Multidrug-resistant plasmids repress chromosomally encoded T6SS to enable their dissemination

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***Acinetobacter baumannii* (*Ab*) is a nosocomial pathogen with one of the highest rates of multidrug resistance (MDR). This is partially due to transmissible plasmids. Many *Ab* strains harbor a constitutively active type VI secretion system (T6SS) that is employed to kill nonkin bacteria. T6SS and plasmid conjugation both involve cell-to-cell contact. Paradoxically, successful conjugation requires the survival of the recipient, which is the target of the T6SS. Thus, an active T6SS in either the donor or the recipient poses a challenge to plasmid conjugation. Here, we show that large conjugative MDR plasmids heavily rely on their distinctive ability to repress the T6SS of their hosts to enable their own dissemination and the conjugation of other plasmids, contributing to the propagation of MDR among *Acinetobacter* isolates.**

Acinetobacter | T6SS | conjugation | MDR | plasmids

Multidrug resistance (MDR) is a growing challenge in the treatment of bacterial infections. In particular, *Acinetobacter baumannii* (*Ab*) is a serious threat to global health due to the increasing prevalence of MDR isolates (1). The *Ab* pangenome is very plastic, with up to 70% of a strain's genome being composed of nonessential, highly variable genetic elements (2). *Ab* strains harbor a plethora of plasmids that play key roles in the dissemination of antimicrobial resistance. However, the basic biology of *Acinetobacter* plasmids is poorly understood (3). Multiple globally distributed *Ab* strains carry large conjugative plasmids (LCPs) ranging in size from 150 to 200 kb and are characterized by three conserved regions: a locus encoding the type IV secretion system (T4SS) conjugative machinery; a region encoding two TetR transcriptional regulators; and a transposon-rich resistance island containing antibiotic resistance genes (*SI Appendix*, Fig. S1) (4). Supporting their role in the emergence of MDR, LCPs from recent clinical isolates carry up to 13 antibiotic (ATB) resistance cassettes (Table 1) (4–6). This is remarkable, considering that LCPs isolated a few decades ago encoded a single antibiotic resistance gene (Table 1). Another family of small plasmids (<11 kb), hereafter referred to as SMPs (for small mobilizable plasmids), are carried in numerous *Ab* clinical isolates worldwide (7). Many SMPs carry antibiotic resistance cassettes against tetracyclines, aminoglycosides, and carbapenems (8, 9). Although these plasmids do not encode their own conjugation machinery, the presence of conserved *oriT* and *mobA* elements suggests SMPs are mobilizable if the conjugative machinery is provided *in trans* (10). Although SMPs and LCPs have divergent genetic origins, they contain a conserved T4SS machinery and/or elements that target them for mobilization, supporting that conjugation plays a critical role in their dissemination. Successful T4SS-mediated conjugation requires close contact between the plasmid "donor" and "recipient" strains and subsequent survival of the recipient (11). This is contrasted with the function of the type VI secretion system (T6SS). T6SS is employed by various gram-negative bacteria ("predator") to inject toxic proteins directly into bacterial competitors ("prey") in a contact-dependent fashion. These toxic proteins, known as effectors, cause death by disrupting the cell wall, cell membrane, or genetic material of the prey bacterium. Resistance to T6SS is achieved by expressing immunity proteins, which bind and prevent the activity of the effectors (12, 13). Prey killing and secretion of Hcp, a structural component of the T6SS machine, are well-established indicators of T6SS activity (14).

Unlike most bacteria, many *Ab* strains carry a constitutively active T6SS (15). This poses a unique challenge to conjugative plasmids, as *Ab* plasmid donors and recipients may kill each other. We previously reported that expression of conserved LCP-encoded TetR transcriptional regulators completely represses the T6SS of their *Ab* host resulting in loss of Hcp secretion (4). Here, we tested the hypothesis that repression of the constitutively active T6SS resolves the conflict between the plasmid and the bacterium favoring conjugation (16) and is essential for the dissemination of MDR conferred by *Ab* plasmids.

Significance

***Ab* has an alarming predisposition to attain multidrug resistance (MDR), and plasmids serve as vehicles for the spread of MDR among *Ab* clinical isolates. Most *Ab* strains harbor a constitutively active type VI secretion system (T6SS) that mediates indiscriminate, contact-dependent killing of neighboring, nonsister bacterial competitors. This poses a unique challenge to conjugative plasmids, as *Ab* plasmid "donors" presumptively kill potential "recipients" and vice versa. However, *Acinetobacter* plasmids are successfully spreading and acquiring additional antibiotic resistance cassettes, suggesting that they have evolved mechanisms to overcome the T6SS-mediated restriction on dissemination. Here, we show that silencing the T6SS is essential for plasmid conjugation. Our work provides new insights into the establishment and evolutionary dynamics of MDR dissemination among *Acinetobacter*.**

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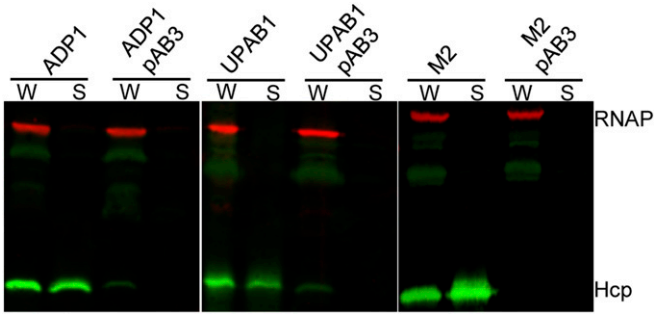

Fig. 1. LCPs repress T6SS in nonrelated *Acinetobacter* spp. Western blot assays probing for Hcp expression and secretion in *A. baylyi* ADP1, *A. nosocomialis* M2, and *A. baumannii* AbCA1 harboring pAB3. RNA polymerase (RNAP) was used as loading control. S, supernatants; W, whole cells.

Table 1. Examples of LCPs encoded by *Acinetobacter* species

Name	Country	Year	ATB resistances	Accession
Plasmid 2	UK	1947	1	LT605060.1
pAB3	France	1951	1	CP012005.1
pA297-3	Netherlands	1984	3	KU744946
pD4	Australia	2006	3	KT779035
pAba7804b	Mexico	2006	4	CP022285.1
pNaval18-131	USA	2006	3	AFDA02000009
pAB04-1	Canada	2012	13	CP012007
pHWBA8_1	Korea	2012	12	CP020596.1
pIOMTU433	Nepal	2013	11	AP014650
pB11911	India	2014	11	CP021344.1

Results

LCP-Mediated T6SS Inhibition Has Broad Species Specificity. *Ab* strains 17978, Ab04, and 1438 each harbor an LCP, which transcriptionally represses their chromosomally encoded T6SS gene cluster, resulting in a lack of Hcp secretion (4). The ability to modulate chromosomally encoded T6SS genes has been reported only for *Ab* plasmids (4). We tested whether LCPs could repress the T6SS of clonally unrelated *Ab* strains and other non-*baumannii Acinetobacter* species that constitutively secrete Hcp. The LCP pAB3 was conjugated from the laboratory strain ATCC17978 (17978) into the *A. baylyi* soil strain ADP1, the *Ab* clinical isolate UPAB1, and the *A. nosocomialis* clinical isolate M2. Tranconjugant derivatives harboring pAB3 did not secrete Hcp (Fig. 1), indicating that LCPs repress T6SS in unrelated strains. This demonstrates that coevolution of plasmids and strains is not required for the regulatory cross-talk between the plasmid and the bacterial host and suggests that T6SS repression provides a selective advantage for the LCP.

An LCP Unable to Repress T6SS Cannot Disseminate via Conjugation Between *Ab* Strains. LCPs encode functional T4SS conjugation machineries, indicating that conjugation is their primary means of dissemination. Thus, we hypothesized that LCP-mediated T6SS repression promotes LCP dissemination by preventing the killing of the recipient cell during the conjugation process. To test this hypothesis, we generated plasmid pAB3*, a pAB3 derivative unable to repress T6SS. This plasmid lacks the previously characterized TetR1 and TetR2 T6SS repressors as well as a third locus (locus I), presumably also involved in T6SS repression. The role of locus I is supported by the identification of

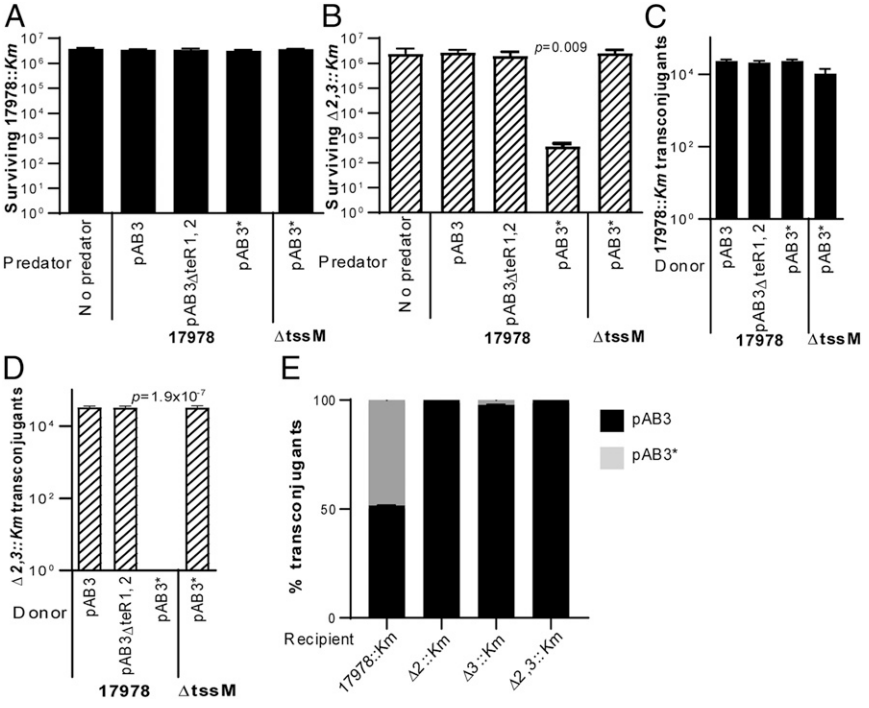

Fig. 2. Inability to repress T6SS abrogates LCPs' capability to conjugate. Data are mean ± SD, $n = 3$ independent replicates. Donor:recipient ratio was 5:1. Recipient/prey strains used were 17978 (immune to T6SS-mediated killing) or 17978Δ*2,3::*K_m (susceptible to T6SS-mediated killing). (*A* and *B*) T6SS-dependent competition assays. Reported are the numbers of surviving prey strains (indicated on the left of each panel); predator strains are indicated in the *x* axis. Parametric one-way ANOVA was performed for 17978 pAB3* compared with the other predator strains. (*C* and *D*) Conjugation assays. Quantification of recovered pAB3 and derivative plasmid transconjugants (indicated on the left of each panel); donor strains are indicated in the *x* axis. Nonparametric Kruskal–Wallis test was performed for 17978 pAB3* versus the other predator strains. (*E*) Competitive conjugation assay; two donor strains carrying pAB3 or pAB3* were simultaneously combined with a recipient strain (indicated in the *x* axis). The plasmid identity in the transconjugants was determined by colony PCR (*Right*).

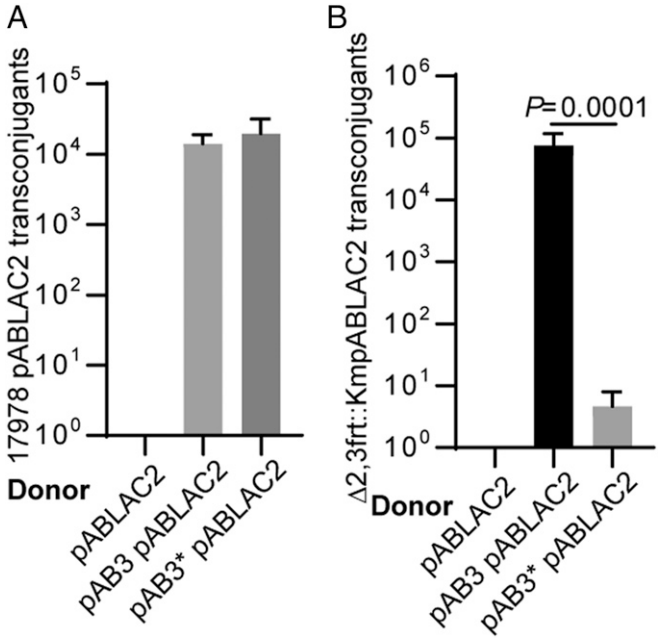

Fig. 3. LCP-mediated SMP mobilization. Data are mean ± SD; $n = 3$ independent replicates. Conjugation assays are at a donor:recipient ratio of 5:1. Quantification of recovered pABLAC2 transconjugants (indicated on the left of each panel); donor strains are indicated in the *x* axis. (*A*) pABLAC2 conjugation is nondetectable if pAB3 is not present in the donor strain. (*B*) pABLAC2 efficiency of conjugation to a T6SS-mediated killing susceptible recipient strain is highly reduced in the presence of an LCP unable to repress T6SS. Statistical analysis was performed using one-way ANOVA and the Tukey–Kramer multiple-comparison test.

another plasmid (pAB3**), carrying a similar deletion (*SI Appendix*, Figs. S2 and S3). To evaluate the impact of the T6SS on LCP dissemination, we compared pAB3 and pAB3* efficiency of conjugation from 17978 to a T6SS-resistant (17978::K_m) or a T6SS-susceptible (17978Δ*2,3*::K_m) recipient strain. Strain 17978 encodes four different effectors, each organized in gene clusters containing *vgrG*, *tse*, and *tsi*, encoding the needle tip of the Hcp syringe, the T6SS effector, and the cognate immunity protein, respectively; however, only two effectors, Tse2 and Tse3, mediate bacterial killing (17). Thus, a 17978 derivative that lacks the gene clusters *vgrG2-tse2-tsi2* and *vgrG3-tse3-tsi3* (17978Δ*2,3*::K_m) is incapable of T6SS killing (lacks Tse2/3 expression) and is susceptible to killing by wild-type (WT) 17978 (lacks Tsi2/3 expression). Accordingly, unlike 17978::K_m, which expresses the immunity proteins for all its effectors and is thus resistant to self-intoxication and T6SS-mediated killing by its parental strain WT 17978 (Fig. 2*A*), strain 17978Δ*2,3*::K_m lacks immunity proteins Tsi2 and Tsi3 and is susceptible to killing by 17978 with an active T6SS, (17978 pAB3*, Fig. 2*B*). Nonetheless, strain 17978Δ*2,3*::K_m is otherwise isogenic to WT 17978, thus preventing killing of the recipient by T6SS-independent bacteriolytic systems (Fig. 2*B*) (18). Our experiments showed that pAB3* was conjugated into WT 17978 as efficiently as pAB3 (Fig. 2*C*), but the conjugation efficiency of pAB3* into the T6SS-susceptible strain (17978Δ*2,3*::K_m) was significantly compromised (Fig. 2*D*). Moreover, pAB3* was efficiently conjugated into both strains from a donor strain with an impaired T6SS (Δ*tssM*) (Fig. 2 *C* and *D*). These results indicate that silencing the T6SS is required for efficient conjugation of LCPs into a T6SS-susceptible recipient strain. To further validate this conclusion, we performed competitive conjugation assays. In these assays, we combined two donor strains, both immune to one another and harboring either pAB3 or pAB3*, with one recipient strain. The presence of pAB3 or pAB3* in the resulting transconjugants was determined by PCR. When the recipient strain was 17978, both pAB3 and pAB3* conjugated at the same efficiency (Fig. 2*E*). However, when the recipient strain was missing one or two immunity proteins, more than 99% of the transconjugants obtained carried exclusively pAB3 (Fig. 2*E*), demonstrating that repression of T6SS in *Ab* is required for LCP conjugation.

LCPs Are Essential for SMP Dissemination. SMPs contribute to the MDR phenotype in many *Ab* clinical isolates around the world, as they encode resistance to tetracyclines, aminoglycosides, and carbapenems (7, 10). The presence of putative *oriT* and *mobA*, a conserved relaxase, indicates that SMPs can be mobilized if the T4SS is provided *in trans*. We hypothesized that LCPs enable SMP dissemination by providing them with conjugative pili and by repressing the T6SS of their host cell. To test this hypothesis we employed two of the most studied SMPs, pABLAC2 and pRAY* (6–8). These plasmids encode the gene *aadB*, which confers aminoglycoside resistance (7, 8, 19), and have been identified in numerous international *Ab* isolates (20, 21). We purified pABLAC2 and pRAY* from their *Ab* host strains LAC-4 and D46, respectively (21, 22), transformed them into 17978, 17978pAB3, and 17978pAB3*, and assayed their mobilization into strains resistant and sensitive to T6SS-mediated killing. Both SMPs were mobilized to an isogenic 17978 recipient strain only from donor strains carrying pAB3 or pAB3* (Fig. 3*A* and *SI Appendix*, Fig. S4*A*), showing that LCPs can mediate SMP dissemination. However, the conjugation efficiency of the SMPs into 17978Δ*2,3* (susceptible to T6SS) was highly reduced in the presence of pAB3* (Fig. 3*B* and *SI Appendix*, Fig. S4*B*), consistent with the inability of pAB3* to silence the donor's T6SS (*SI Appendix*, Fig. S4*C*). SMPs were not mobilized into 17978Δ*2,3* in the absence of pAB3 in the donor strain (Fig. 3*B* and *SI Appendix*, Fig. S4*B*). Thus, LCPs enhance SMP dissemination by supplying a T4SS conjugation machinery and by repressing the T6SS of the donor strain to prevent killing of the recipient.

Constitutively Active T6SS Provides Immunity Against Plasmid Conjugation. Bacteria possess mechanisms of immunity (e.g., CRISPR/Cas9) to protect against foreign genetic elements like plasmids and phage DNA (23). In the previous experiments we analyzed the dynamics of plasmid dissemination when a donor encounters a defenseless recipient strain. However, most *Acinetobacter* strains have a constitutively active T6SS (15). Therefore, we wondered whether an active T6SS in the recipient cell affects conjugation. pAB3 conjugation was highly efficient between isogenic strains; however, when the donor strain was susceptible to T6SS-mediated killing (Fig. 4*A*), conjugation efficiency diminished almost 1,000-fold (Fig. 4*B*). Isogenic strains are not common in nature. Thus, we wondered how the efficiency of plasmid conjugation is affected when donor and recipient strains can kill one another. To this end, we performed conjugation and killing assays using 17978 pAB3 and pAB3* as donor strains and *A. nosocomialis* M2 WT and Δ*hcp* as recipient strains. As expected, both strains can kill each other in a T6SS-dependent manner (Fig. 4*C* and *SI Appendix*, Fig. S5). We found that conjugation of pAB3 from 17978 into *A. nosocomialis* M2 Δ*hcp*, which possesses a nonfunctional T6SS (24), was very efficient. In contrast, conjugation into WT *A. nosocomialis* M2, which possesses a constitutively active T6SS (24), was highly reduced. Our model predicts that the number of transconjugants will decrease if the donor carries a nonrepressing plasmid, irrespective of the T6SS status of the recipient. Indeed, no pAB3* transconjugants were detected in either *A. nosocomialis* M2 WT or Δ*hcp* recipient strains (Fig. 4*D*). We obtained similar results when *A. baylyi* ADP1 WT and Δ*tssM* were used as recipient strains (Fig. 4 *E* and *F*). In addition, efficient conjugation of

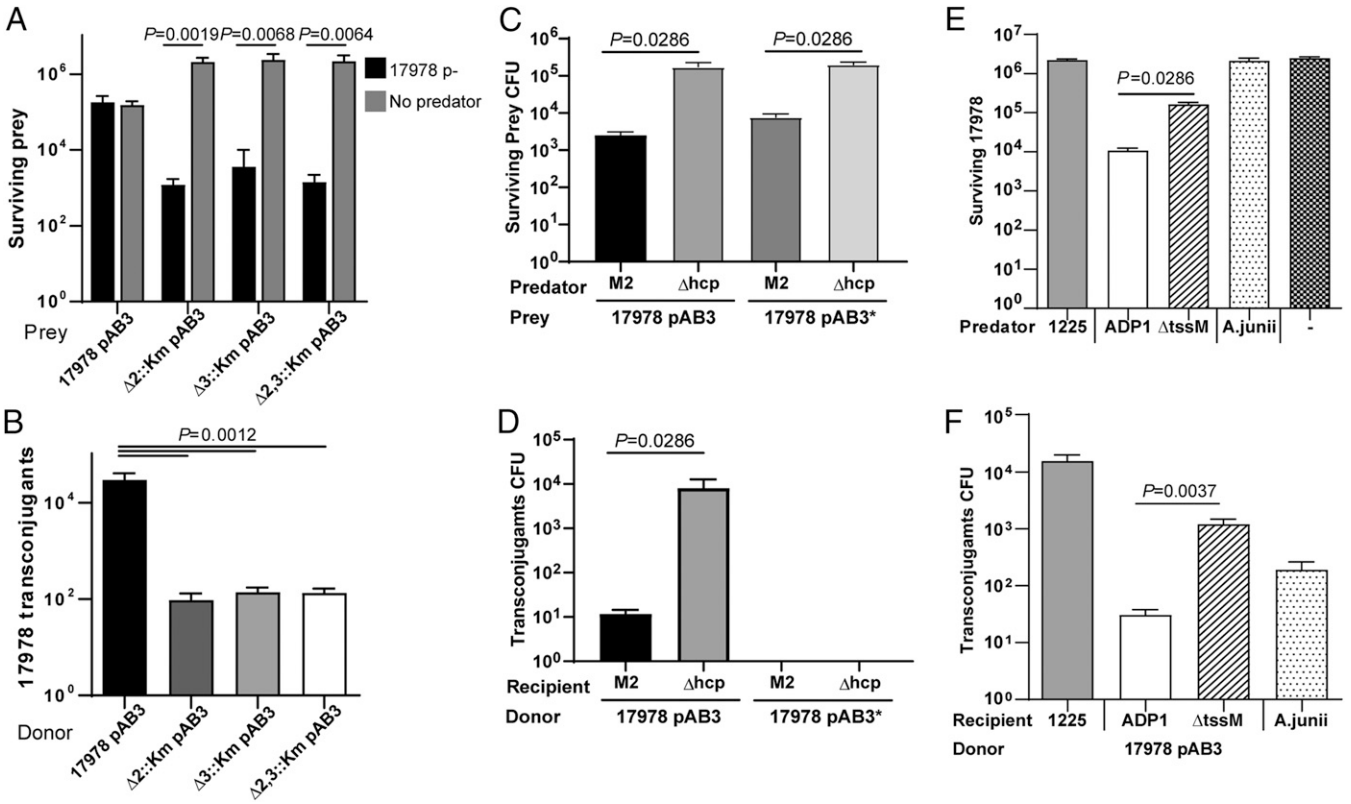

Fig. 4. An active T6SS in recipient strains completely abrogates conjugation. Data are mean ± SD, n = 3–5 independent replicates. Statistical analysis was performed using one-way ANOVA and the Tukey–Kramer multiple-comparison test. (*A*, *C*, and *E*) Quantification of T6SS-dependent competition assays. (*B*, *D*, and *F*) Quantification of conjugation assays. (*A* and *B*) Assays were performed at a donor:recipient ratio of 1:5 for 4 h. Predator/recipient strain is 17978 without pAB3 (17978 p-); prey/donor strains are indicated in the *x* axis. (*C* and *D*) Assays were performed at a donor:recipient ratio of 1:10 for 24 h. Prey/donor strains are 17978 with pAB3 or pAB3*; predator/recipient strains are *A. nosocomialis* M2 WT and M2Δhcp. (*E* and *F*) Assays were performed at a donor:recipient ratio of 1:10 for 24 h. Prey/donor strains are 17978 with pAB3; predator/recipient strains are indicated in the *x* axis.

pAB3 was observed when *Ab* strain 1225 and *A. junii*, which do not exhibit T6SS activity, were used as recipient strains (Fig. 4 *E* and *F*). These results indicate that the T6SS of *Acinetobacter* spp. provides immunity against plasmid conjugation. Together, our results demonstrate that T6SS and conjugation are incompatible processes and that plasmid dissemination relies on the capacity of LCPs to repress T6SS activity in the donor bacterium, independently of the T6SS status in the recipient strain.

Discussion

Plasmids play a key role in the dissemination of MDR in *Ab* and are largely disseminated by conjugation. In only a few decades, LCPs evolved from carrying a single antibiotic resistance cassette to up to 13, and they can now potentially confer MDR to any *Acinetobacter* strain (Fig. 5*A*). The T6SS poses a challenge to plasmids because conjugation and T6SS both require contact between two bacterial cells. However, successful conjugation is dependent on the subsequent survival of the recipient cell, which instead becomes the target of the T6SS. Our experiments demonstrate that LCPs have evolved to repress T6SS to enable their dissemination (Fig. 5*B*). Therefore, LCPs manipulate the host, to overcome a bacterial trait that blocks their horizontal transmission. In addition, we show that LCPs can mobilize the highly distributed SMPs, some of which encode resistance to carbapenems, a class of last-line antibiotics (*SI Appendix*, Fig. S6*B*). Thus, LCPs play a central role in the dynamics of MDR distribution among *Acinetobacter* strains. Various TetR-encoding plasmids have been identified in several gram-negative bacteria encoding T6SS genes; thus, it is possible that our observations may extend to other bacterial species. Indeed, it was recently shown that IncP-1α plasmid pBS228 represses the T6SS of *Pseudomonas aeruginosa* PAO1 (25).

Plasmid dissemination into different *Acinetobacter* strains and species may be dependent on the ability of two nonkin bacteria to coexist. In *Vibrio cholerae*, the T6SS gene cluster is coregulated with competence genes, and T6SS-mediated killing of competing bacteria promotes horizontal gene transfer (26). A similar observation was described in *A. baylyi* (27). Unlike these experiments, here, T6SS appears to limit horizontal gene transfer, through conjugation, rather than promoting it. According to our experiments, the primary mode of dissemination of LCPs and SMPs is conjugation. However, other plasmids lacking a conjugation system have been described. They likely disseminate among competent strains by natural transformation.

In *Pseudomonas aeruginosa*, the conjugative pilus causes a membrane perturbation that triggers the "tit-for-tat" response and activates the T6SS. As a result, the T6SS has been described as an innate immune system against parasitic foreign DNA (28). Our finding that an active T6SS in the recipient cell confers immunity against the conjugation of LCPs supports and expands this model. Unlike in *P. aeruginosa*, most *Acinetobacter* strains with a functional T6SS display constitutive activity. Therefore, most strains are expected to attack a potential donor cell even before the process of conjugation is initiated. As with *P. aeruginosa*, however, this form of immunity is not effective if the donor cell expresses immunity proteins for the T6SS effectors secreted by the potential recipient. We speculate that LCP dissemination can still occur in certain conditions that depend on

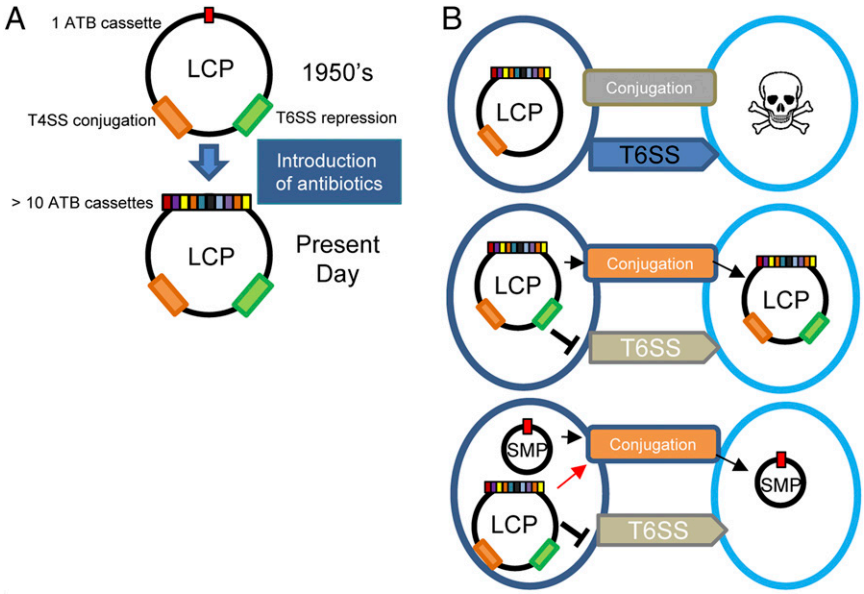

Fig. 5. LCP-mediated conjugation and T6SS. (*A*) Since the introduction of antibiotics into medicine, LCPs have acquired up to 13 antibiotic resistance cassettes, contributing to the increasing MDR phenotype in *Acinetobacter* strains. (*B*) Conjugation and T6SS-mediated killing both require contact between two bacterial cells. Successful conjugation is dependent on the survival of the recipient cell, which is the target of the T6SS. LCPs evolved to repress T6SS to enable their dissemination. Simultaneously, LCPs can mobilize the highly distributed SMPs, which may encode resistance to carbapenems, a class of last-line antibiotics.

the strains involved, the donor:recipient ratio, and the T6SS status of both the donor and recipient bacteria. Altogether, our findings provide insight into the interplay between conjugation, T6SS, and MDR in medically relevant *Acinetobacter* spp. Promoting LCP loss or inhibiting LCP and SMP dissemination may constitute viable approaches to supplement the steadily decreasing treatment options available to combat this nosocomial pathogen.

Materials and Methods

The bacterial strains used in this study are listed in *SI Appendix*, Table S1. To identify a pAB3 derivative unable to repress the T6SS, we performed random transposon mutagenesis on pAB3Δ*tetR1,2* and obtained pAB3*. The strain carrying pAB3* had a chromosomal transposon insertion within gene *prfC*. However, a 17978 *prfC* unmarked deletion mutant carrying pAB3Δ*tetR1,2* does not secrete Hcp. Illumina sequencing revealed that pAB3* lacks locus 1. A subsequent screen identified a second plasmid unable to repress the T6SS, pAB3**, that also carries a deletion in locus 1. pAB3* and pAB3** have been deposited in the BioProject database under accession numbers SAMN08814060 and SAMN08814061, respectively. pRAY* and pABLAC2 plasmids were isolated with a commercial plasmid purification kit from *A. baumannii* D46 and LAC-4 strains, respectively. Plasmids were transformed to 17978, 17978pAB3, and 17978pAB3*, transformants were selected on kanamycin plates, and the presence of the plasmid was confirmed by PCR. Primers used in this study are listed in *SI Appendix*, Table S2. Bacterial killing and conjugation assays were performed as described in *SI Appendix, SI Materials and Methods*. A full description of methods is available in *SI Appendix, SI Materials and Methods*. All data are available in the main text or the *SI Appendix, SI Materials and Methods*.

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