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Sugar inhibits the production of the toxins that trigger clostridial gas gangrene

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ABSTRACT

Histotoxic strains of *Clostridium perfringens* cause human gas gangrene, a devastating infection during which potent tissue-degrading toxins are produced and secreted. Although this pathogen only grows in anaerobic-nutrient-rich habitats such as deep wounds, very little is known regarding how nutritional signals influence gas gangrene-related toxin production. We hypothesize that sugars, which have been used throughout history to prevent wound infection, may represent a nutritional signal against gas gangrene development. Here we demonstrate, for the first time, that sugars (sucrose, glucose) inhibited the production of the main protein toxins, PLC (alpha-toxin) and PFO (theta-toxin), responsible for the onset and progression of gas gangrene. Transcription analysis experiments using *plc-gusA* and *pfoA-gusA* reporter fusions as well as RT-PCR analysis of mRNA transcripts confirmed that sugar represses *plc* and *pfoA* expression. In contrast an isogenic *C. perfringens* strain that is defective in CcpA, the master transcription factor involved in carbon catabolite response, was completely resistant to the sugar-mediated inhibition of PLC and PFO toxin production. Furthermore, the production of PLC and PFO toxins in the *ccpA* mutant strain was several-fold higher than the toxin production found in the wild type strain. Therefore, CcpA is the primary or unique regulatory protein responsible for the carbon catabolite (sugar) repression of toxin production of this pathogen. The present results are analyzed in the context of the role of CcpA for the development and aggressiveness of clostridial gas gangrene and the well-known, although poorly understood, anti-infective and wound healing effects of sugars and related substances.

1. Introduction

Clostridium perfringens is considered to be the most ubiquitous and widely distributed pathogen in nature [1,2]. Two key features that allow for its wide distribution and pathogenicity are its ability to produce numerous virulence-related factors and its ability to form highly resistant dormant spores [1,3,4]. Virulent *C. perfringens* isolates produce as many as thirteen different protein toxins [1–3] and a similar number of other virulence-related proteins (i.e., extracellular matrix binding proteins) [1,5] that are important for the development of different diseases (food poisoning, antibiotic-associated diarrhea and fatal gas gangrene) that are produced by this pathogen [2,4,6].

Clostridium gas gangrene (clostridial myonecrosis) is an acute and devastating infection that develops after the entrance of vegetative *C. perfringens* cells or dormant spores into the body through an injury or predisposing illness (i.e., diabetes, colon cancer) [4,7–9]. Once they are established at the site of infection, *C. perfringens* vegetative cells or their germinated spores grow quickly, resulting in a remarkable invasion and destruction of healthy living tissue due to the action of potent extra-cellular protein toxins that cause the necrosis of the host tissue [1,6,7,10–12].

Two toxins are essentials for the onset and progression of clostridial gas gangrene: alpha-toxin, also known as PLC, which is an exo-enzyme with phospholipase C and sphingomyelinase activities [10,11] and theta-toxin or perfringolysin O (PFO), a thiol-activated cytolysin [6,8]. Both toxins are thought to act synergistically to create an anoxic environment and provide essential nutrients inside the host's infected tissues as gas gangrene disease progresses (several inches per hour), despite appropriate antibiotic therapy [6–11].

Even though the clinical importance and sanitary relevance of human gas gangrene infection, our knowledge of the signals affecting toxin production in the gas gangrene process remains sparse. Many bacteria regulate virulence gene expression in response to cell population density, a phenomenon known as quorum sensing

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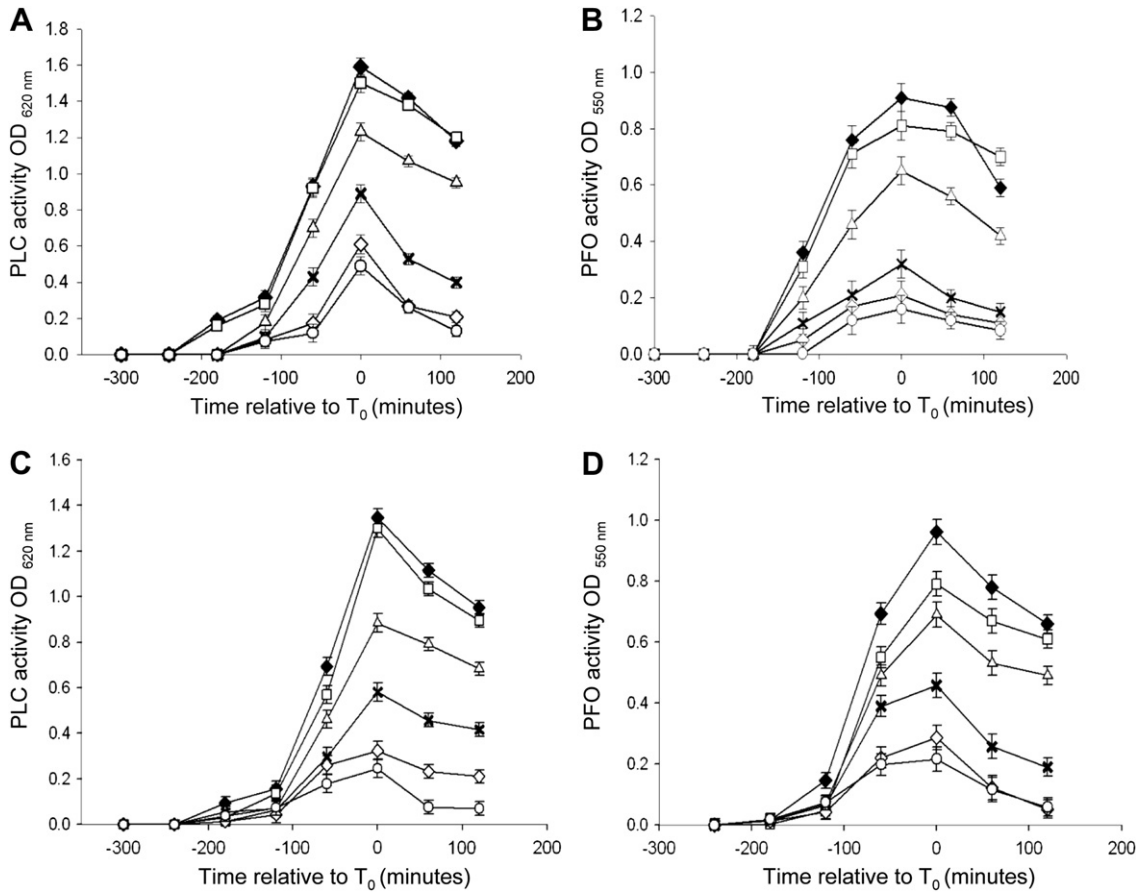

Fig. 1. Sugars regulate PLC and PFO production in *C. perfringens*. Dose-dependent responses of PLC (alpha-toxin) phospholipase activity (A, C) and PFO (theta-toxin) hemolytic activity (B, D) of cell-free supernatants of *C. perfringens* strain 13 grown in TY broth with (white symbols) or without (black symbol) glucose (A–B) or sucrose (C–D) supplementation. The assayed sugar concentrations were as follow: 0.25% (-□-), 0.5% (-△-), 1.0% (-×-), 2.0% (-◇-), and 3% (-○-). Samples were taken at the indicated times and enzymatic reactions were developed as indicated in the Methods section. The addition of glucose or sucrose, at the different concentrations, did not affect the vegetative growth while the final cellular yield of *C. perfringens* strain 13 cultures grown in the presence of sugars was slightly higher (data not shown). T_0 represents the end of the exponential phase of growth. A representative set of results obtained from three independent experiments is shown.

(QS), and environmental signals [13–15]. Recent studies have demonstrated the key role of different QS mechanisms on the toxin production of the human gas gangrene-producer *C. perfringens* strain 13 [5,16–19]. Intriguingly, these QS regulatory systems act positively on *C. perfringens* toxin production while the nature of putative signals acting negatively on toxin production remains completely unknown.

An unexplored global regulatory network that could regulate the homeostasis of gas gangrene-related toxin production is carbon availability [20]. Effectively, sugars and related substances (i.e. honey and molasses) have been used since millenary times to promote wound healing and prevent wound infection [21–26]. The explanation of the role of sugars in the treatment of wounds to prevent infection and accelerate healing is complex and perhaps impossible to reduce to a single mechanism. In a recent study, we demonstrated that sugars (glucose, sucrose and others carbon catabolites) mediated the carbon catabolite repression (CCR) of gliding motility in *C. perfringens*, a social behavior that would have a significant role during gangrene dispersion throughout tissues [4]. CCR is a widespread phenomenon in bacteria where the expression of a number of genes is regulated by the presence of a preferred carbon source such as sucrose or glucose [20]. Because toxin production is crucial for the development of *C. perfringens* gas gangrene [6–12], we explored the possibility that sugars (CCR) might constitute an important nutritional signal that regulates gas gangrene-related toxin production in *C. perfringens*.

Here, we report the effects of CCR, as mediated by sucrose and glucose, on the expression and activity of the major gas gangrene-associated toxins, PLC (alpha-toxin) and PFO (theta-toxin). We also demonstrate that the carbon catabolite control protein CcpA constitutes the unique and primary transcription factor responsible for the inhibition of toxin production in the presence of sugars. These results shed new light to the beneficial effects of sugars and related substances to prevent wound infection and accelerate its healing.

2. Results and discussion

2.1. *C. perfringens* alpha- and theta-toxin production is inhibited in the presence of sugars

To determine the effect of the presence of sugars on PLC and PFO toxin production, we first examined the effect of different concentrations (ranging from 0.25% to 3.0%) of sucrose and glucose, sugars (carbon catabolites) that are rapidly metabolized by *C. perfringens* [1,4]. For the experiments described in this work, we used the *C. perfringens* strain 13 because it is a natural isolate able to cause experimental gas gangrene, it can be easily genetically manipulated, the sequence of its genome is completed and published and is widely used as a reference strain of *C. prefringens* for research studies around the world [1,4,5,16]. Sugar (sucrose or glucose) was added at the beginning of each experience to TY

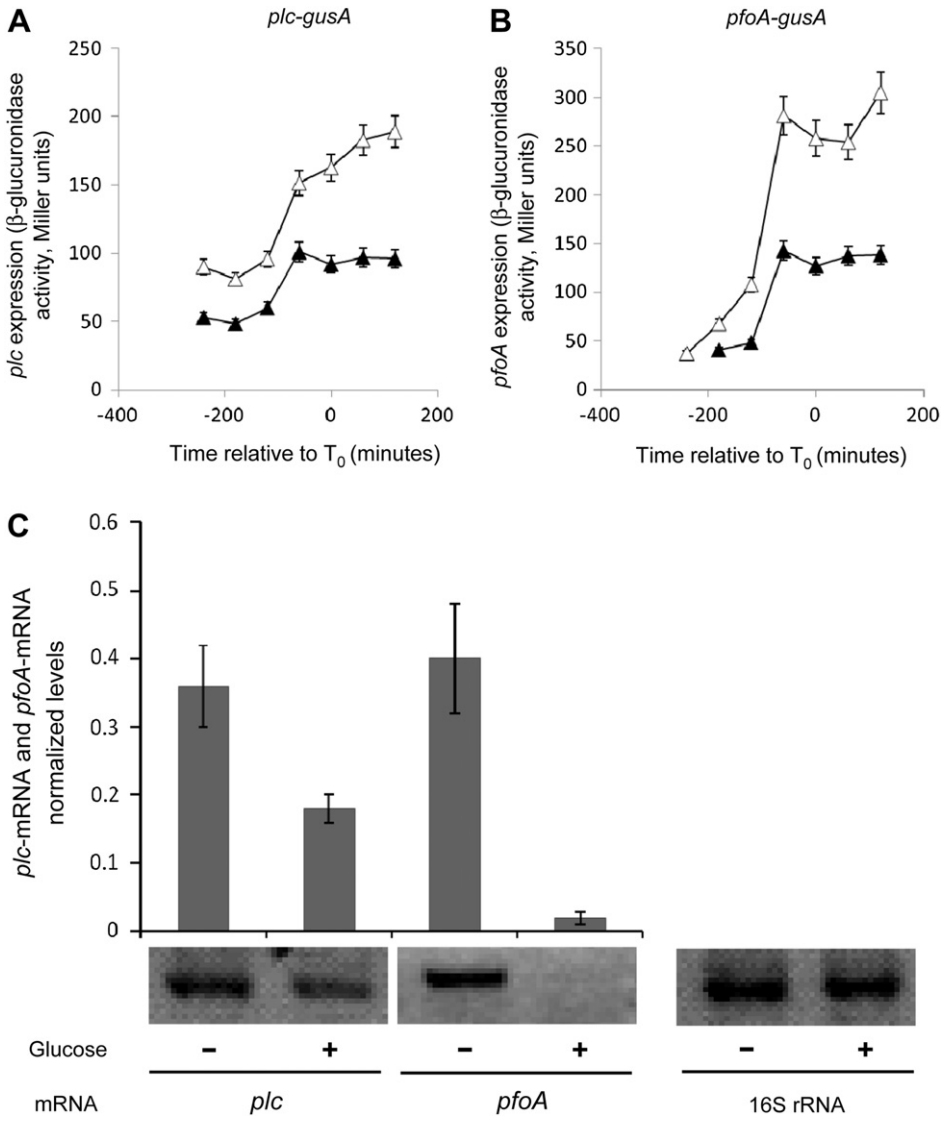

Fig. 2. Sugars produce carbon catabolite repression of *plc* and *pfoA* gene expression in gas gangrene-producing *C. perfringens*. (A–B) β-glucuronidase activity of *plc-gusA* (A) and *pfoA-gusA* (B) transcriptional reporter fusions from *C. perfringens* cultures of strain 13 grown in TY broth with (▲) or without (△) 2% glucose supplementation. At the indicated times, samples were taken and processed as indicated in the Methods to measure β-glucuronidase activity. Similar results were obtained when 2% glucose was replaced by 2% sucrose (data not shown). A representative set of results obtained from five independent experiments is shown. (C) Semi-quantitative RT-PCR of mRNA for *plc* and *pfoA* in the presence or absence of glucose. *C. perfringens* cultures of strain 13 were grown in TY broth with and without 2% glucose supplementation (indicated in the figure by "plus" and "minus" symbols respectively). Samples for RNA extraction were taken at the end of exponential growth (T_0) and processed for RT-PCR amplification and quantification. The relative amounts of specific mRNA produced were normalized against the 16S rRNA amplification band intensity as determined from the gel by optical densitometry (see the Methods section for further details). A representative set of five independent experiments is shown.

medium, a *C. perfringens* broth containing a basal concentration of free sugars lower than 0.1% [3,4]. PLC and PFO activities were measured using filtered supernatants from strain 13 cells (grown with or without sugar supplementation) at different time points during growth. The ability of these supernatants to degrade egg yolk (PLC activity) and lyse red blood cells (PFO activity) was determined as described in the Methods section. As shown in Fig. 1, the production of PLC and PFO, as measured by tittering their enzymatic activities, were not significantly affected by addition of 0.25% glucose (Fig. 1A–B) or 0.25% sucrose (Fig. 1C–D) to *C. perfringens* cultures grown in TY [1,4]. However, higher sugar concentrations, ranging from 0.5 % to 3%, produced a significant and reproducible dose-dependent downregulation in PLC and PFO production throughout the complete growth cycle of *C. perfringens* strain 13 (Fig. 1). The production of PLC and PFO was reduced by 40%, 70% and 90% when *C. perfringens* strain 13 was grown in the presence of 0.5%, 1% and 2% of glucose (or sucrose) respectively in comparison with the levels of toxin produced in the absence of added sugar (Fig. 1). In order to distinguish whether the observed effects on PLC and PFO toxin production were due to the presence of the sugar (carbon catabolite regulation) or the pH variation (due to sugar fermentation), we analyzed the effect of glucose and sucrose on PLC and PFO toxin production in cell-free supernatants derived from *C. perfringens* cultures grown in the presence of sugar plus different concentrations of Tris–HCl or MOPS buffer to regulate the pH of the growth medium (see the Methods section) [3]. Under these experimental conditions (growth in TY-Tris or TY-MOPS), the supplementation with 2% or 3% of glucose or sucrose produced essentially the same downregulation of PLC and PFO activities as shown in Fig. 1 (data not shown). Therefore, carbon catabolites, and not pH, regulated the capacity of *C. perfringens* strain 13 to produce PLC and PFO toxins. A similar result was obtained when glucose and

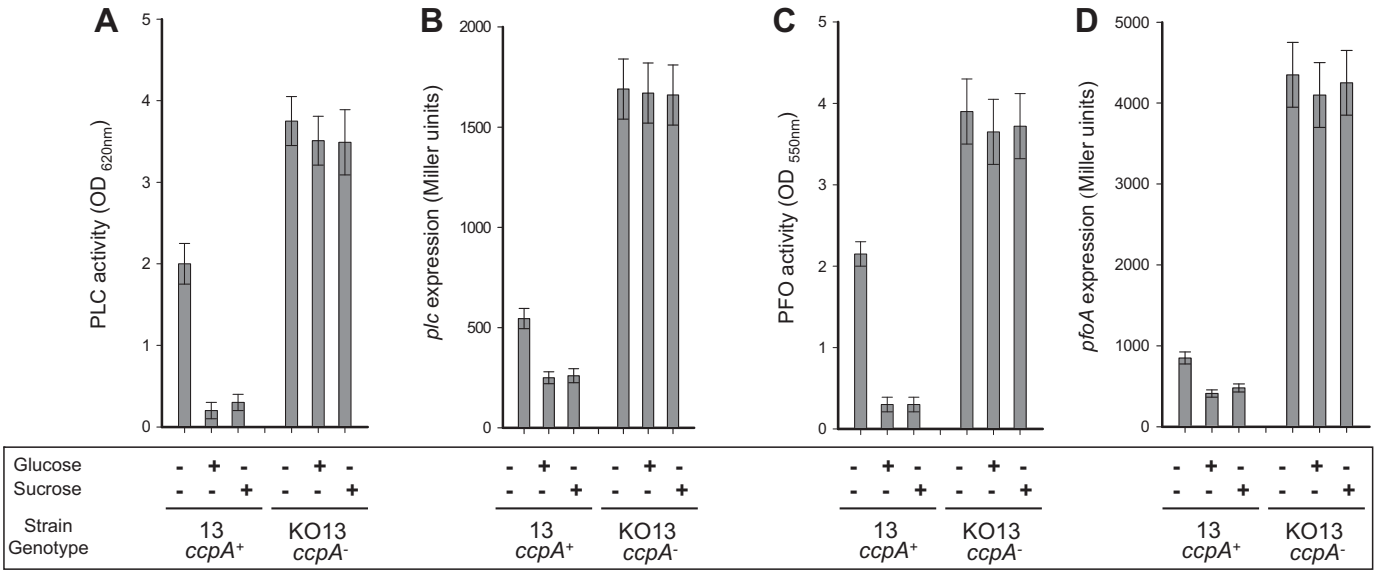

Fig. 3. CcpA mediates the sugar-induced CCR of alpha-toxin (PLC) and theta-toxin (PFO) production in the gas gangrene producer *C. perfringens*. *C. perfringens* cultures proficient and deficient in CcpA production (strains 13 and KO13 respectively), were grown until 1 h after the end of the exponential phase (T_1) in TY broth with or without sugar supplementation (2% glucose or 2% sucrose). From each culture, two samples (at times T_0 and T_1) were taken and processed together as indicated in the Methods section for measuring the accumulated PLC phospholipase activity (A), *plc*-driven β–glucuronidase activity (B), red blood hemolytic PFO activity (C) and *pfoA*-driven β–glucuronidase activity (D), respectively. A representative set of three independent experiments is shown.

sucrose were replaced by fructose, another readily metabolized sugar [4,20,31], but not by complex carbohydrates such as starch (data not shown). These results demonstrated for the first time the carbon catabolite (sugar) regulation of PLC and PFO activities in a human gas gangrene-producing *C. perfringens* strain.

2.2. The expression of alpha- and theta-toxin coding genes, plc and pfoA, is repressed by sugars

We investigated whether the low phospholipase C and red blood cell hemolytic activities of PLC and PFO toxins derived from *C. perfringens* cultures grown in the presence of different sugars (sucrose or glucose) were due to transcriptional repression mediated by the added sugar on toxin gene expression (CCR). To this end, we studied the expression of *plc* and *pfoA*, coding for PLC and PFO toxins, respectively, in *C. perfringens* cultures grown in the presence and absence of catabolite sugars using two different strategies. First, we constructed transcriptional reporter gene fusions of the promoterless *gusA* gene, coding for β-glucuronidase, to the promoter regions of *plc* and *pfoA* (see the Methods section for details). The β-glucuronidase *plc-gusA* and *pfoA-gusA* reporter fusions were introduced separately into *C. perfringens* strain 13 cells by DNA electroporation [3,4]. *Plc-gusA*- and *pfoA-gusA*-harboring cultures of strain 13 were grown separately in TY medium with or without sugar supplementation, and *plc*- and *pfoA*-driven β-glucuronidase activities were assayed as described. For these analyses, we chose a sugar concentration of 2% because it produced a clear inhibitory effect on toxin production (see Fig. 1) and also, this sugar concentration, was previously reported to produce a complete inhibitory effect on the type IV-dependent gliding motility of *C. perfringens* strain 13 [4]. Interestingly, in cultures of *C. perfringens* strain 13 grown in the presence of added glucose (or sucrose, data not shown), there was a substantial and reproducible downregulation (two- to three-fold) of *plc* and *pfoA* promoter activities throughout the growth cycle (Fig. 2A and B, respectively). The observed effect of CCR on gas gangrene-related toxin gene expression (Fig. 2A–B) was confirmed by semi-quantitative RT-PCR for specific *plc* and *pfoA* transcripts from cultures of *C. perfringens* strain 13 grown in the presence or absence of sugar supplementation. As shown in Fig. 2C, supplementing the growth medium with glucose (or sucrose, data not shown) produced a significant downregulation of $mRNA_{plc}$ and $mRNA_{pfoA}$ (two- to five-fold respectively). These results confirmed, for the first time, that the inhibitory effect of catabolite sugars on gas gangrene-related toxin production in histotoxic *C. perfringens* took place at the transcriptional level.

2.3. Carbon catabolite repression of alpha- and theta-toxin activities is under CcpA control in C. perfringens

Carbon catabolite control protein A (CcpA) plays a key role in Gram-positive bacteria with a low G+C content by connecting carbon metabolism with several cellular responses [20,32]. Growing evidence from several pathogens, including pathogenic Streptococci [20,33,34], suggests that CcpA plays an important role in virulence-associated gene expression. In *C. perfringens*, CcpA regulates sporulation, enterotoxin (CPE) production, biofilm formation and gliding motility [4,35,36]. Because of the observed repressive effect of sugars on the expression of *plc* and *pfoA* genes (Fig. 2), we analyzed whether CcpA has a role in the sugar-mediated CCR of *C. perfringens* gas gangrene-related (PLC and PFO) toxin production. We compared the activities and expression levels of both gas gangrene-related toxins (PLC and PFO) in cultures of wild type strain 13 and its isogenic *ccpA* derivative KO13 [4], grown in the presence or absence of added sugar. As expected, when PLC activity was evaluated in the CcpA-proficient strain 13, grown in the presence of 2% glucose or 2% sucrose, low levels of PLC activity were detected (Fig. 3A, left panel). In contrast, the *ccpA* mutant derivative KO13 retained 100% of its PLC activity in the presence of 2% glucose or 2% sucrose (Fig. 3A, right panel). To confirm, using a different approach, the key role of CcpA in sugar-mediated CCR of *C. perfringens* alpha-toxin (PLC) expression, we compared the expression levels of the *plc* promoter (β-glucuronidase activity driven from a *plc-gusA* transcriptional reporter fusion) in isogenic

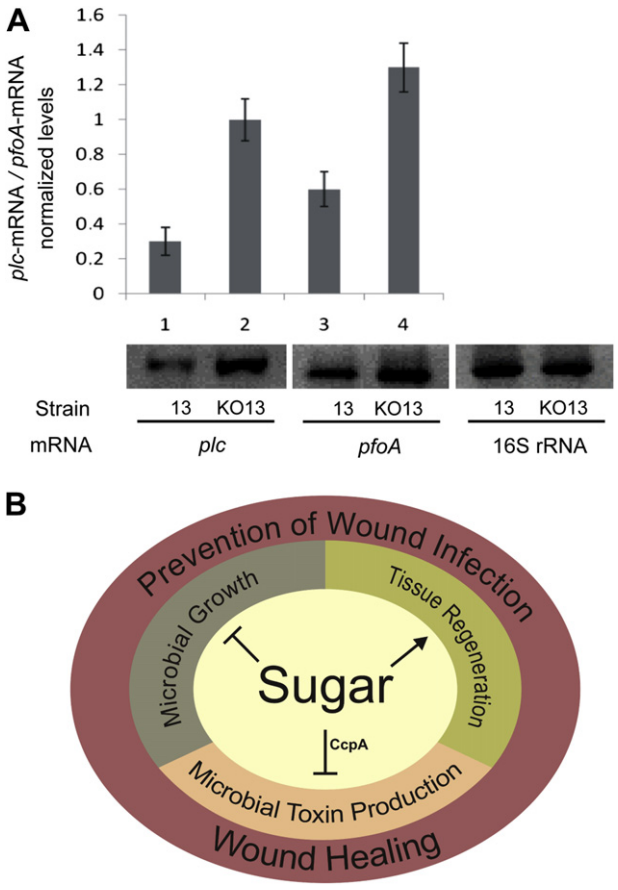

Fig. 4. CcpA plays a key role on gas gangrene-related toxin production in *C. perfringens*. (A) Semi-quantitative RT-PCR of mRNA for *plc* and *pfoA* in the absence of added sugar. *C. perfringens* cultures of strains 13 and KO13 proficient and deficient in CcpA synthesis respectively, were grown in TY broth without sugar supplementation. Samples for RNA extraction were taken at the end of exponential growth (T_0) and processed for RT-PCR amplification and quantification as indicated in the Methods section. The relative amounts of specific mRNA produced were normalized against the 16S rRNA amplification band intensity as determined from the gel by optical densitometry (see the Methods section for further details). A representative set of five independent experiments is shown. (B) A schematic diagram that summarises our current knowledge of how sugars (glucose, sucrose, fructose) and related substances (honey, molasses, sugar paste and other pharmaceutical products) are utilized to treat ulcers of difficult prognosis, prevent wound infection and/or accelerate wound healing (i.e. In diabetic patients). The results described in this work suggest that the sugar-treatment of an injured tissue, where clostridial cells would be present, generates a negative signal, mediate by the transcription factor CcpA, which represses the production of the tissue-degrading toxins PLC and PFO. The inhibition of toxin production by the applied sugar would contribute to stop the development, progression and/or aggressiveness of clostridial gas gangrene. The symbols (↑) and (⊥) indicate stimulation and inhibition respectively. See the conclusion section and [20–26,37–42] for further details.

CcpA-proficient and CcpA-deficient *C. perfringens* cultures grown in TY medium with or without 2% glucose or 2% sucrose. As shown in Fig. 3B, in the CcpA-deficient cultures (right panel), but not in the cultures that expressed CcpA (left panel), *plc* expression was not inhibited by the addition of glucose or sucrose to the growth medium (Fig. 3B, right panel). This conclusion was confirmed by semi-quantitative RT-PCR analysis of $mRNA_{plc}$ in CcpA-deficient (strain KO13) *C. perfringens* cultures grown in TY medium with sugar supplementation (data not shown).

Similar to PLC, there was no significant reduction in PFO activity or *pfoA*-driven β-glucuronidase activity in CcpA-deficient cultures of *C. perfringens* grown in the presence of added sugars (Fig. 3C–D, right panels) compared with the downregulation of PFO activity and *pfoA* expression in CcpA-proficient *C. perfringens* cultures grown in the presence of added sugars (Fig. 3C–D, left panels, respectively). RT-PCR analysis of relative levels of $mRNA_{pfoA}$ also confirmed the complete absence of carbon catabolite regulation on *pfoA* expression of CcpA-deficient (strain KO13) *C. perfringens* cells (data not shown). Because the only difference between strain 13 and KO13 is the ability to produce a functional CcpA [4], we concluded that CcpA is the major, or unique, regulatory protein involved in sugar-mediated CCR of *C. perfringens* PLC and PFO toxin production.

An unexpected but interesting result was that, in the absence of sugar supplementation, *C. perfringens* cultures deficient in CcpA production (KO13 strain) showed a significant increase in PLC and PFO activities and a pronounced induction (three- to five-fold) of *plc* and *pfoA* expression as compared to activity and expression levels of PLC/PFO in isogenic cultures proficient in CcpA production (wild type strain 13) grown under similar conditions (compare lanes without sugar supplementation between strain 13 and KO13 in Fig. 3). Accordingly, as shown in Fig. 4A, there is a clear and reproducible upregulation of the synthesis of the specific mRNAs for *pfoA* and *plc* (three- to four-fold respectively) in the *ccpA* mutant strain KO13 compared with the levels of $mRNA_{plc}$ and $mRNA_{pfoA}$ produced by the isogenic wild type strain 13 grown in the absence of sugar supplementation. These results strongly suggest that, in addition to QS-generated signals that stimulate toxin production [5,16–19], CcpA itself might play an important, direct or indirect, role in the negative regulation of *C. perfringens* pathogenicity. The absence of clear CcpA-binding sites (*Bacillus subtilis*-like CRE sites) on the regulatory regions of all *C. perfringens* genes reported to be affected by CcpA [1,4,35,36] suggests that other CRE-like consensus sequences, different from those reported for *B. subtilis* and other Gram-positive bacteria with low G+C-contents, would exist in clostridia [32]. A transcriptome analysis of CcpA-proficient and CcpA-deficient *C. perfringens* isogenic strains (i.e., strain 13 and KO13, respectively) grown in the presence and absence of carbon catabolites would provide experimental data to uncover the *cre* consensus sequence recognized by CcpA in the promoter regions of toxin-coding genes of *C. perfringens*. Alternatively, it is also possible that the effect of CcpA on toxin production might be undirected and, an unidentified CcpA-related intermediate factor might be directly involved in regulating gas gangrene toxin-gene expression.

3. Conclusions

For long time the therapeutic effects of sugars and related substances (i.e. honey and molasses, mainly constituted by glucose/fructose/maltose and sucrose/glucose respectively), to prevent wound infection and accelerate wound healing relayed on the basis of existing folk therapy [21–26,37–39]. Table sugar (granulated sugar or sucrose) has been used, with a cure success greater than 99%, to treat the wounds of complicated evolution [23,26,39]. Nowadays, there are two scientifically proven effects of the topical application of sugars that accelerate the healing of cutaneous wounds and ulcers. Sugars act as modulator of keratinocytes and fibroblasts to promote re-epithelialisation and new tissue formation [40]. Pure sugar (sucrose 1%) have been reported to promote collagen synthesis from fibroblasts; secretions of urokinase-type plasminogen activator (u-PA), transforming growth factor-α (TGF-α) and Interleukin 1-α (IL-1 α) from keratinocytes, and enhanced integrin expression from both cellular types [40]. These sugar-enhanced effects facilitate the migration of keratinocytes and fibroblasts to the site of the damaged tissue, new extra-cellular matrix formation, re-epithelialisation and new granulation tissue formation [40]. The second mechanism mediated by sugar that promotes wound healing is its antimicrobial effect that prevents wound infections. It is proposed that the treatment of infected

wounds with sugars creates an environment of low water activity, which inhibits microbial growth [41,42]. Here we demonstrated a novel effect of sugar, inhibition of production of tissue-degrading toxins, which might be added to its two-reported beneficial effects to avoid wound infection, impede tissue destruction and accelerate wound healing (Fig. 4B). We demonstrated that sugars regulate the production of the two toxins (PLC and PFO) that are essential for the development and progression of clostridial gas gangrene (Figs. 1,2). We also demonstrated that the regulatory protein CcpA is the main, or unique, factor that is responsible for the sugar-mediated repression of toxin production (Figs. 3 and 4A). Therefore, CcpA links the presence of sugars with carbon metabolism and virulence in the gas gangrene producer *C. perfringens* (Fig. 4B). It is interesting to note that a CcpA-deficient strain of *Streptococcus mutants* (the etiological agent of human dental caries) displays an enhanced capacity to produce acid and tolerate an acidic environment, attributes that would favor its survival and success as a human-pathogen [33]. Furthermore, in Group A Streptococci (GAS), the *sag* operon (encoding for streptolysin S, SLS, production) is under CcpA regulation [34]. A *ccpA* mutant strain of GAS exhibits a higher production of SLS hemolytic activity and a more virulent phenotype as compared to its parental strain in an animal model [34]. These findings have led to the suggestion that, in GAS, CcpA acts to repress toxin production and virulence during systemic infection. From our study, it is also evident that a *ccpA* mutant strain of *C. perfringens* produces higher levels of the toxins PLC and PFO, which are essential for gas gangrene (Figs. 3 and 4A). This finding raises the possibility that, similar to GAS, a CcpA-deficient *C. perfringens* strain, or *C. perfringens* wild type cells with *in vivo* down-regulated CcpA activities (for example, inside the phagosome after phagocytosis, when low levels of sugar are expected to be present), might also show a more aggressive behavior, through the overproduction of cytotoxic and tissue-degrading toxins. If this hypothesis is correct, we envision new avenues of research focusing on the link between carbon metabolism, CcpA and virulence properties (such as toxin production and gliding motility) to design and explore strategies to prevent and/or treat gas gangrene and other diseases (i.e., antibiotic-associated diarrhea) produced by virulent clostridial isolates (Fig. 4B).

4. Material and methods

4.1. Bacterial strains and growth conditions

The *C. perfringens* strains used in this study were the human gas gangrene-producing reference strain 13 and its isogenic *ccpA* mutant derivative KO13 [4]. FTG medium was used for the propagation of *C. perfringens* strains. TY broth, a sugar-free medium [3], with and without sugar supplementation was used for the indicated experiments. To regulate the pH during growth different amounts of 1 M Tris–HCl, pH 8.0 or 2.0 M MOPS buffer pH 7.8 were used as previously described [3]. All cultures were grown under anaerobic conditions in anaerobic jars containing Gas Packs (BD Biosciences, USA) at 37 °C [4].

4.2. Toxin assays

Samples (1 mL each) of filtered supernatants from *C. perfringens* cultures were taken at the indicated times and assayed for phospholipase C (PLC or alpha-toxin) and PFO (theta-toxin or perfringolysin O) production as previously described [8,27]. PLC and PFO activities were measured at 620 nm and 550 nm respectively and expressed as units of absorbance per microgram of protein present in each *C. perfringens* sample.

4.3. Construction of gusA fusion plasmids and the β-glucuronidase assay

The reporter plasmids were constructed by amplifying the *plc* and *pfoA* promoter regions from the gas gangrene-producing strain 13 using the following primers: Pro-*plc*up 5′ATAAGTGAATTCTA GGTTAAAACCTG3′ and Pro-*plc*dw 5′ACAAATCTGCAGCTTACAAA TCTTTCTTTTC3′ for the *plc* promoter, and Pro-*pfoA*up 5′TA GTGAATTCAAATTCCATAAATGGAAC3′ and Pro-*pfoA*dw 5′GCCACT GCAGTACTTGCTATTAATTTTG3′ for the *pfoA* promoter. The PCR products were cloned into pCR®2.1-TOPO® using a TOPO TA Cloning® kit (Invitrogen, USA). The promoter region *EcoR*I-*Pst*I restriction fragments were separately cloned into the pMRS127-derived vector pIH102 (courtesy of Dr. I-hsiu Huang), which harbors a chloramphenicol-resistance (Cm^r) marker [3,4]. The corresponding reporter fusions, p*plc-gusA* and p*pfoA-gusA*, were introduced into *C. perfringens* strain 13 and KO13 by DNA electroporation and Cm^r selection, as previously described [3,4]. The β-glucuronidase activity of the strains carrying the *gusA* fusions were assayed and expressed in Miller Units as previously described [3,4].

4.4. RNA isolation and RT-PCR analysis

C. perfringens RNA extraction was performed as previously described [28,29]. Total RNA was then purified using TRIzol reagent as described by the manufacturer (Invitrogen, USA). To remove contaminating DNA, RNA samples were treated with 1 unit of RQ1 RNase-free DNase I (Promega, USA) in the presence of 0.1 volume of 10× DNase I buffer for 1 h at 37 °C. DNase-treated samples were then re-extracted with TRIzol according to the manufacturer's instructions, using only half of the original reagent volumes. The total RNA from each sample was quantified via spectrophotometry at an absorbance of 260–280 nm using a Genova spectrophotomer (Jenway, UK). The reverse transcription reaction was accomplished using M-MLV retrotranscriptase (Promega, USA) in a Techne thermocycler TC model (Techne, UK) as previously described for *C. perfringens* samples [28–30] using the specific downstream (*plc*dw 5′AGTTAGCTAAAGTTACCTTTGC3′ and *pfoA*dw 5′ TCCATAAGCTACATTTGAAACC3′ for *plc* and *pfoA* expression, respectively) primers. The reverse transcription reactions were stored at −20 °C until use. PCR amplification of cDNAs was accomplished using primers *plc*up 5′CAATTAGGTTCTACT TATCCAG3′ and *plc*dw 5′AGTTAGCTAAAGTTACCTTTGC3′ for *plc* expression, *pfoA*up 5′CATTACAACTTGCAGATAAAGC3′ and *pfoA*dw 5′ TCCATAAGCTACATTTGAAACC3′ for *pfoA* expression. The PCR products were resolved and visualized by 1% agarose ethidium bromide gel electrophoresis, and band intensity from the gel was determined using GelPro image analyzer software. 16S rRNA was used as the internal control, and reverse transcription reactions were performed under the same conditions described above using the primers cp16Sup 5′TTTCGAAAGGAAGATTAATACC 3′ and cp16Sdw 5′CAACTTAATGGTAGTAACTAAC3′.

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