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The *Escherichia coli* colibactin resistance protein ClbS is a novel DNA binding protein that protects DNA from nucleolytic degradation

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ABSTRACT

Cells employ specific and nonspecific mechanisms to protect their genome integrity against exogenous and endogenous factors. The *clbS* gene is part of the polyketide synthase machinery (*pks* genomic island) encoding colibactin, a genotoxin implicated in promoting colorectal cancer. The *pks* is found among the *Enterobacteriaceae,* in particular *Escherichia coli* strains of the B2 phylogenetic group. Several resistance mechanisms protect toxin producers against toxicity of their products. ClbS, a cyclopropane hydrolase, was shown to confer colibactin resistance by opening its electrophilic cyclopropane ring. Here we report that ClbS sustained viability and enabled growth also of *E. coli* expressing another genotoxin, the Usp nuclease. The *recA::gfp* reporter system showed that ClbS protects against Usp induced DNA damage. To elucidate the mechanism of ClbS mediated protection, we studied the DNA binding ability of the ClbS protein. We show that ClbS directly interacts with single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA), whereas ssDNA seems to be the preferred substrate. Thus, the ClbS DNA-binding characteristics may serve bacteria to protect their genomes against DNA degradation.

1. Introduction

To protect the integrity of their genomes against exogenous and endogenous assault, cells employ specific and as well as nonspecific mechanisms. Exogenous factors are radiation, chemical agents and others, while endogenous factors are reactive oxygen species, metabolic products [1] and in a number of bacterial species potentially also genotoxins. Genotoxins such as colibactin are significant bacterial virulence factors that provoke DNA damage in eukaryotic cells. However, the genotoxin producing cell must be protected against its cognate toxin.

Colibactin is encoded by the polyketide synthase (*pks*) genomic island present among the *Enterobacteriaceae,* particularly pathogenic and commensal *Escherichia coli* strains of the B2 phylogenetic group [2]. The prevalence of these strains has in developed countries increased significantly [3,4]. Colibactin generates DNA cross-links that are, during DNA damage repair, converted to double strand breaks [5]. Recently, alkylation of DNA by colibactin has been demonstrated [6]. It has also been shown to induce colorectal cancer [7–11].

Colibactin is initially produced as an inactive prodrug that is activated by ClbP-mediated cleavage upon export to the periplasm [12,13]. Further, it was postulated that ClbS, also encoded by the *pks* island, is a colibactin resistance protein [14] that cleaves the colibactin cyclopropane ring, essential for genotoxicity, via its cyclopropane hydrolase activity [15].

As we previously demonstrated that immunity to the Usp genotoxin is assured by the Imu3 protein via nonspecific DNA binding [16], we postulated that ClbS might also provide direct genome protection. We therefore investigated whether ClbS exhibits DNA binding ability. Our results show that ClbS is a multifunctional protein, that besides directly inactivating colibactin, as shown previously, also associates with DNA to presumably protect the genome against certain genotoxic agents.

2. Material and methods

2.1. Bacterial strains and plasmid construction

Laboratory *E. coli* strains and plasmids used in this study are presented in Table 1. The *clbS* gene was PCR amplified from BAC *pks*$^+$ plasmid [2] with ClbS-F (5`- TGGATCCGCTGTTCCATCATCAAAAGAA

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Table 1
Bacterial strains and plasmids used in this study.

Bacterial strain/plasmid	Description	Source
DH5α (DE3)	Expression host, harboring the lambda DE3.	Thermo Fisher Scientific
E. coli BL21(DE3)	Expression host, harboring the lambda DE3; Cm^R.	Thermo Fisher Scientific
BAC *pks*$^+$	The source of the *clbS* gene.	(2)
pUSP4	The source of the *usp* gene; Ap^R.	(17)
pET8c	*E. coli* T7 expression vector, containing an N-terminal hexahistidine tag (His_6) and thrombin cleavage site.	Novagen
pCLB-his	pET8c with *clbS* gene.	This study
pLysS	pLys with removed lysozyme gene; Cm^R.	Thermo Fisher Scientific
pLCUBI	pLysS with *clbS* and *usp* genes; Cm^R and Ap^R.	This study
pLUBI	pLysS with *usp* gene; Cm^R and Ap^R.	This study
pSC201	Plasmid with *recA::gfp* fusion; Kn^R.	(18)

GAG-3`) and ClbS-R (5`-TTACGCGTTATTCTGCAAGACATTTCTGCAG-3`) primers (Sigma-Aldrich) at 55 °C annealing temperature. The PCR product, digested with *Bam*HI and *Mlu*I, was cloned into the expression vector pET8c, in a fusion with a thrombin-cleavable N-terminal hexahistidine tag (His_6-tag). This plasmid, designated pCLB-his, was subsequently used for ClbS protein isolation and for the construction of vectors, used for *in vivo* studies. Sequencing of the inserted *clbS* fragment was performed to check for potential PCR introduced mutations. For the construction of vectors, the *Bam*HI and *Eco*RV digested and blunted fragment of the low copy number pLysS plasmid was employed. This backbone fragment contained only the replication region and the chloramphenicol resistance gene, with the T7 lysozyme gene removed. The source of the *usp* gene for subcloning into the pLysS fragment was plasmid pUSP4 [17]. Two plasmids were thus constructed, pLUBI, carrying the *usp* gene but without its immunity genes, and pLCUBI harbouring *usp* and *clbS*, and were transformed into *E. coli* BL21 (DE3). Both genes were controlled by independent T7 polymerase promoters. To follow the SOS response, plasmid pSC201 with a *recA::gfp* fusion was used [18], transformed into either *E. coli* BL21 (DE3) with pLCUBI, or *E. coli* BL21 (DE3) with pLUBI.

2.2. Expression, Isolation, purification and detection of the ClbS protein

ClbS was expressed in *E. coli* strain BL21 (DE3) pCLB-his. An overnight culture was used to inoculate liquid Luria Bertani medium supplemented with 25 μg/mL chloramphenicol and grown at 37 °C to an OD_{600} 0.5 when ClbS production was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) at a final concentration of 0.8 mM. The culture was subsequently grown for an additional 4 h when biomass was collected by 10 min centrifugation at 4.000 × g. His_6-tagged ClbS was purified by Ni-NTA metal affinity chromatography (Qiagen) and stored in elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8) at 4 °C. Further, overnight dialysis was performed against a final buffer (50 mM $NaH_2PO_4{\cdot}H_2O$, 200 mM NaCl, 20 mM β-mercaptoethanol, pH 8) at 4 °C. The concentration of the purified ClbS protein was determined using a NanoDrop 1000 (Thermo Fisher Scientific) with the extinction coefficient at 280 nm of 37530 $M^{-1}\,cm^{-1}$ and by Pierce BCA protein assay kit, using Bovine Serum Albumin (BSA) (Sigma-Aldrich) as the standard. Additionally, the His_6-tag was removed from His_6-ClbS with the Thrombin cleavage capture kit (Novagen) and the buffer exchanged by overnight dialysis as described above. To assay the removal of the affinity tag, the thrombin-digested or undigested His_6-ClbS samples were resolved on a 12% acrylamide gel (Fig. S1).

2.3. Electrophoretic mobility shift assays (EMSA)

Various concentrations of ClbS carrying or lacking the affinity His_6-tag (0.15 μM – 2.4 μM) and 60 ng/μL of DNA (Table 2) were used to establish the nucleic acid-binding ability of ClbS. ClbS was incubated with DNA in 1 X FastDigest Green Buffer (Fermentas) at 37 °C for 15 min, prior to the analysis of the protein-DNA complexes by EMSA analysis on 1.5–2 % agarose gels. As a negative control BSA was included at a final concentration of 1.6 μM. Additionally, mixtures of pUC19/*Eco*RI (60 ng/μL) and ClbS (0.15 μM – 2.4 μM) were pre-incubated at 37 °C for 5–15 minutes when colicin E7 (ColE7), isolated from *E. coli* [19] or commercial DNase I (Sigma-Aldrich) were added in final concentrations 0.42 mg/ml and 0.5 mg/mL, respectively, followed by an additional 30 min incubation. As a control, DNA without ClbS was incubated with ColE7 or commercial DNase I. Results were analyzed employing 2% agarose gels.

2.4. Surface plasmon resonance assays (SPR)

Measurements were performed at 25 °C on a Biacore T100 (GE Healthcare, UK) at the Infrastructural Centre for Analysis of Molecular Interactions (University of Ljubljana, Slovenia). To prepare biotinylated DNA for immobilization on the streptavidin sensor chip, plasmid pJBPL-A was used as a matrix DNA to amplify the 403 bp long DNA sequence (from *Salmonella bongori*). Annealing temperature of 55 °C was used to amplify with primers Bong-biotin (5` -CATAAAACCTCGTGTA TGGTGCGC-3 and Bong-R (5`-GAATTCCATTTTACGACATCCGAATAT AAATTAAACGAA-3`) (Integrated DNA Technology). Approximately 50 response units (RU) of the 3′-biotinylated 403 bp long PCR product were immobilized on the second flow cell of the streptavidin (SA) sensor chip (GE Healthcare) in SPR buffer (20 mM Tris (pH 7.4), 140 mM NaCl, 2 mM DTT, 0.005% surfactant P20 and 0.1 mg/ml BSA). The first flow cell was left empty and used to control any non-specific binding to the sensor surface. The interaction between ClbS and chip-immobilized DNA was studied by injecting various concentrations of ClbS in SPR buffer. Injections of ClbS over the immobilized DNA fragment were performed at a flow rate of 10 μl min^{-1} for 2 min with dsDNA and 4 min with ssDNA. The sensor chip with bound DNA was regenerated by injection of SPR buffer containing 0.03% SDS for 15 s at a flow rate of 30 μl min^{-1}. Further, chip-immobilized dsDNA denaturation was applied with 2 pulses of 50 mM NaOH for 30 s to obtain ssDNA. The response after NaOH treatment dropped for approximately 20 RU, indicating that one DNA chain was removed. Injections of various concentrations of ClbS in SPR buffer was applied at a flow rate of 10 μl min^{-1} for 4 min. SPR experiments were performed in triplicate and representative experiments are shown. The data were analyzed with the Biacore T100 Evaluation Software and equilibrium dissociation constants (K_D) were determined by fitting the data to the steady state affinity model.

2.5. Quenching of intrinsic ClbS tryptophan fluorescence by ss- and dsDNA

Intrinsic tryptophan fluorescence of ClbS (40 μg/mL in 50 mM phosphate buffer, 200 mM NaCl, β-mercaptoethanol, pH 8.0) was measured. DNA used for titrations are presented in Table 2. After adding DNA, a 10 s incubation followed when measurements were made in a constantly stirred 1 cm path length quartz cuvette with a scan

Table 2
DNA employed in this study.

DNA	Length (bp or bases)	Application	Reference
pUC19	2686	EMSA	Fermentas
pJBPL-A	3377	Matrix DNA for PCR reaction.	This laboratory
PCR amplicon	403	SPR (ds- and ssDNA), EMSA, Trp fluorescence quenching	This study
ds- and ssDNA phage genome M13	6407	EMSA, Trp fluorescence quenching	[20]

rate of 250 nm/min (37 °C, excitation and emission slits at 5 nm each). The fluorescence emission spectra were recorded in the wavelength range of 300–450 nm after excitation at 295 nm, specifically monitoring the tryptophan fluorescence emission. Fluorescent emissions of the buffer titrated with DNA (background intensities) were subtracted.

2.6. Growth of *E. coli clbS*$^+$ *usp*$^+$ and *E. coli usp*$^+$ strains and cell morphology analysis

The two *E. coli* BL21 (DE3) strains were cultivated at 37 °C in liquid BHI medium with ampicillin and chloramphenicol at final concentrations 100 and 20 μg/ml, respectively. To suppress basal level expression of the T7 polymerase promoter, glucose was added at a final concentration of 0.4%. When cultures reached OD_{600} 0.5, protein synthesis was induced by addition of IPTG at final concentration 0.8 mM. One ml of both cultures was sampled before induction and each hour following induction (at least three hours). The OD_{600} was measured and plotted in time.

Prior to microscopy, the *E. coli* BL21 (DE3) *clbS*$^+$ *usp*$^+$ and *E. coli usp*$^+$ strains were transformed with a plasmid carrying a *recA::gfp* gene fusion and cultured as described above in media supplemented with 30 μg/ml kanamycin. Both cultures were sampled before and every hour following induction. Cells were washed twice with NaCl and attached to glass slides coated with 0.1% (w/v) poly-L-lysine (Sigma). Cell morphology was observed under light and *recA::gfp* expression under fluorescence microscopy, using a Zeiss AxioImager Z1 microscope equipped with a HRc digital camera.

3. Results and discussion

3.1. ClbS binds ss- and dsDNA in a non-specific manner

Initially, EMSA was applied and showed that ClbS causes circular and linear DNA retardation (Fig. 1A) and, that it interacts with ss- or dsDNAs of various lengths and source (Table 2, Fig. S2). Thus, EMSA experiments implied that ClbS binds DNA in a non-specific manner. It is of note that ClbS binding activity was not affected by the His_6 tag (Fig. S3). SPR spectrometry measurements of ClbS binding to ssDNA (Fig. 1B) or dsDNA (Fig. 1C) revealed an approximately 40-times higher affinity of ClbS for ssDNA exhibiting an apparent equilibrium dissociation constant (K_D) of 24.5 ± 1.2 nM compared to dsDNA exhibiting a K_D of 936.8 ± 12.3 nM (Fig. 1 D). Additionally, to confirm that ClbS interacts with DNA we assayed the tryptophan fluorescence spectra of ClbS, which carries five tryptophan residues, in the presence or in the absence of DNA. We observed quenching of intrinsic ClbS tryptophan fluorescence by various ss- and dsDNAs. The most pronounced decrease in intensity of emission maximum was observed upon titration with ssDNA (Fig. 1E). Our results implied that the five ClbS tryptophan residues behave as two populations of fluorophores, one accessible to quenchers and the other buried in the protein structure [21], presented as downward curvature in the Stern-Volmer plot (Fig. 1F).

3.2. ClbS protects DNA from deoxyribonucleases

DNA protection by ClbS was examined by measuring growth of the *E. coli* strain carrying a plasmid encoded *usp* gene and its cognate immunity genes replaced with *clbS,* in comparison to a strain harbouring only the *usp* gene (Table 1). Expression of *usp* and *clbS* was controlled by the T7 polymerase promoter. Following IPTG induction of *usp* and *clbS* the strain encoding only *usp* (lacking its immunity genes or *clbS*), exhibited dramatically reduced growth in comparison to the strain with both *usp* and *clbS* (Fig. 2A). DNA damage induces the SOS response involved in DNA repair. The principal regulators of the SOS response are the LexA repressor and RecA activator. Upon DNA damage, RecA is activated by binding to exposed ssDNA which stimulates LexA autocleavage, derepressing SOS genes. SOS induction inhibits cell division, resulting in cell filamentation [22]. Light microscopy of IPTG-induced cells expressing *usp* revealed pronounced filamentation (Fig. 2B), while typical cell shape and size were detected among *E. coli* expressing both *usp* and *clbS* genes (Fig. 2C). The "two examined strains also carried a plasmid encoded *recA::gfp*, a DNA damage SOS reporter gene fusion. Fluorescent microscopy revealed that *usp*$^+$ cells without *clbS,* additionally exhibited elevated expression of *recA::gfp* (Fig. 2D) when compared to the *usp*$^+$*clbS*$^+$ cells (Fig. 2E). Filamentation and elevated expression of *recA* are hallmarks of induction of the DNA damage inducible SOS response. Thus, typical cell shape and size, as well as basal level expression of *recA* in *usp*$^+$*clbS*$^+$ cells indicates, that ClbS protects cells from Usp nuclease activity.

We subsequently hypothesized that binding of ClbS could provide DNA protection against other deoxyribonucleases. Co-incubation of DNA and ClbS protected plasmid DNA against DNase I (Fig. 2F) and the ColE7 nuclease (Fig. 2G) in a concentration dependent manner, confirming that ClbS provides protection against various nucleases by presumably interacting non-specifically with DNA. ClbS binding to DNA is evident as DNA retardation (Fig. 2G, and F). Other examples of protective bacterial proteins have been characterized previously namely, two *E. coli* nucleoid associated proteins, the multifunctional Dps and CbpA, that non-specifically bind DNA, to drive nucleoid compactation and DNA protection [23–26]. Further, the α/β type small acid-soluble spore proteins that are synthesized during sporulation, bind DNA changing its conformation to an A-like helix, that is more resilient against damage [27,28].

In conclusion, our results imply that ClbS, besides directly interacting with colibactin is also a novel DNA binding protein. Thus, beyond colibactin, ClbS also protects DNA against degradation/genotoxic activity presumably providing a selective advantage for bacterial strains encountering stressful conditions also within the host.

Conflict of interest

The authors have no conflict of interest.

Footnotes

Data accessibility available via Mendeley Data:
https://data.mendeley.com/datasets/4y3wpkrfzc/draft?a=

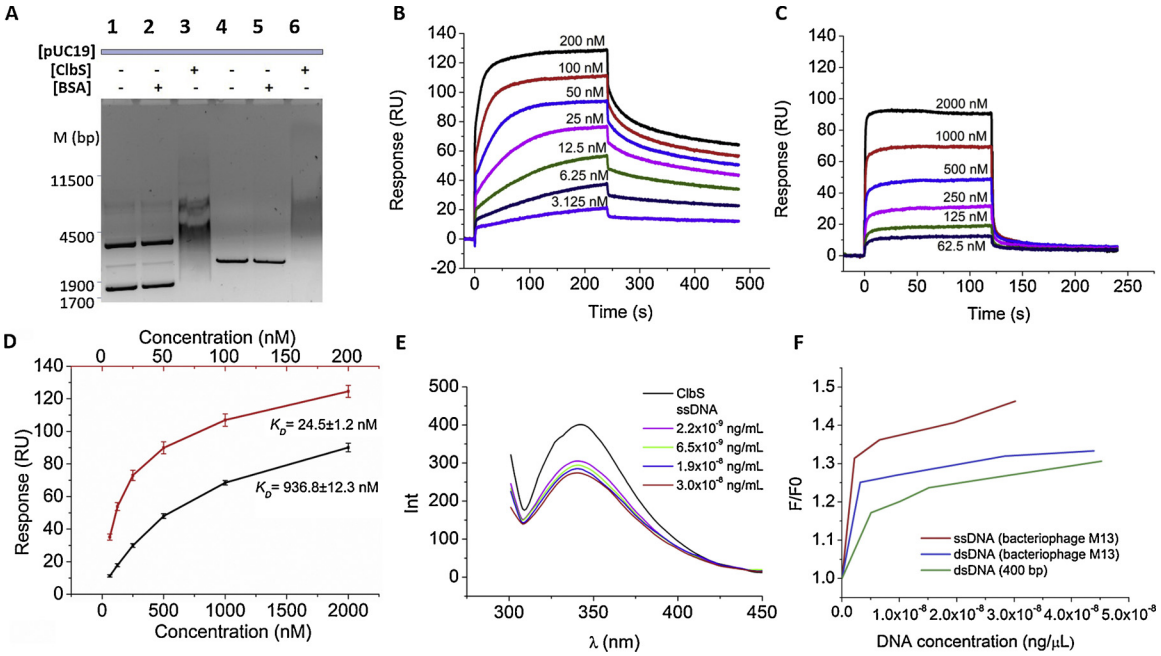

Fig. 1. ClbS is a DNA binding protein. (A) EMSA of ClbS interacting with the DNA. M designates marker (bp). Lane 1 contains pUC19 plasmid DNA, lane 2 pUC19 with BSA and lane 3 pUC19 with ClbS. Lane 4 contains pUC19 linearized with *Eco*RI, lane 5 pUC19/*Eco*RI with BSA and lane 6 pUC19/*Eco*RI with ClbS. (B) SPR sensorgrams showing the interaction of ClbS with 403 bp long chip-immobilized ssDNA or (C) dsDNA. (D) The apparent equilibrium dissociation constant (K_D) values of ClbS interacting with ssDNA, red or with dsDNA, black. The average K_Ds and standard deviations were determined from three titrations of each protein. (E) Quenching of intrinsic tryptophan fluorescence of ClbS titrated with ssDNA. The legend denotes ssDNA concentrations preincubated with ClbS. (F) Stern-Volmer plot of tryptophan quenching within ClbS due to interaction with ssDNA or dsDNA.

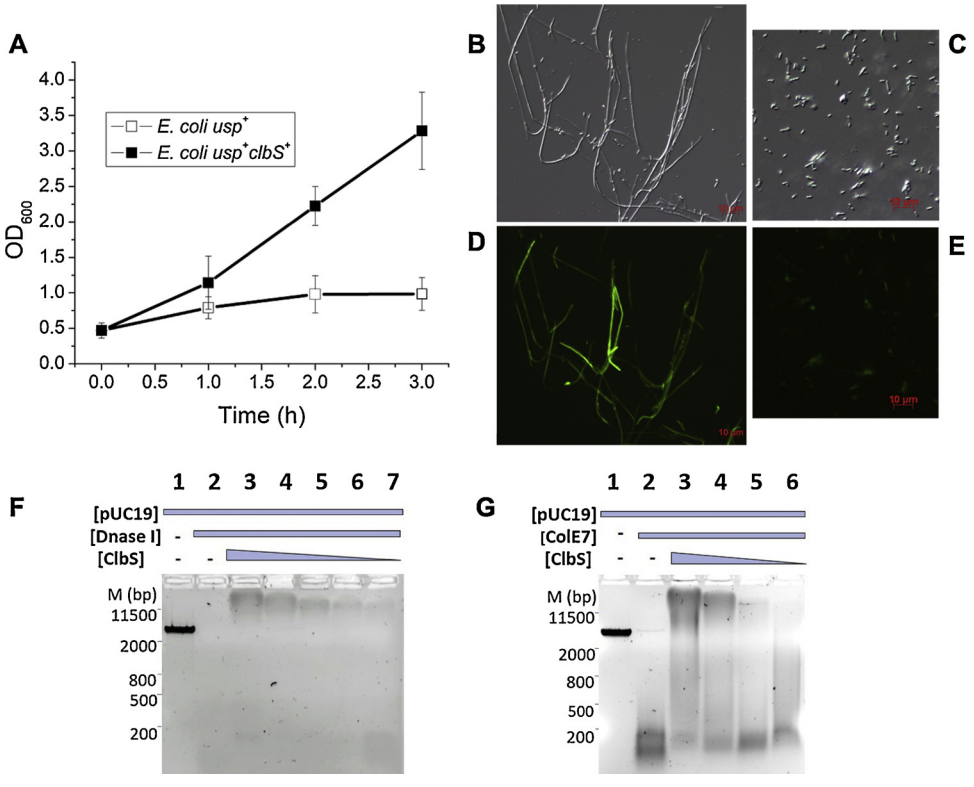

Fig. 2. ClbS protects DNA from deoxyribonucleases. (A) Growth curves of *E. coli usp*$^+$*clbS*$^+$ (black squares) and *E. coli usp*$^+$ strains (white squares). Induction of proteins at time 0 h. The experiment was performed seven times, and the means ± standard errors of the means (error bars) are shown. (B) Light and (D) fluorescent microscopy of the *E. coli usp*$^+$ strain following 3 h - induction. (C) Light and (E) fluorescent microscopy of the *E. coli usp*$^+$*clbS*$^+$ strain following 3 h - induction. (F and G) ClbS protect DNA against DNase degradation. (F) M designates marker (bp). Lane 1 contains pUC19/*Eco*RI plasmid DNA, lane 2 DNA with DNase I (0.5 mg/mL), lanes 3–7 DNA with ClbS (2.4-0.15μM) and DNase I. (G) M designates marker (bp). Lane 1 contains pUC19/*Eco*RI DNA, lane 2 DNA with ColE7 (0.42 mg/ml) and lanes 3–6 DNA with ClbS (2.4-0.15μM) and ColE7.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.dnarep.2019.05.003.

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