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.

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 nonster bacterium. 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.


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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 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.

Table 1. Examples of LCPs encoded by Acinetobacter species

<table>
<thead>
<tr>
<th>Name</th>
<th>Country</th>
<th>Year</th>
<th>ATB resistances</th>
<th>Accession</th>
</tr>
</thead>
<tbody>
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<td>UK</td>
<td>1947</td>
<td>1</td>
<td>LT605060.1</td>
</tr>
<tr>
<td>pAB3</td>
<td>France</td>
<td>1951</td>
<td>1</td>
<td>CP012005.1</td>
</tr>
<tr>
<td>pA297-3</td>
<td>Netherlands</td>
<td>1984</td>
<td>3</td>
<td>KU744946</td>
</tr>
<tr>
<td>pD4</td>
<td>Australia</td>
<td>2006</td>
<td>3</td>
<td>KT779025</td>
</tr>
<tr>
<td>pAbAba704b</td>
<td>Mexico</td>
<td>2006</td>
<td>4</td>
<td>CP022285.1</td>
</tr>
<tr>
<td>pNaval18-131</td>
<td>USA</td>
<td>2006</td>
<td>3</td>
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<td>pAB04-1</td>
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<td>2012</td>
<td>13</td>
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<tr>
<td>pHWBAba8_1</td>
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<td>2012</td>
<td>12</td>
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</tr>
<tr>
<td>pIOMTU433</td>
<td>Nepal</td>
<td>2013</td>
<td>11</td>
<td>AP014650</td>
</tr>
<tr>
<td>pB11911</td>
<td>India</td>
<td>2014</td>
<td>11</td>
<td>CP021344.1</td>
</tr>
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</table>

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.
Bacteria possess mechanisms of immunity (e.g., AadB lacks immunity proteins) that may prevent conjugation from occurring. In clinical isolates around the world, the presence of putative oriT and mobA, a conserved relaxase, indicates that SPMs can be mobilized if the T4SS is provided in trans. We hypothesized that LCPs enable SPM displacement 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 SPMs, pABLAC2 and pRAY, which encode aminoglycoside resistance and have been identified in numerous international Ab isolates (20, 21). We purified pABLAC2 and pRAY from their Ab hosts 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 SPMs were mobilized into an isogenic 17978 recipient strain from donor strains carrying pAb3 or pAb3* (Fig. 3A and SI Appendix, Fig. S4A), showing that LCPs can mediate SPM dissemination. However, the conjugation efficiency of the SPMs into 17978Δ2,3 (susceptible to T6SS) was highly reduced in the presence of pAb3* (Fig. 3B and SI Appendix, Fig. S4B), consistent with the inability of pAb3* to silence the donor’s T6SS (SI Appendix, Fig. S4C). SPMs were not mobilized into 17978Δ2,3 in the absence of pAb3 in the donor strain (Fig. 3B and SI Appendix, Fig. S4B). Thus, LCPs enhance SPM 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. 4A), conjugation efficiency diminished almost 1,000-fold (Fig. 4B). 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 Δhcp as recipient strains. As expected, both strains can kill each other in a T6SS-dependent manner (Fig. 4C and SI Appendix, Fig. S5). We found that conjugation of pAb3 from 17978 into A. nosocomialis M2 Δhcp, 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 Δhcp recipient strains (Fig. 4D). We obtained similar results when A. baylyi ADP1 WT and ΔssM were used as recipient strains (Fig. 4 E and F). In addition, efficient conjugation of another plasmid (pAb3**), carrying a similar deletion (SI Appendix, Figs. S2 and S3), on pAb3 and pAb3* efficiency of conjugation from 17978 to a T6SS-resistant (17978::Km) or a T6SS-susceptible (17978Δ2,3::Km) 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 (17978Δ2,3::Km) 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::Km, 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. 2A), strain 17978Δ2,3::Km lacks immunity proteins Tsi2 and Tsi3 and is susceptible to killing by 17978 with an active T6SS (17978 pAb3*, Fig. 2B). Nonetheless, strain 17978Δ2,3::Km is otherwise isogenic to WT 17978, thus preventing killing of the recipient by T6SS-independent bacteriolytic systems (Fig. 2B) (15). Our experiments showed that pAb3* was conjugated into WT 17978 as efficiently as pAb3 (Fig. 2C), but the conjugation efficiency of pAb3* into the T6SS-susceptible strain (17978Δ2,3::Km) was significantly compromised (Fig. 2D). Moreover, pAb3* was efficiently conjugated into both strains from a donor strain with an impaired T6SS (ΔssM) (Fig. 2C 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. 2E). However, when the recipient strain was missing one or two immunity proteins, more than 99% of the transconjugants obtained carried exclusively pAb3 (Fig. 2E), demonstrating that repression of T6SS in Ab is required for LCP conjugation.
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 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.

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 and SMPs is conjugation. However, other plasmids lacking a conjugation system have been described. They likely disseminate among competent strains by natural transformation.

Our finding that an active T6SS in the recipient cell confers immunity against the conjugation 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.
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 pAB3ΔtetR1,2 and obtained pAB3*. The strain carrying pAB3* had a chromosomal transposon insertion within gene prfC. However, a 17978ΔprfC unmarked deletion mutant carrying pAB3ΔtetR1,2 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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