Creation of a single-vector I-*SceI*-based allelic exchange system

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Abstract

Counter-selection markers are used extensively as genetic tools and have become an essential part of genomic engineering. One broadly applicable counter-selectable system in use is the I-SceI endonuclease, along with its rare 18 bp recognition site. Current systems for I-SceI counter-selection introduce I-SceI and sce sites in two steps because of the high activity of the I-SceI protein. Drawbacks of the two-step system are that they typically require additional selection markers which may not be available for some organisms, and that they require additional time for mutant construction. The purpose of this project was to create a broadly applicable one-step counter-selection system based on a single plasmid containing both an I-SceI gene encoding a temperature-dependent enzyme and sce sites. A pool of mutant I-SceI alleles was generated using mutagenic PCR and screened for temperature-sensitive alleles. The resulting pEXSceM0 and pEXSceMQ vectors carry both a mutated I-SceI structural gene expressing a temperature-sensitive I-SceI enzyme and two sce sites flanking a multiple cloning site. The utility of the pEXSceM0 vector was confirmed by creating a deletion of the amrRAB-oprA region in a Select Agent excluded Burkholderia pseudomallei strain. The newly developed plasmids will expand the utility of the I-SceI/sce counter-selection system.

Introduction

Counter-selection against unwanted DNA sequences is an integral step in many bacterial allele replacement systems. The various methods that have been described over the years employ different counter-selection strategies that are usually based on markers or properties that bestow a deleterious phenotype on the bacteria when they are cultivated under certain conditions. For instance, the widely used *sacB*[1] and *pheS*[2] counter-selection markers are toxic to the bacteria in the presence of the respective substrates sucrose and chlorophenylalanine.

The I-*SceI* system has been engineered as an alternative positive counter-selectable marker for genetic manipulations.[3] The advantage of this system is that it does not rely on addition of substrates and is thus widely applicable in organisms where other counter-selection methods based on substrates such as sucrose and chlorophenylalanine are not applicable. The I-*SceI* gene codes for a homing-endonuclease found in the yeast *Saccharomyces cerevisiae*. I-*SceI* belongs to the LAGLIDADG homing endonuclease family and is a heterodimer that cleaves DNA by creating a double stranded break at an asymmetrical, non-palindromic, rare 18 bp nucleotide recognition site, leaving 4 bp overhangs exposing a 3'OH group open for recombination.[4,5] Double stranded breaks are resolved by either repairing the break resulting in gene conversion or extensive single-strand degradation leading to nonreciprocal crossovers creating a deletion.[6] In its natural host, I-*SceI* is responsible for the mobility of introns in the mitochondria of yeast, initiating gene conversion or crossovers creating deletions. I-*SceI* creates crossover deletions at a higher frequency compared to the frequency of gene conversions.[6]

I-*SceI* restriction enzyme recognition sites are not found in bacterial chromosomes and cleavage of the chromosome will thus only occur at artificially introduced sce sites. Currently, researchers use the I-*SceI* gene and sce sites counter-selection by introducing the two components in a two-step procedure (Figure 1). In standard bacterial allelic exchange strategies, the first step is to generate a merodiploid strain via homologous recombination into the host chromosome. This is done by using a plasmid vector carrying the substitution allele as well as sce site(s) encoded in the vector backbone.[7] The second step introduces the I-*SceI* gene expression plasmid, which results in I-*SceI* cleaving the marked DNA at the sce sites creating a double-stranded break. This step increases rates of recombination and provides selection for a second homologous recombination event to replace the chromosomal allele with the vector carried allele.[3,7] However, by isolating an I-*SceI* allele encoding an enzyme with temperature-dependent activity, the I-*SceI* gene and *sce* site could be combined into a single plasmid, which would be stable at permissive temperatures, greatly simplifying the use of I-*SceI* in genomic engineering.

A library of mutant I-*SceI* alleles has been created by mutagenic PCR and screened proteins encoded by these alleles for their ability to cure plasmids carrying *sce* sites in a temperature-dependent fashion. An I-*SceI* allele with enhanced activity at 42°C but reduced activity at 33°C was used for the creation of the allelic exchange vector, pEXSceM0, containing both an I-*SceI*gene and sce sites. The utility of our temperature-dependent I-*SceI* allelic exchange vector was demonstrated by deleting the *amrRAB-oprA* multidrug efflux pump structural and regulatory genes from the Select Agent-excluded *Burkholderia pseudomallei* strain, Bp82. This generated a strain with enhanced susceptibility to aminoglycosides and macrolides.[8,9]

Results and Discussion

Isolation of mutant I-SceI alleles

A pool of I-*SceI* mutants was initially created for testing with a two-plasmid counter-selection system by mutagenic PCR, followed by cloning the I-*SceI* gene into pARAGW, replacing the Gateway cassette. This provides positive selection for clones with a replaced cassette. Only clones with a replacement could survive due to toxicity of the CcdB toxin carried by the GW cassette. Isolated colonies were picked by a Qpix

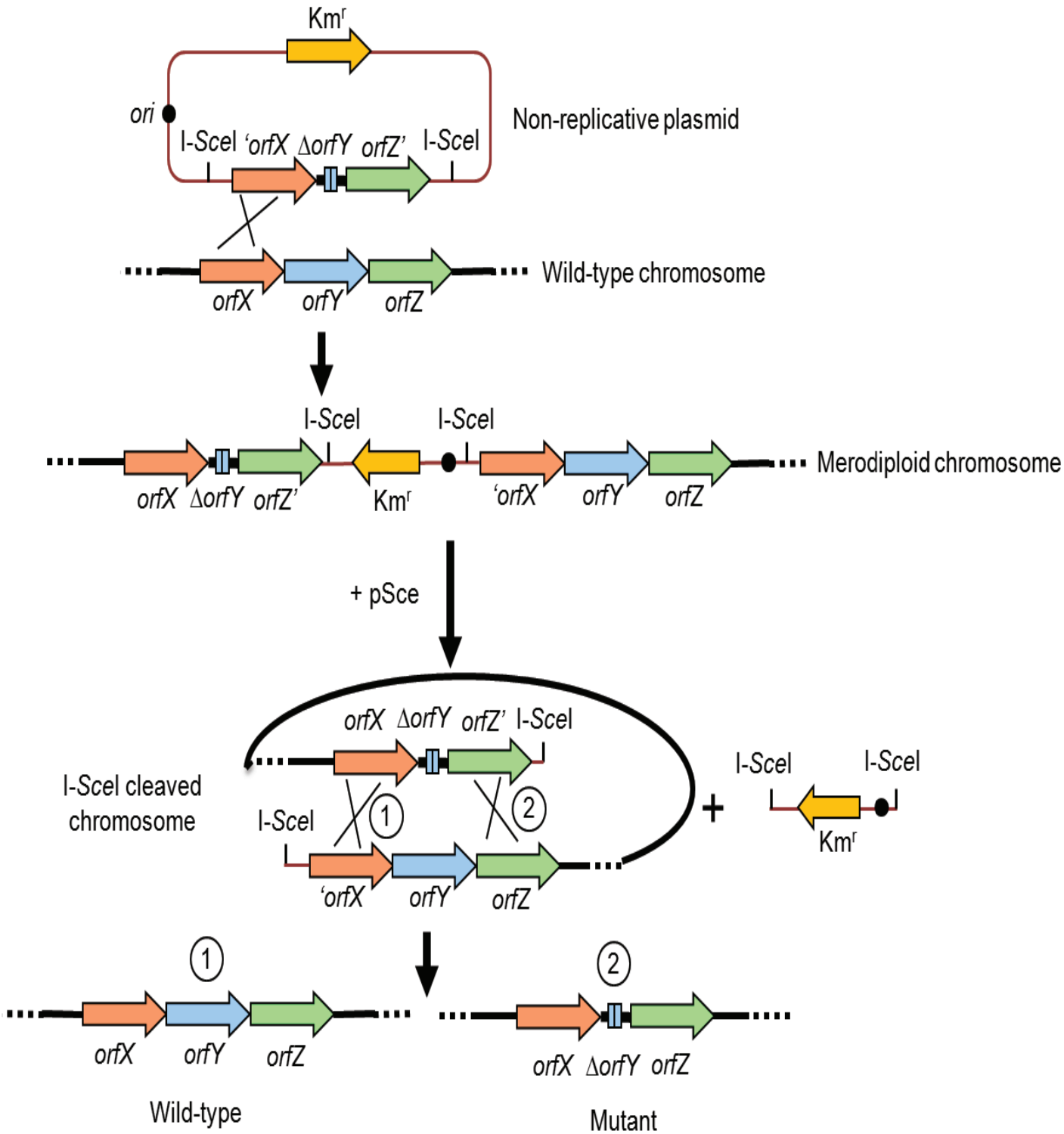

Figure 1. Schematic of two-step I-SceI allelic-exchange procedures. A chromosomal segment containing a deletion of orfY with flanking orfX and orfZ sequences is cloned into an appropriate vector that does not replicate in the target bacterium because it contains a narrow host range origin of replication (ori). This plasmid is transformed into the target bacterium, followed – in this case – by kanamycin resistance (Kmr) selection. This step results in integration of the allelic replacement construct into the chromosome by homologous recombination between cloned and chromosomal sequences. For I-*SceI* catalyzed resolution, the merodiploid is transformed with the I-SceI expression construct which results in double-stranded cleavage of the chromosome and release of most of the plasmid backbone. This event can be monitored by loss of Kmr. Repair of the double-stranded break by homologous flanking repeat sequences leads to formation of a wild-type strain (event denoted by circled "1") or a mutant strain (event denoted by circled "2"). The two events are distinguished by phenotypic analyses and/or PCR.

colony-picking robot and pooled. One allele, Rob3A, was identified from initial tests that allowed partial curing of sce reporter plasmid pR6KSCE (Figure 2B) at 25°C and decreased curing at 33°C. Compared to the wild type I-SceI protein, the Rob3A allele encodes a protein with six amino acid substitutions: K65N, M70V, N154Y, V74I, Y201N, and S204P.

Counter-selection curing with a two-plasmid system

A two-plasmid system is currently the common counter-selection method for I-*SceI*. A two-plasmid counter selection reporter system was employed in this study to determine the curing rates of I-*SceI* mutant alleles. It consists of a plasmid containing an I-*SceI* mutant allele, pARAIS*ceI*mut, and a reporter plasmid, pR6KSCE, containing a single *sce* site (Figure 2A and 2B). The two-plasmid system was utilized to determine the curing rates of the sce site containing reporter plasmid by the I-*SceI* Rob3A mutant allele, compared to spontaneous curing of the reporter plasmid. The I-*SceI* Rob3A allele showed maximal curing rates at 42˚C with decreased cure rates at 25˚C and almost no curing at 33˚C. In contrast, the reporter plasmid was maintained at all three temperatures tested in the absence of I-*SceI* Rob3A (Figure 3). These results provide evidence for the temperature-dependent and endonuclease-based curing of the reporter plasmid containing a *sce* site.

Screening for alleles with improved I-SceI activity

After initial tests the Rob3A allele showed promising results and the I-*SceI* gene was added to an allelic exchange vector carrying two *sce* sites. A one-plasmid system was created by placing the I-*SceI* gene and two sce sites onto a single plasmid also containing a *gusA* indicator gene and a gene encoding Km resistance. A secondary mutant pool was created by PCR mutagenesis in an attempt to identify a one-plasmid system clone expressing an I-*SceI* restriction endonuclease allele with more pronounced temperature-dependent activity. The screening assay consisted of assessing curing of the I-*SceI* plasmid by a decreased blue colony count on X-Gluc plates due to the loss of the *gusA*

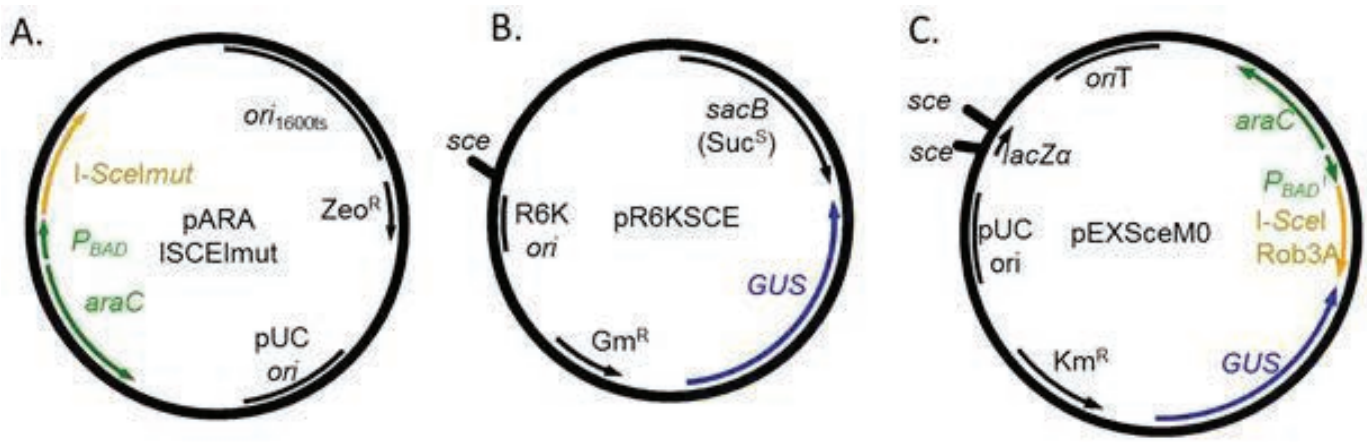

Figure 2. Maps of vectors used for two-plasmid and one-plasmid I-SceI-based curing
A. Plasmid pARAI*SceI*mut contains an I-SceI allele from the pool of mutant alleles, the P_{BAD} promoter which is under control of the araC encoded regulator, a pUC origin of replication, a temperature sensitive origin of replication ($ori1_{600ts}$) and a zeocin resistance (Zeo^R) marker
B. Plasmid pR6KSCE contains the *sacB* counter selection marker, a *gusA* indicator gene, an R6K origin of replication and a gentamicin (Gm^R) resistance marker.
C. Plasmid pEXSceM0 contains an origin of transfer (*oriT*), a *gusA* indicator gene, a pUC origin of replication, a gene expressing the I-SceI Rob3A allele from the P_{BAD} promoter and a LacZα peptide-encoding gene with two I-*SceI* cleavage sites (*sce*).

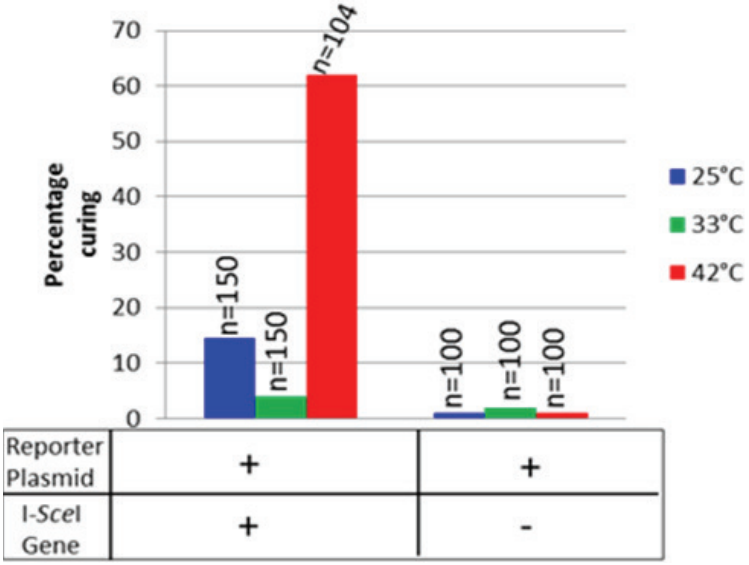

Figure 3. The Rob3A protein causes temperature-dependent plasmid curing at 42°C. DH5α(λpir+) carrying the GmR pR6KSCE sce containing reporter plasmid was transformed with the pARAISceImut vector expressing the Rob3A allele and transformant clones were recovered at 25°C, 33˚C and 42°C. pARAISceImut Rob3A transformants were screened for GmR, which determined percentage curing rates of the pR6KSCE reporter at each temperature. DH5α(λpir+)/pR6KSCE was incubated under identical conditions to determine the percentage rate of pR6KSCE curing in the absence of I-SceI. The sample number of clones (n) screened from each test condition is listed.

gene and susceptibility to Km.

Initial screening on LB plates containing X-Gluc and LB plates containing 35 µg/ml Km produced many candidates that were later proven by PCR and DNA sequencing not to contain an I-*SceI* gene. The reason(s) for these puzzling results remain unclear. After intensive screening of 575 plasmids, one plasmid, pEXSceMQ, carrying I-*SceI* allele Q18 demonstrated increased cure rates and was shown to contain the I-*SceI* gene. The map of pEXSceMQ is the same as that of pEXSceM0 (Figure 2C), but the I-SceI protein encoded by it has 18 additional amino acid substitutions compared to Rob3A, none of which represent reversion back to wild type I-*SceI* sequence. Mutations compared to the wild-type I-SceI protein are as follows (initial Rob3A mutations are indicated by bold letters): N5P, N9Y, **K65N** Y69H, **M70V**, C74T, Y77F, V98E, Q104L, F106S, N116D, L140Q, D152V, **N154Y**, K160E, S161P, F169L, E173G, **V174I**, **Y201N**, **S204P**, M223T, E233V, and K237E.

Induction or repression of PBAD driven I-SceI expression does not affect plasmid curing

Expression of the I-*SceI* gene in pEXSceM0 (Figure 2C) and pEXSceMQ is under control of the PBAD promoter and its cognate regulator encoded by araC. Transcription from PBAD is induced in the presence of arabinose and repressed in the presence of glucose.[10] Both DH5α/pEXSceMQ and DH5α/pEXSceM0 were used to determine possible effects of absence or presence of induction on plasmid curing. DH5α/pEXKm5 was used as a negative control in this experiment because it does not contain an I-*SceI* gene. The results shown in Figure 4 demonstrate an increased cure rate of all three plasmids when cells are grown at 42°C, but cure rates were higher with plasmids encoding I-*SceI*. Absence or presence of inducing or repressing sugars had no effect on plasmid cure rates. These results indicate that plasmid curing is increased in the presence of I-*SceI* but not dependent on its induction. Increased cure rates in cells grown at 42°C are probably due to plasmid instability at higher temperatures.

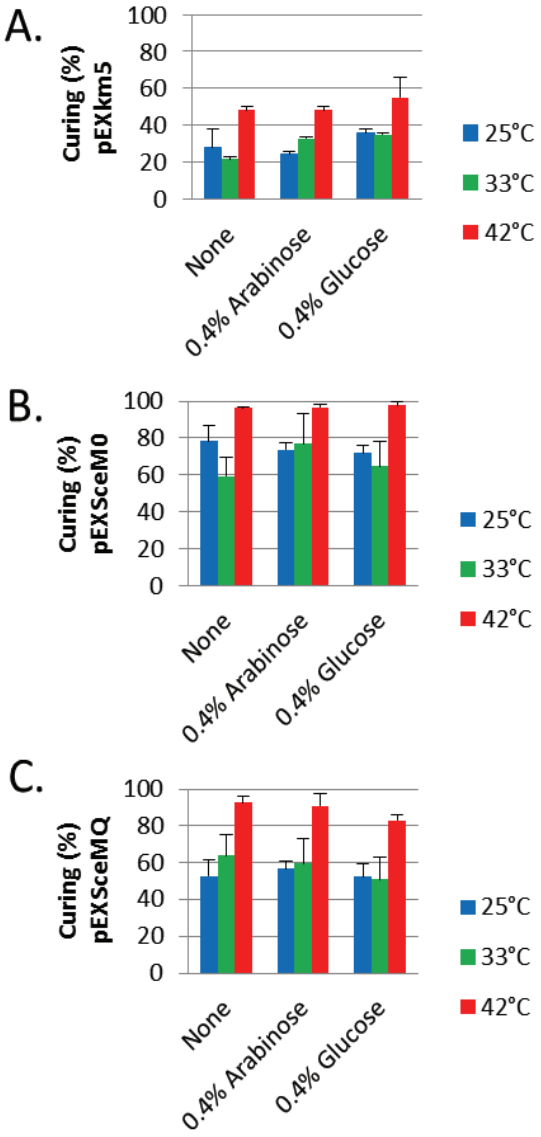

Figure 4. Neither induction nor repression of PBAD driven I-SceI expression effect curing percentages in a one-plasmid system. DH5α with the indicated plasmids, pEXKm5 (A), pEXSceM0 (B) and pEXsceMQ (C), was grown at 25°C, 33°C and 42°C in LB medium with no addition (none) or with 0.4% arabinose (inducing conditions) or with 0.4% glucose (repressing conditions). Ratios of blue to white colonies that grew on LB medium with X-Glux were calculated to determine plasmid curing percentages. All experiments were performed in triplicate.

The new pEXSceM0 vector is functional in allele replacement experiments

After initial experiments demonstrated use of pEXSceM0 in plasmid curing experiments, the use of I-SceI single vector counter-selection system in allelic exchange was tested by attempting to introduce a pEXSceM0-borne amrRAB-oprA deletion construct into the *B. pseudomallei genome*. The construct was integrated into the Bp82 genome after conjugation from E. coli (*Escherichia coli*) via a single crossover, creating a merodiploid exconjugant (Figure 5A and B). Undesired plasmid and chromosomal DNA sequences were then deleted via a second recombination event that was stimulated via I-SceI mediated chromosomal DNA cleavage (Figure 5C). Successful merodiploid resolution was assessed by obtaining white colonies on X-gluc that were Km^S and Gm^S due to the loss of the plasmid backbone that encodes Km^R and the wild-type *amrRAB-oprA* region, which when present confers Gm^R. PCR was performed to confirm the presence of the *amrRAB-oprA* deletion.

The results shown in Figure 5D confirm that the pEXSceM0-borne Δ(*amrRAB-oprA*) mutation was successfully transferred to the Bp82 chromosome. PCR primers used yield an amplicon size of 1,251 bp with DNA from strains containing the Δ(*amrRAB-oprA*) allele and an amplicon size of 215 bp with DNA from merodiploid strains with an oriT in the integrated plasmid backbone. The Δ(*amrRAB-oprA*) deletion allele was present in the merodiploid BP82SceM0-M2 and one of the resolved strains, BP82SceM0-R3. The deletion could not be detected in the

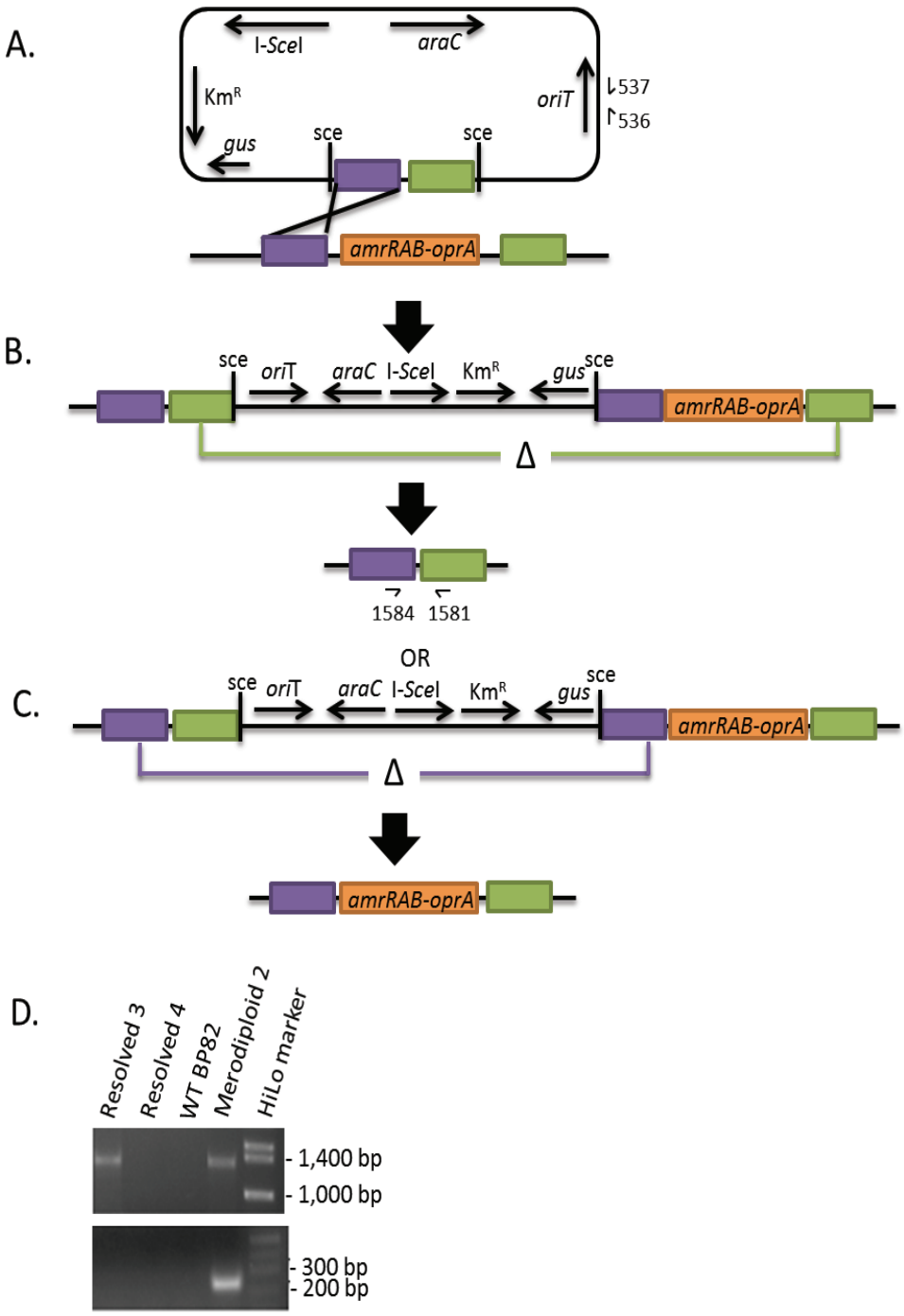

Figure 5. Schematic representation for the steps of allelic exchange at the *B. pseudomallei amrRAB-oprA* locus using pEXSceM0. The pEXSceM0 vector is transferred from *E. coli* to *B. pseudomallei* by conjugation and KmR exconjunats are selected. Because pEXSceM0 cannot replicate in *B. pseudomallei*, KmR colonies represent merodiploids which have the plasmid sequences incorporated in the chromosome via homologous recombination between sequences that flank the *amrRAB-oprA* deletion region. In the illustrated example recombination occurs between the cloned left flanking region and the respective chromosomal sequences (A). I-SceI expression leads to cleavage of chromosomal DNA and stimulates homologous recombination which either leads to incorporation of the plasmid-borne Δ(*amrRAB-oprA*) deletion into the chromosome (B) or restoration of the wild-type configuration of the *amrRAB-oprA* region (C). Inverted half arrows indicate primers used to check for presence of *oriT* sequences (536 & 537) or the presence of the Δ(*amrRAB-oprA*) mutation (1581 & 1584). Although, the 1581 and 1584 priming sites are present in the respective strains, the segment located between them in the intact *amrRAB-oprA* region in the wild-type strain and merodiploid is too large to be amplified using normal PCR conditions. (D). PCR confirmation of allelic exchange with primers 1581 & 1584 (presence of Δ(*amrRAB-oprA*) mutation, top panel) and 536 & 537 (presence of *oriT*, bottom panel). The 1,251 bp amplicon signifying presence of the Δ(*amrRAB-oprA*) mutation was present in the merodiploid strain Bp82SceM0-M2 and resolved strain Bp82SceM0-R3. It could not be detected in the parent strain Bp82 and strain BP82SceM0-R4, indicating that this strain had reverted to the wild-type configuration of the *amrRAB-oprA* region. As expected, the *oriT* sequences were only detected in the merodiploid strain BP82SceM0-M2. Abbreviations: *araC*, AraC protein encoding gene; *gusA*, glucuronidase encoding gene; I-*SceI*, endonuclease encoding gene; KmR, kanamycin resistance gene; *amrAB-oprA*, AmrAB-OprA efflux operon; *amr*R, *amrAB-oprA* efflux operon repressor; *oriT*, origin of transfer; $P_{BAD,}$ *E. coli* arabinose operon promoter; *sce*, I-*SceI* cleavage sites.

resolved strain BP82SceM0-R4 indicating that it had reverted to back to wild-type. The *oriT* sequence could only be detected in the merodiploid strain BP82SceM0-M2.

Materials and Methods

Bacterial strains and culturing conditions

Bacterial strains used in this study are listed in Table 1. Strains were grown in Lennox Broth (LB) media (MO BIO Laboratories, Carlsbad, CA) or agar solidified LB plates at 42°C, 37°C, 33°C, or 25°C, as noted below. Media for *E. coli* were augmented with antibiotics or colorimetric agents at the following final concentrations: 35 µg/ml kanamycin (Km) (Gold Biotechnology, St. Louis, MO); 15 µg/ml gentamicin (Gm)(EMD Biosciences, San Diego, CA); 25 µg/ml zeocin (Zeo)(Life Technologies, Grand Island, NY); 25 µg/ml chloramphenicol (Cm)(Sigma, St. Louis, MO); and 40 µg/ml 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc)(Gold Biotechnology, St. Louis, MO). For growth of E. coli RHO3, media was supplemented with 200 µg/ml diaminopimelic acid (DAP) in liquid cultures or 400 µg/ml DAP for agar solidified plates. B. *pseudomallei* Bp82 strains were grown in LB with 80 µg/ml adenine at 37°C with constant agitation overnight. For recovery of Km resistant Bp82 exconjugant merodiploids, cells were plated on LB plates containing 1000 µg/ml Km.

Strain	Genotype/Relevant Features	Reference or Source
E. coli		
DH5α	F^-Φ80 *lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17*(rK^- mK^+) *phoA glnV44*	(11)
DH5α(λ*pir*$^+$)	DH5α(λ*pir*$^+$)	(12)
MaH1	DH5α::mini-Tn7-*pir116*$^+$	(13)
JM110(λ*pir*$^+$)	λ*pir*$^+$, *rpsL* (Sm^R) *thr leu thi-1 lacY galK galT ara tonA tsx dam dcm glnV44* Δ(*lac-proAB*) [F' *traD36 proAB lacIqZ*ΔM15]; Dam and Dcm methylase-deficient	(14)
DB3.1	F^- *gyrA462 endA1 glnV44* Δ(*sr1-recA*) *mcrB mrr hsdS20*(r_B^-, m_B^-) *ara14 galK2 lacY1 proA2 rpsL20* (Sm^R) *xyl5* Δ*leu mtl1*; strain for maintenance of plasmids containing the $CcdB^R$ Gateway cassette	Invitrogen
KVT6	F^-,Δ(*gpt-lac)5*, *glnV44*(AS), *glnV89*(AS), λ *cI*$^+$, *ara*(FG), *gyrA-0*(NalR), metB, argE (Am), rpoB0(RifR), thiE1, gyrA462; CcdBR Gateway maintenance strain	Schweizer laboratory stock
RHO3	F^-, λ*pir, thi-1, thr-1, leuB6, lacY1, tonA21, glnV44, recA, yfcU*::Mu, Δ*asd*::*FRT*, *glvB*::RP4-2-Tc^R::Mu, Δ*aphA*::*FRT* (Km^S); DAP-dependent conjugation strain	(7)
Burkholderia pseudomallei		
Bp82	1026b Δ*purM*; Select Agent excluded strain, adenine auxotroph	(15)
Bp82SceM0-M2	Bp82::pEXSceM0Δ(*amrRAB-oprA*); merodiplid strain with plasmid integrated at the *amrRAB-oprA* locus; GUS^+, Km^R	This work
Bp82SceM0-R3	Bp82 Δ(*amrRAB-oprA*); aminoglycoside and macrolide susceptible strain	This work

Table 1. Strains used in this study

Descriptive Name	Laboratory Name	Relevant Features	Source [GenBank Accession Number]
pUC18-mini-Tn7T-Gm-GW	pPS1612	Source of Gateway cassete for pARAGW; Amp^R, Gm^R, Cm^R	(16)[AY737004]
pBADSCE	pPS2212	I-*Sce*I expression vector; Zeo^R	(17)[FJ797515]
pARAGW	pPS2875	Source of Gateway cassette for pEXK-mGW; Zeo^R, Cm^R	This study [JX516791]
pEXKmGW	pPS3028	Gateway destination vector; GUS^+, Km^R	This study
pR6KSCE	pPS2842	*sce* counter selectable marker; sucrose curable, GUS^+, Gm^R	This study
pARAISCEImut	pPS2929	Plasmid containing I-*Sce*I Rob3A mutant gene; Zeo^R	This study
pEXKm5	pPS2539	Allelic exchange vector, GUS^+, sucrose curable; Km^R	(7) [GQ200735
pEXSceM0	pPS2931	Allelic exchange vector; GUS^+, I-*Sce*I Rob3A, Km^R	This study
pEXSceMQ	pPS3067	Allelic exchange vector; GUS^+, I-*Sce*I Q18, Km^R	This study
pEXKm5-Δ(*amrRAB-oprA*)	pPS2557	Δ(*amrRAB-oprA*) construct; sucrose curable, GUS^+, Km^R	Schweizer laboratory stock
pEXSceM0-2	pPS3068	Δ(*amrRAB-oprA*) construct; I-*Sce*I curable, GUS^+, Km^R	This study

Table 2. Plasmids used in this study

Plasmid DNA purification

Plasmids used in this study are listed in Table 2. *E. coli* strains were cultured in LB medium with appropriate antibiotics at 33°C with constant agitation overnight. Plasmid DNA was isolated using the Fermentas Life Sciences GeneJet plasmid mini prep kit, (Thermo Scientific, Glen Burnie, MD) according to the manufacturer's instructions.

DNA manipulation

Restriction enzymes and *Taq* DNA polymerase were purchased from New England Biolabs (Ipswich, MA) and used according to manufacturer's recommendations. T4 ligase was purchased from Invitrogen (Grand Island, NY). All oligonucleotides were obtained from Integrated DNA Technologies (Coralville, Iowa) and are listed in Table 3. PCR reactions were performed as previously described in[18] (Table 4.)

Plasmid transformation

Competent cells were prepared using a standard rubidium chloride protocol.[19] Cells were transformed with plasmid DNA using previously described methods.[18] For purposes of two-plasmid curing efficiency

testing, transformation mixtures were evenly split into three tubes of 200 µl each, which were incubated for 1 h without agitation at 25˚C, 33˚C, or 42˚C, respectively.

Plasmid construction

To create pARA-GW, pBADSCE[7] was digested with Acc65I and HindIII, and the 5,980 bp backbone was ligated to the 1,746 bp Acc65I+HindIII digested Gateway (GW) cassette fragment from pUC18-mini-Tn7T-Gm-GW.[17] After transformation into *E. coli* DB3.1, transformants were recovered on LB+Zeo+Cm plates. This step replaced the I-*SceI* gene with a GW cassette. The ribosome-binding site of the I-*SceI* gene was left intact. Correct clones were confirmed by Acc65I+HindIII and Acc65I+SalI digests.

To create pARA I*Sce*Imut (Figure 2A), the I-*SceI* gene was PCR-amplified from pBADSCE using error-prone PCR and oligonucleotide primers 2352 and 2353 (Table 4) that introduce flanking KpnI and XhoI restriction sites. The 100 µl PCR reaction was split into four 25 µl reactions to avoid overrepresentation of early-cycle mutations in the pool. The PCR amplicons representing a library of mutant I-*SceI* alleles were digested with KpnI+XhoI and cloned into the KpnI+XhoI digested pARA-GW arabinose inducible expression vector to create the pARA I*Sce*Imut library. The cloned mutant I-*SceI* alleles replaced the GW cassette which encodes the CcdB toxin encoding gene. By recovering the plasmids in a $CcdB^S$ strain of *E. coli*, this provided selection against any clones that lack an *I-SceI* allele.

The pARA I*Sce*Imut library was then transformed into the I-*Sce*I activity reporter strain DH5α(λ*pir*$^+$)/pR6KSCE and transformants were plated onto LB+Zeo+0.2% arabinose and incubated at 25°C for 3 days. 1,900 isolated colonies were then picked by a Qpix colony picking robot into LB and LB containing Gm and incubated at 37°C to identify Gm^S clones. A total of 19 GmS clones were identified, pooled, and used for plasmid DNA isolation, creating a 19-clone pool. The 19-clone pool was re-transformed into the DH5α(λ*pir*$^+$)/pR6KSCE reporter strain and transformants were plated on LB+Zeo+0.2% arabinose and incubated at 33°C. Colonies from these plates were then patched onto Gm-containing plates in allele was PCR amplified from pEXSceM0, using error-prone PCR and oligonucleotide primers 2352 and 2353 as described above. The mutagenic PCR products were then purified, combined with pEXKmGW, and digested with NheI and ZraI. The digested DNA was purified directly from the digest reaction using the GenElute Gel Extraction Kit and ligated overnight at 14˚C. The ligation was transformed into *E. coli* DH5α and glycerol was added to a final concentration of 15%. The mixture was then divided into 200 µl aliquots and the resulting cell suspensions were frozen at -80˚C, creating a mutagenic pool for screening.

Primer	Laboratory Name	Sequence 5'→3'
ISCE-I mut F	P2352	GGGCTAGCAGGAGGGTACC
Aat-iSCEmut-rev.2	P2522	TCTGACGTCGCTGAAAATCTTCTCTCATCCGCC
ISCE Forward	P2520	GATCGATGTTGATGATCGGTTTGTTTTT
ISCE Reverse	P2521	GCACAAAAAAGAACGTGTTAACCACCT
ISCE-I mut F	P2352	GGGCTAGCAGGAG GGTACC
ISCE-I mutR	P2353	TAACTCGAG ACGTCGGGCCCTTATT
OriT Up	P536	TCCGCTGCATAACCCTGCTTC
OriT DN	P537	CAGCCTCGCAGAGCAGGATTC
amrRAB-oprA-UP-For	P1581	AGGGTGTCCACATCCTTGAA
amrRAB-oprA-DN-Rev	P1584	GAAATACGCCTTGACGCACT

Table 3. Primers and oligonucleotides used in this study

Determining temperature-dependent I-SceI/sce curing rates with a two-plasmid reporter system

pARA I*Sce*Imut was transformed into DH5α(λ*pir*$^+$)/pR6KTSCE and cultures were outgrown, standing for one hour at either 25˚C, 33˚C, or 42˚C in liquid LB. Transformation mixtures were plated onto LB+25 µg/ml Zeo+0.2% arabinose and incubated at either 25˚C, 33˚C, or 42˚C. Recovered colonies were patched onto LB plates containing Gm to check for the presence or absence of the *sce* site containing Gm^R reporter plasmid pR6KTSCE.

Medium throughput screening of I-SceI/sce curing rates

Mutagenic pool aliquots from the secondary pool of mutant I-*Sce*I alleles were removed from the -80˚C freezer, plated onto LB agar plates containing 35 µg/ml Km and the plates were incubated standing overnight at 33˚C. Recovered colonies were inoculated into 200 µl of LB medium and incubated standing overnight at 33˚C. The incubated overnight cultures were diluted 1:100 in 200 µl aliqouts with LB + 0.4% arabinose in wells of three 96 well plates, and the plates were incubated standing overnight at 25˚C, 33˚C, or 42˚C. A 1:100 dilution was made of each overnight culture and 10 µl of each sample was spot-plated onto both LB+Km and LB+X-Gluc plates. The plates were incubated overnight at 33˚C. Spots on LB+Km plates were then checked for decreased colony counts and LB+X-Gluc plates were checked for a decrease in the number of blue colonies. 575 individual plasmids were screened using this procedure.

To verify the presence of an I-*Sce*I gene, candidates were retransformed into DH5α and subjected to a second round of spot testing, and all potential candidates were confirmed via sequencing and standard PCR by amplifying a 280 bp I-*SceI* probe with primers 2520 and 2521. pEXSceMQ (pEXSceM0 derivative from test plate well Q18) was isolated using this procedure.

Quantitative self-curing tests

One ml of LB medium was inoculated with either DH5α/pEXSceM0, DH5α/pEXSceMQ, or DH5α/pEXKm5 (I-*Sce*I lacking, negative control vector), and cultures were incubated standing overnight at 33˚C. A 1:100 dilution was made into three 1.5 ml microfuge tubes containing LB+0.4% arabinose to 1 ml final volume. These cultures were then incubated standing overnight at either2 5˚C, 33˚C, or 42˚C. Tests were also conducted with LB+0.4% glucose (to repress expression from the P_{BAD} promoter) and plain LB. Serial dilutions were made into sterile saline to obtain a 10^{-5} dilution and a 10^{-6} dilution from each temperature variant culture. These dilutions were then plated onto LB+X-Gluc plates. After overnight incubation at 33˚C percentages of blue versus white colonies were recorded.

Alleleic exchange testing

To create a pEXSceM0-2 plasmid vector Bp82 merodiploid strain, 1 ml of RHO3/pEXSceM0-2 and 1 ml of Bp82 overnight culture were harvested by centrifugation, washed twice in equal volumes of fresh LB+adenine and concentrated 5X into 200 µl. 20 µl samples of both cell suspension were combined on a sterile cellulose acetate filter on an LB+DAP+adenine plate alongside individual parental controls and incubated overnight at 33°C. Cells were recovered from the filters by centrifugation and washed 1x in 1 ml LB. Merodiploids were recovered by plating contents of conjugation filters on LB+1,000 µg/ml Km+adenine.

Merodiploids were resolved using the I-*Sce*I counter-selection curing protocol by first inoculating merodiploids into 1 ml of LB and incubating at 33° standing. Second, three 1:100 dilutions in LB were made of the initial overnight and incubated at 25°, 33°, or 42°. Another 1:100 dilution was made for each tube and plated onto LB agar containing X-Gluc. White colonies growing on these plates were patched onto LB+adenine, LB+Gm+adenine, and LB+Km1000+adenine. Patches that were Gm^S, Km^S and grew on the LB were then cultured for DNA boiling preparations.

DNA boiling preparations were performed by combining 10 µl of culture with 30 µl of distilled H_2O followed by boiling on a hot plate for 10 min. PCR was used to check for the presence of the Δ(*amrRAB-oprA*) mutation. Bp82, BP82SceM0-M2, and resolved BP82SceM0-R3 and R4 were confirmed with PCR using primers, allowing detection of the *amrRAB-oprA* deletion construct and the *oriT* from pEXSceM0. Primers 1581 and1584 flanking the deletion construct of the *amrRAB-oprA* region were used for confirmation of the presence of the Δ(*amrRAB-oprA*) mutation. Electrophoresis using 1% agarose gels was performed to identify the expected Δ(*amrRAB-oprA*) 1,251 bp amplicon. Bp82 and wild type revertants should yield an amplicon size of 6,709 bp. Primers 536 and 537 were used to amplify an *oriT* probe amplicon of 215 bp.

Conclusions

Substantial progress has been made towards the development of a temperature dependent I-*Sce*I allele to make the process of counter-selection with I-*Sce*I more broadly applicable and simpler to use. Currently, the most effective counter-selective system that does not require supplementary genetic manipulation is the use of the *Bacillus subtilis sacB* gene along with sucrose, since this combination causes levan-poisoning in many bacterial strains. However, *sacB* is not compatible to work with bacterial species containing endogenous *sacB* genes. The temperature-dependent I-*Sce*I/*sce* system does not require additional genetic manipulation or host-/replicon-dependent mutations and works as counter-selection with a simple shift in temperature. This makes this new genetic tool desirable for researchers working with diverse bacteria. I-*Sce*I generated DNA stranded breaks are highly lethal regardless of the target organism, making this genetic tool highly versatile.

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