Supplementary Information

Rapid Production and Characterization of Antimicrobial Colicins Using *Escherichia coli*-based Cell-Free Protein Synthesis

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Supplementary Table S1. All primers used in this study. '*' indicates phosphorothioated bases.

Primer	Purpose	Sequence (5' → 3')
pJL1-F	pJL1 backbone for Gibson Assembly	CAAAGCCCGAAAGGAAGCTGAGTTG
pJL1-R		CTTAAAGTTAAACAAAATTATTTCTAGAGGG
Gacol-F	Amplification of *cma* gene encoding colicin M	TTTGTTTAACTTTAAGAAGGAGATATACATATG
Gacol-R		TTCCTTTCGGGCTTTGTTAGCAGCCGGTCGACTTA
ColE1-F	Amplification of *cea* gene encoding colicin E1	ATTTTGTTTAACTTTAAGAAGGAGATATACATATGGAAACCGCGGTAGCGTAC
ColE1-R		CGGGCTTTGTTAGCAGCCGGTCGACTTATTTTTCGAACTGCGGATGGCTCCAAATC CCTAACACCTCATTTATAG
ColE2-F	Amplification of *col* gene encoding colicin E2	ATTTTGTTTAACTTTAAGAAGGAGATATACATATGAGCGGTGGCGATGGACG
ColE2-R		CGGGCTTTGTTAGCAGCCGGTCGACTTATTTTTCGAACTGCGGATGGCTCCACTTA CCCCGATGAATATCAATATG
ColIa-F	Amplification of *cia* gene encoding colicin Ia	ATTTTGTTTAACTTTAAGAAGGAGATATACATATGTCTGACCCTGTACGTATTAC
ColIa-R		CGGGCTTTGTTAGCAGCCGGTCGACTTATTTTTCGAACTGCGGATGGCTCCAAATA CCCCAGAACTTATTCG
E2Imm-F	Amplification of *imm* gene encoding E2 immunity	ATTTTGTTTAACTTTAAGAAGGAGATATACATATGGAACTGAAACATAG
E2Imm-R		CGGGCTTTGTTAGCAGCCGGTCGACTCATTTTTCGAACTGCGGATGGCTCCAGCCC TGTTTAAATCC
T7Mega-F	T7 promoter addition	A*G*ATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGAGACCACAACGGTT TCCCTCTAGAAATAATTTTGTTTAACTTTAAGAAGG
TAA-R	Addition of overlap region with T7 terminator	TATTGCTCAGCGGTGGCAGCAGCCAACTCAGCTTCCTTTCGGGCTTTGTTAGCAGC CGG
T7Mega-R	T7 terminator addition	T*A*ATCAGAATTGGCTTTCAGCAAAAAACCCCTCAAGACCCGTTTAGAGGCCCCA AGGGGTTATGCTAGTTATTGCTCAGCGGTGGCAGC
T7-Pro	Sequence checking for colicin amplification	TAATACGACTCACTATAGGG
T7-Ter		GCTAGTTATTGCCTCAGCGG

Supplementary Table S2. DNA sequences of all colicin genes used in this study. T7 promoter, terminator, translocation domain, receptor-binding domain, and cytotoxicity domain are highlighted as blue, red, turquoise, yellow, and green, respectively. Bolded lowercase characters indicate Strep-tag sequences.

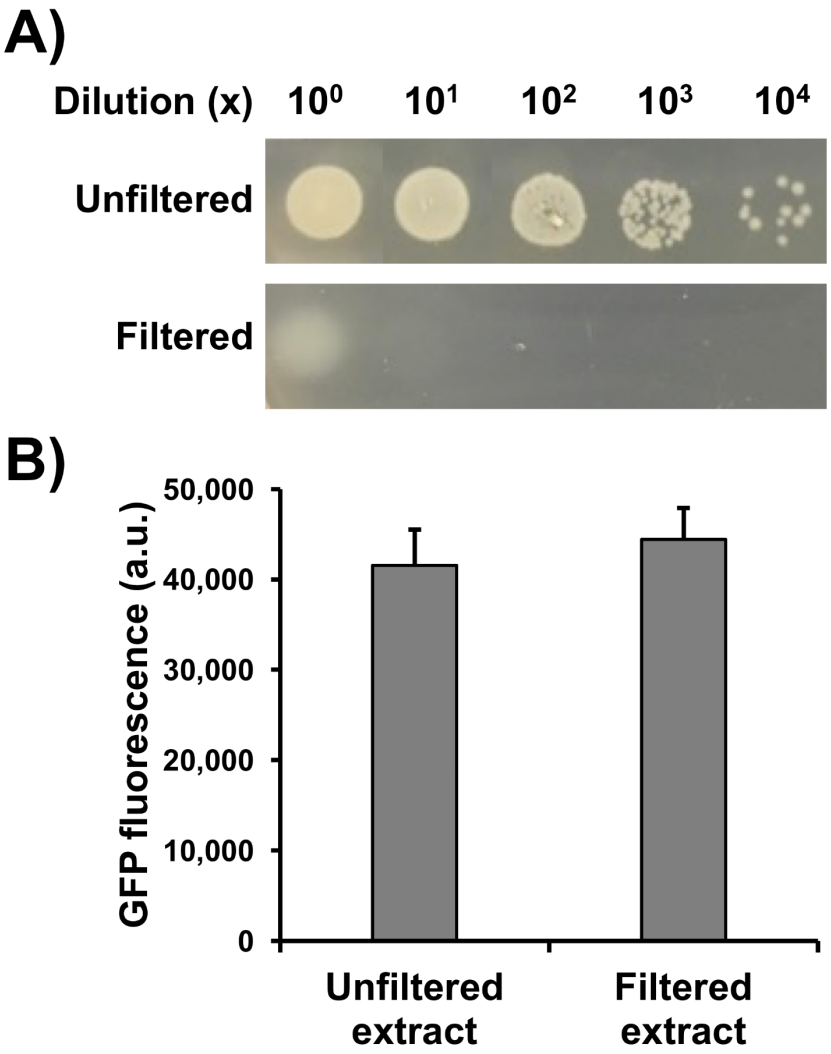
Name	Size (bp)	Sequence 5' → 3'
cma encoding M	1073	AGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTT TAACTTTAAGAAGGAGATATACATATGGAAACCTTAACTGTTCATGCACCATCACCATCAACTAACTTACCAAGTT ATGGCAATGGTGCATTTTCTCTTTCAGCACCGCATGTTCCTGGTGCTGGACCTCTTTTAGTCCAGGTTGTTTATAG TTTTTTCCAGAGTCCAAACATGTGTCTTCAGGCTTTAACTCAACTTGAGGATTACATCAAAAAACATGGGGCTAGC AACCCTCTCACATTGCAGATCATATCGACAAATATTGGTTACTTCTGTAACGCCGACCGAAATCTGGTTCTTCACC CTGGAATAAGCGTTTATGACGCTTACCACTTCTCAAAACCAGCGCCAAGTCAATATGACTATCGCTCAATGAATAT GAAACAAATGAGCGGTAATGTCACTACACCAATTGTGGCGCTTGCTCACTATTTATGGGGTAATGGCGCTGAAAGG AGCGTTAATATCGCCAACATTGGTCTTAAAATTTCCCCTATGAAAATTAATCAGATAAAAGACATTATAAAATCTG GTGTAGTAGGTACATTCCCTGTTTCTACAAAGTTCACACATGCCACTGGTGATTATAATGTTATTACCGGTGCATA TCTTGGTAATATCACACTGAAAACAGAAGGTACTTTAACTATCTCTGCCAATGGCTCCTGGACTTACAATGGCGTT GTTCGTTCATATGATGATAAATACGATTTTAACGCCAGCACTCACCGTGGCGTCATCGGAGAGTCGCTCACAAGGC TCGGGGCGATGTTTTCTGGTAAAGAGTACCAGATACTGCTTCCTGGTGAAATTCACATTAAAGAAAGTGGTAAGCG A**tggagccatccgcagttcgaaaaa**TAAGTCGACCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTG CCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGCCAA TTCTGATTA
cia encoding Ia	2138	AGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTT TAACTTTAAGAAGGAGATATACATATGTCTGACCCTGTACGTATTACAAATCCCGGTGCAGAATCGCTGGGGTATG ATTCAGATGGCCATGAAATTATGGCCGTTGATATTTATGTAAACCCTCCACGTGTCGATGTCTTTCATGGTACCCC GCCTGCATGGAGTTCCTTCGGGAACAAAACCATCTGGGGCGGAAACGAGTGGGTTGATGATTCCCCAACCCGAAGT GATATCGAAAAAAGGGACAAGGAAATCACAGCGTACAAAAACACGCTCAGCGCGCAGCAGAAAGAGAATGAGAATA AGCGTACTGAAGCCGGAAAACGCCTCTCTGCGGCGATTGCTGCAAGGGAAAAAGATGAAAACACACTGAAAACACT CCGTGCCGGAAACGCAGATGCCGCTGATATTACACGACAGGAGTTCAGACTCCTGCAGGCAGAGCTGAGAGAATAC GGATTCCGTACTGAAATCGCCGGATATGACGCCCTCCGGCTGCATACAGAGAGCCGGATGCTGTTTGCTGATGCTG ATTCTCTTCGTATATCTCCCCGGGAGGCCAGGTCGTTAATCGAACAGGCTGAAAAACGGCAGAAGGATGCGCAGAA CGCAGACAAGAAGGCCGCTGATATGCTTGCTGAATACGAGCGCAGAAAAGGTATTCTGGACACCCGGTTGTCAGAG CTGGAAAAAAATGGCGGGGCAGCCCTTGCCGTTCTTGATGCACAACAGGCCCGTCTGCTCGGGCAGCAGACACGGA ATGACAGGGCCATTTCAGAGGCCCGGAATAAACTCAGTTCAGTGACGGAATCGCTTAACACGGCCCGTAATGCATT AACCAGAGCTGAACAACAGCTGACGCAACAGAAAAACACGCCTGACGGCAAAACGATAGTTTCCCCTGAAAAATTC CCGGGGCGTTCATCAACAAATGATTCTATTGTTGTGAGCGGTGATCCGAGATTTGCCGGTACGATAAAAATCACAA CCAGCGCAGTCATCGATAACCGTGCAAACCTGAATTATCTTCTGAGCCATTCCGGTCTGGACTATAAACGCAATAT TCTGAATGACCGGAATCCGGTGGTGACAGAGGATGTGGAAGGTGACAAGAAAATTTATAATGCTGAAGTTGCTGAA TGGGATAAGTTACGGCAAAGATTGCTTGATGCCAGAAATAAAATCACCTCTGCTGAATCTGCGGTAAATTCGGCGA

		GAAATAACCTCAGTGCCAGAACAAATGAGCAAAAGCATGCAAATGACGCTCTTAATGCCCTGTTGAAGGAAAAAGA GAATATCCGTAACCAGCTTTCCGGCATCAATCAGAAGATAGCGGAAGAGAAAAGAAAACAGGATGAACTGAAGGCA ACGAAAGACGCAATTAATTTCACAACAGAGTTCCTGAAATCAGTTTCAGAAAAATATGGTGCAAAAGCTGAGCAGT TAGCCAGAGAGATGGCCGGGCAGGCTAAAGGGAAGAAAATACGTAATGTTGAAGAGGCATTAAAAACGTATGAAAA GTACCGGGCTGACATTAACAAAAAAATTAATGCAAAAGATCGTGCAGCGATTGCCGCAGCCCTTGAGTCTGTGAAG CTGTCTGATATATCGTCTAATCTGAACAGATTCAGTCGGGGACTGGGATATGCAGGAAAATTTACAAGTCTTGCTG ACTGGATCACTGAGTTTGGTAAGGCTGTCCGGACAGAGAACTGGCGTCCTCTTTTTGTTAAAACAGAAACCATCAT AGCAGGCAATGCCGCAACGGCTCTTGTGGCACTGGTCTTCAGTATTCTTACCGGAAGCGCTTTAGGCATTATCGGG TATGGTTTACTGATGGCTGTCACCGGTGCGCTGATTGATGAATCGCTTGTGGAAAAAGCGAATAAGTTCTGGGGTA TT**tggagccatccgcagttcgaaaaa**TAAGTCGACCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCT GCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGCCA ATTCTGATTA
cea encoding E1	1826	AGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTT TAACTTTAAGAAGGAGATATACATATGGAAACCGCGGTAGCGTACTATAAAGATGGTGTTCCTTATGATGATAAGG GACAGGTAATTATTACTCTTTTGAATGGTACTCCTGACGGGAGTGGCTCTGGCGGCGGAGGTGGAAAAGGAGGCAG TAAAAGTGAAAGTTCTGCAGCTATTCATGCAACTGCTAAATGGTCTACTGCTCAATTAAAGAAAACACAGGCAGAG CAGGCTGCCCGGGCAAAAGCTGCAGCGGAAGCACAGGCGAAAGCAAAGGCAAACAGGGATGCGCTGACTCAGCGCC TGAAGGATATCGTGAATGAGGCTCTTCGTCACAATGCCTCACGTACGCCTTCAGCAACAGAGCTTGCTCATGCTAA TAATGCAGCTATGCAGGCGGAAGACGAGCGTTTGCGCCTTGCGAAAGCAGAAGAAAAAGCCCGTAAAGAAGCGGAA GCAGCAGAAAAGGCTTTTCAGGAAGCAGAACAACGACGTAAAGAGATTGAACGGGAGAAGGCTGAAACAGAACGCC AGTTGAAACTGGCTGAAGCTGAAGAGAAACGACTGGCTGCATTGAGTGAAGAAGCTAAAGCTGTTGAGATCGCCCA AAAAAAACTTTCTGCTGCACAATCTGAAGTGGTGAAAATGGATGGAGAGATTAAGACTCTCAATTCTCGTTTAAGC TCCAGTATCCATGCCCGTGATGCAGAAATGAAAACGCTCGCTGGAAAACGAAATGAACTGGCTCAGGCATCCGCTA AATATAAAGAACTGGATGAGCTGGTCAAAAAACTATCACCAAGAGCCAATGATCCGCTTCAGAACCGTCCTTTTTT TGAAGCAACCAGACGACGGGTTGGGGCCGGTAAGATTAGAGAAGAAAAACAAAAACAGGTAACAGCATCAGAAACA CGTATTAACCGGATAAATGCTGATATAACTCAGATCCAGAAGGCTATTTCTCAGGTCAGTAATAATCGTAATGCCG GTATCGCTCGTGTTCATGAAGCTGAAGAAAATTTGAAAAAAGCACAGAATAATCTCCTTAATTCACAGATTAAGGA TGCTGTTGATGCAACAGTTAGCTTTTATCAAACGCTGACTGAAAAATATGGTGAAAAATATTCGAAAATGGCACAG GAACTTGCTGATAAGTCTAAAGGTAAGAAAATCGGCAATGTGAATGAAGCTCTCGCTGCTTTTGAAAAATACAAGG ATGTTTTAAATAAGAAATTCAGCAAAGCCGATCGTGATGCTATTTTTAATGCGTTGGCATCGGTGAAGTATGATGA CTGGGCTAAACATTTAGATCAGTTTGCCAAGTACTTGAAGATTACGGGGCATGTTTCTTTTGGATATGATGTGGTA TCTGATATCCTAAAAATTAAGGATACAGGTGACTGGAAGCCACTATTTCTTACATTAGAGAAGAAAGCTGCAGATG CAGGGGTGAGTTATGTTGTTGCTTTACTTTTTAGCTTGCTTGCTGGAACTACATTAGGTATTTGGGGTATTGCTAT TGTTACAGGAATTCTATGCTCCTATATTGATAAGAATAAACTTAATACTATAAATGAGGTGTTAGGGATT**tggagc** **catccgcagttcgaaaaa**TAAGTCGACCGGCTGCTAACAAAGCCCGAAAGGAAGCTGAGTTGGCTGCTGCCACCGC TGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTGCTGAAAGCCAATTCTGAT TA

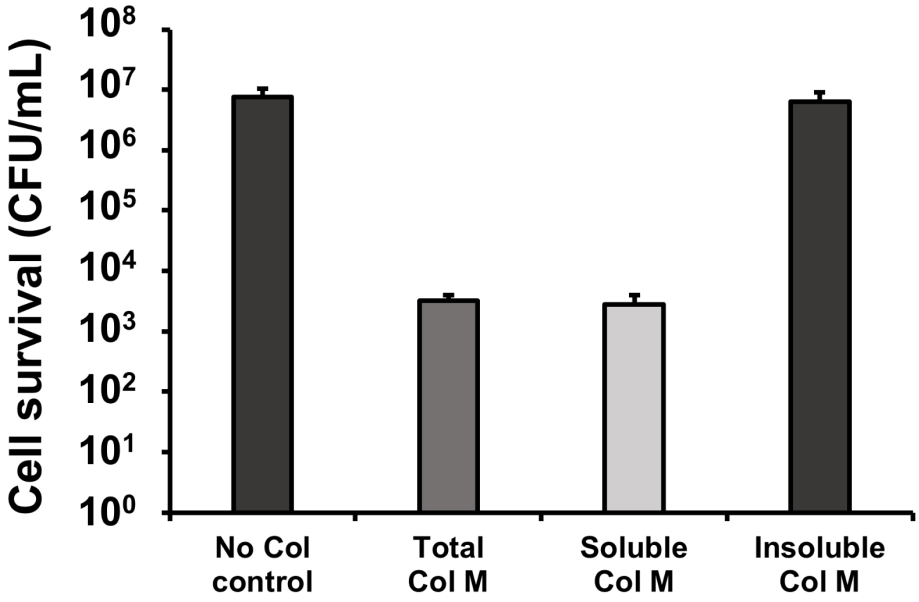
col encoding E2	2003	AGATCTCGATCCCGCGAAATTAATACGACTCACTATAGGGAGACCACAACGGTTTCCCTCTAGAAATAATTTTGTT TAACTTTAAGAAGGAGATATACATATGAGCGGTGGCGATGGACGCGGCCATAACACGGGCGCGCATAGCACAAGTG GTAACATTAATGGTGGCCCGACCGGGCTTGGTGTAGGTGGTGGTGCTTCTGATGGTTCCGGGTGGAGTTCGGAAAA TAACCCGTGGGGTGGTGGTTCCGGTAGCGGCATTCACTGGGGTGGTGGTTCCGGTCATGGTAATGGCGGGGGGAAT AGTAATTCCGGTGGTGGCTCGGGAACAGGCGGTAATCTGTCAGCAGTAGCTGCGCCAGTGGCATTTGGTTTTCCGG CACTTTCCACTCCAGGAGCTGGCGGTCTGGCGGTCAGTATTTCAGCGGGAGCATTATCGGCAGCTATTGCTGATAT TATGGCTGCCCTGAAAGGACCGTTTAAATTTGGTCTTTGGGGGGTGGCTTTATATGGTGTATTGCCATCACAAATA GCGAAAGATGACCCCAATATGATGTCAAAGATTGTGACGTCATTACCCGCAGATGATATTACTGAATCACCTGTCA GTTCATTACCTCTCGATAAGGCAACAGTAAACGTAAATGTTCGTGTTGTTGATGATGTAAAAGACGAACGACAGAA TATTTCGGTTGTTTCAGGTGTTCCGATGAGTGTTCCGGTGGTTGATGCAAAACCTACCGAACGTCCAGGTGTTTTT ACGGCATCAATTCCAGGTGCACCTGTTCTGAATATTTCAGTTAATAACAGTACGCCAGCAGTACAGACATTAAGCC CAGGTGTTACAAATAATACTGATAAGGATGTTCGCCCGGCAGGATTTACTCAGGGTGGTAATACCAGGGATGCAGT TATTCGATTCCCGAAGGACAGCGGTCATAATGCCGTATATGTTTCAGTGAGTGATGTTCTTAGCCCTGACCAGGTA AAACAACGTCAGGATGAAGAAAATCGCCGTCAGCAGGAATGGGATGCTACGCATCCGGTTGAAGCGGCTGAGCGAA ATTATGAACGCGCGCGTGCAGAGCTGAATCAGGCAAATGAAGATGTTGCCAGAAATCAGGAGCGACAGGCTAAAGC TGTTCAGGTTTATAATTCGCGTAAAAGCGAACTTGATGCAGCGAATAAAACTCTTGCTGATGCAATAGCTGAAATA AAACAATTTGATCGATTTGCCCATGACCCAATGGCTGGCGGTCACAGAATGTGGCAAATGGCCGGGCTTAAAGCCC AGCGGGCGCAGACGGATGTAAATAATAAGCAGGCTGCATTTGATGCTGCTGCAAAAGAGAAGTCAGATGCTGATGC TGCATTAAGTGCCGCGCAGGAGCGCCGCAAACAGAAGGAAAATAAAGAAAAGGACGCTAAGGATAAATTAGATAAG GAGAGTAAACGGAATAAGCCAGGGAAGGCGACAGGTAAAGGTAAACCAGTTGGTGATAAATGGCTGGATGATGCAG GTAAAGATTCAGGAGCGCCAATTCCAGATCGCATTGCTGATAAGTTGCGTGATAAAGAATTTAAAAACTTTGACGA TTTCCGGAAGAAATTCTGGGAAGAAGTGTCAAAAGATCCCGATCTTAGTAAGCAATTTAAAGGCAGTAATAAGACG AACATTCAAAAGGGAAAAGCACCTTTTGCAAGGAAGAAAGACCAAGTAGGTGGTAGGGAACGCTTTGAATTACATC ATGATAAACCAATCAGTCAGGATGGTGGTGTCTATGATATGAATAATATCAGAGTGACCACACCTAAGCGACATAT TGATATTCATCGGGGTAAG**tggagccatccgcagttcgaaaaa**TAAGTCGACCGGCTGCTAACAAAGCCCGAAAGG AAGCTGAGTTGGCTGCTGCCACCGCTGAGCAATAACTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGG TTTTTTGCTGAAAGCCAATTCTGATTA

Supplementary Table S3. Effective multiplicity of colicin Ia, E1, and E2 after exposing cells to each colicin with different concentrations for 3 mins and 1 h. Multiplicity (m) was calculated using the formula ($S/S_0 = e^{-m}$), where S is the number of surviving cells after colicin treatment and S_0 is the number of untreated cells.

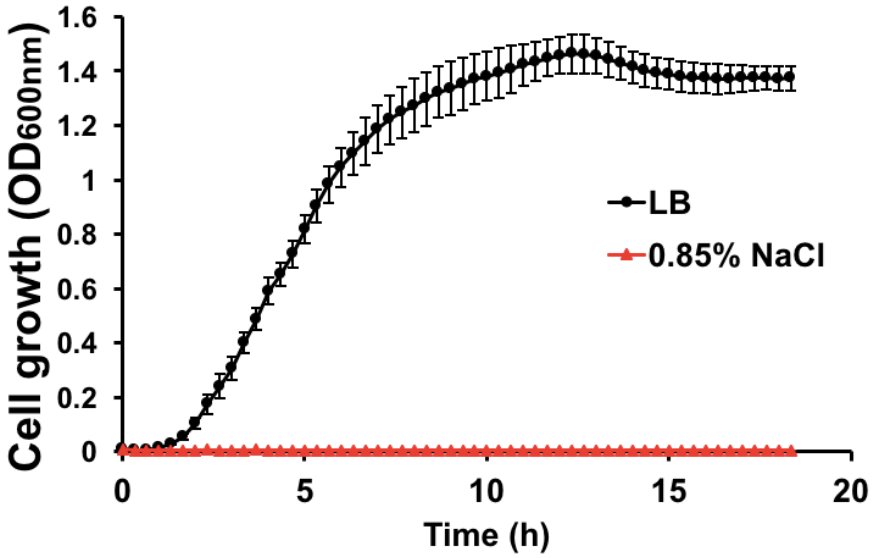
	3 min treatment			**1 h treatment**		
Concentration (ng/mL)	**Ia**	**E1**	**E2**	**Ia**	**E1**	**E2**
2	0.2	0.7	0.2	0.3	0.0	0.1
4	0.2	1.3	0.2	0.2	0.0	0.6
8	0.3	3.2	0.4	0.3	0.6	1.8
16	0.4	4.0	0.8	0.2	1.6	3.5
32	1.1	6.4	0.6	0.5	4.7	3.5
64	1.1	11.3	3.6	1.0	6.4	9.6
128	1.4	12.6	4.3	1.5	11.2	10.8
256	1.3	12.9	4.7	1.6	11.6	11.3
512	1.0	13.2	4.9	1.6	12.2	12.0
1,024	1.3	13.3	4.7	1.3	12.2	12.0



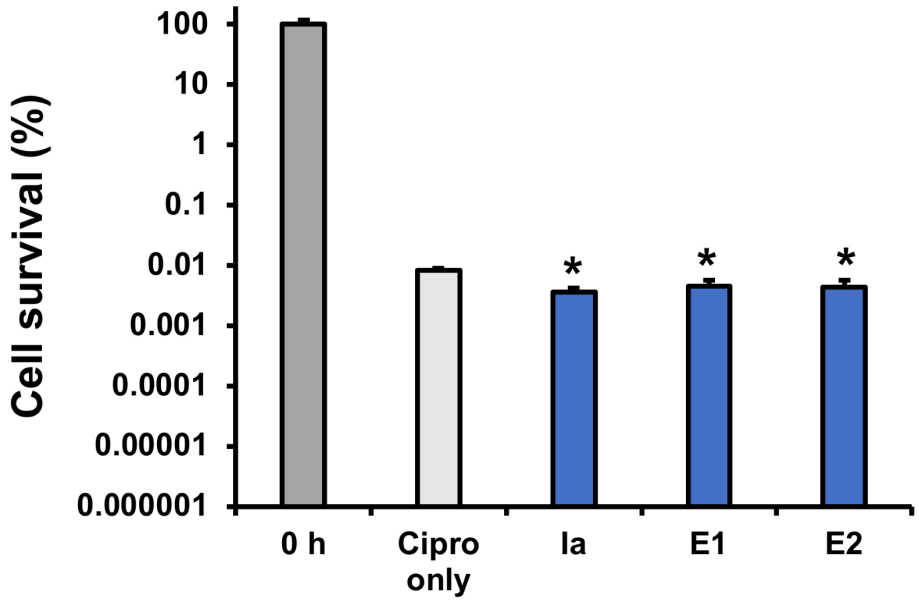
Supplementary Figure S1. Assessment of 'cell-free' crude extract. (**A**) The number of cells in the unfiltered and filtered crude extracts were examined with a serial dilution followed by addition of 10 μL of each sample onto a nutrient agar plate. (**B**) Green fluorescent protein (GFP) production was assessed with unfiltered and filtered extracts after incubating at 37°C for 20 h. Error bars indicate standard deviations from two independent batch reactions with three well replicates each.



Supplementary Figure S2. Cell viability assay of colicin M. K361 cells (initial cell density of $5x10^6$ CFU/mL) were exposed to cell-free synthesized total colicin M (1,500 ng/mL), soluble M (90 ng/mL), and insoluble M (1,390 ng/mL) for 1 h at 37°C at 220 rpm in LB medium. Error bars indicate standard deviations from two independent cultures with three plating replicates each.



Supplementary Figure S3. Cell growth of K361 strain. Overnight K361 cell culture was diluted to OD_{600nm} 0.01 in LB medium or 0.85% NaCl solution, and 200 μL of the adjusted culture was incubated at 37°C with continuous shaking in a 96-well plate. Cell growth was measured every 20 min. Error bars indicate standard deviations from two independent starter cultures with three well replicates each.



Supplementary Figure S4. Persister assay in the presence of ciprofloxacin and colicins with the same multiplicity. Exponential phase cells were exposed to ciprofloxacin (Cipro, 5 μg/mL) and different colicins that share the same effective multiplicity (m~1.6) for 3 h with shaking at 220 rpm at 37°C. Concentrations of Ia, E1, and E2 were 512 ng/mL, 16 ng/mL, and E2 8 ng/mL, respectively (**Supplementary Table S3**). Error bars indicate standard deviations from two independent cultures with three plating replicates each. * represents significant difference compared to Cipro only treated sample with p-value <0.05.