An Efficient Thermoinducible Bacterial Suicide System
Elimination of Viable Parental Bacteria from Minicells

Shingo Tsuji, Ilham Naili, Nathan R. Authement, Anca M. Segall, Veronica Hernandez, Bryan M. Hancock, Matthew J. Giacalone, and Stanley R. Maloy

In manufacturing bacterial-derived biologics, it is essential to eliminate live parental bacteria and free endotoxins before in vivo administration to minimize the risk to patients of infection and septic shock. Purification currently relies on complex combinations of physical and biological methodologies such as differential centrifugation, filtration, irradiation, antibiotic treatment, and heating. Current purification processes can reduce yields and reproducibility, inactivate the biologics produced, increase production costs, and lead to contamination with undesirable products such as free endotoxins. Ideally, contaminating bacteria could be killed by introducing a simple, inducible, genetic suicide mechanism that would eliminate many problems of conventional purification.

Bacterial suicide mechanisms based on conditional cell lysis (1-3) and nucleic acid degradation have been described previously (4, 5). Although these systems are effective in killing bacteria, they are problematic for purification of biologics. Cell lysis releases free endotoxins that can cause septic shock, thereby compromising the safety of bacterial-based biologics. Although nuclease-based suicide systems do not cause cell lysis (4), restriction endonucleases (e.g., EcoRI) and nonspecific nucleases for killing bacteria can interfere with production of nucleic acid-based biologics such as plasmids and interference RNA (RNAi) molecules. An ideal suicide system would consist of a target-specific endonuclease that kills bacteria by introducing irreparable double-stranded DNA (dsDNA) breaks only within chromosomes to ensure minimal cell lysis and maximal killing efficiency. It would need to be easily used in a range of bacterial host strains.

The I-CeuI homing endonuclease from the green alga Chlamydomonas moewusii (also known as C. eugametos) (6) is ideal for such a system. This endonuclease recognizes and creates a staggered dsDNA break in a naturally occurring sequence (7, 8) within the highly conserved 23s rRNA in rrn operons (7-13 sites) on the chromosomes of many bacterial species used in biologics production. For example, Escherichia coli and Salmonella enterica each have seven I-CeuI recognition sites on their chromosomes (9, 10). I-CeuI will not damage plasmid DNA within bacterial cells because the long recognition sequence limits its target to 23s rRNA genes. In addition, the I-CeuI–based suicide system is versatile not only because of its effective host range, but also because it is active in a wide range of pH, temperature, and salt concentrations (11).

In this study, we show that a thermoinducible I-CeuI–based suicide system can facilitate elimination of viable, contaminating bacterial cells in the manufacture of bacterial-based biologics. Because our commercialization goals are centered on the clinical-grade manufacturing of bacterial minicells (12), we have successfully incorporated and used this novel system into a panel of minicell–producing strains (13, 14). As demonstrated here, we used the system to eliminate contaminating...
parent cells in processing clinical-grade minicell preparations. The same system also has broad applicability that would benefit many bacterial-based production schemes.

**MATERIALS AND METHODS**

**Culture Methods and Bacteria Strains:** We routinely cultivated bacterial strains in Luria-Bertani (LB) medium supplemented with 0.2% d-glucose at 30 °C, providing aeration unless otherwise indicated. LB medium was supplemented with 20 µg/mL chloramphenicol (Cam), 50 µg/mL kanamycin (Kan), and/or 100 µg/mL ampicillin (Amp) when appropriate, as detailed below. We used noncarbon E (NCE) medium with 0.2% succinate and E medium containing 0.2% d-glucose and 0.05 mM thiamine as minimal media (15).

The thermoinducible I-CeuI suicide system was built into an antibiotic resistance marker–free, pir⁰, *E. coli* MG1655 derivative with a deletion in the closely linked lactose use and proline biosynthesis genes (Δlac-pro XIII). We constructed the background strain (VAX-6C3) by transducing the Δlac-pro region from *E. coli* JMXAc into *E. coli* MG1655 with P1vir transduction. Transductants that inherited the deletion were selected by demanding growth on medium containing 2 mM of the toxic lactose analog 2-nitrophenyl 1-thio-β-D-galactopyranoside (TONPG) from Sigma (www.sigmaaldrich.com) (16, 17), then screened for proline auxotrophy. The proline-auxotrophic phenotype allows antibiotic resistance gene–free selection by demanding stable restoration of Pro⁺ in minimum media lacking proline.

The integration plasmid pVX-66 (Figure 1) containing the I-CeuI suicide system and minicell–producing VAX-8I3 causes cell death without cell lysis. (top) Relative viability of the VAX-8I3 strain after suicide activation at OD₆₀₀ of 0.1 and 1.0. (middle) Growth curve of VAX-8I3 with or without suicide system induction (the suicide system and minicell production were simultaneously activated at 0.1 OD₆₀₀). (bottom) Relative viability of *E. coli* strains with pVX-55 (L-rhamnose–inducible I-CeuI) and pVX-65 (thermoinducible I-CeuI); the suicide system was induced at 0.1 OD₆₀₀.

**Figure 1:** Plasmid map of integration construct pVX-66 with thermoinducible suicide system and minicell production cassette; the thermoinducible suicide system consists of I-CeuI endonuclease expressed from the heat-sensitive csb57(ts) regulated phage λ P₄₁₆ promoter system. In the minicell production cassette, a lacI⁰-regulated tac promoter drives expression of itsZ. The *E. coli* MG1655 probA⁺ genes with their natural promoters allow antibiotic-free selection of pVX-66 in Pro⁻ strain backgrounds. The pir⁻-dependent R6K origin and attP₈ sites allow integration of pVX-66 into the attB₈ site on the Pir⁻ bacterial strain chromosomes. Tₐ₁₆, Tₐ₅, and Tₐ₆ terminators are used to prevent unwanted transcriptional read-through. Restriction sites used for construction of pVX-66 are also shown.

**Figure 2:** Activation of the I-CeuI–based suicide system in minicell-producing VAX-8I3 causes cell death without cell lysis. (top) Relative viability of the VAX-8I3 strain after suicide activation at OD₆₀₀ of 0.1 and 1.0. (middle) Growth curve of VAX-8I3 with or without suicide system induction (the suicide system and minicell production were simultaneously activated at 0.1 OD₆₀₀). (bottom) Relative viability of *E. coli* strains with pVX-55 (L-rhamnose–inducible I-CeuI) and pVX-65 (thermoinducible I-CeuI); the suicide system was induced at 0.1 OD₆₀₀.
transcriptional termination site and the E. coli and an optimized version of the E. coli Shine-Dalgarno (SD) sequence engineered within the primer sequence is.

Polymerase Chain Reaction (PCR) Primers: Table 1 lists PCR primers we used during strain construction. The table also shows restriction sites from genomic DNA of E. coli trp operon transcriptional termination site engineered in primer sequences. We conducted PCR reactions with error-proof Pfu DNA polymerase from Invitrogen (www.invitrogen.com). All PCR products were blunt-end ligated into a pCR-Blunt II-Topo cloning kit, and proline auxotrophy and maintained replication in pVX-37 and pVX-42, we directionally cloned the minicell-producing construct and a piece of pVX-42 containing the minicell-producing construct into pVX-42 to restore the R6K origin of replication. Clones were selected for loss of proline auxotrophy and maintained using VAX-6C9 strain (Pir' and Pro-).

Construction of Inducible \( ftsZ \) Minicell Production System: We PCR-amplified an optimized isopropyl \( \beta \)-d-thiogalactopyranoside (IPTG) inducible \( ftsZ \)-based minicell-producing cassette (T4, lacI, \( \beta\)-l-acI, \( ftsZ \)) from genomic DNA of MPX1B9 using primers 1B1 and 1B2 (13). We then cloned a minicell-producing cassette with a natural CiaI site downstream of \( ftsZ \) into NdeI-CiaI sites of pir-dependent PKD3 (21) following restriction digestion to construct pVX-37. Functional copies of the \( proBA \) genes with native promoters were PCR-amplified using genomic DNA from E. coli strain MG1655 and primers 1B3 and 1B4. The PCR-amplified construct (\( SphI-proBA-KpnI \)) was digested and directionally subcloned into \( SphI-KpnI \) sites adjacent to \( attP \) in pLacZYattP (pir-dependent) to construct pVX-42. Next, pVX-37 and pVX-42 were digested with HindIII. We ligated together the fragment of pVX-37 containing the minicell-producing construct and a piece of pVX-42 with \( attP \) and \( proBA \) to create pVX-43 (pir-dependent and antibiotic resistance marker-free). Because HindIII cleaves R6K origin of replication in pVX-37 and pVX-42, we directionally cloned the minicell-producing construct into pVX-42 to restore the R6K origin of replication. Clones were selected for loss of proline auxotrophy and maintained using VAX-6C9 strain (Pir' and Pro-).

Construction of Inducible I-CeuI Suicide System: We PCR-amplified the I-CeuI gene with primers 1B9 and 1C1, then cloned it into SalI-XbaI sites of pRHA-67 to express I-CeuI from the RhaRS-regulated pRHA promoter system, which can be induced with L-rhamnose (22). The rhamnose-inducible I-CeuI suicide system was then PCR-amplified with primers 1C1 and 1C2, and the PCR product was blunt-end ligated into a pCR-Blunt II-Topo kit to construct pVX-55.

Table 1: Oligonucleotide PCR Primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>DNA Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1B*</td>
<td>TCTCGTGTCGCTGCTCCGATTACAGGGCCGATTTCAAATCCAAGGCATCAAATTAAACGNorI</td>
</tr>
<tr>
<td>1B2</td>
<td>GCTTAAATACGCGACACTTATCGCCATCTTCGACACGAACAGTC Sphl</td>
</tr>
<tr>
<td>1B3*</td>
<td>ATCTGTAATAAAACCGTAACAGCATGCGATGCTTTGAGTCATCC</td>
</tr>
<tr>
<td>1B4*</td>
<td>CAAGGTTTAAACTAACGTTACCCATGCCTAATGCCCTTGTGAATCATAATG KpnI</td>
</tr>
<tr>
<td>1B9ab</td>
<td>GTGCCGACCCGAGAGCCCCTTTATGCTCAACATTTACTAAACCGGGGGCG SD</td>
</tr>
<tr>
<td>1C1a</td>
<td>TCTAGATATTCTTACTTTTNTATATAATTACGAGC XbaI</td>
</tr>
<tr>
<td>1C2a</td>
<td>TCTAGAATTAACTTCTCGAAATTAGTACG XbaI</td>
</tr>
<tr>
<td>1G1b</td>
<td>GGTTTCGAGTTAGGGGAATATAACTGATGCAACCTTTATAATTAAACCGGGGCG BamHI SD</td>
</tr>
<tr>
<td>1G3a</td>
<td>AGTGGATCCGAATCTATTTATACCTTTATAATTACGAGC BamHI</td>
</tr>
<tr>
<td>1G4b</td>
<td>AAAGGATCCGAAATGCGCGACCGCGACTGGGCGATTAGCTGTCAGCCAAGCT trp terminator CTCTTACGCCCTG</td>
</tr>
<tr>
<td>1G5s</td>
<td>AAATCGTAAACCATGGAAGCTAGGAACTACTTACTTTTTATATATTACGAGC ACGAGCAAAGTC NcoI NheI</td>
</tr>
</tbody>
</table>

* Restriction endonucleases and their recognition sites within primers are bold and underlined.
| 1C1 | TCTAGATATTCTTACTTTTNTATATAATTACGAGC XbaI |
| 1C2 | TCTAGAATTAACTTCTCGAAATTAGTACG XbaI |
| 1G1 | GGTTTCGAGTTAGGGGAATATAACTGATGCAACCTTTATAATTAAACCGGGGCG BamHI SD |
| 1G3 | AGTGGATCCGAATCTATTTATACCTTTATAATTACGAGC BamHI |
| 1G4 | AAAGGATCCGAAATGCGCGACCGCGACTGGGCGATTAGCTGTCAGCCAAGCT trp terminator CTCTTACGCCCTG |
| 1G5 | AAATCGTAAACCATGGAAGCTAGGAACTACTTACTTTTTATATATTACGAGC ACGAGCAAAGTC NcoI NheI |

* Restriction endonucleases and their recognition sites within primers are bold and underlined.

Because pACTAK2 has a pir-dependent R6K origin of replication (20), any unintegrated plasmid is unable to replicate in a pir- strain.

Polymersase Chain Reaction (PCR) Primers: Table 1 lists PCR primers we used during strain construction. The table also shows restriction sites and an optimized version of the E. coli Shine-Dalgarino (SD) sequence and the E. coli trp operon transcriptional termination site engineered in primer sequences. We conducted PCR reactions with error-proof Pfu DNA polymerase from Invitrogen (www.invitrogen.com). All PCR products were blunt-end ligated into a pCR-Blunt II-Topo cloning kit, also from Invitrogen, before subsequent restriction digestion and subcloning.

Construction of Inducible ftsZ Minicell Production System: We PCR-amplified an optimized isopropyl \( \beta \)-d-thiogalactopyranoside (IPTG) inducible ftsZ-based minicell-producing cassette (T4, lacI, \( \beta\)-l-acI, ftsZ20) from genomic DNA of MPX1B9 using primers 1B1 and 1B2 (13). We then cloned a minicell-producing cassette with a natural CiaI site downstream of ftsZ into NdeI-CiaI sites of pir-dependent PKD3 (21) following restriction digestion to construct pVX-37. Functional copies of the \( proBA \) genes with native promoters were PCR-amplified using genomic DNA from E. coli strain MG1655 and primers 1B3 and 1B4. The PCR-amplified construct (SphI-proBA-KpnI) was digested and directionally subcloned into SphI-KpnI sites adjacent to attP in pLacZYattP (pir-dependent) to construct pVX-42. Next, pVX-37 and pVX-42 were digested with HindIII. We ligated together the fragment of pVX-37 containing the minicell-producing construct and a piece of pVX-42 with attP and proBA to create pVX-43 (pir-dependent and antibiotic resistance marker-free). Because HindIII cleaves R6K origin of replication in pVX-37 and pVX-42, we directionally cloned the minicell-producing construct into pVX-42 to restore the R6K origin of replication. Clones were selected for loss of proline auxotrophy and maintained using VAX-6C9 strain (Pir' and Pro-).

Construction of Inducible I-CeuI Suicide System: We PCR-amplified the I-CeuI gene with primers 1B9 and 1C1, then cloned it into SalI-XbaI sites of pRHA-67 to express I-CeuI from the RhaRS-regulated pRHA promoter system, which can be induced with L-rhamnose (22). The rhamnose-inducible I-CeuI suicide system was then PCR-amplified with primers 1C1 and 1C2, and the PCR product was blunt-end ligated into a pCR-Blunt II-Topo kit to construct pVX-55.
We constructed the integrated I-CeuI plasmid (pVX-66) as follows: After selection and validation of pVX-43, we PCR-amplified the I-CeuI endonuclease gene from purified C. moewusii whole-genome using primers 1G1 and 1G3. The PCR product (XhoI-SD-I-CeuI-BamHI) was restriction digested and cloned into XhoI-BamHI sites of pCGV1 to place I-CeuI expression under control of pR\_I and pR\_I dual promoters regulated by the phage λ cI857(Ts) (23, 24). We then PCR-amplified the heat-inducible suicide gene construct — XhoI-cI857(Ts), λ pR\_I-pR\_I-I-CeuI-BamHI — using primers 1G4 and 1G5 and cloned it into XhoI-BamHI sites of pVX-43 to construct the pir-dependent plasmid pVX-66. Clones were selected for loss of proline auxotrophy on E-media at 30 °C and maintained using the VAX-6C9 strain. The PCR-amplified suicide cassette was also blunt-end ligated into a pCR-Blunt II-Topo cloning kit to construct pVX-65.

**TUNEL Assays:** We used a terminal deoxynucleotidyl transferase (TdT)–mediated dUTP nick-end labeling (TUNEL) assay as described elsewhere (25, 26) to detect I-CeuI-dependent dsDNA breaks in the E. coli chromosome. After thermoinducing I-CeuI, we harvested cells and fixed them in 4% paraformaldehyde, permeabilizing them in 0.1% Triton X-100 containing 0.1% sodium citrate. The cells were then treated with dUTP-fluorescein isothiocyanate (FITC) from Roche Applied Science (www.roche-applied-science.com) to enzymatically label 3′-OH DNA ends using TdT and counterstained with the nucleic acid–specific TOTO-3 iodine dye and the cell membrane–specific FM4-64 dye, both from Invitrogen.

We analyzed the TUNEL-labeled and counterstained cells using a flow cytometer with a BD FACS\_Aria cell sorter from Becton-Dickinson (wwwbdbiosciences.com) and examined a total of 50,000 events per sample. The total percentage of dsDNA-damaged (TUNEL-positive) cells was counted as a fraction of the membrane-enclosed (FM4-64 positive) or DNA-containing (TOTO-3 positive) cells. We treated noninduced controls (cultured continuously at 30 °C to suppress I-CeuI expression) identically to the I-CeuI induced cultures. And we substituted TOTO-3 dye with 4′,6-diamidino-2-phenylindole (DAPI) from Sigma for fluorescent microscopic analysis.

**Growth Inhibition Assays:** An overnight culture of VAX-8I3 grown at 30 °C in 2 mL of LB was subcultured into 2 mL LB at a 1/200 dilution and grown to an optical density (OD\_600) of 0.1 or 1.0 at 30 °C. At those OD\_600 values, we added 20 µM IPTG to induce minicell production and raised the incubation temperature to 42 °C to activate expression of I-CeuI. Control cultures were grown continuously at 30 °C to suppress I-CeuI expression. We measured the number of corresponding colony forming units (CFU) by spot plating tenfold serial dilutions of each culture (0–10\(^{-9}\)) onto LB agar with 0.2% D-glucose at various time points up to 24 hours after induction. Following incubation at 30 °C, total CFU were counted so we could calculate the CFU/mL of each original culture. We calculated relative viability by dividing CFU/mL of the suicide-activated cultures (grown at 42 °C) by that without activation (grown at 30 °C).

The growth inhibition experiment for E. coli strains with pVX-55 and pVX-65 was performed as described for the VAX-8I3 strain, but these strains were grown in LB-Kan. We induced I-CeuI in pVX-55 with l-rhamnose (10 mM) at 37 °C.

**Minicell Purification:** For minicell production and purification, VAX-8I3 was cultured as described for growth inhibition assays, but we increased the culture volume to 200 mL LB. Minicells were harvested from primary cultures 24 hours after induction and purified by differential centrifugation and linear sucrose gradients as described elsewhere (12, 14). We examined growth inhibition by counting the CFU at 24 hours postinduction and quantified minicells by measuring OD\_600 and applying this equation (14):

\[
\text{minicells/mL} = \frac{\text{OD}_{600}}{(5.0 \times 10^{-11})}.
\]

To quantify contaminating viable parent cells, we plated 10\(^{10}\) minicells on LB agar with glucose and grew them at 30 °C overnight.

**Statistical Analysis:** Data are presented as the mean ± standard errors of the mean (SEM). We used a two-tailed, paired Student’s t-test to calculate the statistical significance of differences between experimental groups.

![Figure 4: Activation of the I-CeuI suicide system causes accumulation of dsDNA damage and anucleation of E. coli. (Top) Merged fluorescent microscopic image of TUNEL (dsDNA breaks, green), FM4-64 dye (membrane specific, red), and DAPI (nucleic acid specific, blue)–labeled VAX-8I3 cultured 12 hours after induction of the suicide system at 42 °C. (Middle) Merged fluorescent microscopic image of negative control cultures stained with TUNEL, FM4-64 dye, and DAPI at 12 hours after continuous growth at 30 °C. (Bottom) Flow cytometric counts of the percentage of TUNEL-positive cells among FM4-64-positive population. Microscopic images are 1,000×.](image-url)
RESULTS

Using the I-CeuI endonuclease gene from the alga C. moewusii, we constructed an inducible suicide system capable of introducing irreparable chromosomal damage to a panel of Gram-negative bacteria, including an inducible minicell-producing E. coli strain. This suicide system consists of the C. moewusii I-CeuI suicide gene expressed from the phage λ P8 promoter system cloned into a plasmid-based integration construct, pVX-66 (Figure 1). The cI857(Ts) heat-sensitive repressor is tightly regulated, allowing high-level induction of I-CeuI expression at 42 °C and complete repression at 30 °C.

To determine the effect of I-CeuI expression on the viability of VAX-813, we compared viable populations from I-CeuI-induced cultures with those of noninduced cultures by CFU analysis under a range of conditions (Figure 2). Activation of the chromosome-based I-CeuI suicide system immediately decreased the relative number of viable bacteria, and viable cells continued to decrease over a 24-hour period to a significant overall reduction (p < 0.005) of ~10^6-fold (Figure 2, top). All cultures tested for minicell production showed identical levels of parental cell growth inhibition, regardless of the culture volume (≤1.2 L, highest volume tested). Delaying activation of the suicide system until the culture reached an OD_{600} of 1.0 was also bactericidal 24 hours after activation (Figure 2, top).

Based on the OD_{600} readings, VAX-813 continued to grow after suicide system activation (Figure 2, bottom). Although activation resulted in cell death, it did not cause cell lysis — as indicated by the OD_{600} readings of suicide-activated cultures (Figure 2, bottom) and the presence of intact but uniformly filamented cells (Figure 3). In addition, I-CeuI induction alone led to filamented cells (Figure 3, middle).

When I-CeuI expression was induced from high-copy-number plasmid constructs, pVX-55 and pVX-65 (both with pUC origin), 10^5- to 10^6-fold reduction in viable cell populations occurred three to six hours after induction (Figure 2, middle). Thermoinduction of the I-CeuI from pVX-65 caused ~10× more cell death than the l-rhamnose–induced I-CeuI expressed from pVX-55 at the postinduction time point of six hours. However, initial killing efficiency was higher with I-CeuI expression from pVX-55.

TUNEL assays verified that induction of I-CeuI caused dsDNA breaks in the chromosomal DNA (Figure 4). Fluorescent microscopic observations revealed that most cells had dsDNA damage, as represented by green fluorescent TUNEL-positive cells 12 hours after induction of the suicide system (Figure 4, top). By contrast, TUNEL-positive cells were rare in uninduced cultures (Figure 4, middle). We determined the proportion of bacteria with DNA damage (TUNEL labeled) among membrane-enclosed (FM4-64 positive) or DNA-containing (TOTO-3 positive) populations by flow cytometric analysis. The proportion of membrane-enclosed bacteria with dsDNA damage accumulated over time and became saturated by 12 hours postinduction (Figure 4, bottom). The number of TUNEL-positive cells slightly increased in the bacteria that had not been induced for the suicide system (Figure 4, bottom, black bars), which may indicate DNA damage caused by FtsZ overexpression during minicell production (27).

Because our goal is to develop methods to manufacture bacterial minicells as clinical-grade biologics, we used the thermoinducible suicide system to eliminate contaminating E. coli cells from minicells in batch production runs. To demonstrate the effectiveness of the I-CeuI–based suicide system for this process, we produced minicells with and without induction of the suicide system, then purified them by successive differential and sucrose density gradient centrifugation. We then compared minicell purity, determined by live parental cell contamination and yield.

Induction of the suicide system reduced the contaminating viable bacteria population by 10^7-fold (p < 0.05), giving a purified preparation with a mean contamination level of <1 viable bacterium per 10^9 minicells. When the I-CeuI suicide system was activated, minicell yields increased by sevenfold (p < 0.005) from 5.6 × 10^7 ± 3.8 × 10^6 minicells/mL in control cultures (inactivated) to 4.1 × 10^8 ± 1.9 × 10^7 minicells/mL in suicide-system active cultures. Larger-scale minicell production runs (≤1.2 L) gave similar minicell yields and contamination levels (data not shown).

DISCUSSION

To eliminate viable parental E. coli from purified minicell preparations, we constructed a bacterial suicide system based on thermoinducible I-CeuI endonuclease. This system offers several genetic advantages:

- It is stably integrated into the attB site of the E. coli chromosome to prevent segregation of genes and incompatibility problems with plasmids needed for other purposes, such as the delivery of DNA vaccines (14) or production of recombinant proteins.

- It is possible to select for this suicide system by complementation of a proline auxotroph, eliminating the
need for a selectable antibiotic resistance marker. This facilitates compliance with the US Food and Drug Administration (FDA) restriction against the use of certain antibiotic-resistance gene markers.

- The strain is sensitive to kanamycin, so kanR (the only antibiotic resistance marker approved by the FDA) can be used as a selectable marker for plasmids in these strains.

When the single chromosomal copy of I-CeuI was induced, cell death occurred gradually over a 24-hour period, allowing continuous population growth and production of proteins, plasmids, and minicells until cell death (Figure 2, top). TUNEL experiments revealed that activation of the I-CeuI suicide system causes cell death by irreparable chromosomal DNA damage in the form of double-stranded breaks (Figure 4). The system was effective over a wide range of activation time points (OD_{600} of 0.1 and 1), making it useful for a number of different applications. I-CeuI induction from the high-copy-number plasmids pVX-55 and pVX-65 caused faster declines in viable cell population than the chromosomal I-CeuI expression (Figure 2, top and bottom). Although the chromosome-based I-CeuI system is less efficient in causing cell death, both suicide systems ultimately reduced the viable cell population to a similar level.

When I-CeuI was expressed from high-copy-number plasmids pVX-55 and pVX-65 (Figure 2, bottom), we observed more efficient killing after l-rhamnose induction (pVX-55) than after thermoinduction (pVX-65). However, we observed more cell death after six hours of thermoinduction. Increased lethality of the thermoinducible system may have been because I-CeuI-mediated DNA cleavage is more efficient at 42 °C than at 37 °C (11). Nevertheless, it is clear that the I-CeuI suicide gene can be placed under another inducible promoter system, such as the l-rhamnose-inducible pRHA system for expression at lower temperatures for manufacturing heat-sensitive biologics.

I-CeuI-mediated cell death occurred without promoting cell lysis (Figure 2, middle, and Figure 3). This unique feature greatly enhances the safety of minicells or other biologics intended for in vivo administration in animals by preventing the release of free endotoxins. Moreover, the E. coli VAX-813 parent cells became filamented after induction of the suicide system (Figure 3), which enhanced their removal by simple differential centrifugation and filtration.

Simultaneous induction of the I-CeuI suicide and ftsZ minicell production systems increased minicell yields and simultaneously reduced parental cell contamination. Although the exact mechanism for that increased minicell yield is unknown, it is possible that minicell formation is enhanced by the SOS response to the resulting DNA damage (28). Minicell production with suicide system activation in larger culture volumes has demonstrated that minicell production, I-CeuI suicide efficacy, and parental contamination are proportionally scalable.

These results demonstrate that activation of the I-CeuI suicide system caused irreversible chromosomal DNA damage and cell death, reducing the number of contaminating viable bacteria to levels comparable with those observed in previously described suicide systems (10^{-3}–10^{-6}) without their disadvantages (1–5). Our results indicate that the I-CeuI suicide system functions over a wide range of conditions and can be used in number of expression systems. The thermoinducible I-CeuI suicide system greatly enhances the purity and safety of biological products. In addition, it reduces large-scale manufacturing costs because the system requires no antibiotics for selection or expensive inducing agents such as IPTG, and it simplifies downstream purification. The I-CeuI suicide system provides a simple, efficient, and cost-effective tool that should apply to a diversity of bacterial-based manufacturing processes.

**ACKNOWLEDGMENTS**

The majority of this work was supported by Vaxxion Therapeutics, Inc. and a National Institutes of Health (NIH) small-business innovation research (SBIR) phase I grant (No. 1R43GM084517-01). FACS analysis and TUNEL assays were performed in the laboratory of Dr. Anca Segall and supported by NIH grant number R01AI058253.

**REFERENCES**


11. Marshall P, Davis TB, Lemieux C. The I-CeuI Endonuclease: Purification and


Ilham Naili is a graduate student, Nathan R. Authement is a research technician, and Anca M. Segall is a professor at San Diego State University’s department of biology; Shingo Tsuji is a research associate, Veronica Hernandez and Bryan M. Hancock are research technicians, Matthew J. Giacalone is business development manager, and corresponding author Stanley R. Maloy is chief scientific officer at Vaxiion Therapeutics Inc., 11585 Sorrento Valley Road, Suite 105, San Diego, CA 92121, 1-858-792-0799, fax 1-858-792-7078; smaloy@vaxiion.com.