFYEEGKIKDGIKVVNTYLNVRDSICGNIICKVFNGEBSIITWKDGWYYIEYNTNHGKCRG	382
ZP_02640305	RYEGEQNKKFYEN--IQYGIKRVNSSLNVRPNNGEVIKSVYKDEKQVLEKEENGWYIEYSTSKGKCRG	379
ZP_02636955	RYEGEQKERFYR--IC-----	349
ZP_02630819	RYEGEQTERFYK--KGEVVNVRTSLTVRKGPNTNYSNIGSLEPNENVDILEMIGEWYHVEYNTNKGRKRG	409
ZP_02953092	RYEGEQKERFYK--KGKVVNVRTSLTVRKGPNTNYSNIGSLEPNENVDILEKVEGWYHVEYNTNARNERKRG	404
CP25L	YVSKYVEEV- 377	
EIA17471	YVSEKYIEIIQ 377	
ZP_02633232	YVSKYVEEV- 392	
ZP_02640305	YVSKYIELV- 389	
ZP_02636955	----- -	
ZP_02630819	YVSAKYIKEV- 419	
ZP_02953092	YVSEKYIEIIQ 415	

Fig. 1 Alignment of the amino acid sequence of CP25L compared with putative *N*-acetylmuramoyl-L-alanine amidases from *C. perfringens* genomes identified by Blastp analysis. Coloring of letters indicates degree of amino acid conservation between sequences (*black*, 100 %, *dark grey*

80 %, *light grey* 60 %); *lines above* CP25L sequence indicate areas of homology with the PGRP and amidase_2 domains (*black line*) or the SH3_3 bacterial domain (*grey*)

clostridial species. All of the 25 *C. perfringens* strains tested were sensitive to the endolysin, but the speed of lysis varied

greatly (Table 1). For some strains, the drop in turbidity was immediate, while with others there was a lag of up to c. 20 min

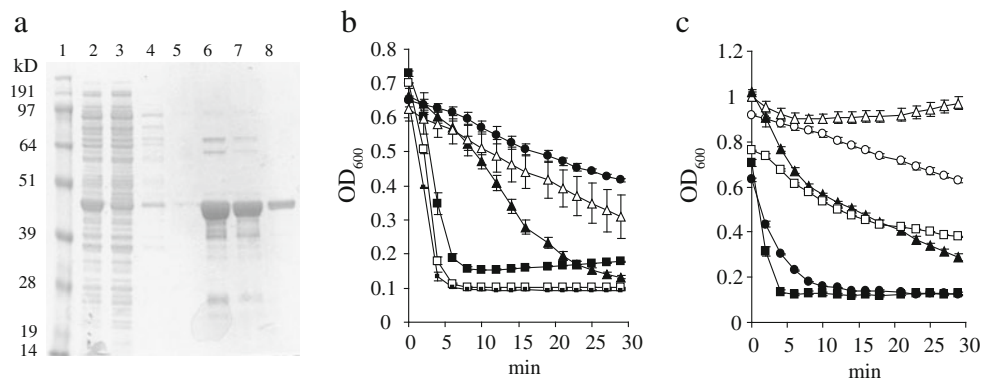


Fig. 2 Expression of CP25L in *E. coli*. **a** SDS-PAGE analysis of products from Ni-NTA column purification. Lanes 1, SeeBlue Plus2 marker, 2, cell lysate, 3, column flow through, 4–5 column washes, 6–8 successive 1 ml column eluates. Lanes contain 3.25 µl samples. **b**, **c** Lytic activity of Ni-NTA-purified protein on *C. perfringens* showing the effect of protein concentration (**b**) and lysis medium (**c**). **b** Closed square,

100 µg/ml; open square, 33 µg/ml; small closed square, 3 µg/ml; closed triangle, 0.3 µg/ml; and open triangle, 0.03 µg/ml of CP25L compared with buffer control (EB, closed circle). **c** Cells were resuspended in PBS (closed circle), BHI+C, (closed triangle), or CM (closed square) and incubated with 33 µg/ml CP25L (closed symbols) or EB (open symbols). Results are the mean of duplicate assays±standard deviation

before lysis was detected. Sensitivity to the positive control lysozyme was also variable, with some strains being insensitive while others exhibited rapid lysis. There was no apparent relationship between sensitivity to the CP25L lysin and to lysozyme. In addition, CP25L also showed activity against *Clostridium bifermentans*, *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Bacillus subtilis*, and *Bacillus cereus* cells (Table 1) but failed to cause lysis of a range of other bacteria (data not shown, strains listed in Table 2).

L. johnsonii as an endolysin delivery vehicle

The *cp25l* gene was cloned into an expression and delivery system which combined expression from the P_{nisA} , in conjunction with the chromosomally located signal transduction *nisRK* genes, with a signal peptide to secrete the lysin. To

confirm lysin expression in this host, crude protein extracts from *L. johnsonii* were used in turbidity reduction assays (Fig. 5). This showed firstly that extracts from *L. johnsonii* carrying the lysin expression construct (FI10744-L) had a lytic activity that was absent from extracts of the empty vector control (FI10744-V) and secondly that lytic activity from cells induced with nisin was equivalent to that from uninduced cells. This demonstrates that the *nisRK-P_{nisA}* system produced in *L. johnsonii* FI10744-L gives constitutive expression without the requirement for nisin induction. Secretion of the endolysin was assessed using *L. johnsonii* culture supernatants (Fig. 6a). The lytic activity associated with the concentrated supernatant was demonstrated in samples prepared from cultures of FI10744-L grown either with or without nisin induction, showing that the SLPmod signal peptide is effective for lysin secretion. Constitutive endolysin expression and secretion was further demonstrated by growing colonies of *L. johnsonii* on plates incorporating autoclaved *C. perfringens* cells, with clear development of zones of lysis around colonies expressing the lysin (Fig. 6b).

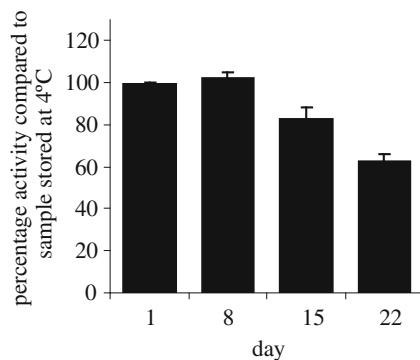


Fig. 3 CP25L stability. Endolysin activity was measured in turbidity reduction assays using 33 µg/ml Ni-NTA-purified protein. Results represent the activity of samples stored at room temperature as a percentage of the activity of endolysin stored at 4 °C assayed at the same time; activity was calculated as the percent decrease in OD₆₀₀ over 4 min of linear lysis using the mean of duplicate assays±standard deviation

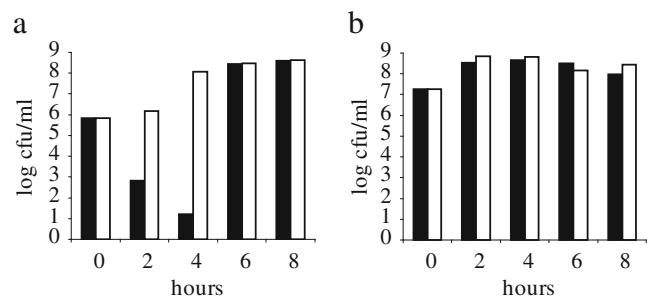


Fig. 4 Viability assays under anaerobic conditions to examine the effect of 33 µg/ml Ni-NTA-purified CP25L (black bars) on the viability of *C. perfringens* in comparison with the buffer control (white bars). Endolysin was added to the media either at point of inoculation (0 h) (**a**), or during the early exponential phase (OD₆₀₀ 0.3, 3.5 h) (**b**)

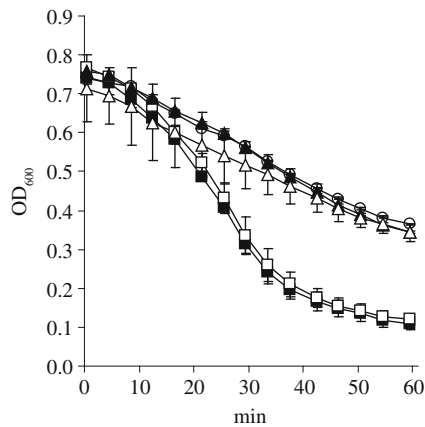


Fig. 5 Endolysin expression in *L. johnsonii*. Frozen cells of *C. perfringens* were incubated with 33 µg/ml crude protein extracts from *L. johnsonii* FI10744-L (filled square) or FI10744-V (filled triangle), or EB (open circle). Filled symbols were from cultures induced with nisin; open symbols had no exposure to nisin. Results are the mean of duplicate assays ± standard deviation

Discussion

C. perfringens is widely distributed in the intestine of animals, especially poultry, and can be pathogenic to the host. It is responsible both for severe infections and important economic losses (Lee et al. 2011; Scallan et al. 2011); targeting the pathogen in poultry will have impacts not just on poultry welfare and production but also on carriage in the food chain and the incidence of human infections. In this study, we have examined the ability of a novel endolysin to kill *C. perfringens* cells and demonstrated endolysin delivery by a probiotic strain of *L. johnsonii*.

The gene for CP25L is located within a prophage which can be released as a bacteriophage from the *C. perfringens* chromosome (Gervasi et al. 2013). In common with three other endolysins which have been demonstrated to show

activity against *C. perfringens*, it is predicted to be an amidase (Simmons et al. 2010; Zimmer et al. 2002). A number of prophage lysin-like enzymes found in the genomes of *C. perfringens* contain an amidase domain, and the presence of an SH3 domain is also very prevalent in these proteins (Schmitz et al. 2011). In CP25L, the predicted SH3_3 domain is present in the C-terminal region commonly associated with endolysin specificity (Fischetti 2010; Schmelcher et al. 2012). The C-terminal and the central region between the amidase domain and the SH3_3 domain showed high similarity to other putative amidases from *C. perfringens*, and it will be interesting to investigate whether these regions are associated with recognition of the *C. perfringens* cell wall.

When expressed from *E. coli*, CP25L showed a potent lytic activity against *C. perfringens* NCTC 3110, a type B strain and was also active against all 24 other *C. perfringens* strains tested. However, activity varied widely between strains, even between two strains of the same type A (NCTC 8238 and 8239). Although sensitivity to the lysozyme positive control also varied to a lesser extent between strains, there was no relationship between the speed of lysis with lysozyme and that with CP25L. This large difference in activity between strains was also found with the amidase endolysins Ply3626, PlyCP390, and PlyCP26F and the glucosyl hydrolase endolysins SM101 and PlyCM (Nariya et al. 2011; Schmitz et al. 2011; Simmons et al. 2010; Zimmer et al. 2002), although CP25L shows a slightly broader host range than most of these lysins. The sensitivity of other selected clostridial and *Bacillus* species might indicate shared binding targets in the cell walls of these organisms.

The ability to lyse cells in complex media at physiologically relevant pH values suggests that the endolysin might be able to function in GI tract conditions, without causing collateral damage to the normal microflora. However, as demonstrated in small cultures, despite initial evidence of control, the *C. perfringens* population was able to recover from endolysin

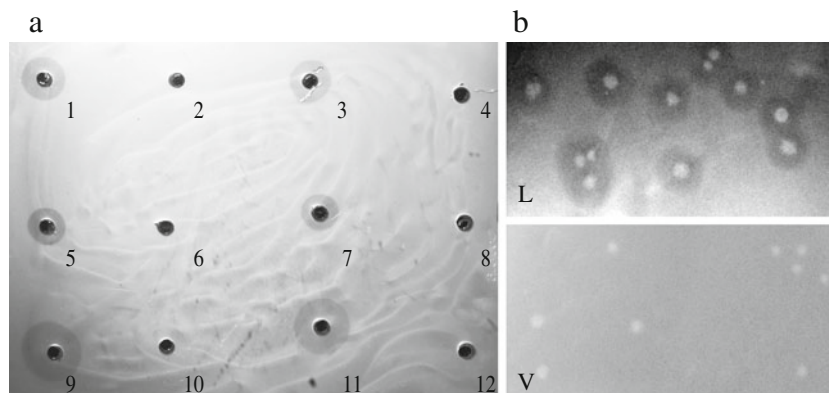


Fig. 6 Endolysin delivery from *L. johnsonii*. **a** Plate assay showing lysis from crude protein extracts (1–4) and concentrated supernatants (5–7, 20× concentrated; 9–12, 166× concentrated) from *L. johnsonii* FI10744-L induced with nisin (1, 5, 9) or uninduced (3, 7, 11), *L. johnsonii* FI10744-

V induced with nisin (2, 6, 10) or uninduced (4, 12); 8, EB control. **b** Lytic zones produced by growing colonies of FI10744-L (L) compared with control FI10744-V (V) on media incorporating autoclaved *C. perfringens*

treatment in a medium which favored rapid growth, while addition to a rapidly growing culture was ineffective. It is possible that the lysin remains attached to the cell walls of lysed cells, leading to a depletion in concentration and allowing rapidly growing cells to outstrip the lytic activity. Control might be more effective under less optimal growth conditions, such as in the competitive environment of the GI tract, and the lysin was shown to function in a complex medium that permits the growth of gut bacteria in simulated colon conditions with pH 6 and incubation temperature of 37 °C. However, survival of the lysin and contact with the *C. perfringens* target are further hurdles to overcome in the GI tract. Together with the finding that the lytic activity is not stable after prolonged incubation at 42 °C, these results indicate that a continuous high production of protein prior to infection would be required for pathogen control. This would be a feasible proposition in an environment where the endolysin was delivered to the gut via a colonizing probiotic.

L. johnsonii FI9785 was previously shown to reduce colonization and shedding of *C. perfringens* in chickens after a single oral dose (La Ragione et al. 2004), making it an ideal tool for preventative treatment. The use of a delivery system incorporating the signal peptide SLPmod, which has previously been demonstrated to be effective in the delivery of interleukin-12 to the mouse gut by *L. lactis* (Fernandez et al. 2009), gave successful production and secretion of a functional enzyme, as demonstrated by the lysis assays. Furthermore, the combination of the nisin A promoter and the chromosomally located signal transduction genes gave constitutive production without the need for nisin induction, which is of great potential benefit for use in animals. Production in *L. johnsonii* was lower than that in *E. coli*, and this is an area which may benefit from improvement. Another possibility is co-delivery or co-application with other antimicrobials—the combination of a phage endolysin with nisin achieved a strong synergistic effect against *Staphylococcus aureus* (Garcia et al. 2010), and the ability of lactobacilli to produce a range of bacteriocins, in addition to their other probiotic properties (Lebeer et al. 2008), make them valuable candidates as vehicles to deliver specific antimicrobials to gut communities. This preliminary study indicates that either large numbers of probiotic bacteria combined with high delivery rates and high activity of one or more antimicrobial or some method of attracting the bacterium to its target will be necessary for effective control.

The probiotic *L. johnsonii* has the capability to exclude *C. perfringens* from the GI tract of poultry birds. In this study, we have demonstrated its ability to express a biologically active anti-*C. perfringens* endolysin, indicating that the engineered strain has the potential to be an even more effective control agent for this pathogen. Future work will focus on the efficacy of the CP25L-secreting *L. johnsonii* FI10744-L strain in controlling *C. perfringens* in a GI tract environment, on its own or in co-application with other antimicrobials.

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