Model colibactins exhibit human cell genotoxicity in the absence of host bacteria

Emilee E. Shine1,2*, Mengzhao Xue3*, Jaymin R. Patel2,4, Alan R. Healy2,3, Yulia V. Surovtseva5, Seth B. Herzon3,6*, Jason M. Crawford1,2,3*

1Department of Microbial Pathogenesis, Yale School of Medicine, New Haven, Connecticut 06536, United States
2Chemical Biology Institute, Yale University, West Haven, Connecticut 06516, United States
3Department of Chemistry, Yale University, New Haven, Connecticut 06520, United States
4Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, Connecticut, 06516, United States
5Yale Center for Molecular Discovery, West Haven, Connecticut 06516, United States
6Department of Pharmacology, Yale School of Medicine, New Haven, Connecticut 06520, United States

Supporting Information Placeholder

ABSTRACT: Colibactins are genotoxic secondary metabolites produced in select Enterobacteriaceae, which induce downstream DNA double-strand breaks (DSBs) in human cell lines and are thought to promote the formation of colorectal tumors. Although key structural and functional features of colibactins have been elucidated, the full molecular mechanisms regulating these phenotypes remain unknown. Here, we demonstrate that free model colibactins induce DSBs in human cell cultures and do not require delivery by host bacteria. Through domain-targeted editing, we demonstrate that a subset of native colibactins generated from observed module skipping in the nonribosomal peptide synthetase-polyketide synthase (NRPS-PKS) biosynthetic assembly line share DNA alkylation phenotypes with the model colibactins in vitro. However, module skipping eliminates the strong DNA interstrand cross-links formed by the wildtype pathway in cell culture. This product diversification during the modular NRPS-PKS biosynthesis produces a family of metabolites with varying observed mechanisms of action – DNA alkylation versus crosslinking – in cell culture. The presence of membranes separating human cells from model colibactins attenuated genotoxicity, suggesting that membrane diffusion limits colibactin activity and could account for the observed bacteria-human cell-to-cell contact phenotype. Additionally, extracellular supplementation of the colibactin resistance protein ClbS was able to intercept colibactins in an E. coli-human cell transient infection model. Our studies demonstrate that free model colibactins recapitulate cellular phenotypes associated with module-skipped products in the native colibactin pathway and define specific protein domains that are required for efficient DNA interstrand crosslinking in the native pathway.

Perturbations in community structure of the gut microbiota coupled with shifts in host-microbe metabolism can lead to a variety of altered host physiologies associated with diseases, such as inflammatory bowel disease and cancer.1–3 A prominent, yet rare, example of a one-pathway, one-phenotype correlation in the microbiome is that of the colibactin pathway.4–8 The colibactin gene cluster (clb) contains 19 genes (clbA–clbS) that encode the biosynthesis of hybrid polyketide synthase–nonribosomal peptide synthetase (PKS-NRPS) secondary metabolites known as colibactins. The clb locus is found in many strains of Enterobacteriaceae including select strains of gut-commensal and extraintestinal pathogenic E. coli (ExPEC) and Klebsiella pneumoniae, among others.9, 19 The pathway is associated with virulence17 and is significantly more prevalent in patients with inflammatory bowel disease (IBD), colorectal cancer (CRC), and familial adenomatous polyposis (FAP).12–14

A growing number of studies support a causative role for the metabolites in colorectal cancer formation. For example, when co-cultured with colibactin-expressing strains, mammalian cells accumulate DNA double-strand breaks (DSBs), activate ataxia-telangiectasia mutated kinase (ATM) signaling, and undergo cell cycle arrest and senescence.9, 15, 16 Clb+ E. coli induce tumor formation in three in vivo models of CRC12, 17, 18. Synthetic colibactin derivatives of metabolites from clb+ bacteria are genotoxic in vitro15. Finally, it was recently shown that incubation of exogenous DNA with colibactin-expressing strains induces DNA interstrand croslinkss in a pathway-dependent manner.20 Characterization of colibactins has been difficult; to date, no colibactin has been directly isolated from any producing strain. This has been attributed to their low levels of natural production and/or putative instability.21 Progress has been made using a combination of biochemical characterization of pathway enzymes22–27, comparative metabolomics21, 28, 29, isolation from large-scale fermentation of genetically-modified mutants,30, 31 and chemical synthesis.7, 19, 29, 32 This body of research has revealed important aspects of colibactin biosynthesis, structure, and function. First, colibactins are assembled by the NRPS-PKS biosynthetic machinery as prodrugs with an appended N-acetyl-asparagine side chain.22, 28, 33 These “precolibactins” are
transported into the periplasm by the 12-transmembrane 
transporter ClbM. The N-acyl-d-Asn residue is then removed 
by the pathway-dedicated membrane-bound peptidase 
(ClbP). Upon deaclylation, spontaneous cyclization reactions 
come into play to generate genotoxic colibactin structures.

Deletion of any one of the biosynthetic genes within the 
colibactin pathway results in abrogation of DSBs in cell culture. 
Access to fully-functionalized colibactins is desirable to understand 
their trafficking from the periplasmic space and mode of action in 
eukaryotic cells. However, to the best of our knowledge, a metabolite that accounts for all of the genes in the 
clb gene cluster has not been isolated or predicted with experimental 
support.

**Figure 1.** Synthetic colibactins exhibit cellular genotoxicity. (A), Structures of synthetic colibactins that previously demonstrated *in vitro* DNA alkylation activity. (B), HeLa cells were treated with 11 µM of compounds 2-6 for 4 hours and subsequently fixed and stained for a marker of cellular DNA damage, γH2AX. As a positive control, 1 µM of etoposide is shown for comparison. Quantification of γH2AX induction relative to that of etoposide is shown in the supplementary information. (C), Of the model colibactins, only dimer 6 shows significant activation of 53BP1, a downstream effector of ATR kinase signaling, a phenotype induced by the native colibactin pathway.

Model (pre)colibactins alkylate and crosslink DNA *in vitro*. Previous studies identified colibactin 1, which was detected in ClbP-proficient strains (Fig. 1A). Derivatives of 1, such as the N-methylamide 2 and dimethylene diamine derivative 3 alkylated DNA *in vitro* by nucleotide addition to the electron deficient cyclopropyl residue.

We have shown that linear biosynthetic products are off-loaded from the pathway and that they undergo spontaneous cyclization to unsaturated imines, such as 1, via intermediates resembling 4. We previously synthesized 4 and found that it is genotoxic *in vitro*, indicating that cyclization to an unsaturated imine is facile. The N-methylamide analog 5, which was designed to block formation of an inactive pyridone isomer, was also genotoxic. Finally, the dimer 6 was synthesized and examined for functional comparison. With the exception of 6, all compounds incubated with linearized plasmid demonstrated extensive alkylation and degradation of duplex DNA, with cyclic imine and cationic cap moieties enhancing potency down to the nanomolar range. Compound 6 crosslinked DNA, as expected.

Herein, we evaluated the abilities of the model colibactins 2-6 to recapitulate the genotoxic phenotypes associated with colibactins produced in transiently infected human tissue cultures. Human cell (HeLa, U2OS) DNA damage was quantified by immunofluorescence imaging of the DNA damage marker phospho-SER139-histone H2AX (γH2AX). The effects of the compounds incubated for 4 h at 16 doses ranging from 230 nM to 100 µM were quantified relative to 1 µM etoposide as a positive control (set at 100% effect) and DMSO vehicle as a negative control (0% effect). All of the compounds induced γH2AX activation and were cytotoxic. Aside from a few exceptions, we could establish reliable half-maximal inhibitory concentration

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<th>Compound</th>
<th>Cell Line</th>
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<tr>
<td>2</td>
<td>HeLa</td>
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<td>n.d.</td>
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<tr>
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<tr>
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Table 1. Half-maximal effective concentrations for γH2AX activation. Effect of compounds on γH2AX foci was quantified relative to 1 µM etoposide and graphed on a XY plot with a nonlinear regression curve fit. IC₅₀ values were calculated unless the fit was ambiguous (n.d.).
we previously observed U2OS cells were transiently infected with 0.2% of very weak DNA in-denaturing in the PKS module of ClbK and in the NaOH percentages red demonstrating ap-10 or 1 nal require-sporters as a DNA a. investigations, we noted that in-vitro c for-alkyl hydroperoxide. not the is as at the 2 transport and Active site p 3 27, 39 genetic screen of bleomycin 19, 24 We hypothe-oint mu-(Fig. S11). Of the suite of compounds evaluated, only this residue may in-, 3 without in-vitro However, revealed mutations in polyamine tran-alkylate DNA, µ, i. in 27 but not in-vitro cing, with high micromolar c- elements detected in cell culture, can crosslink DNA in vitro, although these crosslinks showed different stability compared to DNA crosslinked by native clb+ strains.

Compounds 2–6 are approximately ten-fold less potent than etoposide in tissue culture, yet they induce comparable levels of DNA damage at nanomolar concentrations in vitro. We speculated that this discrepancy is due to issues of transport and membrane permeability and might be related to biosynthetic events not represented in the current model colibactins. The model colibactins employed in this study do not contain a α-aminomalonate extender unit that is known to be incorporated by the PKS module of NRPS-PKS hybrid protein ClbK. While the genes dedicated to the synthesis and incorporation of this residue are essential for genotoxicity in the transient infection model, the only colibactin metabolite known to date that contains this motif has not been reported to harbor geno-toxicity similar to fully functionalized native colibactin. This is in part due to the retention of the N-acyl-D-asparagine side chain, as well as possibly due to the missing biosynthetic addi-tions provided by the largely uncharacterized enzymes ClbO and ClbL in the pathway. Though the exact functional require-ment of the α-aminomalonate unit is as-of-yet unclear, it has been speculated that the basic amine in this residue may in-crease DNA affinity and enhance cellular permeability. Indeed, a genetic screen of bleomycin-resistant Saccharomyces cerevisiae revealed mutations in polyamine transporters as a key bleomycin-resistant determinant, implying that an amino-containing cationic cap could enhance nuclear delivery.

To further explore these functional consequences of the un-accounted clb enzymes, we evaluated the genotoxicity of pathway-mutants both in vitro and in the transient infection model in comparison to our model colibactins. Active site point mu-tations were constructed in the PKS module of ClbK and in the peptidase ClbL based on protein sequence alignments to known ketosynthase and homologous amidase domains, as previously described. U2OS cells were transiently infected as the concentration of base arose during denaturing gel assays (Fig 2A, Fig. S7-S9). However, crosslinks resulting from natively expressing clb+ strains remain stable after denaturing with higher NaOH percentages (Fig. S11). The data indicate that colibactin analogs, which closely resemble known metabolites detected in cell culture, can crosslink DNA in vitro, although these crosslinks showed different stability compared to DNA crosslinked by native clb+ strains.

Figure 2. Model colibactins are able to both strongly alkylate and weakly crosslink DNA, compared to the full biosynthetic path-way capable of producing a major metabolite that strongly crosslinks DNA and minor pathway intermediates that alkylate DNA, as a result of evolutionarily encoded product diversification. (A) Incubation of Compound 3 with linearized pBR322 at pH 5 reveals clear crosslinks under mild denaturing conditions (50 mM/0.2% NaOH, on ice). (B) Incubation of linearized pBR322 with isogenic mutants of the clb pathway for 4 hours demonstrate varying levels of DNA alkylation activity. Denaturing gels were run at 50 mM (0.2%) NaOH.
with DH10B E. coli carrying the full clb pathway, clbK point mutant (C167A), clbL point mutant (S179A), or empty BAC vector control, and γH2AX activation was analyzed by flow cytometry (Fig. S10). Both point mutants ablated the genotoxic effects of the pathway, demonstrating that these specific catalytic activities are essential for clb- E. coli in inducing DSBs in cell culture.

To remove uncharacterized human cell trafficking aspects of the transient infection model, we next analyzed these strains for their ability to damage exogenously supplied DNA using denaturing gel assays (Fig. 2B). E. coli negative controls lacking the pathway (clb-) or fully deleted for the PKS clbO were inactive as expected. However, E. coli with the inactive PKS module in clbK or inactive peptidase clbL exhibited DNA damage, but not crosslinking. Because the complete pathway leads to stable DNA interstrand crosslinks, as described above and elsewhere, relative to the model colibactins (Fig S11), additional structural modifications afforded by the PKS module of ClbK, PKS ClbO, and amidase CblB transform the potent DNA alkylators with weak-to-moderate crosslinking activities into a highly efficient DNA interstrand crosslinker. The activity of the clbK and clbL mutants in vitro is consistent with the extensive DNA alkylation and degradation that we have observed for model colibactins, which are analogs of metabolites produced by biosynthetic PKS module skipping of ClbK. Thus, while model colibactins do not fully reproduce the cellular phenotypes induced by fully functionalized colibactins, they do phenotypically recapitulate a subset of characterized on-pathway intermediates. This illustrates how product diversification evolutionarily encoded by the modular NRPS-PKS biosynthetic enzymes can generate functionally distinct products with varying modes of action. In addition, the alkylation activity for clbK and clbL mutants in vitro, but not in the transient infection model, again suggests that these uncharacterized enzymatic modifications enhance transport and cellular permeability.

The nature of colibactin transport is largely unknown; however, bacterial-human cell-to-cell contact is necessary for genotoxicity. This has largely puzzled researchers, given that expression of the clb locus does not depend on the presence of mammalian cells yet outer membrane vesicles isolated from clb-expressing strains in some studies confer genotoxicity when applied, yet membranes separating clb-expressing bacteria from mammalian cells or thick adherent intestinal mucus layers abolishes genotoxicity. To test whether the genotoxic effects of synthetic colibactins were eliminated by membrane separation, we treated U2OS cells with 50 µM 3 for 4 h in the presence or absence of separating 0.45 µm membranes, and γH2AX activation was analyzed by flow cytometry (Figure 3). While the effect was not drastic in comparison to the complete ablation of genotoxicity caused by the membrane in the presence of clb+ E. coli, the membrane did diminish the γH2AX response of 3 (Figure 3). To test whether the presence of bacteria affected the activity of 3, we pretreated the membranes with clb- or clb+ bacteria for 30 min before adding 3. The γH2AX response was also diminished in this experiment. This suggests that diffusion of 3 across the membrane is slow, or potentially deleterious interactions with the membrane itself impede transport into cellular targets attenuating genotoxic activity.

Given that free model colibactins induce DNA double strand breaks in human cells, we evaluated whether ClbS could protect human cells when delivered extracellularly. We found that when purified ClbS was exogenously supplemented (at 1 µM in

**Figure 3.** (A) 6.0 x 10⁵ U2OS cells were treated with 50 µM compound 3 in the presence or absence of a 0.45 µm membrane separating the bacteria from the U2OS cells for four hours. Cells were immediately fixed and stained for γH2AX and quantified by flow cytometry. (B) Membranes were pretreated with equal numbers of clb- or clb+ bacteria grown to OD=1.0 at MOI = 10 for 30 minutes prior to addition of 3 for four hours. (C) Native colibactins from live expressing clb+ or control (clb-) cells applied with or without membranes to U2OS cells, and subsequently evaluated for DNA double stranded breaks. (D) Percentage of H2AX+ positive foci of each treatment condition, normalized to 5% H2AX+ for the negative control, DMSO vehicle. Results are shown as means and standard deviation (s.d.). P value = 0.0241, (∗ = < 0.05). P value obtained with an unpaired t-test with Welch’s correction.
our studies), U2OS cells were protected from clb- E. coli, as determined by quantification of γH2AX staining (Fig. 4; 10 μM ClbS studies are shown in Fig. S12). While this work was in progress, a similar observation was reported.20 Additionally, we had previously identified the active site Tyr residue Y55 in ClbS as critical for cyclopropane hydrolyase activity.27 When 1 μM of the ClbS Y55F mutant was supplemented to U2OS cells transiently infected with clb- E. coli, protective effects were not observed (Fig. 4). These data indicate that the previous protein biochemical studies using purified ClbS and model colibactins are consistent with the activities observed in cell cultures. These data also suggest that native colibactins can be interrupted by extracellularly supplemented ClbS. The results of these experiments also suggest that direct cell-to-cell contact might not be required for native colibactin activity. We propose that poor diffusion and chemical instability contribute to the observed bacteria-human cell-to-cell contact phenotype.

In conclusion, we have demonstrated that model colibactin analogs can recapitulate cellular genotoxic phenotypes consistent with native clb+ E. coli infection by γH2AX and 53BP1 foci formation. In addition, we have shown that these compounds also reconstitute unstable DNA interstrand crosslinks in vitro. DNA interstrand crosslinking has recently been supported as the primary mode of action of the mature, final pathway product.25 However, several modifications relative to the model colibactins, which are encoded in the PKS module of ClbK, PKS ClbO, and peptidase Clbα, are needed to recapitulate efficient stable crosslinks of the native colibactins produced by the full pathway. Our membrane studies, in combination with the ability of exogenous ClbS to intercept and neutralize native colibactins, call into question the absolute necessity of cell-to-cell contact for colibactin’s genotoxic action. Our work also provides important new insights toward resolving the remaining questions surrounding colibactin’s mature structure and mode of action.

**METHODS**

**Mammalian cell Lines and Reagents.** U2OS and HeLa cell lines were obtained from American Type Culture Collection (ATCC). For microscopy, U2OS cells were cultured in McCoy’s 5A medium (Fisher Scientific) supplemented with 10% fetal bovine serum (FBS) (Life Technologies). For flow cytometry studies, U2OS cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Life Technologies) F12 with 25 mM HEPES and 5% FBS. HeLa cells were cultured in DMEM with 10% FBS. All cells were maintained at 37 °C with 5% CO2. Antibodies for immunofluorescence were purchased from Upstate (phospho-specific H2AX, 05-636), Novus Biologicals (53BP1, NB100-904SS), or Molecular Probes (Alexa Fluor 488-conjugated goat anti-mouse immunoglobulin G (IgG) and Alexa Fluor 647-conjugated goat anti-rabbit IgG). Antibodies for flow cytometry quantification were purchased from Cell Signaling (Ph-histone H2AX S139 rabbit) and Life Technologies (Alexa Fluor 647, REF A21245).

**Bacterial Strains and Growth Conditions.** The E. coli strains used in this study were DH10B containing the colibactin gene cluster on pBelobAC11 (clb+ or empty vector alone (clb-))2. The clbL, clbK, and clbO mutants were constructed as previously described22. Bacteria were grown overnight in LB and diluted into DMEM F12 15 mM HEPES with chloramphenicol (25 μg/mL) for assays. Standard growth conditions were 37 °C with 250-rpm agitation.

**Synthesis of Colibactin Analogs.** Compounds were prepared as previously described.23

**Immunofluorescence Assay.** Cells were seeded at 2500 (HeLa) or 5000 (U2OS) cells per well to achieve total well volumes of 20 μL in 384-well plates (black with optically clear bottom, Greiner Bio One 781091) using a Thermo Combidrop liquid dispenser. Cells were grown for 24 h, followed by the addition of test compounds using Echo acoustic liquid handler (Labcyte). For each tested drug concentration, 20 nL aliquot of the 1000× stock was added to 20 μL of cells to provide final DMSO concentration of 0.1%. Each plate contained 16 negative vehicle control wells (0.1% DMSO) and 16 positive control wells (1 μM etoposide). The cells were incubated with the compounds for 4 h and then fixed and subjected to immunofluorescence.

**Immunofluorescence.** Cells were fixed with 4% paraformaldehyde (PFA) (Electron Microscopy Sciences) in the presence of 0.02% Triton X-100 at room temperature for 20 min and then incubated in permeabilization/blocking solution (10% FBS, 0.5% Triton X-100 in phosphate-buffered saline (PBS)) at room temperature for 1 h. Primary antibodies were diluted 1:500 in permeabilization/blocking solution and used to stain cells at 4 °C overnight. Cells were imaged using the InCell 2200 Imaging System (GE Corporation), and analyzed using InCell Analyzer software (GE Corporation) to quantify the number of γH2AX and 53BP1 foci. The effect of the test compounds was normalized to the mean of positive control wells (1 μM etoposide, set as 100% effect) and the mean of negative control well (0.1% DMSO, set as 0% effect).

![Figure 4. Protection from Genotoxicity by Supplementation of Extracellular ClbS. 6.0 x 10^5 U2OS cells were transiently infected with clb-expressing bacteria at MOI=10 for four hours. Media was replaced and 50 μg/mL gentamycin were added. Cell fixation and γH2AX antibody staining occurred 16 hours after infection to monitor downstream DSBs. γH2AX positive cells were quantified using flow cytometry.](image-url)
DNA crosslinking assay. The 4.163 bp plasmid pBR322 was purchased from NEB and linearized with Sau3A I (NEB). The cut plasmid was purified using PCR clean kit (NEB) and eluted into 10 mM Tris pH 8.0. For each reaction with synthetic colicin A 2-6, 200 ng of linearized DNA (31 μM base pairs) was incubated with compound in a 20 μL total volume. Compounds were diluted in DMSO such that each reaction consisted of a fixed 5% DMSO concentration. Reactions were conducted in 10 mM Tris EDTA buffer pH 8.0 or in 10 mM sodium citrate pH 5.0 as labeled on the figure. Reactions proceeded for 3 hours at 37 °C, unless otherwise noticed. For each reaction with bacteria, 600 ng linearized plasmid DNA was added to 150 μL of DMEM/F12 15 mM Hepes (Invitrogen) inoculated with 6 x 10⁶ bacteria pre-grown to exponential phase in the DMEM/F12 15 mM Hepes (Invitrogen) media. The DNA-bacteria mix were incubated at 37 °C for 4 hours, and the bacteria were then pelleted. The DNA was isolated from the supernatant using PCR clean kit (NEB) and quantified using nanodrop. The DNA concentration was adjusted to 10 ng/μL using water and stored in −20 °C until ready for gel analysis. Pure methyl methanesulfonate (MMS) (Alfa Aesar) and cisplatin (Biovision) stock solutions were diluted into DMSO immediately prior to use. As controls, 200 ng of DNA was treated with 80 μM of cisplatin (Biovision) in 10 mM sodium citrate pH 5 buffer with 5% final DMSO concentration. The DNA was immediately tested with gel electrophoresis after incubation.

DNA gel electrophoresis. For each DNA sample, the concentration was pre-adjusted to 10 ng/μL. 4 μL (40 ng) of DNA was taken out and mixed with 1.5 μL of 6x purple gel loading dye; no SDS (NEB) for non-denatured gel. For denatured gels, 5 μL (50 ng) of DNA was taken out each time and separately mixed with 15 μL of 0.2% denaturing buffer (0.27% sodium hydroxide, 10% glycerol, 0.013% bromophenol blue), 0.4% denaturing buffer (0.53% sodium hydroxide, 10% glycerol, 0.013% bromophenol blue), or 1% denaturing buffer (1.33% sodium hydroxide, 10% glycerol, 0.013% bromophenol blue) on an ice bath. The mixed DNA samples were denatured in 4 °C for 10 min and immediately loaded onto 1% agarose Tris Borate EDTA (TBE) gels for 1.5 hour at 90 V. The gel was post stained with SybrGold (Thermo Fisher) for 2 hours.

Membrane Transport Assay. 6 x 10⁵ U2OS cells were seeded in 6-well plates and treated with 50 μM compound 3 diluted in 300 μL suspended on the surface of a 0.45 μm membrane (Millipore) for four hours. DH10B clb− or clb+ cells at OD₆₀₀ = 1.0 were diluted into 300 μL DMEM at MOI of 10 and suspended on the surface of the 0.45 μm membrane to separate bacteria from the U2OS cells.

ClbS Protection Assay. Expression and purification of ClbS was performed as previously described.²⁷ Briefly, 1 L of BL21 cells carrying either pET28-ClbS-His or pET28-ClbS/S55C were induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and grown overnight at 25°C. Bacteria were lysed in lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 10 mM imidazole, pH 8, 1 mg/mL of lysozyme freshly added) and purified on a 2 mL bed volume of HisPur Ni-NTA resin (Thermo Scientific). Proteins were eluted in 250 mM imidazole, 100 mM Tris, 300 mM NaCl, 10% glycerol (pH 8). Purified proteins were separated over a 15% SDS-PAGE gel to verify size and relative purity, and then buffer exchanged into 50 mM potassium phosphate buffer (pH 8) using PD-10 Desalting Columns (GE). 60 x 10⁵ U2OS were infected with either clb− or clb+ bacteria at OD₆₀₀ = 1.0, MOI 10 with either 1 μM or 10 μM ClbS/ClbS/S55C or BSA and incubated at 37 °C for 4 hours.

Quantification of H2AX: Cells were immediately fixed and stained for γ-H2AX antibody and quantified by flow cytometry. Cells were fixed with 4% paraformaldehyde in PBS for 10 minutes, permeabilized with 90% ice cold methanol for 30 minutes on ice, and blocked in PBS with 1 mM CaCl₂, 1 mM MgCl₂ and 10% FBS for 10 minutes. Primary antibodies were diluted 1:100 in blocking solution and incubated overnight at 4°C. Secondary antibodies were diluted 1:1000 in blocking solution and incubated at room temperature for 2 hours. Cells were washed and resuspended in PBS before analysis on a FACSaria II (BD).

ASSOCIATED CONTENT
Supporting Information
Figures S1-S12 (PDF).

Corresponding Author
* Corresponding authors. Please address correspondence to jason.crawford@yale.edu or seth.herzon@yale.edu.

Author Contributions
* E.E.S. and M.X. contributed equally.

Notes
The authors declare no competing financial interests.

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