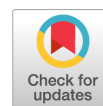


# REGULATION OF *CLOSTRIDIODES DIFFICILE* CRISPR-Cas SYSTEM BY BIOFILM-ASSOCIATED FACTORS AND GLUCOSE



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**Abstract.** *Clostridioides difficile* is a spore-forming enteropathogenic anaerobic bacterium and one of the most common opportunistic human pathogens. Its pathogenicity relies on the production of toxins, sporulation, biofilm formation, and the ability to withstand numerous stresses encountered in the host environment. In addition to these well-known mechanisms, *C. difficile* possesses a remarkably complex CRISPR-Cas system characterized by two *cas* operons and multiple CRISPR arrays. While CRISPR-Cas systems are primarily studied as adaptive immune mechanisms against bacteriophages and mobile genetic elements, accumulating evidence suggests they may also be integrated into broader regulatory networks that contribute to bacterial physiology, adaptation, and virulence. However, the regulation and functional dynamics of this system in *C. difficile* remain largely unexplored. In this study, we investigated the regulation of the *C. difficile* CRISPR-Cas system under biofilm-inducing factors. Quantitative PCR analysis revealed the induction of several CRISPR arrays and the partial *cas* operon under high intracellular levels of the secondary messenger cyclic di-guanosine monophosphate, a key regulator of bacterial phenotypic shifts. These results were partially confirmed by interference efficiency assays. A secondary bile salt, sodium deoxycholate, known to trigger biofilm formation, also increased both *cas* operons and one CRISPR array expression, suggesting its role for CRISPR-Cas system regulation during host-associated stress. Moreover we identified glucose as a regulatory factor for *C. difficile* CRISPR-Cas system. Elevated glucose concentration in the medium induced the expression of the partial *cas* operon and CRISPR 3–4 arrays. However, at the same time it functionally suppressed the interference efficiency of the system. Together, our findings demonstrate that the *C. difficile* CRISPR-Cas system is responsive to biofilm-inducing signals and nutrient availability, linking its regulation to key aspects of bacterial physiology and adaptation to the host. This work also highlights the potential for non-canonical regulatory roles of CRISPR-Cas in *C. difficile* survival and pathogenesis.

**Key words:** CRISPR-Cas, CRISPR-Cas regulation, *Clostridioides difficile*, biofilms, cyclic di-guanosine monophosphate, sodium deoxycholate, glucose.

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## РЕГУЛЯЦИЯ СИСТЕМЫ CRISPR-Cas ПАТОГЕННОЙ БАКТЕРИИ *CLOSTRIDIODES DIFFICILE* ФАКТОРАМИ, АССОЦИИРОВАННЫМИ С БИОПЛЕНКОЙ, И ГЛЮКОЗОЙ

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**Резюме.** *Clostridioides difficile* — анаэробная спорообразующая энтеропатогенная бактерия, являющаяся одним из наиболее распространенных оппортунистических патогенов человека. Ее патогенность обусловлена продукцией токсинов, споруляцией, формированием биопленок и способностью противостоять множественным стрессовым воздействиям внутри организма-хозяина. Помимо этих хорошо известных механизмов, *C. difficile* обладает довольно сложной системой CRISPR-Cas, включающей в себя два *cas*-оперона и множество CRISPR-кассет. Хотя системы CRISPR-Cas преимущественно исследуются с точки зрения их функций адаптивных иммунных механизмов против бактериофагов и других мобильных генетических элементов, накапливающиеся данные свидетельствуют о том, что эти системы также могут быть включены в другие внутриклеточные процессы, участвующие в регуляции физиологии, адаптации и вирулентности бактерий. Однако регуляция и функциональная динамика системы CRISPR-Cas у *C. difficile* остаются в значительной степени неизученными. В настоящем исследовании была изучена регуляция системы CRISPR-Cas у *C. difficile* под действием факторов, индуцирующих образование биопленок. При помощи метода количественной ПЦП была выявлена индукция нескольких CRISPR-кассет и неполного *cas*-оперона при высоких внутриклеточных уровнях циклического ди-гуанозинмонофосфата, вторичного мессенджера и ключевого регулятора перехода к неподвижному фенотипу у бактерий. Данные результаты были частично подтверждены экспериментами по эффективности интерференции. Другой фактор образования биопленок, вторичная желчная соль дезоксихолат натрия, также увеличивала экспрессию двух *cas*-оперонов и одной CRISPR-кассеты, что свидетельствует об ее возможной роли в регуляции данной системы CRISPR-Cas при стрессах, связанных с обитанием внутри организма-хозяина. Кроме того, в данной работе мы выявили, что глюкоза обладает регуляторным эффектом на систему CRISPR-Cas у *C. difficile*. Повышенная концентрация глюкозы в питательной среде индуцировала экспрессию неполного *cas*-оперона и CRISPR 3–4 кассет, но при этом подавляла функциональность данной системы на уровне эффективности интерференции. Таким образом, полученные результаты демонстрируют, что система CRISPR-Cas энтеропатогенной бактерии *C. difficile* реагирует на сигналы, индуцирующие образование биопленок, и на доступность питательных веществ на уровне экспрессии ее составных компонентов и на функциональном уровне. Полученные данные показывают возможную связь между регуляцией системы CRISPR-Cas и ключевыми аспектами физиологии этой бактерии, ее адаптацией к организму-хозяину. Настоящая работа также выявила потенциальное наличие неклассических функций у CRISPR-Cas системы *C. difficile*, которые могут играть важную роль в выживании и патогенезе *C. difficile*.

**Ключевые слова:** CRISPR-Cas, регуляция CRISPR-Cas, *Clostridioides difficile*, биопленки, циклический ди-гуанозинмонофосфат, дезоксихолат натрия, глюкоза.

## Introduction

*Clostridioides difficile* (syn. *Clostridium difficile*) [26] is an anaerobic, Gram-positive, spore-forming bacterium, and one is of the major clostridial pathogens. *C. difficile* causes nosocomial gut infections associated with antibiotic therapy [2]. The disturbance of the microflora by antibiotic therapy leads to the colonization of the intestinal tract by *C. difficile* cells, resulting in infection. During the infection cycle, this enteropathogen produces main virulence factors (toxins TcdA and TcdB), which cause changes in epithelial cells actin cytoskeleton inside the intestine. This leads to diarrhea and pseudomembranous colitis, a potentially lethal disease [8, 16]. Furthermore, *C. difficile* produces spores within the host, which subsequently can be released into the environment and contribute to disease spread. During its infection cycle, *C. difficile* metabolically adapts to changing environments and different stresses [2] and forms biofilms [9]. *C. difficile* vegetative cells also interact with bacteriophages

inside the host gut [25, 37]. Since the last two decades, the number of severe infection forms has been rising due to the emergence of the hypervirulent and antibiotic-resistant strains [4, 12, 30]. Many aspects of *C. difficile* pathogenesis, including molecular mechanisms of its adaptation to changing conditions inside the host, remain poorly understood.

During their life cycles, prokaryotes must cope with a large number of genetic parasites, including bacteriophages. For this purpose, prokaryotes often use various protective mechanisms. Over the past decade, CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas (CRISPR-associated) adaptive immunity systems have become a center of interest among various anti-invader bacterial defense systems [34]. These defensive systems are made up of CRISPR arrays and *cas* gene operons. CRISPR arrays, in their turn, consist of short, direct repeat sequences (20–40 bp) separated by variable spacers. Spacers are often complementary to phages and other mobile genetic elements [33]. The ac-

tion of CRISPR-Cas systems can be divided into two main processes: the acquisition of new spacers (CRISPR-adaptation), and CRISPR-interference, when the CRISPR-Cas system compounds are expressed and subsequently form an effector complex consisting of Cas proteins and CRISPR RNAs (cr-RNAs). This effector complex recognizes the target on the foreign DNA (or RNA, depending on the system type), resulting in degradation of the nucleic acid of the genetic invader [22]. To date, most CRISPR-Cas systems studies were focused on their functionality and the practical usage of these systems in genome editing. However, research on CRISPR-Cas system regulation and its role in bacterial physiology has been progressed significantly in recent years [39]. *C. difficile* has an original CRISPR-Cas system, which is characterized by an unusually large set of CRISPR arrays (12 arrays in the laboratory 630 strain and 9 in the hypervirulent R20291 strain), two type I-B *cas* operons, and the link with toxin-antitoxin type I systems [7, 20]. This bacterium should coordinate the functioning of this complex system in response to various signals of changes in the environment and in the physiology of the cell. To date, the regulatory factors of the *C. difficile* CRISPR-Cas system are still poorly understood.

In the present work we describe the regulation of expression and functioning of the *C. difficile*

CRISPR-Cas system by biofilm-inducing factors and at increased glucose concentration in the nutrient medium.

## Materials and methods

**Bacterial strains and growth conditions.** All bacterial strains used in this study are listed in Table 1. *C. difficile* strains were grown in brain heart infusion (BHI) (BD Biosciences) medium at 37°C under anaerobic conditions (5% H<sub>2</sub>, 5% CO<sub>2</sub>, and 90% N<sub>2</sub>), within Bactron 300 anaerobic chamber (Sheldon Manufacturing). *E. coli* strains were grown in LB medium [5], supplemented with ampicillin (Amp) (100 µg/ml) and chloramphenicol (Cm) (15 µg/ml) when it was necessary. The non-antibiotic analog anhydrotetracycline (ATc) was used for induction of the P<sub>tet</sub> promoter in *C. difficile* CD3 strain.

**RNA extraction and qRT-PCR.** To analyze CRISPR-Cas system expression in high cyclic di-guanosine monophosphate (c-di-GMP) level conditions, total RNA was isolated from *C. difficile* 630Δ*erm* [13] and CD3 strains, grown for 5 and 24 hours in TY medium (tryptone 30 g/L, yeast extract 20 g/L, pH 7.4), supplemented with ATc (250 ng/ml). For the experiments with deoxycholate and glucose, RNA samples were obtained from *C. difficile* 630Δ*erm* cultures grown for 5 and 24 h in BHI

**Table 1. Strains and plasmids used in this study**

Strain	Genotype	Source
<i>E. coli</i>		
HB101 (RP4)	<i>supE44 aa14 galK2 lacY1 Δ(gpt-proA) 62 rpsL20 (StrR)xyl-5 mtl-1 recA13 Δ(mcrC-mrr) hsdSB (rB-mB-) RP4 (Tra+ IncP ApR KmR TcR)</i>	Laboratory stock
<i>C. difficile</i>		
630Δ <i>erm</i>	Sequenced reference strain Δ <i>ermB</i>	Laboratory stock, [13]
CD3	630Δ <i>erm</i> P <sub>tet</sub> - <i>dccA</i> (CD1420)	Laboratory stock
Plasmid	Description	Reference
pRPF185Δ <i>gus</i>	P <sub>tet</sub> - <i>gusA</i> Tm <sup>R</sup> expression and cloning <i>Clostridium-Escherichia coli</i> shuttle vector, pRPF185 vector derivative	[11, 36]
pDIA6435	pRPF185Δ <i>gus</i> with the 5' CCA-PAM protospacer, corresponding to the spacer1 from 630Δ <i>erm</i> CRISPR 3 array	[19]
pDIA6436	pRPF185Δ <i>gus</i> with the 5' CCA-PAM protospacer, corresponding to the spacer1 from 630Δ <i>erm</i> CRISPR 4 array	[19]
pDIA6437	pRPF185Δ <i>gus</i> with the 5' CCA-PAM protospacer, corresponding to the spacer1 from 630Δ <i>erm</i> CRISPR 6 array	[19]
pDIA6438	pRPF185Δ <i>gus</i> with the 5' CCA-PAM protospacer, corresponding to the spacer1 from 630Δ <i>erm</i> CRISPR 7 array	[19]
pDIA6439	pRPF185Δ <i>gus</i> with the 5' CCA-PAM protospacer, corresponding to the spacer1 from 630Δ <i>erm</i> CRISPR 8 array	[19]
pDIA6440	pRPF185Δ <i>gus</i> with the 5' CCA-PAM protospacer, corresponding to the spacer1 from 630Δ <i>erm</i> CRISPR 9 array	[19]
pDIA6441	pRPF185Δ <i>gus</i> with the 5' CCA-PAM protospacer, corresponding to the spacer1 from 630Δ <i>erm</i> CRISPR 10 array	[19]
pDIA6442	pRPF185Δ <i>gus</i> with the 5' CCA-PAM protospacer, corresponding to the spacer1 from 630Δ <i>erm</i> CRISPR 11 array	[19]
pDIA6443	pRPF185Δ <i>gus</i> with the 5' CCA-PAM protospacer, corresponding to the spacer1 from 630Δ <i>erm</i> CRISPR 12 array	[19]
pDIA6444	pRPF185Δ <i>gus</i> with the 5' CCA-PAM protospacer, corresponding to the spacer1 from 630Δ <i>erm</i> CRISPR 17 array	[19]

**Table 2. Oligonucleotides used in this study**

Name	Sequence (5'→3')	Description
QRTBD37	GGGAGACTTGAGTGCAGGAG	16S RNA qPCR F
QRTBD38	GTGCCTCAGCGTCAGTTACA	16S RNA qPCR R
AM289	GAGAGAATTGTATAGATGTAAGTGTG	CRISPR 6 qPCR F
OS679	GCAGTGAGCAATATTTGCGATA	CRISPR 3–4/16–15 qPCR F
OS680	CAAATTTGCAGTGAACCATGA	CRISPR 3–4/16–15 qPCR R
AM290	GTGATGAATGTTTCAAGAGGA	CRISPR 6 qPCR R
AM291	AAGCTTTATCATTTGCACTACTC	CRISPR 7 qPCR F
AM292	CAGTATCTTTAAGAATTGAGTGGTT	CRISPR 7 qPCR R
AM175	TGCAAATTTAAGAGAGTTGTATACG	CRISPR 8 qPCR F
AM176	TATCTTGAGCTGTCAATGTGAAC	CRISPR 8 qPCR R
AM293	GGATTGAGGGTGTGTGATAAA	CRISPR 9 qPCR F
AM294	CTTGCAAGAATGGTTTTAATAATGAG	CRISPR 9 qPCR R
PB152	GGAGATGCTAAGTTATTTTGGGA	CRISPR 10 F
PB153	TTAAGACTAGCAGACTCATAAGC	CRISPR 10 R
OS472	CCATTGATTCTTTTTCAGTTTCG	CRISPR 12 qPCR F
OS473	CGCGTTAGGCAAATACAAGG	CRISPR 12 qPCR R
AM177	TCGCTCACTGCAAATTTTG	CRISPR 17 qPCR F
AM178	AAACGCAGGTCAAACCTTA	CRISPR 17 qPCR R

medium (control), BHI medium supplemented with 0.1 M glucose or/and 240  $\mu$ M sodium deoxycholate. The total RNA isolation, cDNA synthesis and real-time quantitative PCR (qRT-PCR) were performed as previously described in [28] using CFX96™ Real-Time System (Bio-Rad). Primers annealed to the first genes of *cas* operons, to leader regions and first spacers of CRISPR arrays were used in qRT-PCR (Table 2). In each sample, the relative expression was calculated relative to the 16S rRNA [24]. The relative change in gene expression was recorded as the ratio of normalized target concentrations ( $\Delta\Delta$ Ct) [18]. The experiments were performed in three biological replications.

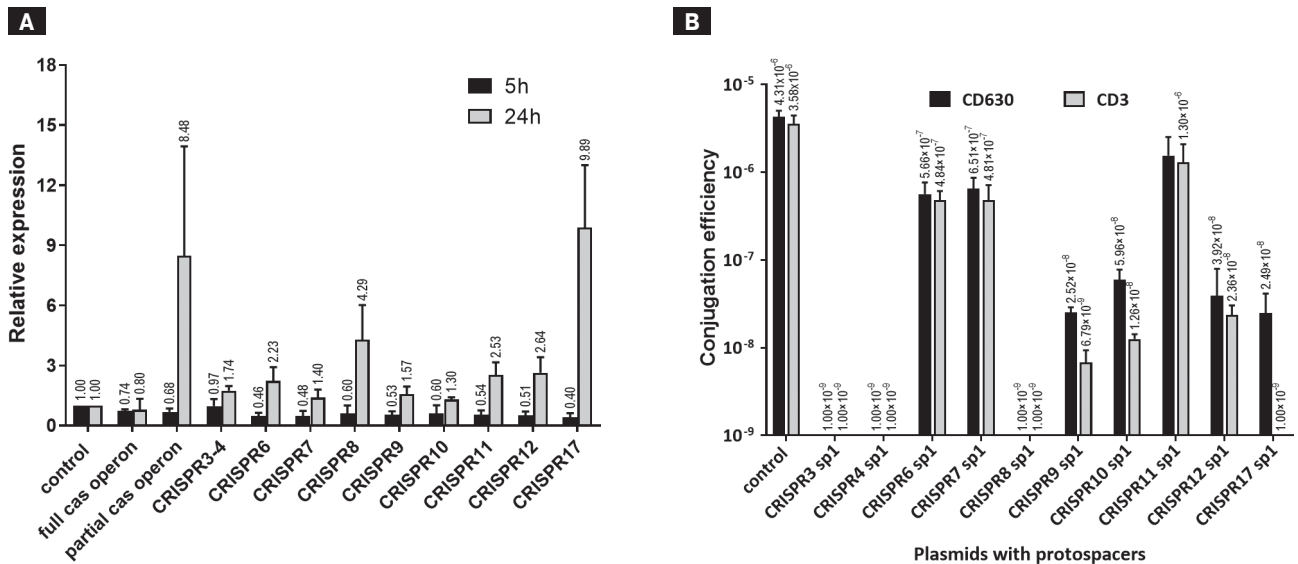
**Plasmid conjugation efficiency assays.** Plasmid conjugation efficiency assays were performed according to the method described in [19]. To evaluate conjugation efficiency, PAM (protospacer adjacent motif)-protospacer carrying conjugative plasmids were transformed into the *E. coli* HB101 (RP4) strain and transferred to *C. difficile* strains by conjugation. BHI plates used at the cultivation stage of conjugative mixtures were supplemented with ATc (250 ng/ml) or 0.1 M glucose or/and 240  $\mu$ M sodium deoxycholate, depending on the purpose of the experiment. The ratio of *C. difficile* transconjugants was counted by subculturing conjugation mixtures on BHI agar supplemented with thiamphenicol (Tm) (15  $\mu$ g/ml), D-cycloserine (Cs) (25  $\mu$ g/ml) and cefoxitin (Cfx) (8  $\mu$ g/ml) and comparing with the number of colony-forming units obtained after plating serial dilutions on BHI agar plates containing Cfx only. The experiments were performed in two technical and two biological replications. All the plasmids used in this work are listed in Table 1.

## Results

*C. difficile* CRISPR-Cas system expression under high cyclic di-guanosine monophosphate intracellular levels. Cyclic di-guanosine monophosphate (c-di-GMP) is a bacterial secondary messenger controlling diverse processes in bacterial cells, and it is mostly known to be an important signal molecule for the transition from the planktonic phenotype to the biofilm state [6]. In this work, we performed experiments to investigate *C. difficile* CRISPR-Cas system regulation under high c-di-GMP intracellular levels. For this proposal, *C. difficile* CD3 strain, carrying a chromosomal diguanylate cyclase gene (*dccA*) [29] under the control of an inducible tetracycline promoter (Ptet) was used. The induction of this gene causes an artificial increase of intracellular levels of c-di-GMP in *C. difficile*.

The qRT-PCR analysis revealed expression levels changes of CRISPR arrays and *cas* operons in the CD3 strain after 5 h and 24 h of cultivation with the ATc inducer (Fig. 1A). The expression of both *cas* operons and all CRISPR arrays decreased after 5 h of growth. An increase in the expression levels of the partial *cas* operon and all CRISPR arrays was observed after 24 h of cultivation (Fig. 1A) with the effect being most pronounced for CRISPR arrays 8 and 17.

**Role of high c-di-GMP intracellular levels on *C. difficile* CRISPR-Cas system interference functionality.** To investigate the role of c-di-GMP on *C. difficile* CRISPR-Cas system functionality, we performed plasmid interference assays with CD3 and 630 $\Delta$ *erm* strains. In these experiments, a set of plasmids containing protospacers corresponding to a selected spacer of *C. difficile* 630 $\Delta$ *erm* CRISPR array, flanked



**Figure 1. C. difficile CRISPR-Cas system regulation under high intracellular levels of cyclic di-guanosine monophosphate (c-di-GMP)**

**Note.** A. qRT-PCR analysis of the *C. difficile* CRISPR-Cas system expression in high c-di-GMP levels conditions after 5 and 24 hours of cultivation. B. Plasmid conjugation efficiencies in *C. difficile* 630 $\Delta$ erm (CD630) and CD3 strains. CRISPR 3–4...17 — indicate CRISPR-arrays. CRISPR 3 and CRISPR 4 arrays are cotranscribed and presented as CRISPR 3–4. Sequences of CRISPR 3–4 and CRISPR 16–15 arrays are identical [7]; therefore, CRISPR 16–15 are not presented. In conjugation experiments, plasmids carrying different protospacers corresponding to each *C. difficile* 630 $\Delta$ erm first spacers (sp1) in different CRISPR arrays and flanked by CCA PAM were used. An empty vector was used as a conjugation control. Lack of transconjugants indicate conjugation efficiencies of less than or equal to  $10^{-9}$ .

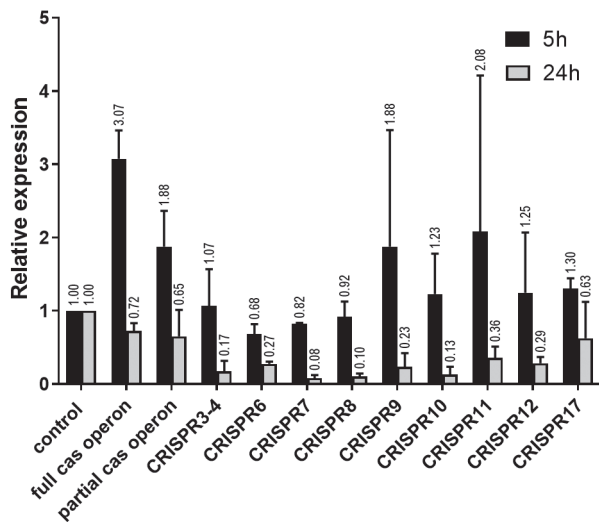
by functional CCA PAM on the 5'-end were used [19]. An empty pRPF185 $\Delta$ gus vector served as a conjugation control [11, 36]. The presence of a protospacer with a correct PAM sequence matching a spacer from one of the CRISPR arrays inhibits conjugation efficiency by several orders of magnitude. Hence, higher conjugation efficiencies correspond to lower CRISPR interference levels. These experiments showed slight induction of CRISPR interference under high c-di-GMP intracellular levels (Fig. 1B). Increased levels of interference were observed in the CRISPR 9, 10, 12, and 17 arrays. The most significant changes in conjugation efficiency were detected in CRISPR 17 array. At the same time, the results of expression analysis showed a significant induction of expression only in CRISPR 17 array. In the cases of CRISPR 9, 10, and 12 arrays, the increase in expression levels was less intense (Fig. 1A).

*Analysis of C. difficile CRISPR-Cas system transcription levels in the presence of sodium deoxycholate in medium.* During its infection cycle, *C. difficile* faces different adverse factors inside the host. Among, these factors there are secondary bile salts deoxycholates. A recent study showed that low concentrations of deoxycholates induce biofilm formation in *C. difficile* [10]. Therefore, deoxycholates can be considered as biofilm-inducing factors, and they could participate in this pathogen CRISPR-Cas system regulation.

To analyze the expression levels of the CRISPR-Cas system, the qRT-PCR was performed with total

RNA isolated from *C. difficile* 630 $\Delta$ erm cells grown in BHI medium supplemented with 0.1 M glucose (control) or with 0.1 M glucose and 240  $\mu$ M sodium deoxycholate. The glucose addition to the nutrient medium was necessary since deoxycholate-induced biofilm forming occurs only in the presence of this sugar [10]. However, qRT-PCR analysis revealed changes in CRISPR arrays transcription levels in control samples (with glucose only) compared to the values obtained previously for *C. difficile* 630 $\Delta$ erm cells grown in BHI medium without glucose [19]. Subsequently, glucose may also contribute to *C. difficile* CRISPR-Cas system regulation. Therefore, further experiments were held with *C. difficile* 630 $\Delta$ erm cells grown in medium supplemented with sodium deoxycholate only. Expression analysis showed a significant increase in both *cas* operons and CRISPR 17 array after 5 h of cultivation with deoxycholate (Fig. 2). On the contrary, expression levels of all CRISPR-Cas system components were decreased after 24 h of growth (Fig. 2). Notably, the growth of *C. difficile* cultures in the presence of deoxycholate was vastly inhibited due to the toxicity of this bile salt. Consequently, it was challenging to assess conjugation efficiency under these conditions.

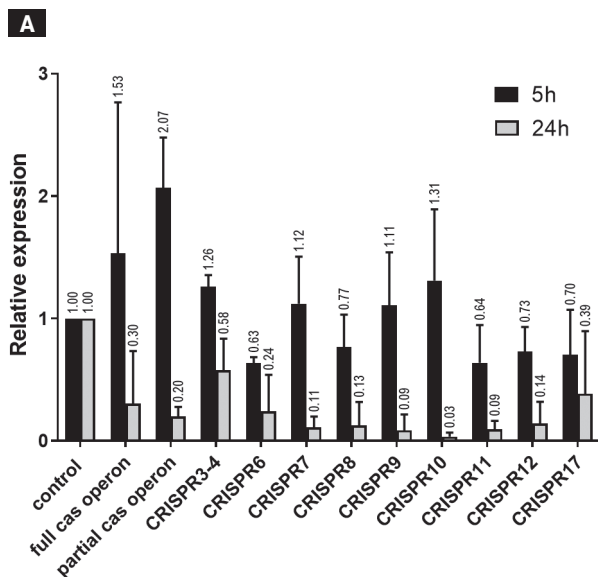
*Role of glucose in C. difficile CRISPR-Cas system expression.* As was revealed above, increased concentrations of glucose in the nutrient medium may be involved in the regulation of *C. difficile* CRISPR-Cas system. To investigate the potential effect of glucose on *C. difficile* CRISPR-Cas system expression, qRT-



**Figure 2. *C. difficile* CRISPR-Cas system expression in the presence of sodium deoxycholate in medium after 5 and 24 hours of growth**

**Note.** CRISPR 3–4...17 — indicate CRISPR-arrays. CRISPR 3 and CRISPR 4 arrays are cotranscribed and presented as CRISPR 3–4. Sequences of CRISPR 3–4 and CRISPR 16–15 arrays of are identical [7]; therefore, CRISPR 16–15 are not presented.

PCR experiments were performed. These assays demonstrated a significant induction of partial *cas* operon and CRISPR 3–4 array expression levels after 5 h of growth in the presence of glucose (Fig. 3A). After 24 h of cultivation, the expression of all CRISPR-Cas components was decreased (Fig. 3A).

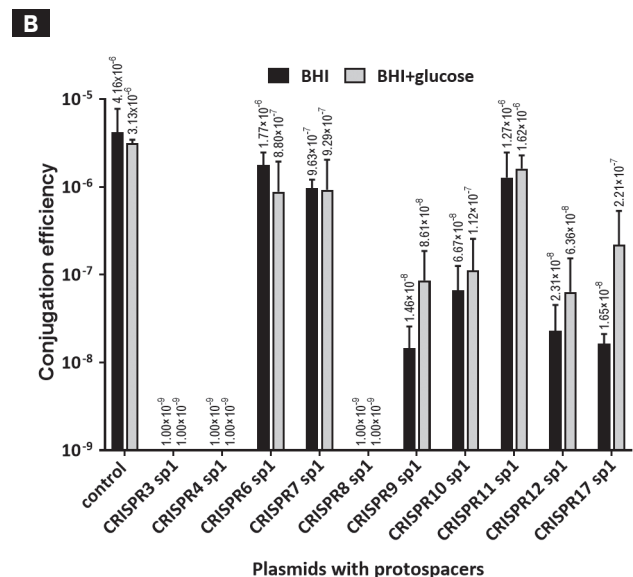


*CRISPR interference assays in increased glucose levels conditions.* Next, we evaluated interference efficiency at increased glucose concentration in the medium. We observed a reduction of CRISPR 9, 12, and 17 arrays interference levels compared to the control (Fig. 3B). This may indicate that the presence of glucose in the medium negatively regulates *C. difficile* CRISPR-Cas system defensive function. At the same time, the expression induction of some CRISPR-Cas components was observed under these cultivation conditions (Fig. 3A).

## Discussion

Human enteropathogenic bacterium *C. difficile* possesses a complex defensive CRISPR-Cas system, composed of many components. This system could be involved in this bacterium infection cycle and its adaptation to changing environments inside the host. Therefore, *C. difficile* CRISPR-Cas system should regulate its activity in response to various physiological and environmental signals. In this work, we performed a study of *C. difficile* CRISPR-Cas system regulation under biofilm-inducing factors. Biofilm mode of bacterial growth is characterized by a high density of the cells and the high possibility of horizontal gene transfer by different mobile genetic elements, including phages [1, 17]. Consequently, the positive regulation of the CRISPR-Cas system expression could be an adaptive strategy of *C. difficile* to increase chances of genetic parasites acquisition.

The secondary messenger c-di-GMP is one of the key components in the regulation of such phenotypic



**Figure 3. *C. difficile* CRISPR-Cas system regulation at high glucose concentration in the medium**

**Note.** A. qRT-PCR analysis of the *C. difficile* CRISPR-Cas system expression in the presence of glucose after 5 and 24 hours of cultivation. B. Plasmid conjugation efficiencies in *C. difficile* 630 $\Delta$ erm under normal growth conditions (BHI) and when 0.1M glucose was added to the growth medium (BHI+glucose). CRISPR 3–4...17 — indicate CRISPR-arrays. CRISPR 3 and CRISPR 4 arrays are cotranscribed and presented as CRISPR 3–4. Sequences of CRISPR 3–4 and CRISPR 16–15 arrays of are identical [7]; therefore, CRISPR 16–15 are not presented. In conjugation experiments, plasmids carrying different protospacers corresponding to each *C. difficile* 630 $\Delta$ erm first spacers (sp1) in different CRISPR arrays and flanked by CCA PAM were used. An empty vector was used as a conjugation control. Lack of transconjugants indicate conjugation efficiencies of less than or equal to  $10^{-9}$ .

shifts in bacteria [31]. Using quantitative PCR, we analyzed expression of all *C. difficile* 630 $\Delta$ erm CRISPR-Cas system components and revealed the induction of several CRISPR arrays and the partial *cas* operon in the presence of high c-di-GMP levels. These results were partially confirmed on the functional level by interference efficiency assays. To date, the research of c-di-GMP role in CRISPR-Cas systems regulation is only at the very beginning of its way. A recent work demonstrated that this secondary messenger negatively regulates the expression of *Erwinia amylovora* type I-E *cas* genes [15]. Thus the present study is, to our knowledge, the first to demonstrate positive regulation of CRISPR-Cas system components and functionality by c-di-GMP. Mechanistically, c-di-GMP works through binding effector proteins or riboswitches to modulate downstream gene regulatory networks [31, 35]. Therefore, in relation to CRISPR-Cas systems, c-di-GMP role appears to be indirect through affecting global regulatory proteins or RNA metabolism rather than direct gene transcription regulation. These mechanisms remain to be elucidated in further research.

Another biofilm formation factor explored in this work is the secondary bile salt deoxycholate [10]. In the presence of sodium deoxycholate in the medium, we detected an increase in expression levels of both *cas* operons and one CRISPR array. Therefore, these secondary bile salts can be a regulation factor for *C. difficile* CRISPR-Cas system. The link between the of presence sodium deoxycholate in medium and CRISPR-Cas system alternative function was observed in *Salmonella* Typhi [23]. This work showed the contribution of the CRISPR-Cas system to sodium deoxycholate resistance through the regulation of porin expression. Moreover, the enhanced biofilm formation observed in the *cas* mutants suggests that this system negatively regulates biofilm-associated genes. The complex *C. difficile* CRISPR-Cas system also may have non-canonical functions in this bacterium physiology [21] and induction of several CRISPR-Cas components by sodium deoxycholate might indicate their potential role in *C. difficile* survival within the host.

In addition, in experiments with deoxycholate and glucose in the medium, a regulatory effect of glucose was found. We observed an increase in expression levels of the partial *cas* operon and one CRISPR array under high glucose concentration in the nutrient

medium. At the same time, the presence of glucose decreased interference levels. This may indicate that increased glucose concentrations in the medium negatively regulate the functioning of the *C. difficile* CRISPR-Cas system despite the expression induction of its components. The regulation effect of glucose was also demonstrated for the CRISPR-Cas systems in *Thermus thermophilus*, *Pectobacterium atrosepticum* and *E. coli* [27, 32, 38]. High levels of cyclic adenosine monophosphate (cAMP) which is associated with glucose starvation have been shown to positively and negatively regulate the expression and activity of these systems. In *C. difficile* catabolite control protein A (CcpA) is a major global transcriptional regulator, and it is involved in response to changes in glucose levels [3]. A recent study revealed that one *cas* operon is directly regulated by CcpA in *Streptococcus mutans* [14]. Global regulators such as CcpA may also participate in the control of CRISPR-Cas activity in *C. difficile*. Further studies are needed to clarify the scope and mechanisms of this possible regulatory involvement.

## Conclusion

Altogether obtained results demonstrate the regulation of the *C. difficile* CRISPR-Cas system under biofilm conditions and in the presence of glucose in the nutrient medium. Our data indicate that *C. difficile* CRISPR-Cas system is subject for complex regulation by environmental and metabolic signals, suggesting its potential role beyond canonical defense functions, especially in the adaptation of this pathogen to the changing conditions inside the host. A more detailed analysis of *C. difficile* CRISPR-Cas system regulation is required, particularly with respect to other biofilm-related stimuli and stresses, as well as the molecular mechanisms underlying these regulatory processes.

## Additional information

**Declaration of competing interest.** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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