Carbon catabolite repression of type IV pili-dependent gliding motility in the anaerobic pathogen Clostridium perfringens

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Abstract

*C. perfringens* is an anaerobic Gram-positive spore-forming bacterium responsible for the production of severe histotoxic and gastrointestinal diseases in humans and animals. *In silico* analysis of the three available genome-sequenced *C. perfringens* strains (13, SM101 and ATCC13124) revealed that genes that encode flagellar proteins and genes involved in chemotaxis are absent. However, those strains exhibit Type IV pili-dependent gliding motility. Since carbon catabolite regulation has been implicated in the control of different bacterial behaviors we investigated the effect of glucose and other readily metabolized carbohydrates on *C. perfringens* gliding motility. Our results demonstrate that carbon catabolite regulation constitutes an important physiological regulatory mechanism that reduces the proficiency of gliding motility of a large number of unrelated human and animal-derived pathogenic *C. perfringens* strains. Glucose produces a strong dose-dependent inhibition of gliding development without affecting vegetative growth. Maximum gliding inhibition was observed at a glucose concentration (1%) previously reported to also inhibit other important behaviors in *C. perfringens*, such as spore development. The inhibition of gliding development in the presence of glucose was due, at least in part, to the repression of the genes pilT and pilD, whose products are essential for TFP-dependent
gliding proficiency. The inhibitory effect of glucose on pilT and pilD expression was under the control of the key regulatory protein CcpA (catabolite control protein A). The deficiency in CcpA activity of a ccpA knock-out C. perfringens mutant strain restored the expression of pilT and pilD and gliding proficiency in the presence of 1% glucose. Carbon catabolite repression of gliding motility of the ccpA mutant strain was restored after the introduction of a complementing plasmid harboring a wild type copy of ccpA. These results point to a central role for CcpA in orchestrating the negative effect of carbon catabolite regulation on C. perfringens gliding motility. Furthermore, we discovered a novel positive role, in the absence of catabolite regulation, of CcpA on pilT/pilD expression and gliding proficiency. Carbon catabolite repression of gliding motility and the dual role of CcpA, either as repressor or activator of gliding, are analyzed in the context of the different social behaviors and diseases produced by C. perfringens.
Introduction

Motility is an important attribute utilized by many pathogenic and non-pathogenic bacteria to colonize new environments, search for nutrients and to allow the formation of complex architectural structures (i.e. biofilms, fruiting bodies) (14, 15, 25). Translocation in liquid medium (swimming) is mediated by flagella and swimming has been traditionally used to describe bacterial motility. However, it is also true that in nature, most bacteria are associated with surfaces and therefore, have evolved diverse mechanisms of translocation on solid or semi-solid biotic and abiotic surfaces which would be important for rapid dissemination and colonization during the course of an infection (21). Among the surface-associated mechanisms of motility that play a key role in cell-cell and cell-surface interactions it is worthy to mention (i) swarming, a flagella-dependent social form of translocation where planktonic cells differentiate into giant and hyper-flagellated cells that move in groups (“swarm”) to explore and colonize new habitats (9); (ii) twitching, a flagella-independent but type IV pili (TFP)-dependent form of intermittent and jerky surface motility (4, 14, 15), which has been demonstrated in the opportunistic pathogen Pseudomonas aeruginosa (5), the pathogenic Neisseiria gonorrhoeae (23), and recently by Haemophilus influenzae, which was previously considered a non-motile bacteria (2); and (iii) gliding motility which has been studied in detail in model organisms such as Myxococcus xanthus,
Synechocystis and Anabaena (19). In M. xanthus, two types of mechanisms for gliding motility are utilized: adventurous (A)-gliding and social (S)-gliding (16). A-gliding motility is observed when cells are isolated or in low number without contact with each other. In this sense, gliding motility is defined as a smooth movement of rod-shaped cells in the direction of their long axis on a surface. It has been proposed that the driving force for A-gliding motility in M. xanthus is generated by the action of a nozzle-like structure that produces a polysaccharide slime trail. S-gliding motility, similar to twitching motility, is dependent on the active extension and retraction of TFP (16). In other bacteria (i.e. Cytophaga, Flavobacterium and Mycoplasma mobile) gliding takes place in the absence of Type IV pili and ATP consumption. Instead, they generate motility with motility proteins anchored to the membrane that in turn use proton motive force (PMF) and/or polar polysaccharide extrusion to drive cellular motility over surfaces; all these gliding examples point out the extreme diversity of gliding mechanisms (4, 14, 15, 22, 24). Extracellular TFP appendages are polymers of the small protein PilA or pilin. In P. aeruginosa, PilA is initially translated as a pre-pilin with a short leader peptide cleaved by PilD (48); PilB provides energy for assembly. PilT and PilU are nucleoside-triphosphate (NTP) binding proteins that have been implicated in pili retraction during twitching motility (4). In P. aeruginosa, almost 40 genes are involved in the biosynthesis and function of
TFP (23). Besides the key role of TFP in twitching and gliding, these appendages also mediate other important phenomena such as adherence, fruiting body formation, bacteriophage absorption, DNA uptake, cytotoxicity, activation of host cell responses and biofilm development (1, 14, 23, 24, 31).

*Clostridium perfringens* is a Gram-positive, anaerobic, spore-forming bacterium that causes severe gastrointestinal and histotoxic infections in humans and animals (28, 32). This pathogen has been traditionally described as a non-motile bacterium, as no genes that encode flagellar proteins or genes involved in chemotaxis were identified in the complete genomic sequences of the three human-pathogenic *C. perfringens* strains 13, SM101 and ATCC13124 (26, 36). However, sequence analysis (data not shown) suggests that the three strains carry genes which code for type IV pili (TFP) components (such as *pilA-D* and *pilT*) (26, 36). Recently, Varga *et al.* demonstrated that *C. perfringens* strain 13, SM101, and ATCC13124 exhibited social gliding motility on BHI (Brain Heart Infusion) agar plates, and TFP were detected on the surface of the bacteria (41). This study also demonstrated that *pilT* and *pilC* mutants failed to produce detectable pili and were non-motile on BHI agar (41). Other putative TFP biosynthesis genes found in *C. perfringens* remains to be characterized and the exact mechanism of TFP assembly, its physiological role, importance and regulation are currently unknown.
To this respect, diverse bacterial behaviors; such as biofilm development, sporulation, fruiting body formation and surface-associated motility; are regulated by environmental, metabolic and quorum sensing signals (9, 10, 16, 28). In particular, carbon catabolite regulation 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 glucose or fructose (27, 39, 43). In *C. perfringens*, and many low G+C Gram-positive bacteria, carbon catabolite control is under the regulation of CcpA (catabolite control protein A), a pleiotropic transcriptional regulator belonging to the LacI/GalR family of transcription factors (27, 39, 43). CcpA functions as a DNA binding protein, either activating or repressing genes generally in the presence of a preferred carbon source (35, 43). More precisely, surface-associated motility is an interesting example of social bacterial behavior that could be regulated by nutrient (i.e. carbon) availability in nature. Interestingly, in *P. aeruginosa*, swarming (but not twitching) motility is carbon source-regulated; poor swarming activity is observed in the presence of glucose (37). Therefore, the ability of the pathogen to carry out surface-associated social motility during the course of the infection is of crucial importance (2, 9, 14, 21, 24). In this work, we have investigated the effects of glucose, and other readily metabolized carbohydrates, on the type IV pili (TFP)-dependent social gliding motility of a collection of pathogenic *C. perfringens* strains isolated
from human and animal sources. Our results clearly demonstrate that carbon catabolite repression is a general and common regulatory mechanism of gliding motility in *C. perfringens*, independent of the source of isolation (human or animal) or type of infection (diarrhea or myonecrosis). We demonstrate that the repressive effect of glucose on gliding motility is partially due to the CcpA-mediated down regulation of TFP biosynthesis genes. In addition we have discovered and analyzed a novel carbon catabolite-independent positive role of CcpA in *C. perfringens* gliding motility.

**Material and Methods**

**Strains and culture conditions.** The *C. perfringens* strains used in this study are listed in Table 1. The growth media employed to propagate strains were FTG (Fluid Thioglycolate) and TGY (3% Tryptic soy broth, 2% Glucose and 1% Yeast extract, 0.1% Cysteine) (8, 32). TY medium results from the omission of glucose from TGY formula. All cultures were grown under anaerobic conditions in anaerobic jars containing GasPak (BD) at 37º C.

**Motility assays.** *C. perfringens* strains were grown overnight in FTG at 37º C, and 300 µl of this culture were inoculated and propagated in TGY for 5 h at the same temperature. Next, 1 ml of culture was centrifuged and concentrated 2-fold. Five
microlitres of the concentrated cell suspension were spotted onto pre-dried (1 h at 37° C) Brain Heart Infusion agar (BHIA), TGY agar (TGYA) or TY agar (TYA) plate. All plates were supplemented with 0.7% agar. The inoculated plates were then incubated anaerobically for 48-96 h at 37° C. Photographs of the plates were taken with a Canon Power Shot SD550 digital camera.

Construction of gusA-fusion plasmids and the β-glucuronidase assay. The PCR-amplified product carrying the upstream region of pilD or pilT was first cloned into the pCR®-XL-TOPO® vector using the TOPO®-XL cloning kit (Invitrogen). Briefly, the DNA fragments carrying the promoter region of each of pilD or pilT from SM101 or strain 13 were amplified by PCR using primers CPP53/CPP55, and CPP230/CPP231, respectively (Table 2). The SalI site was incorporated into the forward primer and PstI site into the reverse primer of each primer pairs. These PCR products were then cloned into the pCR®-XL-TOPO® vector. The recombinant clones carrying the expected DNA fragment were confirmed by restriction enzyme digestion, PCR analysis and then DNA sequencing. The SalI-PstI fragments carrying the promoter regions of pilD or pilT from pCR®-XL-TOPO® clones were then re-cloned into the SalI/PstI sites of pMRS127 to create pilD-gusA or pilT-gusA fusion constructs derived either from the strain SM101 or strain 13. These reporter plasmids were then
introduced by electroporation and Cm\(^{r}\) selection (8) into the corresponding wild type 
*C. perfringens* strains 13, SM101 and the isogenic derivates deficient in CcpA.

The strains carrying the *gusA* fusions were grown overnight in FTG at 37º C, and
150 µl of these cultures were inoculated into TY and TGY. At various time points, 1 
ml of each culture was centrifuged for 2 min at 9000 rpm, and the pellet was stored
frozen at -20º C until used. The cell pellets were resuspended with 1 ml of Z-buffer 
(per liter: 8.54g of Na\(_{2}\)HPO\(_{4}\), 5.5g of NaH\(_{2}\)PO\(_{4}\).H\(_{2}\)O, 0.75g of KCl, 0.25g of MgSO\(_{4}\)
and 1.4 ml of β-mercaptoethanol.7H\(_{2}\)O, pH 7) (13, 28). Adequate aliquots of the
resuspended cells were brought to a final volume of 730 µl with buffer Z. Next, 10 µl 
of 10 mg/ml lysozyme was added and the mixture was incubated for 30 min at 37º C,
followed by the addition of 10 µl 10% Triton X-100 (Sigma). The enzymatic 
reactions were initiated by adding 100 µl of 6 mM 4-nitrophenyl β-D-glucuronide 
(Sigma). After 15 min of incubation at 37º C, the reaction was stopped by adding 150 
µl of 1M Na\(_{2}\)CO\(_{3}\). The absorbance was measured at 405 nm and the β-glucuronidase 
activity was calculated using the following equation: \((\text{Abs}_{405\text{ nm}} \times 1000) / (\text{OD}_{600\text{ nm}} \times \text{culture volume [milliliters]} \times \text{X time (minutes)})\) (19).

**Insertional inactivation of *ccpA* gene in strain 13.** To disrupt *ccpA* in strain 13, an
internal 760-bp fragment of the *ccpA* gene was amplified by PCR using primers 
CCP-F and CCP-R (Table 2) and cloned into the SmaI site of pUC18 to create pKO1.
For selection, an erythromycin resistance cassette (ermBP) was ligated into the HincII site of pKO1, creating pKO2. The plasmid pKO2 (which has no origin of replication for C. perfringens) was transformed into strain 13 by electroporation and Em' selection. One clone Em' (KO13) was analyzed (see SFig.1) for correct integration by a single cross-over event involving homologous recombination of the suicidal mutator plasmid (pKO2) on ccpA and utilized in the present study.

The insertional disruption of wild-type ccpA in KO13 was first demonstrated by PCR analysis of DNA isolated from the mutant (see supplemental Figure 1). As expected, a 1.6-kb product was amplified from KO13 DNA using primers P1 and M13F, and 2.5-kb using primers P2 and M13R. However, no PCR product was obtained with either P1 and M13R or P2 and M13F. These PCR results are consistent with the suicidal mutator plasmid pKO2 having been inserted into the wild-type ccpA gene in KO13. Southern blot analyses showed that a 2.4-kb HindIII DNA fragment from wild-type strain 13 hybridized with our ccpA-specific probe. However, two hybridizing bands, of 2.9 and 4.3 kb, were observed with DNA from mutant strain, MO13. This profile is consistent with the expected result since the inserted pKO2 has a HindIII site.

**Southern blot analysis.** A 350-bp internal ccpA DNA fragment was amplified from strain 13 by PCR using primers KO-F and KO-R (Table 2) and labeled with alkaline
phosphatase using the Gene Images™ AlkPhos Direct™ Labeling and Detection System (Amarsham Bioscience) (28, 44). Isolated \textit{C. perfringens} DNA samples, prepared as described (8, 38) were digested with HindIII, separated by electrophoresis on 1\% agarose gels and transferred by Southern blotting. The blot was hybridized with the AlkPhos-labeled \textit{ccpA} probe and hybridized probe was then detected by CDPstar chemiluminescence (Amersham Bioscience).

\textbf{Construction of the CcpA complementing plasmid pIH100.} A 1539-bp fragment containing the \textit{ccpA} ORF and its 450-bp upstream region was amplified by PCR using primers CPP265 and CPP266 (Table 2) and then cloned into pCR®-TOPO-XL® (Invitrogen) to generate pCcpA-comp-XL. Next, the ~1.5 kb \textit{KpnI/XbaI} fragment was cloned into the \textit{KpnI/XbaI} sites of shuttle vector pJIR750 to generate the \textit{ccpA} complementing plasmid pIH100.

\textbf{Results}

\textbf{Glucose represses the gliding motility of wild \textit{C. perfringens} strains isolated from human and animal infections.} A recent study demonstrated type IV pili (TFP)-dependent gliding motility in three human-derived \textit{C. perfringens} strains for which a genome sequence have been previously determined (41). TFP-dependent
gliding motility constitutes an important bacterial behavior also known as twitching

(14, 15, 24). Taking into account that the referred study (41) was limited to the
analysis of only three strains, all of human origin, when there exits a great diversity of

C. perfringens isolates causing diverse infections (i.e. gas gangrene, food poisoning,
antibiotic associated diarrhea, etc.) in human beings and animals, we considered of
interest to evaluate whether gliding motility was an intrinsic and general property of
wild and undomesticated C. perfringens isolates. To this end, we performed a gliding
motility analysis on a collection of seventeen different pathogenic human and
animal-derived C. perfringens strains (including those strains whose gliding behavior
was previously reported) (Table 1). In our study, motility is defined as the ability of
cells to spread away from the edge of inoculation point by at least 0.4 cm in a curved
flare pattern after 72 h at 37º C. When culture aliquots (see Material and Methods for
details) of strain 13, SM101 and NCTC8239 were spotted onto BHIA or TYA plates,
the pattern of colony translocation was similar to the gliding motility previously
observed, (41), showing a distinctive curved flare pattern (Fig. 1A). Interestingly,
both on TGYA and on BHIGA plates (which contained 2% glucose), the spotted
cultures of strain 13, SM101 and NCTC8239 remained in the inoculation point
indicating that gliding motility was inhibited. In addition, we noted that the degree of
motility was more evident on TYA rather than on the BHIA that was used in the
previous study (41) (Fig. 1A and data not shown). Therefore, all subsequent motility experiments were conducted using TYA plates.

When the gliding motility assay was performed on the entire collection of *C. perfringens* isolates (Table 1), all the strains exhibited full proficiency in social gliding motility on TYA plates. Gliding motility was, in contrast, completely blocked in the presence of glucose (Fig. 1B and data not shown). Therefore, and taking into consideration that glucose is a known inhibitor of other bacterial social behaviors such as sporulation (28, 30) and biofilm formation (39), we concluded that the glucose added to the BHI and TY agar plates, yielding BHIG and TGYA respectively, was responsible for the repressive effect on gliding motility of all the surveyed *C. perfringens* isolates.

To determine how gliding motility was affected by different glucose levels, a glucose gradient was generated on a TYA plate (see Material and Methods). Five microliters (5 µl) of a concentrated middle-log phase culture of the food poisoning strain SM101 were inoculated at various positions distributed along the TYA glucose-concentration testing plate (see Material and Methods). As observed in Fig. 2A, the extent of gliding motility exhibited by the pathogenic food poisoning SM101 strain was inversely proportional to the glucose concentration; as the concentration of glucose decreases, the extent of gliding motility increases.
To determine the minimum concentration of glucose required to inhibit gliding motility, five different concentrations of the sugar were independently assayed: 0.1%, 0.25%, 0.5%, 1% and 2%. As shown in Fig. 2B, at a glucose concentration of 1%, no gliding motility was observed in the gas gangrene producer strain 13 or in the food poisoning isolate NCTC8239. In contrast, these two strains were able to glide slightly on TYA plates supplemented with 0.5 % glucose. No inhibition of gliding motility was observed when these strains were spotted on TYA plates supplemented with 0.25% and 0.1% glucose (Fig. 2B and data not shown). Therefore, glucose repression of social gliding motility in *C. perfringens* was concentration-dependent and apparently triggered at a glucose concentration of 0.5%.

**Carbon catabolite repression of *C. perfringens* gliding motility.** In order to determine if the observed inhibitory effect of glucose on gliding motility was a general phenomenon of carbon catabolite regulation (repression), other readily metabolized carbohydrates such as galactose, fructose, lactose and sucrose were tested. The motility of the strains NCTC8239 (food poisoning) and 13 (gas gangrene) were inhibited when growing on plates containing 2% of either fructose, galactose, lactose, or sucrose (Fig. 3). Next, we analyzed the effect on gliding of complex carbohydrates (i.e. raffinose and starch) which are slowly metabolized but required for the efficient sporulation of *C. perfringens* and enterotoxin (CPE) production (17, 18). When 2%
raffinose was added to TYA plates no inhibition of gliding motility was observed (Fig.3). With 2% starch, the extent of motility in both strains (13 and NCTC8239) was suppressed to a minor degree, although gliding was not affected in the presence of 0.4% starch (Fig. 3). When similar experiments were performed on the rest of the *C. perfringens* isolates listed in this work (Table 1), no gliding motility was observed by all tested strains grown on TYA plates supplemented with 2% of each fructose, galactose, lactose or sucrose, while gliding motility was observed with 2% raffinose supplementation. However, on TYA plates supplemented with 2% starch, all tested strains were non-motile on the agar surface, with the exception of the strains NCTC10239 (food poisoning), which exhibited partial gliding motility, and JGS1807 (diarrheic pig) which was highly motile even in the presence of 2% starch (data not shown). The overall results indicated that the complex carbohydrates raffinose and starch did not affect gliding motility at the concentrations routinely used (2% and 0.4% respectively) for *C. perfringens* spore formation and CPE production (17, 18, 28). More importantly, we demonstrate for the first time that *C. perfringens* gliding motility is subject to carbon catabolite repression.

**Kinetics of type IV pili-dependent gliding motility in *C. perfringens***. Proficiency in surface-associated motility is an important feature of many human and animal pathogens (2, 9, 14, 21, 24). In the case of virulent and invasive *C. perfringens*
isolates, an efficient mechanism of translocation on solid or semi-solid surfaces could be favorable for escaping from host defenses and for the rapid dissemination of the infection (i.e. an aggressive gangrene can progress at a rate of 1 cmh⁻¹). Therefore, we consider it of interest to characterize the kinetics and efficiency of gliding motility of strain 13 (gas gangrene producer) on semi-solid TYA plates in the absence and presence of added-glucose. As observed in Fig. 4, it is possible to visualize two clear phases of colony growth and expansion. Initially, the growing colony expanded on the TYA surface from the inoculation point at a rate comparable with the speed of colony expansion expected from non-swimming bacteria. During the first 20 h of incubation, the size of the growing colony was evenly increased by a factor of 1.75 as consequence of cell division, reaching a speed of colony spreading of approximately 125µmh⁻¹, (Fig. 4 and data not shown). After this initial phase of even colony spreading, a striking change occurred: social gliding was initiated by groups of cells located at the edge of the colony (see marked burgeoning cells in Fig. 4 at a developmental time of 20 h). After the appearance of these first signs of active surface gliding motility (the emergence of up and coming cells from the budding sites), a robust and uniform gliding was strongly induced, reaching a maximal speed of 600-700 µmh⁻¹ (Fig. 4), a value for the gliding speed comparable with the average
values (200µmh⁻¹ to 1,000 µmh⁻¹) of social gliding and twitching speed previously reported for other microorganisms (9, 14, 24).

Interestingly, in the presence of 2% glucose, gliding motility was not generated even after long incubation times (Fig. 4 and data not shown). The morphology of the C. perfringens colony developed on TYA plates supplemented with glucose (gliding deficient phenotype) was quite similar to the colony morphology developed on TYA plates without glucose supplementation just before the onset of active gliding; it was also very similar to the central part of a mature colony that has glided for more than 40 h on TYA plates (Fig. 4 and data not shown). These observations indicate that glucose and other sugars (data not shown) did not affect the initial phase of vegetative colony spreading. However, they do show that the sugar completed blocked (carbon catabolite repression) the onset of the second phase of surface translocation that relayed on the development of active gliding.

Effect of glucose on pilT and pilD expression of gas gangrene and food poisoning-producing C. perfringens strains. The recent study of Varga et al. demonstrated that the gliding of C. perfringens strain 13 depended on the products of pilT and pilC, which are required for TFP assembly (41). The pilT mutant of strain 13 does not spread out from the inoculation spot as the wild type does, and no pili were detected using Field Emission-Scanning Electron Microscopy (FE-SEM) (41). Since
glucose and other readily metabolized carbohydrates completely suppressed gliding motility in all the surveyed *C. perfringens* strains (Table 1) that we analyzed (Figs. 1-4 and data not shown), we considered the scenario where the transcription of *pil* genes might be affected by carbon catabolite repression. Therefore, to test this hypothesis, we measured the expression of two other genes required for TFP-dependent motility, *pilD* and *pilT*, in TY medium with and without glucose supplementation (Fig. 5). The β-glucuronidase *pilD-gusA* and *pilT-gusA* reporter fusions were introduced separately into strain 13 and SM101 by DNA electroporation and *pil*-driven β-glucuronidase activity was assayed (see Material and Methods for details). There were no significant differences in the growth of strains carrying the *pil-gusA* fusions in medium with or without added-glucose (data not shown), although the final cellular yield was slightly higher in TY media supplemented with glucose than in non-supplemented TY media (data not shown). Interestingly, in the presence of 1% glucose, there was a dramatic downregulation of *pilD* and *pilT* promoter activities in both *C. perfringens* strains (Fig. 5). The expression of *pilT* was reduced by 60% in the gas gangrene producer strain 13 and by 75% in the food poisoning isolate SM101 when grown in the presence of 1% glucose (Fig.5A). Similarly, a significant reduction in *pilD-gusA* expression was observed in cultures of strain 13 (≈80% reduction) and SM101 (≈75%) when grown in the presence of 1% glucose.
compared with their expression levels in the absence of glucose (Fig. 5B). These results demonstrated that glucose strongly down-regulates the expression of the pilT and pilD genes. They also suggest that the inhibitory effect of carbon catabolite regulation on gliding motility took place, at least partially, at the level of TFP expression.

**Dual role of the carbon catabolite protein CcpA in C. perfringens gliding motility.**

It is known that the catabolite control protein A (CcpA) plays a key role in low G+C Gram-positive bacteria, interconnecting carbon metabolism with several cellular responses such as virulence, spore formation and biofilm development (39, 43). In *C. perfringens*, CcpA is required for efficient sporulation and expression of the enterotoxin CPE that is responsible for the symptoms of food poisoning and diarrhea in humans and animals (42). Due to the observed repressive effect of glucose on the expression of pil genes (Fig. 5) that are essential for the ability of *C. perfringens* to glide on a solid substrate (41), we were tempted to analyze whether CcpA has a role on the carbon catabolite repression of *C. perfringens* gliding motility. To explore this possibility, we constructed a *C. perfringens* ccpA− mutant strain (see Material and Methods and Supplemental Fig. 1) to assay the gliding phenotype and the activity of TFP genes (pilT and pilD) of isogenic ccpA+ and ccpA− *C. perfringens* strains grown in the presence or absence of added-glucose. When gliding proficiency was evaluated
in the presence of 1% and 2% glucose, the CcpA-proficient strain 13 was, as expected, unable to move at both sugar concentrations, remaining situated at the point of inoculation (round and smooth colony morphology). In contrast, the isogenic ccpA− mutant derivate KO13, deficient in CcpA production, inoculated on the same plates was able to retain more than 50% of its gliding ability in the presence of 1% glucose and also showed a small, but perceptible and reproducible, gliding proficiency on TYA plates supplemented with 2% glucose (Fig. 6A). Under similar conditions, no gliding motility was observed in the presence of 1% glucose with the ccpA− mutant strain harboring the CcpA-complementing plasmid pIH100 (Table 1 and data not shown), suggesting that functional CcpA was required for carbon catabolite repression of C. perfringens social gliding motility.

In order to further confirm the role of CcpA on carbon catabolite repression of C. perfringens gliding motility, we compared the expression of pilT and pilD in isogenic CcpA-proficient and CcpA-deficient C. perfringens cultures grown in TY broth plus / minus 1% glucose. As shown in Fig. 6B the CcpA-deficient cultures, but not the cultures that express CcpA, showed active expression of pilT and pilD in the presence of glucose at almost the same level observed for wild type cultures grown in the absence of glucose. Collectively, these results demonstrate that CcpA was actively
involved in the carbon catabolite repression of *C. perfringens* gliding motility and the glucose-induced down-regulation of TFP biosynthetic genes.

An intriguing observation was that the gliding ability of the CcpA-deficient *C. perfringens* strain KO13 grown in the absence of sugar supplementation was delayed in comparison with the gliding proficiency of the wild type (*ccpA*+) strain 13 (see right panel, no added-glucose, in Fig. 6A). This observation could uncover an additional, and unexpected, carbon-independent positive requirement of CcpA for full proficiency of *C. perfringens* in gliding motility. It is worthy to indicate that the slight but reproducible catabolite-independent positive effect of CcpA on social behaviors has been previously documented during biofilm formation in *B. subtilis* (39) and spore development in *C. perfringens* (42). In these bacteria the absence of CcpA activity was reflected in a lower proficiency in biofilm development and spore formation comparing with the ability of the wild type (CcpA-proficient) strains (39, 42).

We confirmed our hypothesis by quantifying the gliding developed by the *ccpA*+ (strain 13) and its isogenic *ccpA*− mutant derivate on TYA plates in the absence of glucose supplementation. Previously, we determined that introduction of the *ccpA* mutation in strain 13 did not affect its growing ability and the final cellular yield after growth in liquid TY medium (Fig. 7A). In contrast to what occurred in broth, where
the growth phenotypes of the CcpA-proficient and CcpA-deficient cultures were the same, we observed clear differences in the speed and kinetics of gliding between the \( \text{ccpA}^+ \) and \( \text{ccpA}^- \) strains. The gliding proficiency of the \( \text{ccpA}^- \) mutant strain showed a reduced speed compared with the velocity of gliding of the wild type strain- 250 \( \mu \text{m h}^{-1} \) and 670 \( \mu \text{m h}^{-1} \)- for the CcpA-deficient and CcpA-proficient strains, respectively (Fig. 7B). Also, gliding of the \( \text{ccpA} \) mutant strain stopped before the gliding of the wild type strain did (Figs. 6A-right panel and 7B). These observations strongly suggest a novel and overlooked positive role of CcpA, in the absence of sugar supplementation, on the proficiency of gliding motility of \( C. \text{perfringens} \). To confirm this conclusion we measured the expression of \( \text{pilD} \) and \( \text{pilT} \) (whose products are essential for TFP-dependent gliding motility) in wild type and CcpA-deficient \( C. \text{perfringens} \) cultures grown in TY broth without sugar supplementation. As observed in Fig. 7C, there is an unambiguous downregulation of \( \text{pilT} \) and \( \text{pilD} \) expression in the cultures deficient in CcpA production. This positive role of CcpA on TFP expression (Fig. 7C) and social gliding proficiency (Figs. 6A and 7B) in the absence of sugar supplementation plus its opposite (negative) effect on the same social behavior (gliding motility) under conditions of carbon catabolite regulation (presence of sugar, Fig. 6) suggest a novel, dual (activating and repressing) role of CcpA in regulating \( C. \text{perfringens} \) gliding motility (Fig. 8).
Discussion

Our current study shows several significant contributions towards the understanding of the physiology and regulation of type IV pili-dependent gliding motility in *C. perfringens*. First, we extended the analysis to a total of seventeen different *C. perfringens* strains isolated from diverse infections (diarrhea, food poisoning, myonecrosis) produced not only in human beings but also from animal origin (Table 1). Interestingly, all the analyzed strains exhibited an active proficiency in social-gliding motility on agar surface (Fig. 1 and data not shown). These results significantly consolidate and strengthen the idea that gliding motility is an intrinsic property of pathogenic *C. perfringens*, regardless of their origin of isolation (41).

Our understanding of the environmental and metabolic factors that control surface-associated translocation in pathogenic bacteria is very limited. Precisely, the main contribution of our work is the demonstration that carbon catabolite repression (20, 39, 43, 50) regulates social gliding motility in *C. perfringens*. In fact, all the surveyed isolates exhibited social gliding motility on BHIA plates (with no glucose supplementation), but not on TGYA medium which contained 2% glucose, suggesting that glucose is capable of inhibiting social gliding motility. The removal of glucose from TGYA allowed the cells to exhibit social motility while the addition of glucose
in BHIA resulted in the inhibition of gliding motility, confirming that glucose plays a crucial role in inhibiting gliding motility (Figure 1 and data not shown).

In addition to glucose, gliding motility was also inhibited by other rapidly-metabolized sugars such as fructose, lactose, sucrose and galactose (Fig. 3). This finding confirmed that the repression of gliding in *C. perfringens* was due to a general process of carbon catabolite repression (43). Interestingly, two complex carbohydrates, raffinose and starch, behaved differently from the single sugars: raffinose did not inhibit motility at any of the assayed concentrations and starch inhibited gliding only at concentrations higher than 2% (Fig. 3 and data not shown). These results are consistent with previously reported findings that other social behaviors present in *C. perfringens* such as sporulation and enterotoxin (CPE) production were also repressed by rapidly metabolized single sugars such as glucose and lactose (28, 42), while complex carbohydrates, raffinose and starch, were found to induce both events (17, 18). The correlation between carbon catabolite repression of sporulation and surface-associated motility suggests that the two social processes might share, at least in part, a common regulatory network (Fig. 9).

We demonstrate that carbon catabolite repression of gliding motility in *C. perfringens* occurs through the repression of at least two genes involved in TFP production and functionality, namely, *pilD* and *pilT*. As observed in Fig. 5, the
addition of 1% glucose to growing cultures of the reference gliding-proficient strains 13 and SM101 resulted in a dramatic decrease in pilD-gusA and pilT-gusA expression. The maximum reduction in transcription due to the added-glucose occurred approximately after 24 h of growth, which is consistent with the observation that gliding motility on agar plates begins only after 18-20 h of growth (Fig. 4 and data not shown).

In low G+C Gram-positive bacteria, carbon catabolite regulation is under the control of the key transcription factor CcpA (carbon catabolite protein A) a member of the LacI-GalR family of transcriptional regulators (43). In the better known cases of CcpA-mediated carbon catabolite regulation (i.e. in *Bacillus subtilis* and *Lactococcus lactis*), a complex and sophisticated signaling network is present (20, 29, 50). Basically, the CcpA-dependent regulatory network utilizes sugar transporters, glycolytic enzymes, an ATP-dependent, metabolite-activated protein kinase (HprK) and two small HprK-target proteins: the phosphotransfer protein Hpr of the phosphotransferase system (PTS) and the Hpr-homologue Crh (35, 43). Moreover, a central role has been reserved for CcpA, which binds to DNA sequences (CRE sites) present on the regulatory region of its target genes (20, 29, 35, 43). For the activation of CcpA binding to the CRE elements, it is necessary, although not essential (20, 29, 35), for CcpA to bind to the phosphorylated forms of Hpr and/or Crh produced by
HprK (35, 43). In *C. perfringens*, orthologs of *ccpA*, *hpr* and *hprK* (but not for *crH*) are present on the chromosomes of all the sequenced strains, suggesting that the basic elements for CcpA-mediated carbon catabolite regulation are present in this pathogen (43 and data not shown). In fact, we demonstrated that the repression of *C. perfringens* gliding motility by glucose was mediated, in large part, by the action of CcpA. As observed in Fig. 6, the inactivation of *ccpA* significantly restored gliding proficiency (Fig. 6A) and *pil* expression (Fig. 6B) in the presence of glucose. The reversion to the gliding-deficient phenotype of the *ccpA* mutant strain in the presence of glucose was obtained after the introduction (by DNA electroporation) of the plasmid pIH100 that harbored a wild type copy *ccpA*, which provided direct genetic evidence supporting the strong linkage between CcpA expression and carbon catabolite repression of gliding motility in *C. perfringens*. These results suggest that CcpA could act as a transcriptional regulator of TFP biosynthesis genes. However, this effect might be indirect since no putative *cre* sites have been identified in any of the TFP biosynthesis genes analyzed so far (data not shown). It might be possible that other *cre*-like consensus sequences, different that the ones reported for *Bacillus* and other low G + C Gram-positive bacteria, exist in Clostridia (20, 42, 43). Another possibility is that, apart from CcpA, an unidentified intermediate factor might be involved in regulating TFP gene expression. This suggestion received support from
the observation that the *ccpA* mutant strain was not able to restore, in the presence of sugar supplementation, a full gliding proficiency and *pil* expression as the levels reached that of the wild type strain in the absence of added-sugars (Fig. 6 and data not shown).

A final and unexpected finding of our study was the observation that, in the absence of added-sugar, CcpA has a positive role on gliding motility. As observed in Fig. 6, in the absence of added-glucose, the *ccpA* mutant strain glided on the agar plate to a lesser extent than the isogenic wild type strain. As observed in Fig. 7B, the wild type strain (CcpA-proficient) reached a maximum speed of gliding of 630-670 µm h\(^{-1}\), while its isogenic *ccpA* derivate (CcpA-deficient) only reached a maximal speed of gliding of 220-250 µm h\(^{-1}\). Two observations argue strongly for a positive role of CcpA on gliding development: firstly, the *ccpA*\(^{-}\) mutant strain did not show any growth defect on liquid medium, reaching essentially the same final O.D. and viable cell number than the wild type strain (Fig. 7A); furthermore, the initial phase of growth colony (before the onset of gliding) was very similar between both *ccpA*\(^{+}\) and *ccpA*\(^{-}\) strains (Fig. 7B and data not shown). This hypothesis was reinforced by the demonstration that CcpA production was required for an efficient expression of *pilT* and *pilD* in growth media without sugar supplementation (Fig. 7C). These findings indicate that CcpA has a dual role in controlling gliding motility in *C. perfringens*.
(Fig. 8). In the presence of rapidly metabolized sugars (i.e. glucose), CcpA has a negative role on the onset of gliding, an effect that is partly mediated through repression of pilT and pilD expression (Fig. 6). In the absence of added sugars, CcpA switches to a positive role on gliding, a novel property that is uncovered by the deficient gliding phenotype and poor pilT and pilD expression of CcpA-deficient cells cultured in the absence of added glucose (Fig. 7). In agreement with our finding, a similar positive role of CcpA under conditions of non-catabolite regulation (absence of added-sugars) on spore formation and cpe expression has been reported for C. perfringens (42).

Excess glucose in the environment of C. perfringens not only affects stationary phase phenomena, such as sporulation-linked CPE production (25, 28, 42) and gliding motility (this study), but can also act as a catabolic repressor of collagenase production during vegetative growth (42). Moreover, in the other intestinal pathogenic Clostridium bacterium C. difficile, glucose represses toxin production (11). Importantly, within the context of the development of a clostridial infection, it is plausible to envision that proficiency in gliding associated with toxin production and tissue damage would contribute to the progression of the infectious process (Fig. 9). Luminal glucose concentrations in the small intestine of mammals are in the range of 0.006 % to 0.4 % (12). Interestingly, in our study the catabolite repression of gliding
motility by glucose was concentration-dependent; surface motility was only observed when the glucose concentration was less than 0.5% (Fig. 2). This finding opens the possibility that gliding willingly would occur during the course of a clostridial infection (Fig. 9). We are just grasping the regulatory network of surface-associated motility in pathogenic clostridia, the understanding of how carbon catabolite repression inhibits known and potential virulence processes (sporulation, toxin production, gliding motility) in *C. perfringens* (6-8, 21, 26, 36) will contribute to prevent and combat clostridia diseases (Fig. 9).

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References


FIGURE LEGENDS

FIGURE 1: Glucose represses the gliding motility of *C. perfringens* strains isolated from different mammalian hosts.

(A) Gliding phenotype of the three genome-sequenced human pathogen *C. perfringens* strains 13, SM101 and NCTC8239. Gliding was developed after inoculation of a 5µl drop of a concentrate middle-log phase culture of the corresponding strain on BHI or TY agar media with or without 2% glucose supplementation. Top-bottom photographs were taken after 72 h of anaerobic incubation at 37°C. BHI, TY agar media without glucose; BHIG and TGY, media supplemented with 2% glucose. Black dotted circles show the diameter of the initial inoculation spot (see Material and Methods for details).

(B) Gliding phenotype of a collection of human and animal pathogenic *C. perfringens* strains: 1, NB16; 2, JGS1818; 3, 294442; 4, NCTC 10239; 5, 317206; 6, AHT327; 7, B11; 8, B41; 9, F5603; 10, F4969; and 11, AHT2911 (see Table 1 for origin and type of infection produced for each pathogenic strain).

FIGURE 2: Dose-dependent repressive effect of glucose on *C. perfringens* gliding proficiency.
(A) Glucose gradient effect on gliding motility of the enterotoxigenic cpe+ food poisoning C. perfringens strain SM101. One-third of a TYA plate was cut out and replaced with 7 ml of melted TGYA medium, which contained 2% glucose (see text and Material and Methods for details). After solidification of the added-TGYA a natural glucose gradient was generated (from left to the right in the figure) due to the diffusion of glucose molecules from the TGYA section (that contained 2% of added glucose) to the TYA portion (which contained 0% of added glucose) of the plate.

(B) Evaluation of the minimum glucose concentration required to inhibit gliding proficiency of the food poisoning and gas gangrene producer strains NCTC8239 and 13.

For (A) and (B) strains were grown on TYA plates as indicated in Fig. 1 and supplemented with glucose as shown in the figure. Top-bottom photographs were taken after 72 hours of anaerobic incubation at 37°C.

FIGURE 3: Effect of simple and complex carbohydrates on C. perfringens gliding motility.

Rapidly metabolized carbohydrates (Fru, fructose; Gal, galactose; Lac, lactose; Suc, sucrose) and complex carbohydrates (Raf, raffinose and starch) commonly used to enhance sporulation and CPE production in Spo0A-proficient C. perfringens strains
were assayed for their ability to affect gliding motility. Gliding phenotype of the gas gangrene producer and Spo0A-deficient strain 13 and the Spo0A-proficient and food poisoning strain NCTC8239 are shown. Top-bottom photographs were taken after 96 hours of anaerobic incubation at 37°C on TYA plates supplemented with the corresponding concentration of sugar as indicated.

FIGURE 4: Kinetics of gliding development of the gas gangrene producer *C. perfringens* strain 13 in the absence and presence of added glucose.

Five microliters (5 µl) of a concentrated middle-log phase culture of strain 13 grown in TY broth were inoculated in the center of 100 mm Petri dishes containing TYA medium with and without supplementation with 2% glucose. Inoculated Petri dishes were anaerobically incubated at 37°C and gliding proficiency was recorded at different times, measuring the distance traveled (in mm) from the initial inoculation point (black dotted circles) to the edge of the expanding colony (white dotted circles). Open and closed symbols represent the experiments performed in the absence and the presence of 2% added-glucose, respectively. White arrows indicate the onset (under the influx of unknown signals, see text for details) of gliding development. The onset of gliding was only observed in TYA plates without glucose supplementation while in
the presence of glucose gliding was never initiated. Photographs are representative of six independent experiments and plotted values are the average of those repetitions.

FIGURE 5: Glucose represses pilT and pilD transcription in gas gangrene and food poisoning C. perfringens strains.

Transcription of pilT (A) and pilD (B) promoters measured by β-glucuronidase assay of C. perfringens strain 13 and SM101 harboring reporter pilT-gusA (A) and pilD-gusA (B) transcriptional fusions. Strains were grown on TY broth with or without the addition of 1% glucose as indicated in the figure (+ and – respectively). Accumulated β-glucuronidase activity was measured after 30 h of growth. A representative result from three independent assays is shown. Gene arrangement of pilT (A) and pilD (B) chromosomal regions in C. perfringens strains 13 and SM101 are shown at the bottom of the figure with the indication of the repressive effect of glucose on gene transcription (see text for details).

FIGURE 6: CcpA mediates carbon catabolite repression of C. perfringens gliding motility.

(A) Gliding motility phenotype of the CcpA-proficient (ccpA+) C. perfringens strain 13 and its isogenic CcpA-deficient (ccpA−) derivate KO13 (see Material and Methods
for details). Motility assay was performed according to the protocol described in the above figure legends. Top-bottom photographs were taken after 40 h of anaerobic incubation on TYA plates supplemented or not supplemented with glucose as indicated in the figure. Black dotted circles indicate the initial size of the colony immediately after drop inoculation.

(B) Expression of pilT-gusA and pilD-gusA reporter fusions in ccpA⁺ and ccpA⁻ cultures of isogenic C. perfringens strains 13 and KO13 grown during 30 h on TY broth in the absence (-) or presence (+) of 1% glucose. β-glucuronidase activity was calculated as indicated in Fig. 5.

For (A) and (B) a representative set of results obtained from five independent experiments is shown.

FIGURE 7: CcpA has a novel catabolite-independent positive role on C. perfringens gliding motility.

(A) Growth curves of the ccpA⁺ C. perfringens strain 13 and its isogenic ccpA⁻ derivate KO13. Growth was monitored over time, measuring the O.D. at 600 nm of both cultures developed on TY broth at 37°C. A representative experiment from five independent repetitions is shown. Closed symbols (wt), open symbols (ccpA mutant)
(B) Kinetics of gliding motility of strain 13 (wt) and its isogenic CcpA-deficient KO13 derivate (ccpA⁻) developed on TYA plates without sugar supplementation. Gliding was recorded as indicated in Fig. 4. Average values of gliding obtained from five independent experiments are plotted in the figure.

(C) Requirement of CcpA activity for full expression of pilT and pilD genes of C. perfringens strain 13 grown on TY broth without glucose supplementation. β-glucuronidase activity driven from CcpA-proficient and CcpA-deficient C. perfringens cultures (strains 13 and KO13 respectively) harboring reporter pilT-gusA (left) and pilD-gusA (right) transcriptional fusions is shown. The four cultures were grown on TY broth without adding glucose; accumulated β-glucuronidase activity was measured at the times indicated in the figure. Closed and open symbols represent CcpA-proficient and CcpA-deficient isogenic cultures, respectively. A representative experiment from five independent repetitions is shown.

FIGURE 8: A dual (positive and negative) role of CcpA on C. perfringens gliding development.

The present cartoon depicts one hypothetical model that might explain our actual knowledge of the gliding phenotype of CcpA-proficient and CcpA-deficient C. perfringens strains grown in the presence and absence of sugar supplementation. In
the absence of glucose (left part of the figure) or other catabolite-repressing sugars, CcpA by itself might be able to bind to the positive regulatory regions of genes (\textit{pil}) involved in Type IV-pili expression (i.e. \textit{pilT} and \textit{pilD}), producing a positive effect on transcription and hence stimulating gliding proficiency. Supporting this view, it has also been reported that \textit{in vitro} and \textit{in vivo}, CcpA-DNA mediated interactions do occur in the absence of added sugars (20, 29, 35). In the presence of catabolite repressing amounts of glucose (right part of the figure) the phosphotransferase enzyme of the PTS sugar system Hpr-Ser would be phosphorylated by HprK (35). Hpr-Ser-Pi would bind to CcpA and the newly formed Hpr-Ser-Pi::CcpA complex would interact with repressor sites located on the regulatory regions of \textit{pil} (\textit{pilT} and \textit{pilD}) and therefore interfering with gliding proficiency. Also shown in the picture is the possibility that the co-effectors fructose 1,6-bisphosphate (FBP) and glucose 6-phosphate (G6P) would function as adjunct co-repressors to enhance and to fine-tune the response of CcpA to the metabolic needs of the cell (35, 43). Other possibility (indirect effect of CcpA) that is not illustrated in this model is that CcpA would dually regulate an unidentified factor responsible to switch \textit{on} and \textit{off} expression of \textit{pill} genes.
FIGURE 9: A workable model linking carbon catabolite regulation of social behaviors (gliding, sporulation, and toxin production) with disease progression in *C. perfringens*.

In this hypothetical, but realistic, scenario toxigenic *C. perfringens* vegetative cells that reach the lumen of a human or animal gastrointestinal (GI) tract, where the basal luminal concentrations of glucose are normally lower than 0.5 % (12), have the possibility to undergo at least two different differentiation pathways: sporulation and/or gliding development. In the first case, the activation of the key transcription factor Spo0A by inorganic phosphate (Pi) present in the intestinal lumen triggers spore morphogenesis and enterotoxin (CPE) production (28). In the case of gliding development (left part of the figure), unknown signals that might be linked to cell-cell and cell-surface interactions (indicated by double arrows) orchestrate the spatial and temporal organization of the cells to the onset of gliding. The progression of any of both developmental programs (sporulation or gliding) would not exclude the occurrence of the other alternative pathway: sporulation and CPE production would take place in the lumen of the GI tract while gliding motility and vegetative toxin synthesis (i.e. collagenase production) would take place in association with the intestinal mucosa. It is indicated, in the figure, the key role of glucose (representing the occurrence of CcpA-mediated carbon catabolite regulation when the level of the
sugar is at least 1%) as a repressor of sporulation (25, 28) and gliding (this study) development. This regulatory blockage derives in the inhibition of enterotoxin (25, 28) and vegetative-linked toxin production (11, 42) in Clostridium. It is also shown the novel role of CcpA as activator of sporulation (42) and gliding proficiency (this study). The development of inhibitors (i.e. monosaccharide-analogs) that block the onset of gliding and/or sporulation; or antagonists that interfere with the positive role of CcpA on toxin production would contribute to combat the outbreak and dissemination of clostridia diseases.
Table 1. *C. perfringens* strains and plasmids used in this study

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<td>M13-F</td>
<td>5' GTA AAA CGA CGG CCA GT 3'</td>
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<td>PUC18 vector</td>
<td>PCR</td>
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<tr>
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<td>PUC18 vector</td>
<td>PCR</td>
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</tbody>
</table>

<sup>a</sup> Restriction sites that have been added are underlined.

<sup>b</sup>The nucleotide position numbering begins from the first codon and refers to the relevant position within the respective gene sequence (36).

<sup>c</sup>GUS, construction of plasmid for β-glucuronidase assay; MP, construction of mutator plasmid; PROBE, construction of DNA probe for southern blot analysis; COMP, construction of complementing plasmid; PCR, Polymerase chain reaction.
A

Strain SM101

Motility

Glu gradient

B

Without Glu

0.25% Glu

0.5% Glu

1% Glu

2% Glu

Strain13 NCTC8239
A. pilT-gusA

B. pilD-gusA

β-glucuronidase activity

Glucose

Strain 13  SM 101

- + - +

- + - +

pilT  sigK

pilM  pilA2  pilC  pilD  pilA1

Glucose
A

Strain 13

<table>
<thead>
<tr>
<th>Genotype</th>
<th>2% glu</th>
<th>1% glu</th>
<th>0% glu</th>
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</thead>
<tbody>
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<td>ccpA+</td>
<td></td>
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<td></td>
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<tr>
<td>ccpA-</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>ccpA+</td>
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<tr>
<td>ccpA-</td>
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B

- pilT-gusA
- pilD-gusA

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<th>ccpA+</th>
<th>ccpA+</th>
<th>ccpA-</th>
<th>ccpA+</th>
<th>ccpA+</th>
<th>ccpA-</th>
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<td>Glucose +</td>
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</table>

\[
\text{\( \beta \)-glucuronidase activity}
\]

\[\text{ACCEPTED on September 23, 2017 by guest}
\]

http://jb.asm.org/
CcpA

- glu

+ glu

HPr-Ser

HprK

HPr-Ser-P

(FBP)

(GP)

Gliding development

 ppl

ACCEPTED on September 23, 2017 by guest

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