A high-efficiency scar-free genome-editing toolkit for Acinetobacter baumannii

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Received 18 April 2022; accepted 5 September 2022

Background: The current mutagenesis tools for Acinetobacter baumannii leave selection markers or residual sequences behind, or involve tedious counterselection and screening steps. Furthermore, they are usually adapted for model strains, rather than for MDR clinical isolates.

Objectives: To develop a scar-free genome-editing tool suitable for chromosomal and plasmid modifications in MDR A. baumannii AB5075.

Methods: We prove the efficiency of our adapted genome-editing system by deleting the multidrug efflux pumps craA, cmlA5 and resistance island 2 (RI2), as well as curing plasmid p1AB5075, and combining these mutations. We then characterized the susceptibility of the mutants compared with the WT to different antibiotics (i.e. chloramphenicol, amikacin and tobramycin) by disc diffusion assays and determined the MIC for each strain.

Results: We successfully adapted the genome-editing protocol to A. baumannii AB5075, achieving a double recombination frequency close to 100% and routinely securing the construction of a mutant within 10 working days. Furthermore, we show that both CraA and p1AB5075 are involved in chloramphenicol resistance, and that RI2 and p1AB5075 play a role in resistance to amikacin and tobramycin.

Conclusions: We have developed a versatile and highly efficient genome-editing tool for A. baumannii. We have demonstrated it can be used to modify both the chromosome and native plasmids. By challenging the method, we show the role of CraA and p1AB5075 in antibiotic resistance.

Introduction

Acinetobacter baumannii is an aerobic Gram-negative bacterium that is widespread in the environment and inhabits different niches.¹⁻³ However, it can also be an opportunistic pathogen that infects immunocompromised patients.³⁴ Nowadays, it is estimated that up to 10% of nosocomial infections in the USA and 2% in Europe are caused by this pathogen, with these frequencies almost doubling in Asia and the Middle East. Furthermore, around 45% of A. baumannii isolates in global terms exhibit MDR (i.e. resistance to at least three classes of antibiotics), with local rates rocketing to 70% in Latin America and the Middle East.⁴⁻⁷ Due to this, A. baumannii has been included among the most concerning MDR pathogens under the acronym ESKAPE (Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, A. baumannii, Pseudomonas aeruginosa and Enterobacter spp.).⁸ Moreover, a WHO report highlighted carbapenem-resistant A. baumannii as a priority pathogen, for which novel therapeutic approaches urgently need to be developed.⁹

The recalcitrance of this species to treatment is due to its capacity for resistance and persistence,¹⁰ aided by its multiple MDR mechanisms. These include the cell envelope as a barrier, multidrug efflux systems and mutations in genes coding for porins and antibiotic targets (e.g. ribosomal proteins, PBPs, DNA replication enzymes and the lipid A biosynthetic pathway), as well as enzymes that degrade/inactivate antibiotics.³ Oftentimes, these features can spread among the population through mobile genetic elements and the ability of A. baumannii to be naturally competent.¹¹⁻¹²

With technological advances, genome-editing tools have evolved, allowing precise genome editing (i.e. insertions and deletions), from a single nucleotide to dozens of kilobases. However, this progress is often uneven, with tools being developed in a biased way for a few well-established model organisms. In the case of A. baumannii, many simple targeted genetic tools have...
been adapted for use in model strains of this pathogen (reviewed by Sykes et al.13). Mutagenesis in A. baumannii was firstly approached by gene disruption by plasmid insertion in a single recombination event and mutation by antibiotic resistance marker insertion.14 Next-step strategies include recombinase-based gene disruption followed by removal of the selection marker by site-specific recombination, allowing the use of the same marker for subsequent rounds of mutation to construct multiple mutants.15 Even more refined, some protocols allow scarless gene modification by double recombination aided by a counter-selectable marker, with strategies taking advantage of the ability of A. baumannii to be naturally competent.16–18 Moreover, after the emergence of clustered regularly interspaced short palindromic repeats (CRISPR)-Cas systems as a molecular biology tool, a CRISPR-based two-plasmid system for genome editing and a CRISPR interference (CRISPRi) kit for knocking down gene expression have been developed for A. baumannii.19,20

However, depending on the purpose they are intended for, these genetic editing methods can have some limitations. Gene disruption is not always desirable due to the limited amount of selection markers available and possible polar effects within operons. Strategies including marker removal are usually based on site-specific recombinases that leave a scar in the genome.15,21 However, this recombinogenic sequence may cause genomic instability after successive rounds of mutation.22 These drawbacks can be prevented by counterselection-mediated scar-free strategies, which allow more complex genome manipulation (i.e. targeted point mutations, domain truncations, allele exchange, deletion of whole clusters), but complex genome manipulation (i.e. targeted point mutations, domain truncations, allele exchange, deletion of whole clusters), but counterselection (usually based on sucrose sensitivity conferred by sacB, which is often unstable in A. baumannii) frequently requires passing under pressing selection and tedious screening for clones that underwent a second recombination event.16,23 Furthermore, the current tools are mainly developed for model A. baumannii strains, which can be less representative compared with the prevalent clinical isolates. Another major limitation to the application of these tools is that MDR A. baumannii strains are resistant to many of the selection markers used in these protocols.13

In our efforts to implement state-of-the-art methodologies for standardization of genome editing in non-model MDR A. baumannii strains, we have adapted an accelerated highly efficient SceI-based mutagenesis method24–27 developed and optimized for Pseudomonas putida,22,28 to MDR A. baumannii AB5075.5 For this, we have modified the two plasmids used in this system with selectable markers that can be used in this strain and subsequently adapted the protocol pipeline. As a proof of concept, we have constructed an in-frame deletion mutant in craA, a gene encoding a dedicated chloramphenicol-specific efflux pump. Afterwards, we have attempted to address the function of cmlA5, a putative plasmid-borne chloramphenicol efflux pump-coding gene inferred from homology, by comparison with the craA mutant. As a result, we have validated the utility of this system for scar-free chromosomal and plasmid editing in A. baumannii AB5075.

Materials and methods

Bacterial strains and culture media

A. baumannii AB5075 (VIR-O colony morphotype),5,29,30 its derivate mutants and Escherichia coli host strains (DH5α and DH5α apr) were routinely grown in liquid or solid LB (Miller) at 37°C (180 rpm or static, respectively)22,31 When necessary, LB was supplemented with kanamycin (25 mg/L), ampicillin (100 mg/L), apramycin (60 mg/L for E. coli, 200 mg/L for A. baumannii), tetracycline (5 mg/L) or tellurite (6 mg/L for E. coli, 30 mg/L for A. baumannii). A summary of strains used in this work is shown in Table S1, available as Supplementary data at JAC Online.

Plasmid construction

A list of plasmids and primer sequences used in this work can be found in Table S1. All plasmid derivatives were constructed using standard restriction-based molecular cloning.

pEMG-Tel (pEMG) was constructed by cloning a DNA fragment from pMol30-TelR (Addgene, #50799) (bearing the Tel resistance marker) digested with Smal in pEMG cut with AffIII and blunted with Klenow.16,22 For construction of pSW-Apr and pSW-Tc, PCR fragments amplified from pFLAG-attP (Addgene, #110095) with primers Apr fw/Apr rv and from pSEVA524 with primers teta fw/teta rv, respectively, using Q5 High-Fidelity Master Mix (New England Biolabs) were cloned into pSW-1 digested with ScaI.22

For in-frame deletion of craA (ABUW_0337) and cmlA5 (ABUW_4059), pEMG-craA and pEMG-cmlA5 were constructed. For pEMG-craA, 1 kb upstream and downstream homologous regions were amplified from purified AB5075 genomic DNA with primers craA up fw/craA up rv and craA down fw/craA down rv, respectively, and assembled together by joining PCR. The same procedure was followed for assembly of the cmlA5 deletion construct using primer pairs cmlA5 up fw/cmlA5 up rv and cmlA5 down fw/cmlA5 down rv. Both constructs were cloned into pEMG digested with Smal.

Constructs for ΔcraA complementation experiments were generated by amplifying the cmlA5 coding region plus the upstream homologous region (primer pair cmlA5 up fw/cmlA5 rv) and the upstream region alone as a control (primer pair craA up fw/craA up rv) and cloning either of them in pEMG digested with Smal. The resulting plasmids were designated as pEMG-up-cmlA5 and pEMG-up-craA, respectively. Complemented strains were constructed by conjugating either pEMG-up-craA or pEMG-up-cmlA5 as a control and selecting a single recombination event.

All plasmid derivatives were checked by colony PCR using DreamTaq Green PCR Master Mix (Thermo Fisher), restriction patterns and eventually by Sanger sequencing.

Triparental mating

For transfer of plasmid DNA into A. baumannii AB5075 and derivative strains, a standard triparental mating protocol was followed, using pRK2013 (in a DH5α host) as helper plasmid and a DH5α apr donor bearing the plasmid of interest.13 A detailed mating protocol is provided in Text S1. When necessary, DNA deletions were assessed by colony PCR and eventual Sanger sequencing from PCR-amplified genomic DNA. Conjugation frequency was calculated as the number of transconjugant colonies divided by the number of viable cells.

Antibiotic disc diffusion assay (DDA)

Antibiotic susceptibility assays were performed in cation-adjusted Mueller–Hinton (CAMH) medium (pH 7.4, CaCl2 2 mM, MgCl2 1 mM) (Sigma–Aldrich). Overnight cultures of A. baumannii AB5075 or the respective mutant derivatives were diluted to 0.5 McFarland units in CAMHB and spread with a cotton swab on CAMH agar plates. When plates were dry, chloramphenicol, amikacin or tobramycin discs (Oxoid) were placed in the middle of the CAMH agar plate. Plates were incubated at 37°C for 24 h before measuring the diameter of the inhibition zone. Results are shown as averages of three biological replicates.
**MIC determination**

Saturated overnight cultures were diluted in PBS to get an OD_{600} of 0.2. Cells were washed three times and resuspended in 1.2 mL of CAMHB. Ten 2-fold serial dilutions of each antibiotic, starting with 2500 μg/mL, were prepared in CAMHB. In order from highest to lowest antibiotic dilution, cell suspensions and antibiotic-supplemented CAMHB were mixed in a 1:1 proportion in a 96-well plate. The plate was then incubated at 37°C, 200 rpm. MICs were assessed by visual examination, defining them as the lowest antibiotic concentration that led to the absence of visible bacterial growth.

**Data analysis**

For every experiment, three independent replicates were performed. Results are shown as averages of the three measurements (±SD) or as representative images of the replicates. Result representation and statistical analyses were performed using GraphPad Prism 9.

**Results and discussion**

**Rationale of the strategy**

To adapt an efficient genome-editing system for MDR *A. baumannii* AB5075, we built our strategy on that developed by Martínez-García and de Lorenzo\(^\text{22}\) for *P. putida*, further optimized to an accelerated version at the Nickel laboratory.\(^\text{28}\) To perform this strategy, plasmids pEMG and pSW-I needed to be used.\(^\text{22}\) pEMG is a cloning suicide vector bearing two target sites for the endonuclease SceI flanking its polylinker. Once the homologous regions flanking the desired modification are cloned into pEMG, the resulting plasmid is transferred to the target strain and integration in the genome is selected. Subsequently, the broad-host-range pSW-I plasmid, with the SceI coding gene under an inducible XylS-dependent promoter, is introduced in the co-integrate strain. Inducing the expression of sceI triggers a double-strand break in the genome that is eventually repaired by homologous recombination, generating the reversion to the parental strain genotype or the desired mutation. Apart from improvements to make the screening more efficient, Wirth et al.\(^\text{28}\) introduced on-plate induction of sceI expression, reducing the second recombination to one single mating and selection step.

In the case of *A. baumannii* AB5075, one of the disadvantages for its genetic manipulation is its resistance to most available antibiotic selection markers, including those in pEMG and pSW-I. Hence, we constructed a pEMG derivative bearing a tellurite resistance cassette as well as its original kanamycin resistance gene, obtaining pEMG-TelR (pEMGT) as a result (Figure 1). For the second part of the strategy, we produced two variants of the pSW-I plasmid, each bearing either an apramycin resistance marker or a tetracycline resistance gene, namely pSW-Apr (Figure 1) and pSW-Tc, respectively. To validate the method and demonstrate its versatility and robustness, we attempted the construction of scar-free mutants in the chromosome-encoded gene *craA* and the plasmid-borne gene *cmlA5*. *craA* (identified in AB5075 by sequence similarity to the *craA* orthologue characterized in *A. baumannii* ATCC 17978) is an efflux pump previously thought to be specific to chloramphenicol.\(^\text{34,35}\) However, it was recently shown to have a broader substrate range.\(^\text{36}\) On the other hand, *cmlA5* is a putative chloramphenicol efflux pump inferred from homology and encoded within resistance island 2 (RI2) on the native plasmid, p1AB5075.\(^\text{29}\)

**Deletion of the chromosomally encoded *craA***

For the first trial of this genome-editing method, we attempted the construction of an in-frame deletion mutant in *craA* (ABUW_0337). A visual outline of the strategy can be followed in Figure 2. Once the pEMGT derivative bearing the flanking homologous regions of *craA* was constructed (pEMGT-craA), it was conjugated into the AB5075 parental strain and transconjugants bearing the plasmid inserted by recombination were selected in the presence of tellurite. Five candidates were confirmed to carry

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**Figure 1.** Schematic representation of plasmids pEMGT and pSW-Apr. All relevant features borne in each plasmid are presented and named. SceI target sites in pEMGT are circled in dotted lines. Adapted from ‘Custom Plasmid Maps 2’, by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates. This figure appears in colour in the online version of JAC and in black and white in the printed version of JAC.
Figure 2. Schematic outline of the genome-editing strategy adapted for A. baumannii AB5075 applied to the deletion of craA. Plasmid features are represented in Figure 1. When indicated, LB agar plates were supplemented with ampicillin 100 mg/L (Ap), apramycin 200 mg/L (Apr) and/or tellurite 30 mg/L (Tel). For confirmation of craA deletion, colony PCR was performed using primers craA fw seq and craA down rv. As controls, WT AB5075 (WT) and pEMGT-craA (p) were used. M, DNA molecular weight marker, with band sizes indicated (kb). For simplicity, only the events occurring if the first recombination happened in the upstream homologous region is shown. Created with BioRender.com. This figure appears in colour in the online version of JAC and in black and white in the printed version of JAC.
the plasmid integrated into the chromosome by PCR (Figure S1), and transconjugants appeared with a frequency of 10^{-8}.

Three colonies were selected from the candidates and brought forward for performing the second recombination event. To check the effectiveness of both pSW-Apr and pSW-Tc in forcing the second recombination event, both of them were transferred by mating in biological triplicates to the AB5075-pEMGT-craA parental strain and transconjugants were selected in the presence of either antibiotic. We attempted the on-plate sceI induction by adding the inducer 3-methylbenzoate (3MB) to the selective plates. However, the presence of this compound affected A. baumannii growth (Figure S2). Nevertheless, this strategy has been applied before without addition of the inducer,37-39 which also proved successful for A. baumannii AB5075. In the case of pSW-Apr recipients, clear individual colonies grew with a frequency around 10^{-6}. However, although pSW-Tc recipients grew with a similar frequency, colonies appeared with a mucoid phenotype (which we had previously observed when selecting tetracycline resistance) that made selection difficult (Figure S3).

To assess the second recombination, we screened for the loss of tellurite resistance. This screening resulted in 98.0\% ± 1.7\% of clones that achieved a second recombination triggered by the presence of pSW-Apr (Figure 2) and 72.3\% ± 3.2\% of clones by pSW-Tc.

To select a double recombinant carrying the in-frame deletion of craA instead of a reversion to WT genotype, 10 random candidates among all the pSW-Apr transconjugants were streaked to obtain individual colonies and analysed by PCR. Although the theoretical probability of obtaining a second recombination toward WT configuration or deletion is 50\%, the screening resulted in 100\% deletion frequency in this case, according to the size of the PCR product, indicating the high efficiency of this mutagenesis strategy.

As a final step in the protocol, the resulting mutant strain had to be cured of pSW-Apr. For this, one mutant clone was inoculated in LB broth in the absence of apramycin and two passages were given after the cultures reached stationary phase. After this, individual colonies were isolated and screened for apramycin-susceptible clones. Chromosomal deletion was validated by sequencing (Figure S4, File S1). Also, since AB5075 bears three native plasmids (p1AB5075, p2AB5075 and p3AB5075), we checked their maintenance after the first recombination event and after the stabilization of a pSW-I derivative by PCR (Figure S5). This showed that all three plasmids can be maintained over the course of this procedure. To facilitate the use of this strategy, a detailed step-by-step laboratory protocol in 7-9 days is shown as Text S1.

**Deletion of plasmid-borne cmlA5, RI2 and p1AB5075 curation**

In order to demonstrate the versatility of this mutagenesis toolkit, we attempted the editing of p1AB5075, a native plasmid borne in AB5075. Firstly, we challenged our method by deleting cmlA5 (ABUW_4059). This gene encodes a putative chloramphenicol efflux pump and is located within RI2, a region encoding multiple aminoglycoside resistance genes.29,40,41

For the deletion, we performed a similar strategy as for the mutation of craA. Once the respective flanking homologous regions were cloned into pEMGT (pEMGT-cmlA5), the plasmid was transferred to AB5075 and its integration was selected. For the second recombination, we leaned toward using pSW-Apr, given its better performance compared with pSW-Tc. After screening for a second recombination event, we checked 20 candidates by PCR. In this particular case, we found that, whereas 35\% of the clones had suffered a second recombination by the homologous regions upstream and downstream cmlA5 (they gave a PCR of either WT or mutant size), the remaining 65\% did not yield any amplification product. This would indicate that either a rearrangement in the plasmid had occurred, removing the region

![Figure 3](https://academic.oup.com/jac/advance-article-fig/10.1093/jac/dkac328/6755507/5)
RI2 comprises a 7.8 kb region in p1AB5075 delimited by two homologous integrase coding sequences. Homologous recombination between the two integrase regions has been documented before, amplifying the copy number of RI2 and producing aminoglycoside heteroresistance. In our attempts to delete \( \text{cmlA5} \), the strategy required the introduction of the upstream and downstream homologous region of this gene, by which a recombination can happen to repair the SceI double-strand break. As the SceI target sequences would be inserted between those two pairs of homologous regions, the recombination repair could happen either between the cmlA5-flanking regions, deleting the gene, or by the RI2-delimiting regions, thus deleting the whole resistance island. We could validate the latter case by PCR in those clones that did not yield any amplification with the primer pair used to validate the cmlA5 deletion, but still showed the presence of the rest of p1AB5075 (Figure S6), thus obtaining a \( \Delta \text{RI2} \) mutant.

**Figure 4.** Quantification of antibiotic resistance and mucoid phenotype of the multiple mutant strains compared with AB5075. Quantifications were performed by DDAs using chloramphenicol (50 \( \mu \)g), amikacin (30 \( \mu \)g) and tobramycin (10 \( \mu \)g) discs according to the experiment. (a) Chloramphenicol resistance was measured for all mutant strains generated and compared with that of the WT AB5075. The susceptibility phenotypes observed for the \( \Delta \text{craA} \) and \( \Delta \text{p1AB5075}\Delta \text{craA} \) mutants were complemented by reintroduction of the craA coding sequence as compared with the WT and the parental strains bearing the control construction (see Materials and methods). (b) In the case of the mutants affecting RI2 or the whole p1AB5075 plasmid, as well as their combinations with the \( \Delta \text{craA} \) mutation, resistance was also assessed for the aminoglycosides amikacin and tobramycin. (c) For the mutants that produced it, the mucoid zone observed around chloramphenicol discs was measured and compared with the zone of inhibition using the WT strain (no mucoid zone formed) as control. The average zone of inhibition in millimetres (mm) measured from three biological replicates (±SD) is shown. Statistical significance was assessed from \( P \) values obtained from a t-test (* \( P \leq 0.05 \), ** \( P \leq 0.01 \), **** \( P \leq 0.0001 \)).
The MIC assays revealed an increase in the potential to facilitate their study. Whereas there are reports about the role of delete genes linked to antibiotic resistance, whose phenotype can be measured easily.

Curing native plasmids, usually of unknown function, often involves tedious counterselection screenings. Otherwise, spontaneous plasmid-cured strains can be found serendipitously. Given the high frequency of A. baumannii strains bearing multiple native plasmids and the difficulties entailed by mutating and manipulating them, this methodology shows a remarkable potential to facilitate their study.

**Phenotypic characterization of the ΔcraA and ΔcmlA5 mutants**

To assess the efficiency of this mutagenesis method, we chose to delete genes linked to antibiotic resistance, whose phenotype can be measured easily. Whereas there are reports about the role of CraA in chloramphenicol resistance, the RI2-encoded CmlA5 has only been annotated as a chloramphenicol efflux pump based on homology (Figure S7). The phenotypic characterization and comparison of both mutants would help us elucidate the relative contribution of CraA and CmlA5 to antibiotic resistance in AB5075.

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<th>AMK</th>
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<tr>
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<tr>
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**Phenotypic characterization of the ΔRI2 mutant and the p1AB5075-cured strain**

Apart from cmlA5, RI2 encodes four aminoglycoside resistance genes. Due to this, we aimed to quantify the resistance of the plasmid-related mutants (ΔRI2, Δp1AB5075) to the aminoglycosides amikacin and tobramycin [Figure 4(b), Table 1, Figure S8]. We observed that the ΔRI2 mutant demonstrated an increased susceptibility to both aminoglycosides. Furthermore, curing the WT strain of the p1AB5075 led to a much greater increase in susceptibility to both amikacin and tobramycin compared with the sole deletion of RI2. This can be explained by the presence of multiple aminoglycoside resistance genes at other locations outside of RI2 in p1AB5075. The deletion of craA in the plasmid-related mutant did not affect aminoglycoside resistance [Figure 4(b), Table 1].

Regarding chloramphenicol resistance of the p1AB5075-related mutants, we could observe different levels of resistance according to the MIC results (Table 1), all of them showing greater susceptibility than the WT. Apart from the inhibition zone, a zone of mucoid, translucent biomass appeared in chloramphenicol DDA assays, which was also observable for the ΔcmlA5 single mutant [Figure 4(c), Figure S9]. Strikingly, the Δp1AB5075ΔcmlA5ΔcraA double mutant exhibited a zone of inhibition of approximately double the diameter of that observed for the ΔcraA single mutant or the p1AB5075-cured strain. Moreover, it covered an equivalent area to the mucoid zone shown by the rest of the mutant strains. This phenotype could be complemented by reintroducing the craA coding region [Figure 4(a), Figure S10, Table S2], recovering the inhibition zone and mucoid phenotype of the Δp1AB5075 single mutant. When biomass from the mucoid zone was restreaked in the absence of chloramphenicol, the phenotype reverted to non-mucoid, opaque colony morphology in all cases, indicating that the phenotype was not caused by additional mutations nor phase variation. This suggests there might be an interplay between CraA and p1AB5075 in conferring full resistance to chloramphenicol. Unravelling this plasmid–chromosome regulatory interplay may shed light on the capacity of this pathogen to overcome chloramphenicol treatment in the clinic and will be the focus of future work.

**Table 1. MIC for the acraA and ΔcmlA5 mutants and the p1AB5075-cured strain (Δp1AB5075) compared with the WT AB5075.**

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resistance, we show that mutations affecting p1AB5075, even the sole mutation of cmiAS, lead to the formation of a mucoid phenotype. Previously, it was reported that chloramphenicol may trigger a mucoid phenotype by inducing capsule production.\(^{{49}}\) Furthermore, the greater inhibition zone of the Δp1AB5075ΔcraA compared with the Δp1AB5075 and the ΔcraA single mutants suggests a synergistic effect between p1AB5075 and CraA in chloramphenicol resistance. However, the implication of p1AB5075 in this phenotype remains to be understood.

All in all, we showcase an efficient and robust genome-editing toolkit that can be used to modify both the chromosome and the native plasmids harboured by MDR A. baumannii.

**Acknowledgements**

We would like to thank Dr Esteban Martinez-Garcia and Prof. Victor de Lorenzo (Centro Nacional de Biotecnología, Madrid, Spain) for providing pEMG, pSW-1 and pSEV524 as a kind gift.

**Funding**

R.R.M. is supported by the British Society for Antimicrobial Chemotherapy (BSAC-2018-0095). R.R.M.C. and R.D. are supported by a BBRC New Investigator Award (BB/V007823/1). R.R.M.C. is supported by the Academy of Medical Sciences/the Wellcome Trust/the Government Department of Business, Energy and Industrial Strategy/the British Heart Foundation/Diabetes UK Springboard Award (SBF0061040).

**Transparency declarations**

The authors declare no competing interests.

**Author contributions**

R.D. and R.R.M. designed the strategy and the experimental work. R.D.D. and K.G. performed the experiments and analysed the results. R.D.D., K.G. and R.R.M. wrote and reviewed the manuscript.

**Data availability**

All plasmids are available through request to the corresponding author.

**Supplementary data**

Figures S1 to S10, Tables S1 and S2, Text S1 and File S1 are available as Supplementary data at JAC Online.

**References**

5. Jacobs AC, Thompson MG, Black CC et al. AB5075, A highly virulent isolate of Acinetobacter baumannii, as a model strain for the evaluation of pathogenesis and antimicrobial treatments. mBio 2014; 5: e01076-14. https://doi.org/10.1128/mBio.01076-14
Scar-free mutagenesis in *Acinetobacter baumannii*


