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Characterization of ColE1 Production for Robust *tolC* Plate Dual-Selection in *E. coli*

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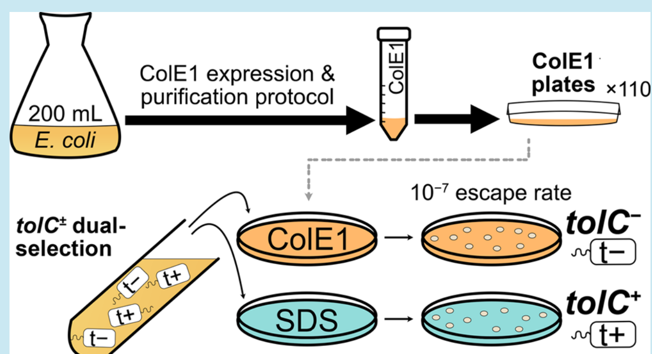
ABSTRACT: Bacterial selection is an indispensable tool for *E. coli* genetic engineering. Marker genes allow for mutant isolation even at low editing efficiencies. *TolC* is an especially useful *E. coli* marker: its presence can be selected for with sodium dodecyl sulfate, while its absence can be selected for with the bactericidal protein ColE1. However, utilization of this selection system is greatly limited by the lack of commercially available ColE1 protein. Here, we provide a simple, plate-based, ColE1 negative-selection protocol that does not require purification of ColE1. Using agar plates containing a nonpurified lysate from a ColE1-production strain, we achieved a stringent negative selection with an escape rate of 10^{-7}. Using this powerful negative-selection assay, we then performed the scarless deletion of multiple, large genomic loci (>10 kb), screening only 12 colonies each. We hope this accessible protocol for ColE1 production will lower the barrier of entry for any lab that wishes to harness *tolC*'s dual selection for genetic engineering.



KEYWORDS: *ColE1, tolC, dual-selection, negative-selection, recombineering, genetic engineering*

Selective markers are a key tool in genetic engineering and synthetic biology. They are commonly used in prokaryotic and eukaryotic cells to maintain plasmids and facilitate deletions and insertions into the genome. Most common marker genes are used for positive selection, for example, introducing antibiotic resistance genes such as ampicillin. Negative-selection markers, also known as counter-selection markers, have a toxic effect that is useful for ensuring the absence or removal of a gene, for example, for curing the cell of plasmids and to remove selection markers so they can be reused.[1] Single genes that enable both positive and negative selection, or "dual-selection markers", are highly desirable since they can be easily and repeatedly inserted and removed for "scarless" manipulation of multiple loci in the same cell. Currently, only a few examples of dual-selection markers are available in bacteria. Beyond recombineering, such dual-selection cassettes have also been used in a range of applications, including the evolution of genetic circuits[2] or the engineering of allosteric transcription factors.[3]

Various types of negative-selection genes for recombineering are currently available.[2,4−12] A critical parameter for negative selections is the rate at which incorrect cells escape selection and form colonies (escapee cells). Among the published systems tested in common *E. coli* lab strains: the inducible toxin systems, the *TetA-SacB* cassette, and the *tolC* marker stand out as the most stringent markers used in *E. coli* recombineering, with selection escape rates as low as 1×10^{-8}, 6×10^{-7}, and 4×10^{-11}, respectively.[5,6,13] However, the *TetA-SacB* cassette requires specialized counter-selection conditions that inhibit cell growth significantly,[6] and inducible toxin systems often require minimal media for counter selection. *PheS* and *rpsL/Strep* are alternatives for negative selection in rich media but require the addition of a positive marker to allow for dual selection. These issues limit the use of these selections in growth-deficient cells.

The *tolC* gene enables highly stringent dual selections in rich LB media. It encodes an outer membrane efflux protein involved in the expulsion of a diverse range of molecules from the cell.[14] ColE1 is a bactericidal colicin protein that allows its bacterial host to kill vulnerable cells and gain a growth advantage. Cells producing ColE1 harbor an immunity gene, also expressed from the same colicin plasmid, to protect itself from the toxic effect.[15,16] Because ColE1 requires TolC to enter and lyse the target cell, cells are safe from ColE1 if they lack TolC. These properties enable *tolC*'s use in a simple dual-selection scheme. In the presence of ColE1 toxin, *tolC*⁺ cells die and *tolC*⁻ cells remain viable (negative selection). In the presence of the surfactant sodium dodecyl sulfate (SDS), *tolC*⁺

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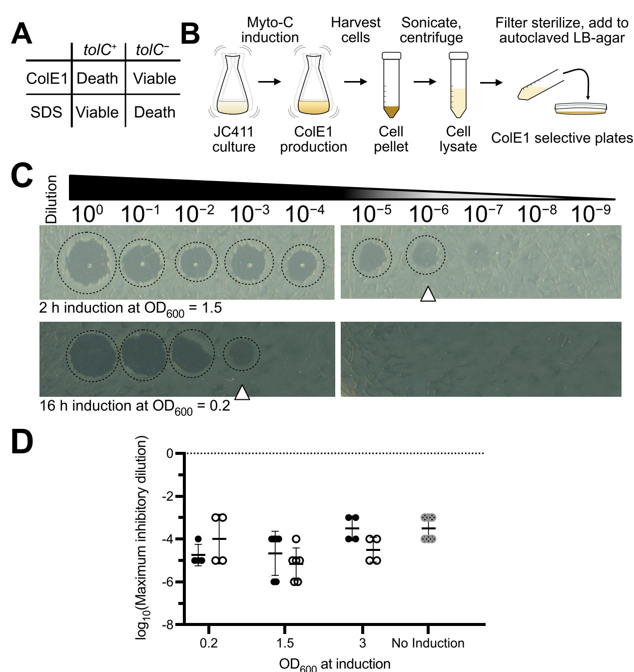

Figure 1. Optimization of ColE1 production. (A) ColE1 lysate and SDS enable a dual-selection scheme for cells containing (*tolC*$^+$) or lacking (*tolC*$^-$) the *tolC* gene. (B) Overview of ColE1 production protocol steps. (C) Two representative spot assays of different ColE1 preparations dropped onto *tolC*$^+$ covered plates were then incubated. Top: 2 h expression at OD_{600} = 1.5. Bottom: 16 h expression at OD_{600} = 0.2. Spots of inhibited growth are circled. Δ indicates the maximum inhibitory dilution. (D) ColE1 expression was induced at three different OD_{600} values and harvested postinduction. Dark circles represent 2 h induction. Open circles represent 16 h induction. Gray circles represent no-induction negative control. Maximum inhibitory dilutions were assessed using the spot assay. Data were log_{10}-transformed and all replicates, means, and standard deviations are represented.

cells are protected, while *tolC*$^-$ die by membrane disruption (positive selection) [Figure 1A].

While *tolC* has been utilized in several studies,[1,13,17−19] the system has so far not been widely adopted due to the difficulty in procuring the ColE1 protein. While methods to obtain pure ColE1 in small amounts for protein analysis are well documented, a protocol for its bulk production as a selective reagent is not well characterized. We are currently unaware of any commercially available sources. Published purification methods[20,21] employ fractionation and multiple chromatography steps for protein purification. In addition, the *tolC* system has been primarily used in liquid selections, complicating the isolation of single colonies. Gregg et al.[13] described a plate-based *tolC* selection scheme using cell lysate for isolation of single colonies from specific recombineering strains. Recently, Tamer et al.[21] described an alternate purification method employing a customized expression vector and both size-exclusion and cation chromatography.

Here, we expand upon, simplify, and improve the reported ColE1 production protocol[13] for a plate-based selection using cell lysate from the ColE1-producing *E. coli* strain JC411.[20] Unlike previous protocols, this assay only requires basic microbial culturing and plate preparation techniques [Figure 1B]. We characterize the effect of ColE1 induction conditions on selection stringency and dynamic range. We demonstrate the repeatable nature of *tolC* dual selection by scarlessly deleting multiple loci across the *E. coli* genome. We believe this work helps reduce the expertise barrier and promotes adoption of *tolC* as a dual-selection marker for microbial genome engineering.

■ RESULTS AND DISCUSSION

The major bottleneck for using *tolC* dual selection is the ColE1 preparation. Without commercially available ColE1 protein, one would have to purify ColE1 to make selective media, rendering the process laborious and expensive. To address this bottleneck and develop a cheaper, more accessible assay, we decided to characterize and optimize ColE1 selections that utilize unpurified cell lysate rather than purified protein.

The use of the ColE1-producing *E. coli* strain JC411 has been previously reported.[20] The ColE1 gene (*cea*) is encoded on the ColE1 plasmid. Under normal conditions, its promoter is inhibited by LexA. Upon exposure to mitomycin-C, the cellular SOS response initiates autocatalytic cleavage of LexA resulting in ColE1 production. It was previously shown that the majority of colicin molecules are kept in a cell's cytosol and on a cell's membrane than are released freely into the media.[22−24] We thus chose to proceed with lysing the cell pellet, rather than harvest the media [Figure 1B]. Briefly, cells are lysed using sonication, the lysate is centrifuged, and the resulting supernatant containing ColE1 is sterile-filtered and incorporated into LB-agar plates. Our optimized protocol provides a robust ColE1 negative selection for *tolC*.

We adopted a simple spot assay to assess the potency of each lysate preparation and compare between batches, since it is difficult to precisely measure the amount of ColE1 in crude cell lysate. In this assay, the crude ColE1-containing lysate is spotted on top of wild type *E. coli* (*tolC*$^+$) spread on an LB^M plate without selection. After the cells are incubated, the toxic presence of ColE1 in the lysate results in clear spots in the lawn. We used "maximum inhibitory dilution"—the lowest dilution of lysate causing cell death—as a relative indicator for the amount of ColE1 in the lysate [Figure 1C, Methods]. When we tested the media of the JC411 culture (instead of the lysed pellet) with the spot assay, we found very little to no selective action (data not shown). We used this assay to compare the strength of lysates prepared with different protocols.

We investigated the effect of two induction parameters on ColE1 production: the OD_{600} at induction, and the duration of ColE1 expression [Figure 1D]. We found cell density at the time of induction had no statistically significant effect on lysate potency (OD_{600} of 0.2, 1.5, or 3). Surprisingly, we did not find significant change in lysate potency using short (2 h) or long (16 h) inductions with mitomycin-C either. We thus chose a short 2 h induction protocol at OD_{600} = 1.5 for subsequent experiments. Using this protocol, 200 mL of JC411 culture routinely produces a hundred negative-selection plates.

We then tested the stringency of ColE1 negative selection by quantifying the number of escape colonies on plates containing different ColE1 amounts. It was previously shown that combining ColE1 with vancomycin[25] yielded a mixture with a lower escape rate compared to using ColE1 alone.[13] We therefore included vancomycin in our characterization of ColE1 selective plates.

First, we performed control experiments confirming that cells lacking *tolC* (*tolC*$^-$) are not affected by ColE1 toxicity, as expected, by forming lawns on all lysate-containing plates,

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noting the plates vary in coloration [Figure 2A]. Alternatively, only individual escapee colonies were detected when plating wild type cells carrying the *tolC* gene ($tolC^+$) on lysate-containing plates.

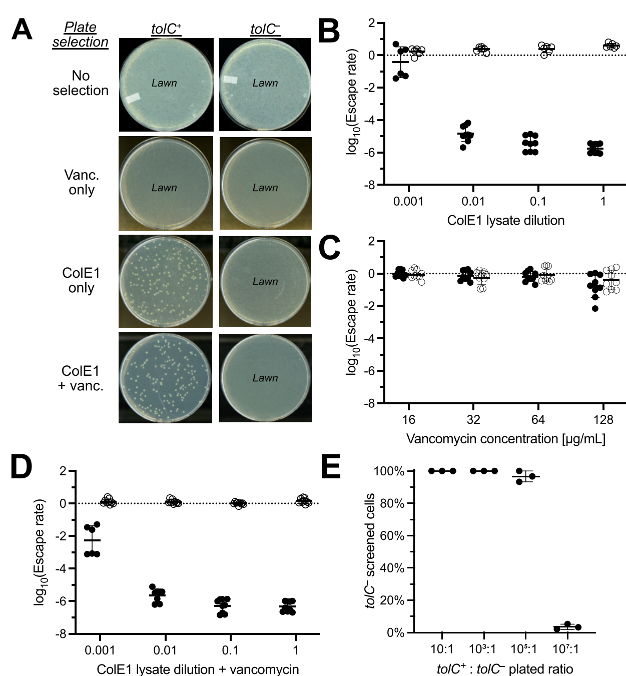

Figure 2. Selection stringency as a function of ColE1 and vancomycin. (A) Stationary-phase $tolC^+$ or $tolC^-$ cultures incubated at 37 °C for 16 h on the indicated plates containing ColE1 and/or vancomycin. (B–D) Escape rate of strains (● $tolC^+$, ○ $tolC^-$) on plates containing ColE1 lysate. (B) Plates containing ColE1 lysate only. ColE1 lysate dilution of 1× refers to 14 μL/mL of cell lysate (see Methods). (C) Plates containing vancomycin only. (D) Plates containing both ColE1 lysate and vancomycin. A constant amount of 128 μg/mL vancomycin was added to varying dilutions of ColE1 cell lysate. Data were $\log_{10}$-transformed and all replicates, means, and standard deviations are represented. (E) Mock selection. Stationary-phase $tolC^+$ and $tolC^-$ cell cultures were mixed at the indicated ratios, plated on ColE1 lysate + vancomycin, and grown overnight. Each dot represents one experiment in which 96 colonies were screened; means and standard deviations are represented.

To measure escape rate, we prepared plates containing undiluted lysate or 10-fold dilutions of the lysate. We then calculated the ratios of CFU/mL between nonselective and selective plates to find the "escape rate" in each case. We used 14 μL of lysate per 1 mL of LB-agar (used previously by Gregg et al.[13]) as the definition of 1× concentration. We found that plates made with this amount of lysate provided a stringent selection with a mean escape rate of 2.15×10^{-6} (without vancomycin) [Figure 2B]. Selective stringency was maintained even when diluting the lysate in the plates by 10- or 100-fold (5.67×10^{-6} and 2.35×10^{-5}, respectively). Selection was completely lost when the lysate was diluted 1000-fold. These results indicate ColE1-containing lysate alone reproducibly provides a robust *tolC* negative selection.

Vancomycin alone had little to no significant inhibitory effect on either $tolC^+$ or $tolC^-$ cells at concentrations below 128 μg/mL. At 128 μg/mL, vancomycin showed a moderate inhibitory effect on both $tolC^+$ and $tolC^-$ (4.00×10^{-1} and 8.31×10^{-1}, respectively) [Figure 2C]. When vancomycin was combined with ColE1 lysate, we observed an increase in *tolC* selection stringency, reaching mean escape rates of 5.91×10^{-7} (undiluted) and 7.2×10^{-7} (10-fold diluted lysate). When using very diluted lysate with vancomycin, mean escape rates showed a slight improvement (from 5.67×10^{-6} to 3.10×10^{-6} using 100-fold lysate dilution, and from 1.49×10^{0} to 1.87×10^{-2} using 1000-fold diluted lysate) [Figure 2D].

Additionally, we challenged our lysate-containing selective plates by isolating a small number of $tolC^-$ resistant cells from a large background of $tolC^+$ cells. In a mock selection experiment, cells were mixed at $tolC^+$:$tolC^-$ ratios ranging from 10:1 to 10^7:1, and the mixture was plated on ColE1 lysate with vancomycin. Ninety-six colonies of each plate were PCR-tested to determine the presence of the *tolC* gene, expecting only $tolC^-$ cells to grow under ColE1 selective conditions [Figure 2E]. The total numbers of cells plated on each plate were from 10^2 to 10^3 $tolC^-$ cells, and from 10^4 to 10^9 $tolC^+$ cells. We found the expected $tolC^-$ cell compositions when using up to a 10^5:1 ($tolC^+$:$tolC^-$) ratio. At the ratio of 10^7:1 (10^9:10^2 plated), we observed that only 4% of screened cells were $tolC^-$. This computes to a $tolC^+$ escape rate of $\sim 2.4 \times 10^{-6}$ as $10^9 \times 2.4 \times 10^{-6} = 2400$ $tolC^+$ escapees, compared to the 100 $tolC^-$ expected cells found ≈4% $tolC^-$. It is possible the reduction in selection stringency was due to the large number of cells on the plate, diluting or shielding $tolC^+$ cells from ColE1 protein. Overall, this demonstrates that a simple ColE1 lysate + vancomycin plate can be used for negative selection to find one successful recombinant cell out of a background of 10^7 with a single 96-well screen.

Finally, we demonstrated the utility of negative selection on ColE1 lysate plates in a *tolC* recombineering assay: targeting the deletion of three different genomic regions in *E. coli* [Figure 3]. We designed several cassettes [Supporting Figure S3, S7], all containing the *tolC* gene, for removing these loci [Figure 3A]. Cassettes were transformed into $tolC^-$ *E. coli* following expression of λ-Red recombinase [Methods]. First, we selected for the integration of *tolC* cassettes into the genome by a positive selection for the presence of *tolC* using SDS-containing plates. PCR screening was performed at each step to identify recombinants, followed by Sanger sequencing [Figure 3C, Supporting Figure S6]. Next, to remove *tolC* from the genome, we performed recombineering of a short deletion oligo (90 bp), which targets homologous recombination at both ends of the inserted *tolC* cassette. The final colonies that carry a scarless deletion of the desired region were isolated by negative selection on ColE1 lysate + vancomycin plates.

The genomic regions targeted for deletion were the Qin/Kim (Q) and CP4–57 (C) cryptic prophages, and the *Flg* operon (F), ranging in size from 11.5 kb to 22.0 kb [Figure 3B]. By repeatedly inserting and then removing the *tolC* cassette, we were able to sequentially perform scarless deletion of all three loci in the same *E. coli* strain. We further showed these deletions can be performed in multiple orders with similar efficiency [Supporting Figure S4]. Overall, this shows that *tolC* dual selection can be efficiently used multiple times in a row with the ease of plating directly for colonies at every step [Supporting Figure S5].

Overall, we present an efficient method to prepare ColE1-based, negative-selection plates at laboratory scale using cell lysate. Our one-day lysate preparation protocol is shorter than previously reported,[13] reaching negative-selection stringency of 10^{-6} using lysate alone, and up to 10^{-7} in the presence of

Figure 3. Scarless deletion of three loci with a dual-selectable *tolC* cassette. (A) Two-step scarless recombineering. *tolC* cassette diagramed in the Deletion step: Boxes represent the 100 bp homology regions, upright perpendiculars represent terminators, bent arrows represent a promoter, and ⇀ represent binding sites for deletion-screening primers. Insertion of the cassette (*tolC*$^{+}$) was positively selected with SDS, then removal of the cassette (*tolC*$^{-}$) was negatively selected with ColE1 lysate + vancomycin. Carbenicillin maintained the λ-Red recombineering plasmid. (B) Three genomic loci (11.5−22.0 kb) were targeted for scarless deletion: the *Flg* operon, and the Qin/Kim and CP4−57 cryptic prophages. (C) Electrophoresis gel showing all steps of PCR deletion screening. No band is expected before deletion, a larger band postdeletion (due to cassette insertion), and a smaller band at the scarless stage.

vancomycin, all in the absence of enhancing genotypes. We show that the stringency of selection is maintained even when the lysate is diluted 10-fold from its originally reported concentration,[13] producing over a hundred plates per batch (see Methods). We also show that ColE1 lysate can be conveniently kept at 4 °C for 6 weeks [Supporting Figure S1], suggesting that *tolC* negative-selection plates can be made in large batches and stored. Further characterization is required to assess frozen lysate stability.

It was previously shown that other genetic modifications, such as duplication of the *tolQRA* locus,[13] can further enhance the stringency of *tolC* negative selection. In this work, we show that the *tolC* gene alone provides a robust negative selection suitable for most routine applications in wild type *E. coli*, including insertion and deletion of large genomic regions. Further work is required to quantify the contribution of such modifications to recombineering.

Our work provides a streamlined, thorough, and accessible method to utilize the full potential of *tolC* negative selection with minimal specialized equipment or protocols. Being relatively short (1.5 kb), the *tolC* gene can be easily cloned and expressed to enable efficient plasmid curing and multistep, scarless recombineering. As the scale and complexity of genome editing projects continues to increase, the mining and characterization of additional dual-selection markers will greatly accelerate microbial engineering and synthetic biology applications.

■ METHODS

Strains and Culture Methods. ColE1 was expressed from strain JC411: *Escherichia coli* (Migula) Castellani and Chalmers (ATCC #27138) (*Escherichia coli F−, leuB6(Am), fhuA2::IS2, lacY1, gln X44(AS), gal-6, λ−, hisG1, rfbC1, galP63, argG6, rpsL104, malT1(λR), xyl-7, mtlA2, metB1*).[20] All strains were grown in liquid culture using the Miller formulation of lysogeny broth (LB^{M}). TOP10 ("*tolC*$^{+}$") cells from Invitrogen and TOP10·*tolC*$^{-}$ ("*tolC*$^{-}$") were used for testing. TOP10·*tolC*$^{-}$ was created from TOP10 using λ-Red recombineering and a 200 bp nonselectable Ultramer (from IDT) to scarlessly delete the endogenous *tolC* gene. It was directly selected for on LB-agar plates containing 14 μL/mL of ColE1 lysate. It is important to mention that *tolC* is part of the major efflux system for many commonly used antibiotics. The minimum inhibitory concentrations (MIC) of antibiotics may be altered when *tolC* is deleted. We found that chloramphenicol was lethal to *tolC*$^{-}$ cells regardless of the presence of a resistance marker or concentration used.

Preparation of ColE1 Lysate and LB^{M} Plates Containing Lysate + Vancomycin. JC411 was grown from a frozen glycerol stock overnight (∼16 h) in 3 mL of LB^{M} at 37 °C. A 200 mL LB^{M} production culture in a glass 1 L baffled flask was inoculated with 400 μL of the overnight culture and grown in a 37 °C shaking incubator (Innova 42) at 250 rpm. At OD_{600} = 1.5, ColE1 production was induced by the addition of mitomycin-C to a final concentration of 0.5 μg/mL. The flask incubated further for 2 h. (Other expression parameters were compared [Figure 1].) The culture was then aliquoted into four 50 mL Falcon tubes and the cells were harvested by centrifugation at 5250 rcf for 40 min at 4 °C (Beckman Coulter model Allegra X-15R). The cell pellets were frozen overnight at −20 °C, if necessary. The pellets were pooled and washed in 5 mL total of 50 mM K_2HPO_4 buffer (previously titrated to pH = 7.6 with HCl). The cells were then centrifuged at 4500 rcf for 15 min at 4 °C and resuspended in 3 mL of buffer. Sonication (QSonica model Q125, set at 50% power) was then performed on ice in a 15 mL Falcon tube with a 2 mm diameter probe, outputting 7 W, in 10 s on/20 s off cycles for 5 min of sonication (15 min total time). The resulting cell lysate was aliquoted into 1.5 mL microcentrifuge tubes and clarified by centrifugation at 21 130 rcf for 30 min at 4 °C to remove cell debris (Eppendorf model 5424 R). Finally, this lysate's supernatant (containing ColE1) was filter-sterilized through a 0.22 μm sterile filter (Corning cat. 431219) to remove any surviving JC411 cells. This lysate was stored directly at 4 °C. ColE1 agar plates (each 25 mL) were prepared by adding lysate (1.4 μL/mL) and vancomycin (128 μg/mL) directly to molten, autoclaved LB-agar (LB^{M} 25 g/L, agar 15 g/L) once the bottle had cooled to approximately 60 °C, so as to not denature the ColE1. Premixing vancomycin and lysate could cause precipitation and uneven mixing. Control experiments using *E. coli* lacking ColE1 production (made from TOP10) did not inhibit growth in the spot assay (data not shown). Protocol is formatted for convenience in Supporting Figure S2.

ColE1 Spot Assay. 100 μL of a confluent TOP10 (*tolC*$^{+}$) culture at OD_{600} = 3 were spread per prewarmed 37 °C LB-agar plate. After 30 min at room temperature, a 10-fold serial dilution of lysate (each dilution made with a clean pipet tip) was made in 50 mM K_2HPO_4 buffer (previously titrated to pH = 7.6 with HCl) and spotted (1 μL per spot) on the plate, left

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at room temperature for 30 min upright until the spots were dry, and then incubated upside-down at 37 °C for 16 h. The number of spots showing clearly inhibited growth (a truly empty, not hazy region) was recorded, and the maximum inhibitory dilution was thereby inferred. The spot assay was used to compare the potency of ColE1 lysate which had been produced from JC411 cells induced at either OD_{600} = 0.2, 1.5, or 3.0, for either 2 or 16 h [Figure 1D].

Escape Rate Assay. The selection stringencies of lysate-only, vancomycin-only, and lysate + vancomycin LB-agar plates at different concentrations were tested. For lysate-only: the maximum concentration of lysate (1×) was defined as 14 μL/mL and 10-fold dilutions down to 10^{-3} were tested. Vancomycin-only: plates were tested at 128 μg/mL and in 2-fold dilutions down to 16 μg/mL. Lysate + vancomycin: The same range of dilutions of lysate was used as in the lysate-only experiment, while 128 μg/mL vancomycin was maintained throughout. The number of *tolC*$^+$ cells which escaped negative-selection and formed colonies was determined by incubating dilutions of confluent cell culture on these series of plates for 16 h at 37 °C. Plating dilutions on LB-agar without selection allowed for redetermination of the total CFU/mL for each biological replicate. To calculate an escape rate, the total number of escapees/mL was divided by the total number of CFU/mL without selection.

Mock Selection. In order to quantitatively model a recombinogenic screen, *tolC*$^+$ and *tolC*$^-$ cultures were grown overnight in LB^M at 37 °C and their OD_{600} was measured to determine the number of cells. The cultures were diluted and mixed to create cell ratios (*tolC*$^+$:*tolC*$^-$) of 10^1:1, 10^3:1, 10^5:1, and 10^7:1. These mixtures were plated in triplicate on LB-agar plates containing 14 μL of ColE1 lysate and 128 μg vancomycin per 1 mL of LB-agar, and grown overnight at 37 °C. From each plate, 96 colonies were PCR screened to infer what percentage of them were the expected *tolC*$^-$ cells. PCR primers targeted the endogenous *tolC* locus and the size of the amplified band determined if the cell was *tolC*$^+$ or *tolC*$^-$ [Supporting Figure S3].

Scarless Deletion Demonstration. Three loci were chosen for scarless deletion in the TOP10·*tolC*$^-$ genome: the *Flg* operon and the Qin/Kim and CP4−57 cryptic prophages [Figure 3B]. The *tolC* cassette without homology regions was ordered from Genewiz as a clonal gene (TurboGENE). Extension primers (from IDT) were used to PCR amplify each *tolC* cassette and add 100 bp of genomic homology on each side [Supporting Figure S3, S7]. Amplified cassettes were DpnI restriction digested (NEB #R0176) and their sizes were confirmed with gel electrophoresis (Invitrogen E-gel EX 1%). Three 90 bp Ultramers (from IDT) were ordered, each consisting of 45 bp from both outer ends of the homology regions joined together [Figure 3A, Supporting Figure S3]. The TOP10·*tolC*$^-$ strain was transformed with pORTMAGE-2 ($Carb^R$) plasmid (AddGene #72677). A single colony was grown overnight in 3 mL of LB^M + carbenicillin (30 μg/mL) on a roller drum (New Brunswick model TC-7) at 60 rpm, 32 °C. 500 μL of this culture was passaged into 50 mL of LB^M + carbenicillin (30 μg/mL) in a 250 mL baffled flask and grown in a 32 °C shaking incubator (Innova 44) at 250 rpm until visibly turbid (OD_{600} < 0.6). Expression of λ-Red recombinase from the pORTMAGE-2 plasmid was induced by heat-shock in a shaking 42 °C water bath (Thermo Scientific model MaxQ 7000) at 180 rpm for 14 min, then the culture was pelleted at 5250 rcf for 14 min at 4 °C (Beckman Coulter model Allegra X-15R). The supernatant was discarded, and the pellet was resuspended in 1 mL of 4 °C ddH_2O. The culture was spun down at 6500 rcf for 2 min 30 s at 4 °C (Eppendorf model 5424 R), then washed identically three more times. Finally, the pellet was resuspended in 150 μL of 4 °C ddH_2O, and 40 μL of those cells were mixed with 2 μL (1−2 ng) of *tolC* cassette. The culture was then electroporated in a 1 mm gap cuvette (Bio-Rad cat. 1652089) with an exponential decay protocol of 1.8 kV, 200 Ω, and 25 μF (Bio-Rad GenePulser Xcell). The culture was immediately resuspended with 1 mL of prewarmed 37 °C SOB medium (a.k.a. Hanahan's Broth). The culture was incubated overnight at 32 °C. The following day, 10% dilutions (100 μL of culture), 10^{-3} dilutions (1 μL culture + 99 μL LB^M) and 10^{-5} dilutions (100 μL from a mix of 1 μL culture + 10 mL LB^M) of the culture were plated on LB-agar plates containing 50 μg/mL sodium dodecyl sulfate (SDS) and 30 μg/mL carbenicillin. After two nights of incubation at 32 °C, 12 colonies were directly PCR screened for the deletion of the target locus. A successful colony was chosen for overnight growth at 32 °C in 3 mL LB^M + SDS + carbenicillin (same concentrations). The induction and transformation processes were repeated but starting with a 250 mL LB^M + SDS + carbenicillin culture and using 2 μL of Ultramer stock solution (6 ng). After recovery in SOB, they were plated in dilutions (as above) on LB-agar plates containing (per mL of LB-agar): 1.4 μL/mL ColE1 lysate, 128 μg/mL vancomycin, and 30 μg/mL carbenicillin and grown for two nights at 32 °C. Twelve colonies were PCR screened for removal of the *tolC* cassette. This process was iterated thrice in the same strain to scarlessly delete all three targeted loci [Supporting Figure S4]. Geneious Prime 2022.0.1 (https://www.geneious.com) was used for Sanger sequencing alignment and analysis [Supporting Figure S6].

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.2c00061.

Additional experimental data relating to cold storage of lysate and quantification of *tolC* recombination, along with all DNA sequences and printable step-by-step instructions for making ColE1 lysate (PDF)

DNA sequence list and TolC cassette (ZIP)

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Author Contributions

M.S.B., S.B.O., and N.O. wrote the manuscript and contributed to the conception and design of the project. M.S.B. and S.B.O. carried out the experiments. G.M.C. supervised the project.

Notes

The authors declare the following competing financial interest(s): M.S.B., S.B.O., and N.O. declare no conflict of interests. For a complete list of G.M.C.'s financial interests, see http://arep.med.harvard.edu/gmc/tech.html.

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