Published in final edited form as:

Arch Microbiol. 2013 November ; 195(0): 675–681. doi:10.1007/s00203-013-0916-4.

Expression of a *Clostridium perfringens* genome-encoded putative *N*-acetylmuramoyl- α -alanine amidase as a potential antimicrobial to control the bacterium

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Abstract

Clostridium perfringens is a gram-positive, spore-forming anaerobic bacterium that plays a substantial role in non-foodborne human, animal, and avian diseases as well as human foodborne disease. Previously discovered *C. perfringens* bacteriophage lytic enzyme amino acid sequences were utilized to identify putative prophage lysins or autolysins by BLAST analyses encoded by the genomes of *C. perfringens* isolates. A predicted *N*-acetylmuramoyl- α -alanine amidase or MurnAc-IAA (also known as peptidoglycan aminohydrolase, NAMLA amidase, NAMLAA, amidase 3, and peptidoglycan amidase; EC 3.5.1.28) was identified that would hydrolyze the amide bond between *N*-acetylmuramoyl and α -amino acids in certain cell wall glycopeptides. The gene encoding this protein was subsequently cloned from genomic DNA of a *C. perfringens* isolate by polymerase chain reaction, and the gene product (PlyCpAmi) was expressed to determine if it could be utilized as an antimicrobial to control the bacterium. By spot assay, lytic zones were observed for the purified amidase and the *E. coli* expression host cellular lysate containing the amidase gene. Turbidity reduction and plate counts of *C. perfringens* cultures were significantly reduced by the expressed protein and observed morphologies for cells treated with the amidase appeared vacuolated, non-intact, and injured compared to the untreated cells. Among a variety of *C. perfringens* strains, there was little gene sequence heterogeneity that varied from 1 to 21 nucleotide differences. The results further demonstrate that it is possible to discover lytic proteins encoded in the genomes of bacteria that could be utilized to control bacterial pathogens.

Keywords

Enzybiotic; Alternative antimicrobial; Bacteriophage; Prophage; Autolysin; Animal/human health

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Conflict of interest none.

Electronic supplementary material The online version of this article (doi:10.1007/s00203-013-0916-4) contains supplementary material, which is available to authorized users.

Introduction

Clostridium perfringens is a gram-positive, spore-forming, anaerobic bacterium commonly present in the intestines of people or animals and the environment that is classified into one of five types (A, B, C, D, or E) based on toxin production (Smedley et al. 2004; Sawires and Songer 2006). The bacterium can cause food poisoning, gas gangrene (clostridial myonecrosis), enteritis necroticans, and non-foodborne gastrointestinal infections in humans, and it is a veterinary pathogen causing enteric diseases in both domestic and wild animals (Smedley et al. 2004; Sawires and Songer 2006; Scallan et al. 2011). *C. perfringens* is estimated to be the second most common bacterial cause of foodborne illness in the United States, causing one million illnesses each year wherein meat and poultry outbreaks accounted for 92 % of outbreaks (Grass et al. 2013). Sampled retail chicken livers (Cooper et al. 2013) and retail foods (Lin and Labbe 2003; Wen and McClane 2004) can be culture-positive for the bacterium, although with a low prevalence of enterotoxigenic isolates. Concerns over antimicrobial resistance and use of antibiotic growth promoters during food animal production may be justified with increasing incidences of antibiotic resistance among bacterial pathogens (NAS 2006; Gyles 2008; Prescott 2008), including bacteria from healthy animals (Persoons et al. 2010). Consequently, there is a need for developing novel intervention methods including narrow-spectrum antimicrobials and probiotics that selectively target pathogenic organisms while avoiding killing of beneficial organisms (NAS 2006).

Clostridial bacteria encode autolysis proteins involved with cell growth and division as well as germination of spores (Paredes et al. 2005; Uehara and Bernhardt 2011). An extracellular initiation protein (IP; spore germination enzyme) produced by *C. perfringens* that hydrolyze spore cortical fragments with the release of free amino groups was identified as an amidase (Tang and Labbé 1987). Subsequently, a spore cortex-lytic enzyme was purified in an active form from fully germinated spore exudates of the bacterium with a reported partial amino acid sequence of VLPEPVVPEYIVVHN identified as SleC (Miyata et al. 1995a, b). Several germination-related enzymes, CspA, CspB, CspC, SleC, and SleM, are synthesized during sporulation of the bacterium (Masayama et al. 2006), and SleC was reported as a bifunctional enzyme possessing lytic transglycosylase activity and *N*-acetylmuramoyl-L-alanine amidase activity (Kumazawa et al. 2007). A peptidoglycan hydrolase (Acp) with *N*-acetylglucosaminidase activity produced mainly during vegetative growth of *C. perfringens* has been reported with lytic activity on the cell walls of several gram-positive bacteria (Camiade et al. 2010). Putative lysin-encoding open reading frames (ORFs) can be identified in lysogenized prophage regions, and this approach was utilized to express a muramidase (PlyCM) that lysed the bacterium, but had little or no activity against other clostridial or non-clostridial species (Schmitz et al. 2011).

Bacteriophage-encoded lytic enzymes have been reported that lyse *C. perfringens* (Zimmer et al. 2002; Simmons et al. 2010; Oakley et al. 2011), and at our laboratory, we have also utilized a genomics approach to identify lytic proteins in bacterial genomes (Simmons et al. 2012). Consequently, we have taken a genomics approach using previously discovered *C. perfringens* bacteriophage lytic enzyme amino acid sequences to identify putative prophage lysins or autolysins by BLAST analyses encoded by the genomes of *C. perfringens* isolates

(Shimizu et al. 2002; Myers et al. 2006). Subsequently, a putative amidase (PlyCpAmi) was cloned from the bacterium's genomic DNA by polymerase chain reaction (PCR) and expressed as a recombinant protein to lyse *C. perfringens* isolates.

Materials and methods

Bacterial cultures and propagation of strains

E. coli were cultivated in LB broth utilizing standard methods, and *E. coli* transformants were selected with 100 µg/ml ampicillin and/or 25 µg/ml chloramphenicol (Studier and Moffatt 1986). *C. perfringens* strains were cultivated and typed by previously reported anaerobic techniques (Siragusa et al. 2006).

Cloning of a putative peptidoglycan hydrolases gene

Using bacteriophage lysins as query subjects (EU588980, YP002265435; Simmons et al. 2010) for BLAST analysis (Schäffer et al. 2001) in NCBI, a putative peptidoglycan hydrolase gene within the genome of *C. perfringens* 13124 (NCBI Accession Number NC008262) was identified encoded by the bacterial genome (Gene Product Accession No. 110800924). As previously described for cloning phage endolysin genes (Pritchard et al. 2004; Donovan et al. 2006; Simmons et al. 2010), oligonucleotide primers were designed to amplify the gene with an *Nde*I restriction site in the forward primer and an *Xho*I restriction site in the reverse primer. The forward primer sequence was 5'-GCA CTA CAT ATG AAG ATA GCA GTA AG-3', and the reverse primer sequence was 5'-GTG GTG CTC GAG ATC TAA ACT TAT AT-3' (restriction sites underlined).

DnA was extracted from *C. perfringens* strain CP509 for amplification of the putative gene as described previously (Simmons et al. 2012). PCR products and pET21a plasmid vector (Novagen, Inc) were digested with restriction enzymes, *Xho*I and *Nde*I. The pET21a vector encodes a 6x-histidine tag to the C-terminal portion of the expressed protein. The digested insert was cloned into the expression vector pET21a and transformed into *E. coli* DH5 cells (Invitrogen, Carlsbad, California USA) following manufacturer's instructions. The insert-containing plasmid was isolated from the DH5 cells and transformed into *E. coli* rosetta 2(DE3) cells (Novagen, Inc.) to optimize codon utilization for low G/C content Gram-positive organisms. Rosetta cells were stored in brain-heart infusion (BHI) broth with 15 % glycerol at -80 °C.

Expression and purification of the *C. perfringens* peptidoglycan hydrolase

The protein was expressed and purified as described previously (Simmons et al. 2010). Briefly, cells from the expression host harboring the plasmid constructs were propagated in 100 ml Luria-Bertani (LB) broth supplemented with 50 µg/ml ampicillin and 34 µg/ml chloramphenicol at 37 °C (shaking at 200 rpm) until the OD₆₀₀ reading was 0.4–0.6 (log-growth phase). The broth was held on ice for 1 h and then treated with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) for induction of peptidoglycan hydrolase gene expression under control of the T7 promoter from the pET21a plasmid. The induced cells were then held overnight at 20.25 °C (slow shaking). The culture was centrifuged for 20 min at 4,000 rpm. The supernatant was removed, and the pellet was suspended in extraction buffer (50

mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8.0). The resuspended cells were lysed with the FastPrep 24 tissue homogenizer (MP Biomedicals, California, USA) for 4 pulses (4.0 M/S for 10 s). A portion of the resultant supernatant was purified via Nickel-NTA purification column (Crowe et al. 1994; Qiagen, Maryland USA) following manufacturer's instructions. The remaining cellular lysate supernatant was tested for lytic capability as well. The purified protein and the cellular lysate were run on SDS-PAGE gel and stained with Coomassie Blue to confirm the presence of the expressed protein (Hames 1990). Additionally, the purified protein (PlyCpAmi) was amino acid sequenced by Edman degradation (Niall 1973) and MALDI-TOF analyses (Rosenfeld et al. 1992) to confirm identity of the protein.

Assessing lytic capability of the expressed proteins

The in vitro lawn assay was used to test the lytic capability of the expressed amidase against *C. perfringens* strains as described previously (Zimmer et al. 2002; Donovan et al. 2006; Simmons et al. 2010). The lawn plate was prepared by anaerobically incubating a culture of *C. perfringens* at 35 °C in 50 ml BHI broth overnight. Following centrifugation at 4,000 rpm for 20 min, the pelleted cells were resuspended in 1.0 ml Lysin Buffer A (50 mM ammonium acetate, 10 mM CaCl₂, 1 mM DTT, pH 6.2). A 0.5-ml aliquot was pipetted into 10 ml of liquid BHI agar (50 °C) and poured into sterile petri dishes to solidify. A 10-μl aliquot of the purified protein was spotted onto the plate to assess lysis. Controls were cellular lysate from host cells transfected with the pET21 plasmid without the gene insert (negative control) and lysozyme. A positive lytic reaction was determined by a visible clearing of the turbidity of the *C. perfringens* cells on the plate. The plates were examined for lysis at 1 h and after overnight at an incubation temperature of 37 °C.

Assessment of cell viability following treatment with recombinant proteins

The ability of the recombinant protein to reduce cell turbidity (Zimmer et al. 2002; Donovan et al. 2006) was assessed using purified proteins. Strains CP26 and CP39 were grown overnight in BHI broth to an OD₆₂₀ of 0.4–0.6. Aliquots of 200 μl of each culture were treated in triplicate wells with the 50 μl of purified amidase (treated) or 50 μl NPI-10 elution buffer (control) for 1–3 h at 37 °C in a 96-well plate. At each hourly time point, the density (OD₆₂₀) of the culture suspension was measured using the Multiskan FC plate reader (Fisher Scientific, Pittsburgh, Pennsylvania USA).

To measure the reduction of viable cells following treatment, 200 μl of log-growth phase culture was treated with 20 μl of the purified protein or 20 μl of water (control). Treatments were performed in triplicate tubes and incubated anaerobically overnight at 37 °C for 24 h. The cultures were serially diluted tenfold and plated (100 μl) in duplicate onto tryptic soy agar with 5 % sheep blood. Plates were incubated anaerobically at 37 °C and counted on the following day. Additionally, treated and untreated cells were spotted in 10-μl aliquots onto glass slides and gram-stained to observe any morphological changes following treatment.

Gene sequence analysis of the putative amidase PlyCpAmi

A partial coding region (925 bp) of the putative amidase gene excluding the primers' sequences from the following *C. perfringens* strains was sequenced: CP26, CP39, CP509,

CP297, CP697, CP726, CP776, CP1036, and CP1113. DNA was extracted from each strain using the MoBio UltraClean DNA purification kit (Carlsbad, California USA). Each 25 µl PCR reaction consists of 0.2 µM (final concentration) of each primer (SM101F and SM101R), nuclease-free water, and one OmniMix HS (per two reactions) lyophilized PCR master mix bead (TaKaRa Bio Inc., Otsu, Japan), which contains 1.5 U TaKaRa Hot Start Taq Polymerase, 200 µM dNTP, 4 mM MgCl₂, and 25 mM HEPES buffer. DNA template was added in 2.5 µl volumes to 22.5 µl of mastermix. Reaction conditions were as follows: 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 120 s. The presence of a band was verified by running the product on a 1 % agarose gel for 1 h at 90 volts in 0.5X Tris–borate buffer. All products were cleaned using the QIAquick PCR Purification kit (Qiagen, Maryland USA) and concentrations and purity verified using the NanoDrop 2000 (Thermo Scientific, Wilmington, Delaware USA). Cleaned PCR products were submitted to retrogen, Inc. (San Diego, California USA) for sequencing (Kristensen et al. 1998). Sequences were edited and aligned using Geneious Pro Version 5.4.6 (Bio-matters Ltd., Auckland, new Zealand) and MEGA Version 4 (Tamura et al. 2007).

Results and discussion

Sequence analysis of the amidase gene

Nine *C. perfringens* Type A strains were chosen for sequencing the putative gene following BLAST analyses using previously described bacteriophage lysins as a query subjects. Oligonucleotide primers used to clone in the coding region of the gene were used for sequencing PCR products directly. Quality sequence data excluding the primer sequences were obtained for 942 bases of the approximately 1,014 bp gene. Aligning the sequences demonstrated high sequence similarity among the strains (Fig. 1). The number of nucleotide differences between strains varied from 1 nucleotide difference to 21 differences (Suppl. Table 1). Search results using nucleotide BLAST (nr/nt) database in blastn for the sequences align with a putative type 3 *N*-acetylmuramoyl-*L*-alanine amidases (pfam01520; COG0860) with the resultant enzymatic domain of the predicted protein to be between residues 1 and 160 (Suppl. Fig. 1).

The predicted lysin was identified as an amidase that hydrolyzes the amide bond between *N*-acetylmuramic acid and an *L*-amino acid, most often *L*-alanine, in the bacterial cell wall peptidoglycan. As opposed to autolysins, almost all endolysins have no signal peptides, and their translocation through the cytoplasmic membrane is thought to proceed with the help of phage-encoded holin proteins. The amidase catalytic module is fused to another functional module (cell wall-binding module or CWBM) either at the N- or C-terminus, which is responsible for high-affinity binding of the protein to the cell wall (Vollmer et al. 2008).

Cloning of the putative amidases gene for expression

The gene for the putative amidase was successfully cloned into the pET21 plasmid vector. Subcloning of the insert-containing vector into the *E. coli* Rosetta cells (DE3) was used to alleviate codon bias associated with expression of a gene originating from a gram-positive organism (reviewed by Kane 1995; Pritchard et al. 2004). As shown in Fig. 2a, the putative amidase was present at approximately 40 kDa when gel electrophoresis was performed

following expression and purification. Furthermore, mass spectrometry and N-terminal amino acid sequencing supported that the protein was expressed in-frame with the correct amino acid sequence for a predicted *N*-acetylmuramoyl-L-alanine amidase (Suppl. Fig. 1 and Suppl. Table 2). The size of the product observed by gel electrophoresis also agreed with the predicted size (38.969 kDa; pI of 8.7) based on translation of the nucleotide sequence of the cloned gene with six histidines at the C-terminal portion of the protein.

Lytic capability of the putative amidase

The purified amidase (concentration of 800 µg/ml) designated PlyCpAmi and the *E. coli* cell lysate were spotted in 10-µl aliquots onto agar plates containing a lawn of *C. perfringens* cells of various strains. By 1-h incubation, lytic zones were observed for the CP509 (α, β2) Type A strain with the purified amidase and the expression host cellular lysate containing the amidase (Fig. 2b). No inhibition growth zones were observed for *E. coli* host cells without the cloned gene or with lysozyme. The *C. perfringens* Type A strains CP26 (α, β2), CP39 (α, β2), CP776 (α, β2), CP1036 (α, β2), and CP12916 (α, CPE +) also showed lytic zones when the purified amidase or lysate was applied to confluent plates; however, those zones of lysis were observed after overnight incubation as reported previously (Simmons et al. 2010). Also, no lysis zones were observed for other clostridial species.

A turbidity reduction assay (Fig. 3) was conducted to assess the effect of the purified amidase on cells grown in broth culture. After 1-h incubation of the cell suspensions with purified amidase, strains CP26 ($p < 0.0001$) and CP39 ($p < 0.0001$) each had a significant reduction of cell turbidity compared to the untreated *C. perfringens* suspensions. The turbidity of strain CP26 was also significantly reduced ($p < 0.0001$) for the 2-h time point versus the 1-h time point. Strain 39 showed no significant reduction in turbidity for 2- or 3-h time points versus the 1-h time point. This is similar to results obtained previously for lysin turbidity reduction assays with the bacterium (Zimmer et al. 2002; Simmons et al. 2010).

Plate counts were used to determine the number of viable, cultivable cells following treatment of the culture with the purified amidase. There was a reduction in viable cells following 24-h treatment with a significant 2.5 log reduction ($p < 0.0001$) of viable *C. perfringens* cells (strain CP509) following treatment with the purified amidase compared to the untreated cells (Fig. 4) and represents at least a 95 % reduction of viable cells. Consequently, the amidase PlyCpAmi can kill stationary phase cells even though they may have a higher resistance to stress. Previous data from our laboratory demonstrated a reduction of approximately 3 logs following treatment with purified phage lysin after 1-h treatment (Simmons et al. 2010). Cells from the treated and untreated suspensions were gram-stained, and morphologies were observed. The observed morphologies depicted in Fig. 5a for the cells treated with the amidase appear vacuolated and non-intact, and therefore were injured compared to the untreated cells in Fig. 5b.

Conclusion

In the European Union (EU), antimicrobial growth promoters have been banned from animal feeds because of concerns over the spread of antibiotic resistances among bacteria (Gyles 2008; Prescott 2008), and the EU-wide ban on the routine use of antibiotics in animal feeds

became effective on January 1 2006 (Regulation 1831/2003/EC; Millet and Maertens 2011; Koluman and Dikici 2013). This has been accompanied by a need for developing novel intervention methods including narrow-spectrum antimicrobials that can selectively target pathogenic organisms in a species-specific manner while avoiding killing of beneficial organisms (NAS 2006). Also, recombinant DNA produced enzymes as feed additives for food production animals such as phytases and carbohydrases are commercially produced and sold for feed additive purposes during monogastric food–animal production (Adeola and Cowieson 2011). Consequently, research at our laboratory has been the development of bacteriophage or prophage-encoded lysins to specifically lyse pathogenic bacteria that do not affect other potentially beneficial organisms (Simmons et al. 2010, 2012). Utilizing a genomics approach another lysin, a putative amidase designated PlyCpAmi, capable of species-specific hydrolysis of *C. perfringens* was cloned and expressed such that the protein could eventually be utilized as an alternative antimicrobial to control the bacterium.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Support for the research was provided by the US Department of Agriculture, Agricultural research Service (ArS CrIS project #6612-32000-060). The authors acknowledge primary amino acid sequencing and mass spectrometry analyses of the recombinant protein by Ms. rebekah Woolsey and Dr. Kathleen Schegg at the Nevada Proteomics Center which operates under the auspices of grants from the national Center for research resources (5P20rr016464-11) and the national Institute of general Medical Sciences (8 P20 gM103440-11) from the national Institutes of Health.

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Figure 1. Phylogenetic relationships demonstrating sequence similarity for partial amidase gene sequences among nine *C. perfringens* strains

Figure 2. SDS-PAGE of the expressed putative amidase and representative spot assay on a lawn of *C. perfringens*. The expressed protein designated PlyCpAmi was present at the predicted size of 40 kDa when expressed and purified from *E. coli* Rosetta cells (a). Using 10 μ l of purified amidase (column elutions) or expression host cell lysate (+lysate) with the insert containing the amidase gene produced zones of clear lysis on lawns of *C. perfringens*

Figure 3. Turbidity reduction assays for two *C. perfringens* strains following treatment with purified amidase for 1–3 h

Figure 4. Treatment with purified amidase leads to a reduction in viable *C. perfringens* cells. A significant reduction ($p < 0.0001$) in viable cells occurred following treatment for 24 h

Figure 5. Gram-stain image of *C. perfringens* following treatment with amidase. The bacterium *C. perfringens* was treated (a) or untreated (b) with the purified recombinant protein and photographed at $\times 1,000$ with a light microscope