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# One-Step Engineering of a Stable, Selectable Marker-Free Autoluminescent *Acinetobacter baumannii* for Rapid Continuous Assessment of Drug Activity S

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The rising cases of multidrug-resistant *Acinetobacter baumannii* (*Ab*) and the lack of effective drugs call for quick attention. Here, based on a Tn7 transposon and Xer/*dif* system, we constructed a stable, selectable marker-free autoluminescent *Ab* capable of producing visible light without extra substrates. Utilization of this autoluminescent reporter strain has the potential to reduce the time, effort and costs required for the evaluation of activities of anti-*Ab* drug candidates in vitro.

**Keywords:** Autoluminescent, marker-free, *Acinetobacter baumannii*, Tn7, Xer/*dif* recombination system

*Acinetobacter baumannii* (*Ab*) is an important gram-negative bacteria causing opportunistic infections [1]. It is ubiquitous and one of the common causes of nosocomial infections [2–4]. The increasing infections caused by this microorganism and the emergence of multidrug-resistant (MDR) *Ab* strains pose a continuous threat to public health [5]. Considering the lack of effective drugs, there is an urgent need to develop new tools for rapid and efficient screening and evaluation of new drug candidates. Bioluminescence-based approaches such as using the *luxCDABE* operon as a reporter have been applied for the evaluation of antimicrobial drugs with significant benefits over conventional methods, *e.g.* solid agar method and liquid broth dilution method with turbidity values as the end points [6]. LuxAB catalyze the luminescence reaction involving the oxidation of reduced flavin mononucleotides and a long-chain fatty aldehyde substrate concomitant with the emission of blue-green light at 490 nm [7]. The *luxCDE*

genes encode the fatty acid reductase complex required for the synthesis of the aldehyde substrate and thus contribute to the recycling of aldehyde [8]. Tn7 transposon inserts, at a relatively high frequency, into a specific site named *att*Tn7 which was shown to be located downstream of *glmS* [9, 10]. More importantly, it is worth noting that the Tn7 insertion has no impact either on the expression of genes or on the growth of bacteria [11]. As such, the Tn7 transposon is a valuable and convenient tool for site-specific tagging in bacteria. Here, the previously described backbone vector pUC18T-mini-Tn7T-lux-Tp (Fig. S1A) containing both the *luxCDABE* operon and Tn7 [12] was utilized. Although this vector harbors a Flp recombinase target (FRT) cassette which can be used to obtain marker-free Tn7 insertions upon introduction of a Flp recombinase [13], the inconvenience of introduction and subsequent removal of the Flp recombinase renders the Flp/FRT system less appropriate. Instead, we utilized the Xer site-specific recombination system in which the removal of resistance genes is dependent on *dif* sequences and the endogenous Xer proteins only [14, 15]. Thus, based on the Tn7 transposon and Xer/*dif* system, we constructed a selectable marker-free autoluminescent *Ab* (UAlAb) expressing the native *luxCDABE* operon for rapid screening and evaluation of potential anti-*Ab* agents by continuously monitoring the light strength as the dead UAlAb can not give out light.

The autoluminescent *Ab* was engineered using one *Ab* clinical isolate that was confirmed by 16S rRNA gene sequencing using primers $P_{27F}$ and $P_{1492R}$ detailed in Table 1 (the sequence obtained shared approximately 99.79% identity relative to the reference *Ab* sequence: Accession No. MG234437.1). Importantly, the clinical strain displays a MDR phenotype (Table S1).

Based on the pUC18T-mini-Tn7T-lux-Tp, we first constructed a recombinant plasmid named pUC18T-mini-Tn7T-lux-Ab-dif-apr. This plasmid contained apramycin (APR)-resistance gene, *apr*, flanked by *dif* sequences as shown in Figs. S1B and S2. To amplify the *apr* gene, polymerase chain reaction (PCR) was performed using primers $P_{Ab\text{-}dif\text{-}Apr\text{-}F}$ and $P_{Ab\text{-}dif\text{-}Apr\text{-}R}$, designed to include the previously described *dif* sequences [16] and restriction sites (Table 1), with the plasmid pMABH1 (Fig. S1C) as template. Notably, since the difference of the four types of *dif* sequences predicted in *Ab* [16] was rather small, by taking into account that each *dif* candidate may work and the only difference may be that they have different DNA dissociation efficiency, we tested only the sequence "TGTTCGTATAAT GTATATTATGTTAAAT" with the highest score as the *dif* sequence in our experiment (Table 1). The amplicon was thereafter digested and inserted into pUC18T-mini-Tn7T-lux-Tp between XbaI and BamHI sites, giving rise to the pUC18T-mini-Tn7T-lux-Ab-dif-apr (Fig. S1B). After verification by sequencing (BGI, Shenzhen, China), this plasmid was co-electroporated with a helper plasmid pTNS3 (Table S2) into *Ab* competent cells freshly prepared as described earlier [17], followed by selection on LB solid media with 100 μg $ml^{-1}$ APR. The resulting colonies were subject to detection of the relative light units (RLU) and PCR analysis using primers $P_{glmSF1}$ and $P_{Tn7R}$ (Table 1) to identify the insertion site. We considered clones yielding an RLU value of $\geq 10^5$ and a PCR product of 368 bp as autoluminescent *Abs* (AlAb) [13] (Figs. 1A and 1B). All the plasmids and bacterial strains used in this study are listed in Table S2.

Since the resistance gene may cause cross resistance to potential active compounds in drug screening, we next removed the *apr* gene, as described previously [18], to engineer the marker-free autoluminescent *Ab* (UAlAb) (Fig. S2). Briefly, the AlAb strain was subcultured in APR-free LB medium to late log phase to allow excision of the *apr* gene by endogenous XerC and XerD [19], followed by serial dilution and plating on APR-free LB agar plates.

**Table 1.** Primers used in this study[#].

| Primers | Nucleotide sequence (5′-3′) |
|---|---|
| $P_{27F}$ | AGAGTTTGATCCTGGCTCA |
| $P_{1492R}$ | GGTTACCTTGTTACGACTT |
| $P_{Ab\text{-}dif\text{-}Apr\text{-}F}$ | CG*GGATCC*ATGGTGTTCGTATAATGTATATTATGTTAAAT CACCACCGACTATTTG |
| $P_{Ab\text{-}dif\text{-}Apr\text{-}R}$ | TGC*TCTAGA*AGCTTATTTAACATAATATACATTATACGAACA AGCTCAGCCAATCGAC |
| $P_{glmSF1}$ | TATGGAAGAAGTTCAGGCTC |
| $P_{Tn7R}$ | CACAGCATAACTGGACTGATTTC |

[#]The *dif* sequences and restriction sites are underlined and in italic, respectively.

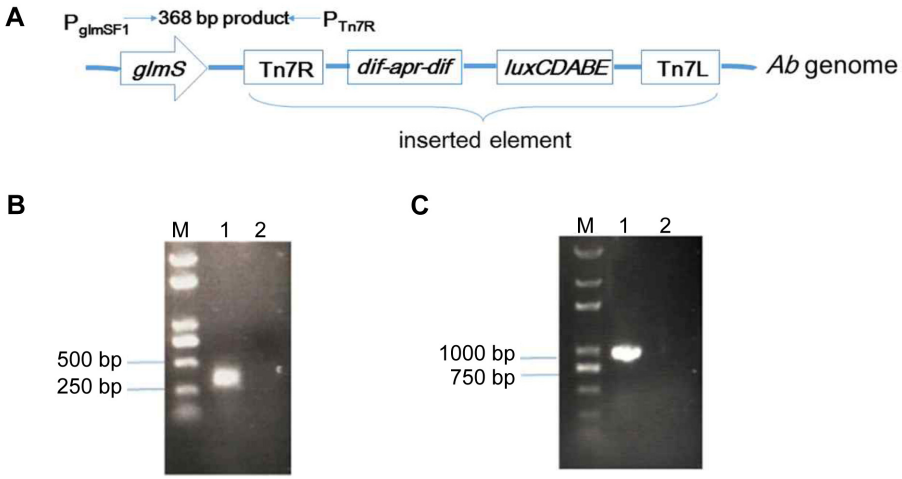


**Fig. 1.** Confirmation of pUC18T-mini-Tn7T-lux-Ab-dif-apr genomic insertion in the *Ab* strain and the loss of *apr* by PCR.
(**A**) Localization of primers for PCR analysis in Fig. 1B. (**B**) Verification of the pUC18T-mini-Tn7T-lux-Ab-dif-apr genomic insertion into the *Ab* strain. Positive clones yielded an amplicon of 368 bp. Lane M, trans 2K plus DNA Marker (Transgene); lane 1, DNA of *Ab* containing pUC18T-mini-Tn7T-lux-Ab-dif-apr as the template; lane 2, DNA of the parental *Ab* as the template; (**C**) The primer pair $P_{Ab\text{-}dif\text{-}Apr\text{-}F}$ and $P_{Ab\text{-}dif\text{-}Apr\text{-}R}$ was used to verify the loss of *apr*. Lane M, trans 2K plus DNA Marker; lane 1, positive control for *apr*; lane 2, lack of band indicating successful removal of *apr* in UAlAb.

Individual colonies were picked, re-subcultured and plated on APR-free LB agar plates for a total of 5 rounds. To calculate removal efficiency (RE) of the *apr* gene, fifty single colonies from each round were randomly picked and replica streaked on both APR-free and APR-containing (100 μg $ml^{-1}$) LB plates. Colonies that could only grow on APR-free but not on APR-containing LB plates were UAlAbs. The loss of *apr* in UAlAbs was further confirmed by PCR using primers $P_{Ab\text{-}dif\text{-}Apr\text{-}F}$ and $P_{Ab\text{-}dif\text{-}Apr\text{-}R}$ (Fig. 1C). The RE was calculated as per the formula shown below:

$$RE = \frac{\text{Number of colonies on plain media} - \text{Number of colonies on APR} - \text{containing media}}{\text{Number of colonies picked (50)}} \times 100\%.$$

We observed that the RE values gradually elevated with the increase of the rounds (Table S3), indicating that the *dif-apr-dif* sequence (Fig. S1B) was recognized and cleaved during the incubation in the absence of APR. To assess whether the obtained UAlAb could produce strong light stably, we grew the UAlAb cells for 30-35 generations, after which the culture was serially diluted and plated on APR-free LB agar plates. We randomly picked 200 colonies for RLU detection and observed that the percentage of autoluminescent clones, judged by RLU values of $\geq 10^5$, was 100%. Therefore, the UAlAb that we obtained is a stable reporter strain.

To assess whether the inserted element interfered with bacterial growth, we compared growth between UAlAb and its parental strain. As shown in Fig. 2, the growth of UAlAb, judged by both $OD_{600}$ (Fig. 2A) and CFUs (Fig. 2B), is comparable to that of its parental strain, suggesting that the inserted DNA element did not influence the growth of the bacteria under the tested condition. We also monitored RLU levels over the incubation period. It was observed that the RLU curve of the UAlAb culture fitted well with its CFU curve (Fig. 2B). Therefore, RLU can be used as an indicator of bacterial growth in UAlAb.

To test whether the UAlAb could be used for drug susceptibility assay, we first assessed the effect of the DNA element insertion on *Ab*'s drug susceptibility using various antibiotics with distinct mechanisms of action (*i.e.* tigecycline, levofloxacin, APR and polymyxin B). Briefly, 500 μl of serial dilutions of *Ab* and UAlAb cultures were separately plated onto LB plates containing serial concentrations of drugs. As shown in Table 2, the MICs of the tested drugs, determined by solid agar method [20], were identical between UAlAb and *Ab* strains, suggesting that the general antibiotic susceptibility is not altered in UAlAb strain. Therefore, we next determined the MICs using Mueller Hinton (MH) media by either RLUs for the UAlAb strain or standard liquid broth method [21] for the parental strain. MICs determined by RLUs were performed as follows. One hundred μl of each drug dilution was

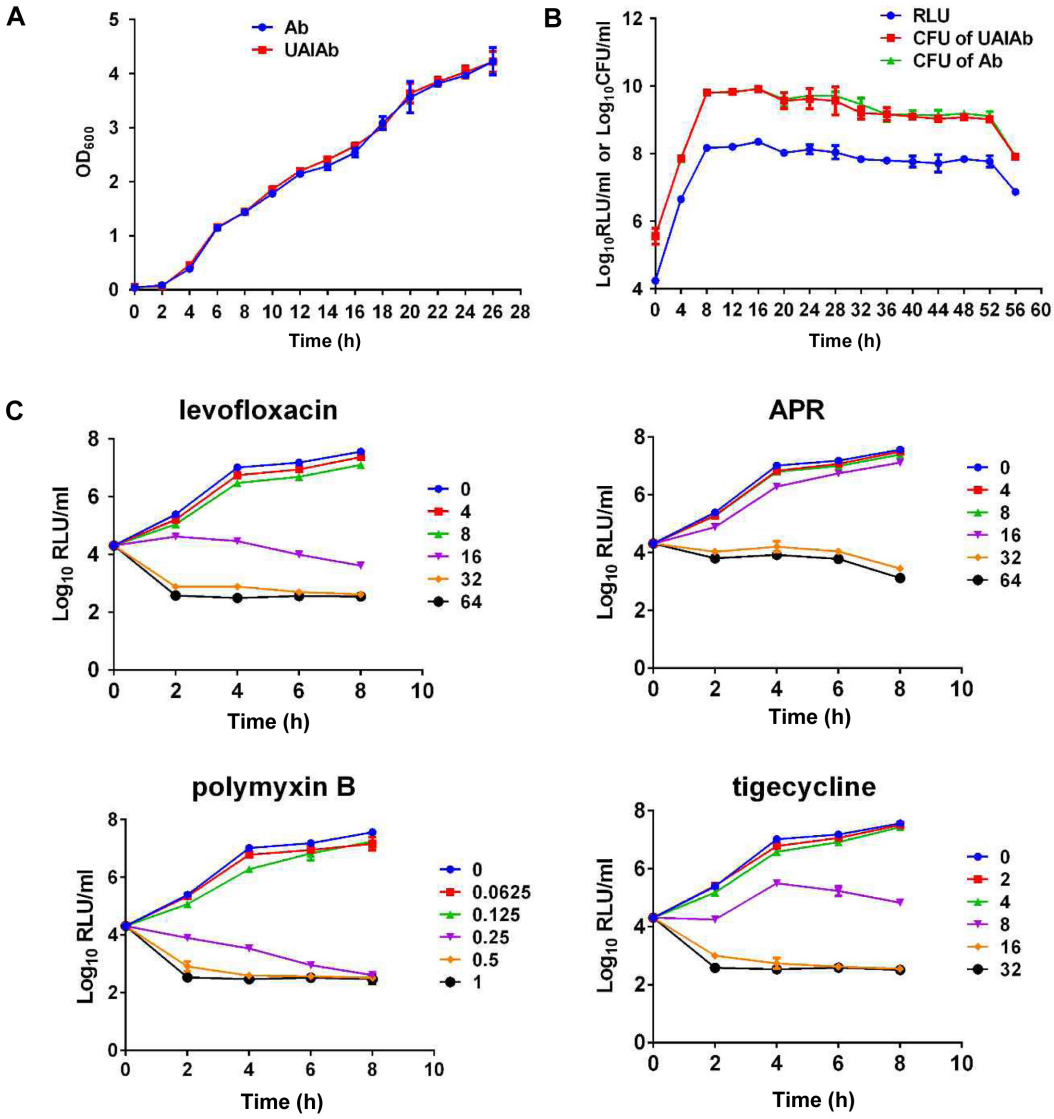


**Fig. 2.** Growth curves of *Ab* and UAlAb.

(**A**) Growth curves of UAlAb and *Ab* by $OD_{600}$. (**B**) Growth curves by CFUs and RLUs of UAlAb. (**C**) MIC determination by real-time RLU measurement using UAlAb. Drug concentrations (μg $ml^{-1}$). Means ± standard deviation (SD) of data from three repeated experiments are shown.

added to a 1.5 ml micro-centrifuge tube containing 100 μl UAlAb, followed by RLU measurement for 4 times using the GloMax 20/20 Luminometer (Promega) at a 2-h interval. The MICs of the tested drugs determined by RLU were comparable to those by the conventional broth method (Table 2). Furthermore, the MICs against the UAlAb strain, measured by either RLUs or by standard broth assay, were also identical. Together, these results demonstrate that RLU measurement using the UAlAb strain as an alternative methodology for antibiotic susceptibility testing is both feasible and valid. Given that autoluminescence can offer additional advantages, such as real-time detection and quicker kinetic monitoring of drug activities [22], we performed real-time RLU measurement for the UAlAb culture in the presence of varying concentrations of drugs. As shown in Fig. 2C, the MICs of the drugs (Table 2) could be obtained as early as 2 h post inoculation by monitoring RLU. Therefore, we propose that UAlAb can be used as a useful surrogate reporter strain in future screening and evaluation of new anti-*Ab* candidates.

In conclusion, based on a Tn7 transposon and Xer/*dif*

**Table 2.** MICs of tigecycline, levofloxacin, APR and polymyxin B for UAlAb and *Ab*.

| Drug | MIC (μg ml$^{-1}$) solid[a] | | MIC (μg ml$^{-1}$) liquid | |
|---|---|---|---|---|
| | UAlAb | *Ab* | UAlAb[b] | *Ab*[c] |
| Tigecycline | 32 | 32 | 4-8 | 4-8 |
| Levofloxacin | 64 | 64 | 16-32[d] | 16-32[d] |
| APR | 32 | 32 | 32 | 32 |
| Polymyxin B | 4 | 4 | 0.25 | 0.25 |

The results are based on three independent experiments.

[a]The MIC was defined as the lowest drug concentration inhibiting at least 99% of bacterial growth observed for drug-free control plates.

[b]The MIC was determined by RLU measurement in Mueller Hinton (MH) media and defined as the lowest drug concentration that decreased ≥ 90% RLU relative to that of the drug-free control.

[c]The MIC was determined in MH media defined as the lowest drug concentration inhibiting visible growth after 18 h of incubation at 37°C.

[d]The MICs obtained were 16 or 32 μg ml$^{-1}$ based on repeated experiments.

system, we successfully constructed a stable, selectable marker-free autoluminescent *Ab* strain by one step that could be valuable for drug susceptibility assay in vitro.

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## Conflict of Interest

The authors have no financial conflicts of interest to declare.

## Transparency Declarations

The selectable marker-free autoluminescent *Acinetobacter baumannii* and the techniques used for its construction were filed for a China invention patent in Jan 2019 (Application number: 201910051035.0).

## References

1. Visca P, Antunes LCS, Towner KJ. 2014. *Acinetobacter baumannii*: evolution of a global pathogen. *Pathog. Dis.* **71:** 292-301.
2. Beggs CB, Kerr KG, Snelling AM, Sleigh PA. 2006. *Acinetobacter* spp. and the clinical environment. *Indoor. Built. Environ.* **15:** 19-24.
3. Morgan DJ, Liang SY, Smith CL, Johnson JK, Harris AD, Furuno JP, *et al.* 2010. Frequent multidrug-resistant *Acinetobacter baumannii* contamination of gloves, gowns, and hands of healthcare workers. *Infect. Control Hosp. Epidemiol.* **31:** 716-721.
4. Lambiase A, Piazza O, Rossano F, Del Pezzo M, Tufano R, Catania MR. 2012. Persistence of carbapenem-resistant *Acinetobacter baumannii* strains in an Italian intensive care unit during a forty-six month study period. *New Microbiol.* **35:** 199-206.
5. Maraki S, Mantadakis E, Mavromanolaki VE, Kofteridis DP, Samonis G. 2016. A 5-year surveillance study on antimicrobial resistance of *Acinetobacter baumannii* clinical isolates from a tertiary Greek hospital. *Infect. Chemother*. **48**: 190-198.
6. Vocat A, Hartkoorn RC, Lechartier B, Zhang M, Dhar N, Cole ST, *et al*. 2015. Bioluminescence for assessing drug potency against nonreplicating *Mycobacterium tuberculosis*. *Antimicrob. Agents Chemother*. **59:** 4012-4019.
7. Hakkila K, Maksimow M, Karp M, Virta M. 2002. Reporter genes *lucFF*, *luxCDABE*, *gfp*, and *dsred* have different characteristics in whole-cell bacterial sensors. *Anal. Biochem.* **301:** 235-242.
8. Brodl E, Winkler A, Macheroux P. 2018. Molecular mechanisms of bacterial bioluminescence. *Comput. Struct. Biotechnol. J*. **16:** 551-564.
9. Choi KH, Gaynor JB, White KG, Lopez C, Bosio CM, Karkhoff-Schweizer RR, *et al*. 2005. A Tn7-based broad-range bacterial cloning and expression system. *Nat. Methods*. **2:** 443-448.
10. Choi K-H, Schweizer HP. 2006. Mini-Tn7 insertion in bacteria with single *att*Tn7 sites: example *Pseudomonas aeruginosa*. *Nat. Protoc*. **1:** 153.
11. Mitra R, McKenzie GJ, Yi L, Lee CA, Craig NL. 2010. Characterization of the TnsD-*att*Tn7 complex that promotes site-specific insertion of Tn7. *Mob. DNA*. **1:** 18-18.
12. Damron FH, McKenney ES, Barbier M, Liechti GW, Schweizer HP, Goldberg JB. 2013. Construction of mobilizable mini-Tn7 vectors for bioluminescent detection of gram-negative bacteria and single-copy promoter *lux* reporter analysis. *Appl. Environ. Microbiol*. **79:** 4149-4153.

13. Ducas-Mowchun K, De Silva PM, Crisostomo L, Fernando DM, Chao T-C, Pelka P, *et al*. 2019. Next generation of Tn7-based single-copy insertion elements for use in multi- and pan-drug-resistant strains of *Acinetobacter baumannii*. *Appl. Environ. Microbiol*. **85:** e00066-00019.
14. Bloor AE, Cranenburgh RM. 2006. An efficient method of selectable marker gene excision by Xer recombination for gene replacement in bacterial chromosomes. *Appl. Environ. Microbiol*. **72:** 2520.
15. Cascioferro A, Boldrin F, Serafini A, Provvedi R, Palù G, Manganelli R. 2010. Xer site-specific recombination, an efficient tool to introduce unmarked deletions into mycobacteria. *Appl. Environ. Microbiol*. **76:** 5312-5316.
16. Kono N, Arakawa K, Tomita M. 2011. Comprehensive prediction of chromosome dimer resolution sites in bacterial genomes. *BMC Genomics* **12:** 19-19.
17. Yildirim S, Thompson MG, Jacobs AC, Zurawski DV, Kirkup BC. 2016. Evaluation of parameters for high efficiency transformation of *Acinetobacter baumannii*. *Sci Rep*. **6:** 22110.
18. Yang F, Tan Y, Liu J, Liu T, Wang B, Cao Y, *et al*. 2014. Efficient construction of unmarked recombinant mycobacteria using an improved system. *J. Microbiol. Methods*. **103:** 29-36.
19. Yang F, Njire MM, Liu J, Wu T, Wang B, Liu T, *et al*. 2015. Engineering more stable, selectable marker-Free autoluminescent mycobacteria by one step. *PLoS One* **10:** e0119341.
20. Zhang T, Bishai WR, Grosset JH, Nuermberger EL. 2010. Rapid assessment of antibacterial activity against *Mycobacterium ulcerans* by using recombinant luminescent strains. *Antimicrob. Agents Chemother*. **54:** 2806-2813.
21. Clinical and Laboratory Standards Institute. 2017. *Performance Standards for Antimicrobial Susceptibility Testing*, M100-27. 27th Ed. Clinical and Laboratory Standards Institute, Wayne, PA.
22. Zhang T, Li SY, Nuermberger EL. 2012. Autoluminescent *Mycobacterium tuberculosis* for rapid, real-time, non-invasive assessment of drug and vaccine efficacy. *PLoS One* **7:** e29774.