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Short communication

Dynamics of *plc* gene transcription and α-toxin production during growth of *Clostridium perfringens* strains with contrasting α-toxin production☆

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ABSTRACT

The aim of the present study was to investigate transcription dynamics of the α-toxin-encoding *plc* gene relative to two housekeeping genes (*gyrA* and *rplL*) in batch cultures of three *Clostridium perfringens* strains with low, intermediate, and high levels of α-toxin production, respectively. The *plc* transcript level was always low in the low α-toxin producing strain. For the two other strains, *plc* transcription showed an inducible pattern and reached a maximum level in the late exponential growth phase. The transcription levels were however inversely correlated to α-toxin production for the two strains. We propose that this discrepancy is due to differences in *plc* translation rates between the strains and that strain-specific translational rates therefore must be determined before α-toxin production can be extrapolated from transcript levels in *C. perfringens*.

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1. Introduction

Necrotic enteritis (NE) is a common disease in broiler chickens caused predominantly by *Clostridium perfringens* (Songer, 1996). Usually, NE occurs 2–6 weeks after hatching and is characterized by the sudden onset of diarrhea and mucosal necrosis caused by overgrowth of *C. perfringens* in the small intestine (Ficken and Wages, 1997; Fukata et al., 1991). The mere presence of *C. perfringens* does not necessarily lead to disease but at a critical population size, toxin production results in necrotization of the gastrointestinal epithelium from random foci (Cowen et al., 1987; Engberg et al., 2002; Ficken and Wages, 1997; Pedersen et al., 2003). The mechanisms behind establishment of infection are not fully determined and several new virulence factors and mechanisms have been suggested recently (Keyburn et al., 2008; Kulkarni et al., 2008; Olkowski et al., 2008). The α-toxin (phospholipase C) is however believed to play an important role in the severity of the disease (Al-Sheikhly and Truscott, 1977a, 1977b; Awad et al., 1995; Fukata et al., 1988; Thompson et al., 2006; Williamson and Titball, 1993) and immunization with recombinant α-toxin has been shown partly to protect broilers in experimental challenges with *C. perfringens* (Cooper et al., 2009; Kulkarni et al., 2007). The dynamics of α-toxin production during growth of different *C. perfringens* strains are poorly understood. The goal of this study was therefore to investigate the relationship between growth phase, *plc* gene transcription, and α-toxin production in three *C. perfringens* strains representing low, intermediate and high levels of α-toxin production, respectively.

2. Materials and methods

2.1. Bacterial strains and growth conditions

In previous studies, 60 strains of *C. perfringens* with different pulsed-field gel electrophoresis profiles were

☆ Nucleotide sequence data reported are available in the GenBank databases under the accession numbers EU834132–EU834179.

Abbreviations: NE, necrotic enteritis; RT, reverse transcription; MDAP, maximum detected α-toxin production.

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isolated from chickens, analyzed for their α-toxin sequence type (Abildgaard et al., 2009; Pedersen et al., 2003) and 35 isolates were further screened for production of α-toxin in batch cultures (Abildgaard et al., 2009) similar to the approach used by Gholamiandekhordi et al. (2006). Based on this screening, the following three strains were selected for more detailed studies: (i) strain T2 (sequence type: VIe (Abildgaard et al., 2009); source: NE chicken; α-toxin production level: low, <1% of maximum detected α-toxin production (MDAP) of the 35 strains); (ii) strain S3 (sequence type: Vb; source: healthy chicken; α-toxin production level: intermediate, 57% of MDAP of the 35 strains); and (iii) strain T9 (sequence type: Vb; source: NE chicken; α-toxin production level: high, 85% of MDAP of the 35 strains). The strains were maintained in Anaerobe Basal Broth (CM0957; Oxoid Ltd., Basingstoke, Hampshire, UK) at 30 °C; 500-ml infusion bottles with 400 ml broth were inoculated (0.1%) from overnight cultures, flushed (80% N_2, 10% CO_2, and 10% H_2) and incubated at 37 °C under agitation (120 rpm) to avoid aggregation and subcultures in different growth phases. At regular intervals, growth was measured as OD_{600}, and samples were withdrawn for quantification of *plc* mRNA by reverse transcriptase quantitative PCR (RT-qPCR; see below) and of α-toxin by the ELISA method previously described (Abildgaard et al., 2009). All experiments were performed in triplicates. The three selected strains were further analyzed for their α-toxin production pattern (α-toxin accumulation in overnight batch cultures) to check for potential inter-strain differences in intracellular pools and/or differences in protease degradation of the α-toxin. For this purpose, batch cultures of the strains were inoculated (0.1%) from pre-cultures as outlined above and grown overnight (approximately 16 h) at 37 °C in 10 ml anaerobe basal broth. The suspensions were centrifuged and the supernatants and the pellets were immediately frozen separately in liquid nitrogen and stored at −80 °C. The frozen pellets were re-suspended in 1 ml TES buffer (define) and 500 μl was transferred to Eppendorf tubes containing 200 mg glass beads (0.18 mm) and amended with 5 μl 100× Protease Inhibitor Cocktail Set I (539131, CALBIOCHEM®, San Diego, CA). The suspensions were Vortexed (1 min), 5 μl lysozyme (10 mg ml^{-1}) added and the tubes were incubated 30 min at 37 °C. The tubes were Vortexed (1 min) again, centrifuged and the supernatants were frozen in liquid nitrogen and stored at −80 °C. The supernatants from the overnight cultures as well as from the lysed cells were analyzed for α-toxin content by the previously described ELISA method (Abildgaard et al., 2009), where the former were amended with the protease inhibitor as well prior to the analysis.

2.2. TaqMan probes and primers

Published primers and probes for qPCR (Skånseng et al., 2006) showed several mismatches to the recently extended *plc* gene sequence set (Abildgaard et al., 2009). To exclude target underestimation caused by destabilizing mismatches (Werbrouck et al., 2007), a new primer/probe set (Table 1) was designed using the Primer Express software package (v. 2.0; Applied Biosystems, Stockholm, Sweden), and checked for potential cross-reactivity against the GenBank database (http://www.ncbi.nlm.nih.gov). Positions 339–340 were avoided as target sites, since an intron between these positions had been observed in strain S3 and other strains of *C. perfringens* (Abildgaard et al., 2009; Ma et al., 2007). Two constitutively expressed genes were selected for normalization of *plc* transcript levels: *gyrA*, encoding DNA gyrase (Huang, 1996), and *rplL*, encoding the ribosomal protein L7/L12 (Ramakrishnan and Moore, 2001). Sequences for *gyrA* and *rplL* were retrieved from 24 *C. perfringens* strains (GenBank accession numbers: EU834132–EU834179) isolated from chickens using published primers (Rooney et al., 2006), and were used, together with 124 *gyrA* and 244 *rplL* sequences of *C. perfringens* isolated from various sources (Rooney et al., 2006), for probe and primer design (Table 1).

Table 1
Primers (F and R) and probes (T) for RT-qPCR.

Target	Name	Sequence (5′–3′)
Plc	398F	CTA GAT ATG AAT GGC AAA GAG GAA ACT A
	475R	AAC ATT GCA GGA TGA TAT GGA GTA GTA TCT AT
	430T	CAA GCT ACA TTC TAT CTT GGA GAG GCT ATG CAC TAT TT
RplL	3F	GAC AAA AGA GCA AAT CAT AGA AGC TAT AA
	125R	TCC GCC TAC AAC AGC AAC TG
	69T	TGT GAA GAA GAA TTC GGA TTC GGA GTA AGC GCT GC
GyrA	693F	CAT TAA AGG ACC AGA TTT CCC TAC AG
	771R	TTT TCC AGT TTC ATA AGC AGC TCT T
	720T	AGG AAT AAT AAT GGG TAA ATC AGG AA

2.3. Quantitative real-time RT-PCR amplification

Samples of RNA from strains S3, T2, and T9 were preserved by transferring aliquots of 0.5 ml culture to 2 ml microcentrifuge tubes containing 1 ml RNA protect (QIAGEN). Total RNA was isolated using the RNAprotect protocol (QIAGEN) which includes enzymatic lysis, proteinase K digestion, and mechanical disruption, and purified using the RNeasy mini kit (QIAGEN) according to the manufacturer's recommendations. The RNA was eluted twice in 20 μl RNase free water. The QIAGEN RNase-Free DNase Set was applied during RNA extraction to remove DNA. The concentration and purity of the RNA was determined as absorbance at 260 and 280 nm in a NanoDrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). The RNA was stored at −80 °C for a maximum of 1 week before cDNA synthesis to minimize differential RNA degradation reported for longer storage (Alifano et al., 1994; Rudi et al., 2003). Reverse transcription (RT) was performed using the first-strand cDNA protocol and SuperScriptTMIII reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommended protocol with 100 ng RNA and 100 ng random hexamer primers (Invitrogen) per reaction. The thermal profile was as follows: 25 °C for 5 min, 50 °C for 60 min, and 70 °C for 15 min. The resulting cDNA (1 μl in a total of 10 μl reaction volume) was amplified with TaqMan Universal PCR Master Mix (Applied Biosystems, Stockholm, Sweden) with 0.4 mg ml^{-1} bovine serum

albumin (Fermentas, Helsingborg, Sweden) and 0.5 pmol μl^{-1} primers and 0.1 pmol μl^{-1} probe (Table 1). The probes contained 6-carboxyfluorescein (FAM) at the 5′ end and either 6-carboxytetramethylrhodamine (TAMRA) (*plc* and *rplL*) or a non-fluorescent quencher with the MGB protein (Applied Biosystems) at the 3′ end (*gyrA*). A 7900HT sequence detection system (Applied Biosystems) was used for qPCR with the following temperature settings: 2 min start up at 50 °C, 10 min initial denaturation at 95 °C, and 40 cycles of denaturing at 95 °C (15 s) and annealing/extension at 60 °C (1 min). Data were analyzed with the SDS 2.1 application software (Applied Biosystems). A standard curve (7×10^5 to 70 genomes μl^{-1}) was prepared by serial dilution of *C. perfringens* genomic DNA of known concentration, using the genome size of *C. perfringens* strain 13 and Avogadro's number for calculations. Since *plc*, *gyrA*, and *rplL* are single copy genes in *C. perfringens* (Shimizu et al., 2002), cycle thresholds (C_T values) were directly converted to gene copy numbers for the standards; results are expressed as number of *plc* transcripts relative to *gyrA* or *rplL* transcript numbers.

3. Results

3.1. Levels of α-toxin production

The relative levels of extra cellular α-toxin accumulation in batch cultures of the three strains (T9: high; S3: intermediate; and T2: low/insignificant) as illustrated in Fig. 1D were highly reproducible and could be repeated with months interval (data not shown). In a control experiment it was further observed that the intracellular pools of α-toxin were insignificant. Thus, α-toxin could only be detected in lysate from the high producing strain T9 and at a concentration 500× lower than in the supernatant. The samples, taken from the control experiments, were all amended with protease inhibitor during the analysis to avoid any bias caused by differences in α-toxin degradation between the strains.

3.2. Design of RT-real-time PCR protocol

While all primers had a perfect match to their target site, the large *gyrA* sequence variation caused problems for designing a suitable probe: despite the design as minor groove-binding (MGB) probe, which forms an extremely stable duplex with single-stranded DNA and therefore allows shorter probe lengths (Kutyavin et al., 2000), one mismatch remained to *gyrA* of strain T2; *plc* transcript levels normalized against *gyrA* might therefore be slightly overestimated for T2 (Fig. 1B). The log-linear range for all qPCR reactions spanned five orders of magnitude with an $r^2 > 0.99$ (Fig. S1). The regression curves for *plc*, *gyrA*, and *rplL* had slopes of 3.33, 3.37, and 3.32, corresponding to amplification efficiencies of 0.99, 0.98, and 1.00, respectively. The contribution of genomic DNA to the cDNA signal was assessed by determination of the ΔC_T for the RT-qPCR with and without addition of reverse transcriptase. During exponential growth, all samples tested had a $\Delta C_T > 4$, indicating a contribution of genomic DNA of <10% to the cDNA signal (calculated from the slope of the regression

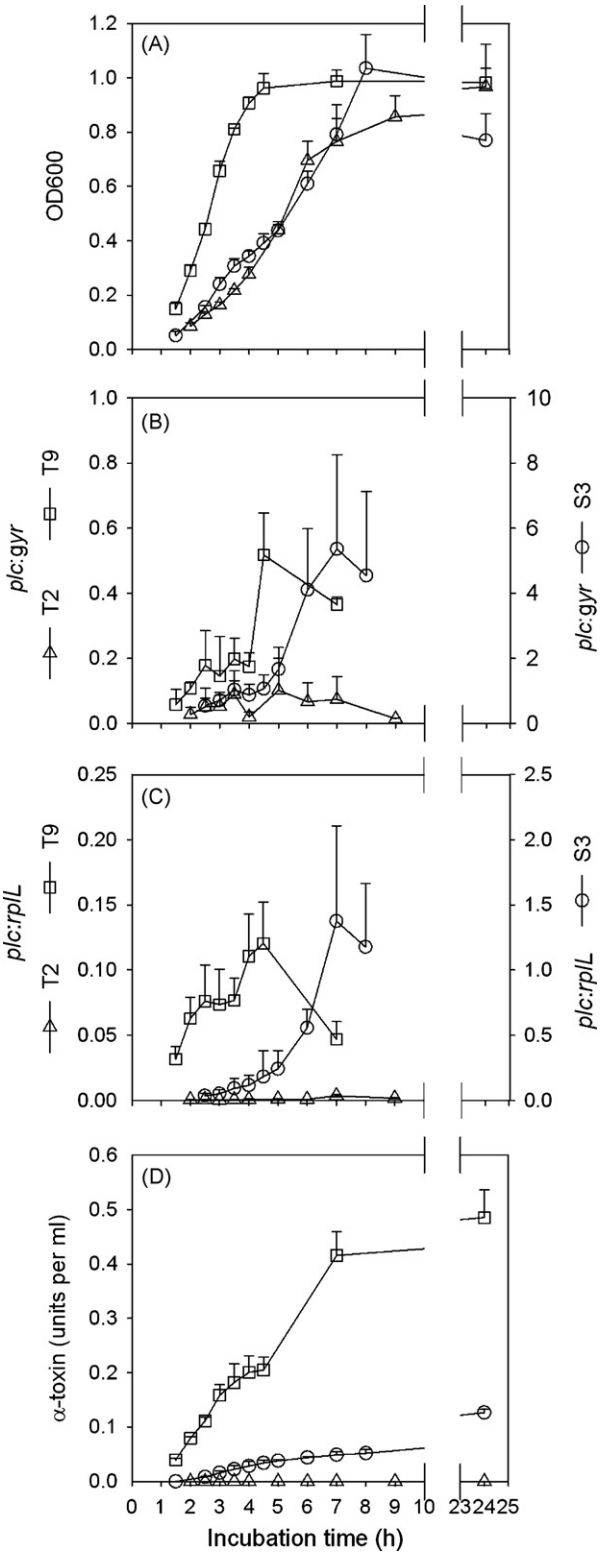

Fig. 1. Growth measured as OD_{600} (A), transcription of *plc* relative to *gyrA* (B) and *rplL* (C), and α-toxin production (D) of *C. perfringens* strain S3 (○), T9 (□), and T2 (△). Error bars, standard deviations ($n = 3$). Error bars smaller than symbols are not shown.

line). This value is within experimental variation and does not interfere with the transcript quantification. In contrast, the ΔC_T was <0.5 in late stationary phase, resulting in transcript levels below the detection limit of the assay.

3.2.1. Transcript levels of plc and production of α-toxin during growth of C. perfringens

The growth of the three different strains reached the early exponential phase and early stationary phase after 1–2 and 5–9 h, respectively (Fig. 1A).

The *plc* transcription dynamics showed the same trend irrespective which transcripts (*gyrA* or *rplL*) were used for normalization (Fig. 1B and C). Transcript levels of *plc* strongly differed between the three strains; for strain T2, it was just around the detection limit throughout the entire growth phase, and no toxin was detected in the culture supernatant, although low levels of α-toxin production had been observed for this strain in an earlier study (Abildgaard et al., 2009). Strains T9 and S3 showed much higher *plc* transcript levels and a 4- to 30-fold increase from early to late exponential phase (Fig. 1B and C). Although following the same dynamics, the relative transcript level of strain S3 was however up to 10 times higher than the level observed for strain T9 (Fig. 1B and C).

4. Discussion

4.1. Levels of α-toxin production

The reproducibility of the levels of α-toxin accumulation measured in supernatants of the batch cultures combined with the fact that only insignificant levels of the toxin were detectable in cell lysates, support the use of secreted α-toxin as a measure of the strain-specific production pattern in accordance with previously reported observations (Abildgaard et al., 2009; Gholamiandekhordi et al., 2006). It is however important to emphasize that comparison should only be made between strains grown under comparable conditions, since, e.g., culture media and pH have been reported to influence toxin production levels of *C. perfringens* significantly (Fernandez-Miyakawa et al., 2007; Möllby et al., 1976).

4.1.1. Transcript levels of plc and production of α-toxin during growth of C. perfringens

Comparison of Fig. 1A–C indicates that transcription of *plc* is induced in exponential phase rather than being constitutive. Peaking of α-toxin accumulation in exponential phase has previously been reported (Bullifent et al., 1996), but to our knowledge, the present study is the first to demonstrate induced *plc* transcription in direct comparison to housekeeping genes. The expression pattern of *plc* could potentially reflect an evolutionary strategy to increase substrate availability in crowded environments by increasing toxin (phospholipase) production. A similar up-shift in transcription of the gene encoding botulinum neurotoxin (*cntA*) has been observed in *Clostridium botulinum* (Sharkey et al., 2005; Shin et al., 2006). A difference in transcript levels between strains T9 and S3 by up to a factor 10 was shown in Fig. 1B and C. An earlier study using Northern hybridization of *plc* mRNA had also reported large variations in transcript levels between strains, up to 40-fold (Tsutsui et al., 1995). Surprisingly, strain T9, with the lower transcript levels, produced the higher amount of α-toxin, i.e., 0.49 U ml^{-1} in late stationary phase vs. 0.13 U ml^{-1} produced by the highly *plc*-transcribing strain S3 (Fig. 1D). This is in contrast to earlier findings of high correlation between plc mRNA and α-toxin levels also in strains showing different levels of phospholipase C activity (Bullifent et al., 1996). The *plc* gene of strain S3 contains an 834 bp-intron (Abildgaard et al., 2009), previously found also in other *C. perfringens* strains (Ma et al., 2007), which theoretically could decrease translation efficiencies due to the need for mRNA processing (splicing). However, no un-spliced mRNA has been detected in these strains (Abildgaard et al., 2009; Ma et al., 2007), indicating rapid mRNA processing. The most probable explanation for the discrepancy in apparent transcription and translation levels between strains T9 and S3 is therefore that both features are strain-specific.

4.2. Implications for monitoring the effect of NE treatment

Transcript levels of toxin genes have been used to monitor the efficiency of treatments against *Salmonella* (Lim et al., 2007), *Vibrio* (Lim et al., 2007), and *C. botulinum* (Sharkey et al., 2005; Shin et al., 2006). Likewise, *plc* gene copy numbers and transcript levels may be used to evaluate the effect of treatments on number and α-toxin production of *C. perfringens* in pure cultures of known strain types. Towards that end, our revised qPCR probe/primer set offers a reliable tool to determine α-toxin gene copy numbers and, combined with RT and normalization against *gyrA* or *rplL* mRNA, α-toxin gene transcript levels. However, due to the putative induction of *plc* transcription during exponential growth, different strains need to be in the same growth phase when comparing treatment effects on *plc* transcript levels. Finally, it is crucial to note that according to our findings, *plc* gene transcript (mRNA) levels can only be used to predict the amount of α-toxin produced when knowing the degree of translational regulation for the strains in question. The protocol can be further developed to measure the effect on NE treatments on *plc* transcription and α-toxin production in intestinal content of broilers during challenge trials. Again for this purpose the relationship between *plc* transcription and α-toxin production of the strain(s) used for infection should be assessed *in vitro*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetmic.2009.05.014.

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