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ClbS Is a Cyclopropane Hydrolase That Confers Colibactin Resistance

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Abstract

Certain commensal *Escherichia coli* contain the *clb* biosynthetic gene cluster that codes for small molecule prodrugs known as precolibactins. Precolibactins are converted to colibactins by *N*-deacylation; the latter are postulated to be genotoxic and to contribute to colorectal cancer formation. Though advances toward elucidating (pre)colibactin biosynthesis have been made, the functions and mechanisms of several *clb* gene products remain poorly understood. Here we report the 2.1 Å X-ray structure and molecular function of ClbS, a gene product that confers resistance to colibactin toxicity in host bacteria and which has been shown to be important for bacterial viability. The structure harbors a potential colibactin binding site and shares similarity to known hydrolases. *In vitro* studies using a synthetic colibactin analog and ClbS or an active site residue mutant reveal cyclopropane hydrolase activity that converts the electrophilic cyclopropane of the colibactins into an innocuous hydrolysis product. As the cyclopropane has been shown to be essential for genotoxic effects *in vitro*, this ClbS-catalyzed ring-opening provides a means for the bacteria to circumvent self-induced genotoxicity. Our study provides a molecular-level view of the first reported cyclopropane hydrolase and support for a specific mechanistic role of this enzyme in colibactin resistance.

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Author Contributions

P.T. conducted structural biology, mutagenesis, and sequence alignment experiments and carried out associated analyses. E.E.S. conducted analytical and preparative biochemical assays on model colibactins and carried out associated analyses. A.R.H. synthesized model colibactins. C.S.K. characterized ClbS product 5 by NMR and computation. S.B.H. oversaw synthetic efforts. J.M.C. oversaw biochemical assay and product characterization efforts. S.D.B. oversaw structural biology and bioinformatics efforts.

Notes

The authors declare no competing financial interest.

The human gut microbiota constitute a complex, diverse community of microorganisms that influence human physiology, clinical responses to drugs, and disease progression.[1] The identification of causative relationships between microbial products and host phenotypes is an outstanding challenge in the field. Colibactins are secondary metabolites produced by certain strains of gut-commensal *Escherichia coli* that contain a 54-kb hybrid nonribosomal peptide synthetase−polyketide synthase (NRPS−PKS) biosynthetic gene cluster termed *clb* (or *pks*). *clb*$^+$ *E. coli* have been implicated in colorectal cancer (CRC) progression: These bacteria induce persistent double-strand DNA breaks and cellular senescence in eukaryotic cells,[2] promote tumor formation in colitis mouse models, and are epidemiologically correlated to CRC patients.[3]

Multiple resistance mechanisms, including target modification, sequestration, and effiux allow cytotoxin-producing bacteria to protect themselves from the toxicity of their own products.[4] In the *clb* pathway, known self-immunity strategies include the *N*-acyl-D-asparagine prodrug motif,[5] whose removal in the periplasm is required to generate the active colibactin genotoxins, the unique 12-transmembrane MATE inner-membrane transporter ClbM,[6] and the resistance protein ClbS (Figure 1). Phenotypic experimental evidence has shown that intracellular ClbS can protect both prokaryotic and eukaryotic cells from *clb*-mediated genotoxicity, although the mechanism of this protection was not defined.[7] ClbS (previously termed c2450) was first isolated from an activity-based protein profiling (ABPP) assay using electrophilic probes containing an α-methylene-γ-butyrolactone moiety.[8] Cys167 of ClbS was demonstrated to be the site of alkylation. Additionally, bacteria lacking the *clbS* gene showed an enhanced SOS response along with growth arrest leading to the proposal that ClbS is a self-resistance protein acting via sequestration or modification of reactive colibactins.[7]

To gain an understanding of how ClbS functionally confers colibactin resistance, we incubated purified ClbS with the unsaturated imine **2** (*m/z* 485.1430, *obs*). This imine has been synthesized, fully characterized, and shown to extensively damage DNA *in vitro*.[9] This substrate is the *N*-methylamide analog of metabolite **1**, which was detected in *clbP*-encoding bacterial cultures *in vivo* and which has also been characterized by synthesis.[10] The structures of fully-elaborated (pre)-colibactins, accounting for all of the genes in the *clb* gene cluster, are unknown. Owing to its ease of organic extraction, **2** was used for analytical and preparative purposes. The products formed on treatment of **2** with substoichiometric amounts of ClbS were analyzed by high-resolution quadrupole time-of-flight liquid chromatography-mass spectrometry (Figure 2). Rather than ClbS alkylative suicide conjugation, we observed catalytic depletion of **2** and accumulation of a signal corresponding to **2** + H_2O (*m/z* 503.1528, *obs*, Figure 2A). Tandem MS of this signal was consistent with hydrolysis of the electrophilic warhead moiety, as in proposed structure **3** (Figure S1). **3** is unstable and converts to other products during longer incubation times (see herein and Figure S2).

A preparative-scale experiment was performed (5.00 mg of **2**) to facilitate the full characterization of **3**. However, though the ring-opened product **3** was detectable, as in the analytical experiments above, **3** reproducibly underwent peroxidation to a mixture of diastereomeric structures consistent with proposed **4a**/**4b** (*m/z* 535.1435, *obs*, supporting

MS/MS is shown in Figure S3). The oxidation products **4a/4b** underwent further transformation and peroxide decomposition events (order unknown) to provide the tetrahydrofurans **5a** and **5b** (Figure 2B,C, *m/z* 519.1477, *obs*; supporting MS/MS is shown in Figure S3). The tetrahydrofurans **5a** and **5b** were isolable and fully characterized by 1- and 2-D NMR spectroscopy (see Figures S4−S9 and Table S2). The NMR data and DP4 computational analysis[11] supported the production of the two diastereomers **5a** and **5b** in approximately equimolar amounts (Figures S10−S12, Tables S3−S4). The relative configurations of the newly formed bicyclic systems were confirmed as *cis*,[12] based on ROESY analysis (Figure S4). Interestingly, position C4 of the five-membered lactam behaves as a second electrophilic site in the formation of these byproducts. C4 was previously proposed to be electrophilic,[13] although **2** did not lead to observable DNA interstrand cross-links *in vitro* with plasmid DNA.[9] It is also interesting that decomposition likely proceeds through the reactive organic peroxide. A role for this *in vitro* sequence in DNA alkylation and genotoxicity remains unknown, but many natural products damage DNA via the generation of reactive oxygen species (ROS).[14]

Mature precolibactins are deacylated by the membrane-anchored peptidase ClbP after transport to the periplasm, which allows their conversion to cytotoxic colibactins.[16] When genetically modified *E. coli* lacking a functional *clbP* gene are cultured, alternative cyclizations leading to pyridone-containing precolibactin scaffolds manifest.[1d,17] However, these deacylated pyridones lack any observable DNA alkylation activity *in vitro*.[9] This suggests that these pyridones should be inert toward ClbS. To assess this, we incubated ClbS with the pyridone **6** and monitored its degradation (Figure 3). The unsaturated imine **2** was used as a positive control. As expected, 95% conversion of **2** to the hydrolysis product **3** was complete after 30 min. However, hydrolysis products of **6** could not be detected, even after longer incubation times (up to 1 h). This result is consistent with our hypothesis that unsaturated imines underlie the genotoxic effects of the *clb* gene cluster and that pyridones resembling **6** are nongenotoxic.

To elucidate the catalytic mechanism of ClbS, we determined its X-ray crystal structure at 2.1 Å resolution (Figure 4A, Figures S13−15, Table S5). The structure reveals an unusual four-helix bundle topology and places ClbS in the DinB_2 family. The functions of members of this superfamily are largely uncharacterized; however, several are metalloenzymes with active sites comprising a triad of histidine residues that chelate nickel or zinc. Limited evidence also suggests that this family is involved in detoxification of xenobiotic small molecules through thiol conjugation.[15] Although globally similar in structure, ClbS lacks the metal binding motif of the DinB_2 family. Additionally, the Cys167 residue shown to be alkylated with electrophilic γ-butyrolactone probes[8] is not present in any obvious active site pocket, but instead is located at the C-terminus on a partially disordered loop. Cys167 is also not conserved among ClbS proteins in homologous *clb* gene clusters (Figure S16). These data call into question its predicted role for covalent capture of genotoxic colibactins, as previously observed for γ-butyrolactone probes.[8] The ABPP-based isolation of enzymes by nonspecific probe labeling at residues not directly associated with the catalytic site is a known limitation of this technique.[18]

We then examined the ClbS structure to identify residues with a potential role in the observed cyclopropane hydrolase activity. Structure-based alignment of ClbS with DinB_2 metal-loproteins identified a conserved tyrosine (Tyr55) residing in the same position as the active metal of several homologues (Figure S17), suggesting this residue could play a role in catalysis. Indeed, automated docking of **2** into the ClbS structure produced models where **2** is in close proximity to Tyr55 (Figure 4B and Figure S18). Attempts to cocrystallize ClbS containing **2** or **3** using these conditions have not been successful, suggesting a potential structural change upon binding.

To evaluate a potential catalytic function of Tyr55, a conservative mutation (Tyr55Phe) was generated (Table S6). When Tyr55Phe ClbS was tested in the hydrolysis assay (2 → 3), no conversion was observed (Figure 4C), implicating Tyr55 as an essential catalytic residue. Although further mechanistic studies are required, Tyr55 could promote ring-opening by forming a hydrogen bonding interaction with the amide carbonyl or by activating a water molecule within the binding pocket. To assess a possible role of Cys167 in catalysis, we also assayed Cys167Ala ClbS along with the double mutant Tyr55Phe/Cys167Ala. Cys167 was dispensable whereas the double mutant was inactive, further supporting a role for Tyr55 in the hydrolysis chemistry (Figure S19). In light of these results, the previously described electrophilic-probe modification by ClbS could be the spontaneous byproduct of a reactive substrate with the surface-exposed Cys167 residue.[8]

Based on a growing body of data, the genotoxicity of colibactins is postulated to derive from DNA alkylation by nucleotide addition to the electrophilic cyclopropane. O^6-Alkylguanine bases, for example, are known to be highly mutagenic and genotoxic, and failure to repair these lesions lead to highly toxic double-strand DNA breaks.[19] A common biological marker for DNA double-strand breaks, phospho-SER139-H2AX (γH2AX),[20] is known to be elevated in established colibactin genotoxicity assays.[2] However, the precise mode of DNA binding and sequence selectivity of colibactins are currently undefined. The identification and structural characterization of the ClbS products **5a** and **5b** here suggest that genotoxic colibactins might damage DNA through additional mechanisms including the generation of a second electrophilic site and a ROS, as DNA-mediated cyclopropane ring opening may result in the formation of these reactive moieties localized to the DNA backbone (Figure S20). ROS are mutagenic, and such an organic peroxide could provide additional damage. The electrophilicity observed at C4 in the lactam could also contribute to the genotoxic phenotype. Detailed mechanistic investigation is needed to assess this model using fully mature colibactins.

In conclusion, we have described the structure and function of the colibactin resistance protein ClbS. To our knowledge, ClbS is the first reported cyclopropane hydrolase. Our data suggest ClbS abrogates colibactin cytotoxicity by hydrolysis of the electrophilic cyclopropane. This finding is consistent with a growing body of literature supporting a role for unsaturated imines resembling metabolite **1** in the genotoxicity of the *clb* gene cluster. In addition, the stability of the pyridone **6** toward ClbS is consistent with the prior determination that these compounds are nongenotoxic byproducts accumulating from the use of unnatural *clbP* deletion strains.[9] Our data suggest a potential role of ROS in colibactin

cytotoxicity and that delivery of ClbS could constitute a new therapeutic strategy to abrogate *clb*$^{+}$ *E. coli*-induced genotoxicity through cyclopropane hydrolysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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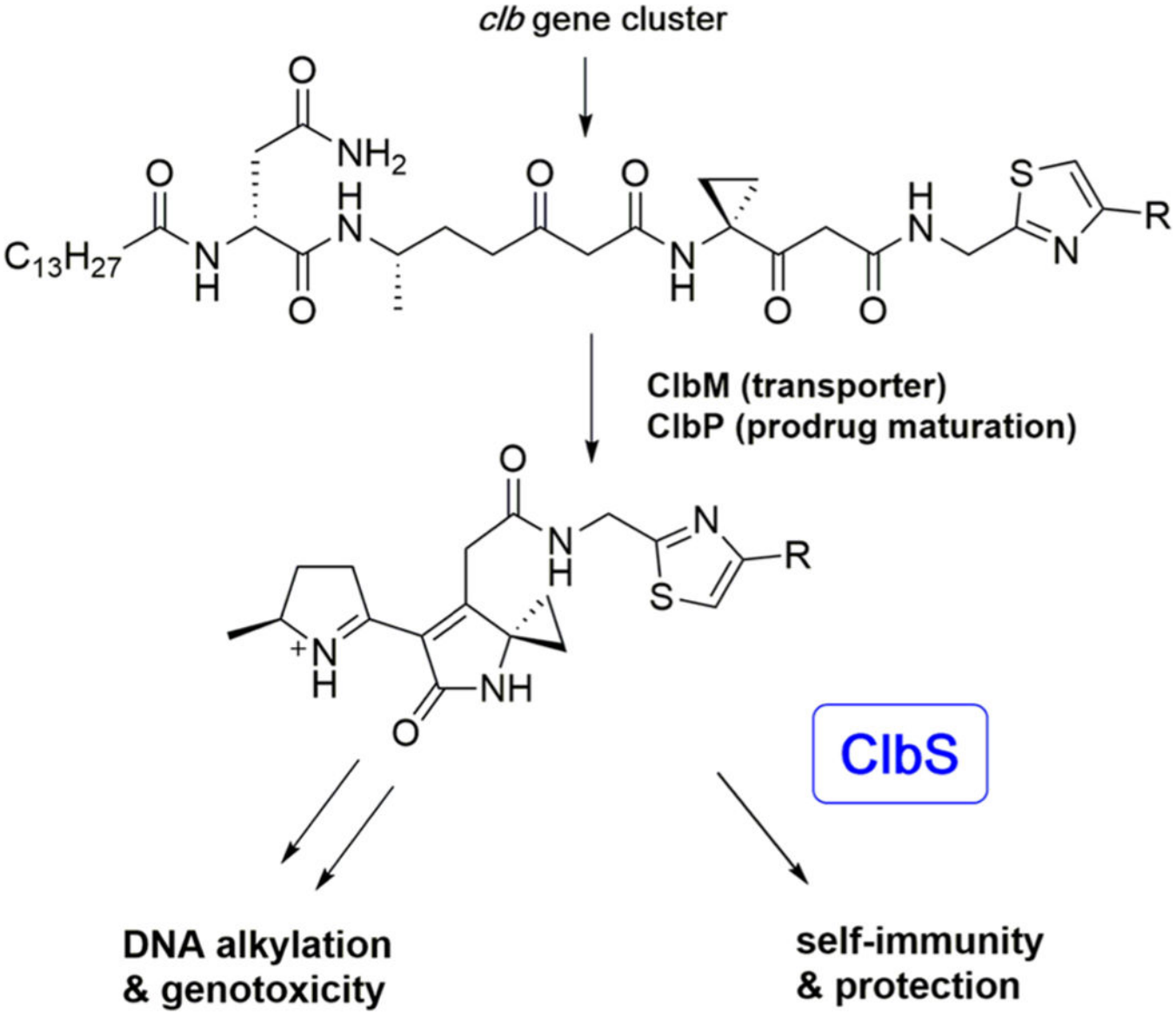

Figure 1.

Generalized prodrug activation of colibactins includes ClbM-mediated inner membrane transport of precolibactins and ClbP peptidase activation. The cyclopropane-containing colibactin warhead alkylates DNA, and ClbS provides protection against genotoxicity. R represents variable region of the mature natural product.

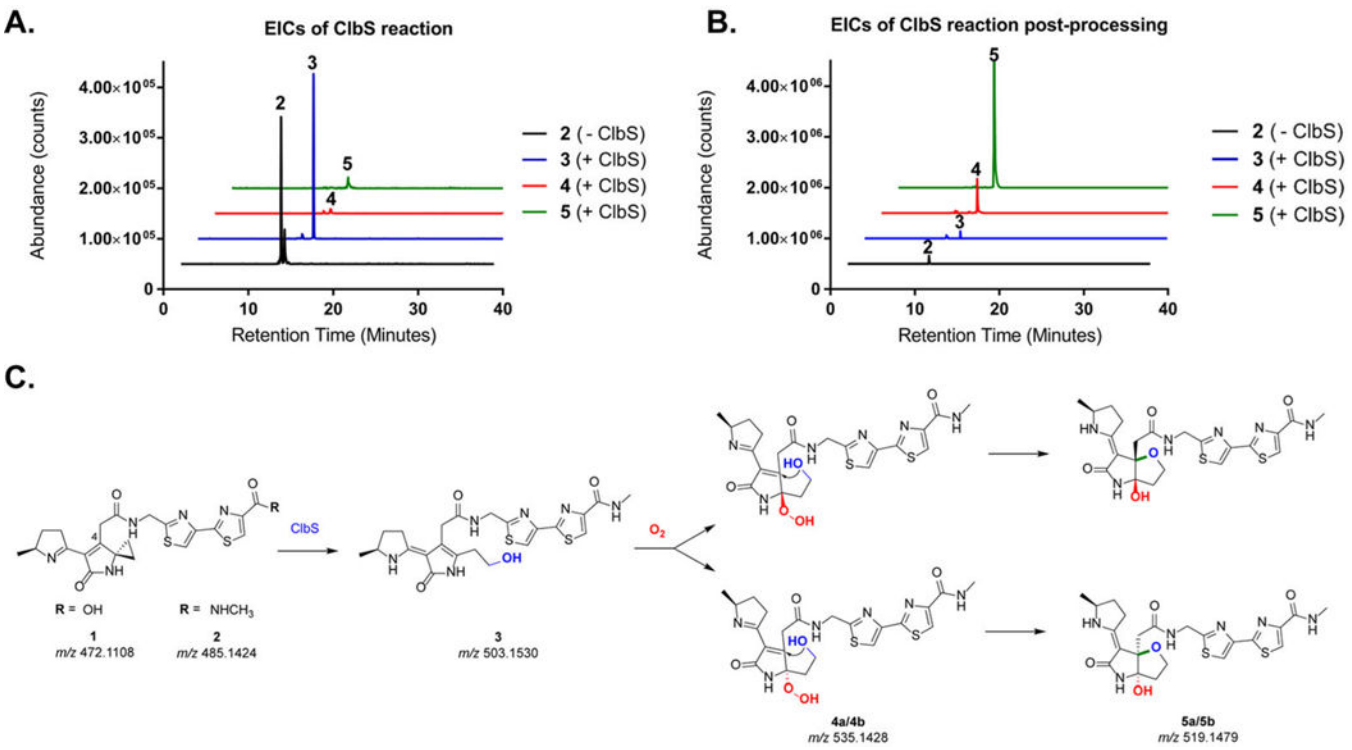

Figure 2.

(A) Extracted ion chromatograms (EICs) of the ClbS reaction (30 min end point assays), displaying representative no-enzyme control (*black*) peak for starting material **2**, and major proposed enzyme-dependent product **3**. The hydrolysis product **3** converts slowly to the tetahydrofuranol **5** after longer incubation times (16 h, Figure S2) and during isolation. The conversion of **3** to **5** may involve the alkyl hydroperoxides **4a/4b**, which were detected by HRMS but were not isolable. Ions extracted with a 10-ppm window around calculated *m/z* values are shown. Observed *m/z* values (corresponding to M + H) are within 2 ppm of calculated, as measured by QTOF-LC-MS (Table S1). **(B)** EICs of large-scale ClbS reaction (16 h end point assay) after attempted purification of **3**. Conversion of peaks for **3** to **5** via **4** is observed. **(C)** Proposed transformations of observed ions. Supporting MS/MS spectra for products **2**−**5** and NMR spectroscopic data for **5** are presented in the Supporting Information.

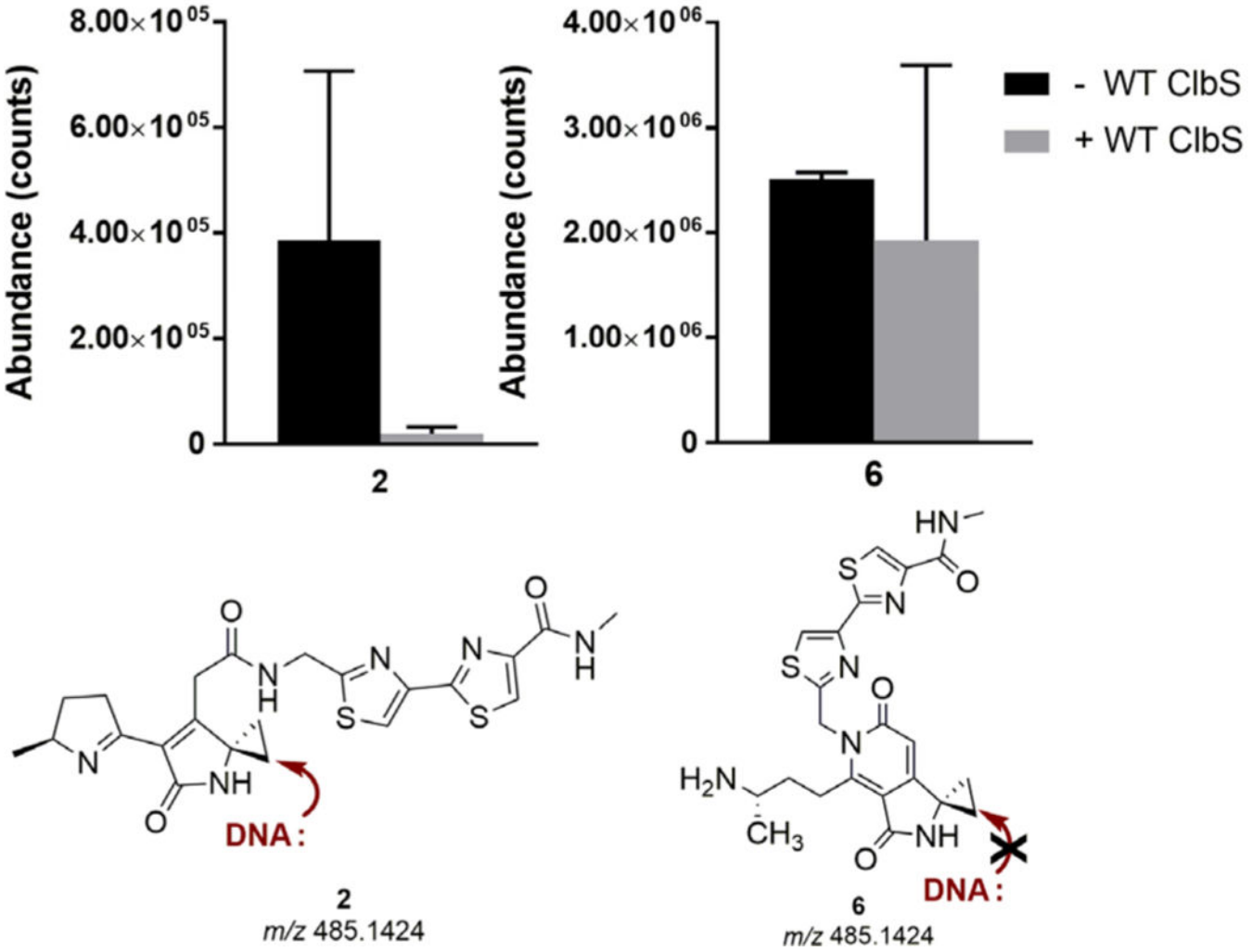

Figure 3.

Comparison of ClbS-mediated hydrolysis, illustrating marked substrate selectivity that discriminates between lactam-containing colibactin model **2** and isomeric pyridone-containing compound **6**. Averages with standard deviations for substrates are plotted. Hydrolysis products of **6** were not detected.

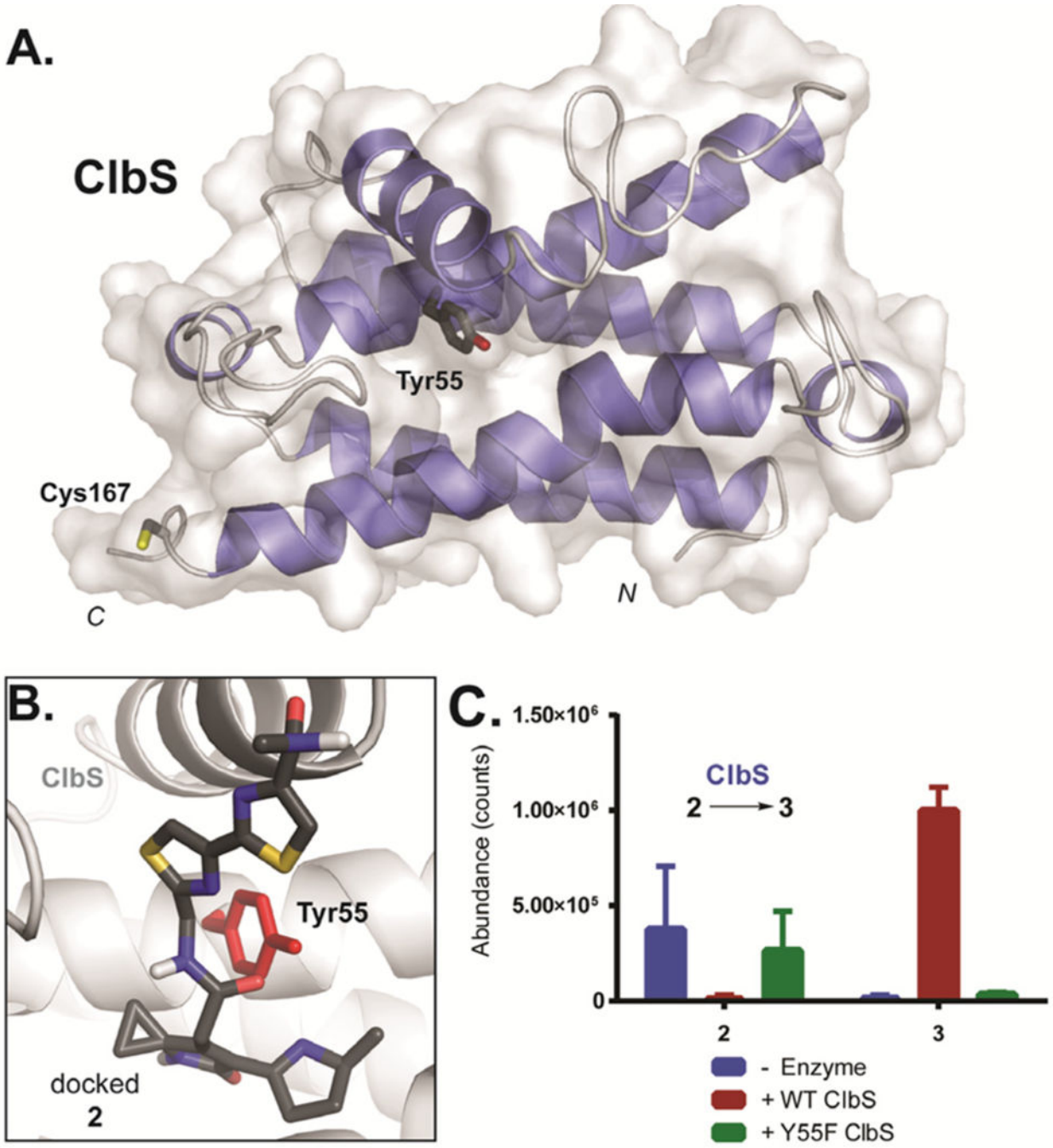

Figure 4.

(A) X-ray structure of ClbS showing active site residue Tyr55 and Cys167. **(B)** Automated docking model of **2** binding ClbS places the substrate in proximity to Tyr55. **(C)** WT ClbS and Y55F ClbS display different hydrolytic activity toward **2.**