Collateral Changes in Cell Physiology Associated with ADC-7 \(\beta\)-Lactamase Expression in *Acinetobacter baumannii*

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**ABSTRACT**

The ADC (AmpC) \(\beta\)-lactamase is universally present in the *Acinetobacter baumannii* chromosome, suggesting it may have a yet-to-be-identified cellular function. Using peptidoglycan composition analysis, we show that overexpressing the ADC-7 \(\beta\)-lactamase in *A. baumannii* drives changes consistent with altered L,D-transpeptidase activity. Based on this, we tested whether cells overexpressing ADC-7 would exhibit new vulnerabilities. As proof of principle, a screen of transposon insertions revealed that an insertion in the distal 3′ end of *canB*, encoding carbonic anhydrase, resulted in a significant loss of viability when the *adc-7* gene was overexpressed. A *canB* deletion mutant exhibited a more pronounced loss of viability than the transposon insertion, and this became amplified when cells overexpressed ADC-7. Interestingly, overexpression of the OXA-23 or TEM-1 \(\beta\)-lactamases also led to a pronounced loss of viability in cells with reduced carbonic anhydrase activity. In addition, we demonstrate that reduced CanB activity led to increased sensitivity to peptidoglycan synthesis inhibitors and to the carbonic anhydrase inhibitor ethoxzolamide. Furthermore, this strain exhibited a synergistic interaction with the peptidoglycan inhibitor fosfomycin and ethoxzolamide. Our results highlight the impact of ADC-7 overexpression on cell physiology and reveal that the essential carbonic anhydrase CanB may represent a novel target for antimicrobial agents that would exhibit increased potency against \(\beta\)-lactamase-overexpressing *A. baumannii*.

**IMPORTANCE**

*Acinetobacter baumannii* has become resistant to all classes of antibiotics, with \(\beta\)-lactam resistance responsible for the majority of treatment failures. New classes of antimicrobials are needed to treat this high-priority pathogen. This study had uncovered a new genetic vulnerability in \(\beta\)-lactamase-expressing *A. baumannii*, where reduced carbonic anhydrase activity becomes lethal. Inhibitors of carbonic anhydrase could represent a new method for treating *A. baumannii* infections.

**KEYWORDS**

*Acinetobacter*, \(\beta\)-lactamase, carbonic anhydrase, antibiotic resistance

The continual rise in antimicrobial resistance is a major threat to human health. In 2019, there were an estimated 4.95 million global deaths associated with bacterial antimicrobial resistance (AMR) (1). Specifically, the opportunistic pathogen *Acinetobacter baumannii*...
High-level expression of β-lactamases is the most conserved and threatening antibiotic resistance mechanism present in MDR A. baumannii. Recent reports estimate that 70 to 99% of modern clinical isolates display stable high-level expression of at least one intrinsic and/or acquired β-lactamase enzyme to inactivate and survive treatment with β-lactams, cephalosporins, and carbapenems (17, 18). Furthermore, A. baumannii universally encodes two β-lactamases on its chromosome, \( \text{bla}_{\text{ADC}} \) (AmpC) and \( \text{bla}_{\text{OXA-51}} \), suggesting they may have cellular housekeeping functions. In the Ambler classification, β-lactamases are grouped into four classes—A, B, C, and D—according to primary sequence motifs (19, 20). Additionally, classes A, C, and D utilize serine as the enzyme active site, whereas class B requires a zinc ion to perform hydrolytic activity. As mentioned previously, A. baumannii encodes class C (AmpC) and class D (OXA-51) β-lactamases on its chromosome. Although the array of horizontally acquired β-lactamases described to date in A. baumannii is nearly endless, two of the most common representatives are class A (such as TEM-1) (21, 22) and class D (such as OXA-23) (23–25) β-lactamases. β-lactamases are believed to have evolved from penicillin-binding proteins (PBPs), which function in peptidoglycan (PG) synthesis for cell integrity and shape (26–28). Although β-lactamase evolution studies have shown that these enzymes have lost their \( \text{D,D} \)-carboxypeptidase activity in favor of increased β-lactam hydrolysis rates, a small but detectable level of \emph{in vitro} \( \text{D,D} \)-peptide hydrolysis has been maintained in all β-lactamase classes (29–31). These studies indicate that β-lactamases may still bind to and act upon a peptidoglycan substrate, although to a much lesser extent than their PBP relatives.

The cellular impact associated with intrinsic β-lactamase overexpression is not well understood. Studies have shown that overexpression of antibiotic resistance genes can be energetically costly and cause metabolic alterations within the bacterial cell, indicating a link between antibiotic resistance and bacterial homeostasis (32, 33). For example, AmpC overexpression in Salmonella enterica type Typhimurium was detrimental to growth rate and cell invasion. However, the fitness cost of AmpC overexpression is not conserved in other bacteria, as recent studies in Pseudomonas aeruginosa and Escherichia coli showed no impact on growth rate or biofilm formation (34, 35). Other studies provide evidence that β-lactamases may maintain residual PG cross-linking activity from their penicillin, i.e., penicillin-binding protein (PBP) ancestors (29–31, 36); therefore, overexpression may have potential off-target effects on PG metabolism. For example, deletion of AmpC in E. coli resulted in aberrant cell morphology, which was further exacerbated by deletion of a low-molecular-weight PBP, AmpH (37, 38). Additionally, recent studies in P. aeruginosa have shown that overexpression of AmpC coupled with mutations in peptidoglycan recycling enzymes, such as \emph{ampG} or \emph{ampD}, displayed impaired motility and virulence (34, 35). Specifically, in A. baumannii, we recently demonstrated the collateral effects of \( \text{bla}_{\text{OXA-23}} \) overexpression on PG composition and identified conditionally essential genes associated with PG metabolism.
with bla<sub>OXA-23</sub> overexpression. These gene products may represent novel antimicrobial targets specific to bla<sub>OXA-23</sub>-overexpressing, but not wild-type, A. baumannii (39).

Carbonic anhydrases (CAs) are zinc-metalloenzymes that are found in all kingdoms of life. Specifically, CAs convert carbon dioxide (CO<sub>2</sub>) and water to bicarbonate, HCO<sub>3</sub><sup>-</sup>, and a proton (40). This reaction plays a central role in many metabolic processes such, as amino acid, nucleotide precursor, fatty acid, and PG biosynthesis as well as central metabolism (41, 42). Because of their crucial role in essential pathways, these enzymes have been considered potential antimicrobial targets (43, 44). In fact, inhibition or loss of CA activity has resulted in decreased survival/fitness in several pathogens, including *Vibrio cholerae* (45), *Mycobacterium tuberculosis* (46–48), *Salmonella enterica* (49, 50), *Pseudomonas aeruginosa* (51), *Helicobacter pylori* (52), and *Escherichia coli* (53).

The goals of this study are to understand the cellular impact of bla<sub>ADC-7</sub> overexpression on *A. baumannii* physiology and identify genes that become conditionally essential when bla<sub>ADC-7</sub> is overexpressed. We report that ADC-7-overexpressing *A. baumannii* cells exhibited an increase in the synthesis of an otherwise rare 3,3-cross-linked muropeptide tripeptide species. However, the growth rate was not impacted when ADC-7 was overexpressed. Additionally, we identified a transposon insertion that resulted in partial loss of carbonic anhydrase activity and conferred conditional lethality when either of three different classes of β-lactamases were overexpressed. Finally, we demonstrate that cells compromised in carbonic anhydrase CanB function display increased sensitivity to PG synthesis and carbonic anhydrase inhibitors. Overall, our results support that the chromosomal ADC β-lactamase confers antibiotic resistance when overexpressed, but at the cost of normal PG synthesis in *A. baumannii*. Moreover, CanB may serve as a future antimicrobial target with increased potency against β-lactamase-expressing isolates.

### RESULTS

**Characterization of the effects of ADC overexpression in A. baumannii.** To assess the effects of ADC-7 β-lactamase overexpression in *A. baumannii*, we generated an ATCC 17978 strain containing an IPTG (isopropyl-β-d-thiogalactopyranoside)-inducible bla<sub>ADC-7</sub> gene (ATCC 17978 Tn7-bla<sub>ADC-7</sub>) and confirmed that this strain displayed a >4-fold increase in the MIC of ampicillin upon ADC-7 overexpression, whereas the MICs were unchanged between the wild-type parent and the uninduced strain (Table 1). ADC-7 overexpression had no impact on the MIC to imipenem. The increase in bla<sub>ADC-7</sub> expression with IPTG induction was confirmed by reverse transcription-quantitative PCR (qRT-PCR) in three

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relative expression compared to 17978 Tn7-bla&lt;sub&gt;ADC-7&lt;/sub&gt;</th>
<th>MIC (µg/mL)</th>
<th>Known β-lactamases present</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ADC</td>
<td>OXA-23</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>17978 empty Tn7</td>
<td>ND</td>
<td>ND</td>
<td>64</td>
</tr>
<tr>
<td>17978 Tn7-bla&lt;sub&gt;ADC-7&lt;/sub&gt;</td>
<td>1.00</td>
<td>Not present</td>
<td>64</td>
</tr>
<tr>
<td>17978 Tn7-bla&lt;sub&gt;ADC-7&lt;/sub&gt; + IPTG</td>
<td>16.59</td>
<td>Not present</td>
<td>&gt;256</td>
</tr>
<tr>
<td>17978 Tn7-bla&lt;sub&gt;ADC-7&lt;/sub&gt; + IPTG</td>
<td>ND</td>
<td>Not present</td>
<td>64</td>
</tr>
<tr>
<td></td>
<td>MU1956</td>
<td>47.30</td>
<td>Not expressed</td>
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<tr>
<td></td>
<td>PR322</td>
<td>69.32</td>
<td>Not present</td>
</tr>
<tr>
<td>17978 Tn7-bla&lt;sub&gt;OXA-23&lt;/sub&gt;</td>
<td>ND</td>
<td>1.00</td>
<td>64</td>
</tr>
<tr>
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<td>80.94</td>
<td>&gt;256</td>
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<td>Not present</td>
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<td>&gt;256</td>
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<tr>
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<td>322.15</td>
<td>&gt;256</td>
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<tr>
<td>AB5075R7-bla&lt;sub&gt;OXA-122&lt;/sub&gt;</td>
<td>ND</td>
<td>ND</td>
<td>24</td>
</tr>
</tbody>
</table>

*ND, not determined; Not present, gene not detected by whole-genome sequencing (WGS) or PCR; not expressed, gene detected by PCR but no RNA transcripts detected by qRT-PCR.*

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**TABLE 1 β-Lactamase transcript levels and MICs of A. baumannii IPTG-inducible β-lactamase strains and β-lactamase-overexpressing clinical isolates**
TABLE 2 Muropeptide composition of peptidoglycan isolated from the 17978 Tn7-blaADC strain (ADC) and the empty Tn7 control strain (WT)*

<table>
<thead>
<tr>
<th>Fraction (no.)*</th>
<th>Retention time (min)b</th>
<th>m/z (M + H)c</th>
<th>z</th>
<th>Molecular mass</th>
<th>Concentration (%)d</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Observd</td>
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<tr>
<td>1</td>
<td>42.1</td>
<td>869.37</td>
<td>1</td>
<td>868.37</td>
<td>868.36</td>
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<tr>
<td>2</td>
<td>59.7</td>
<td>470.7</td>
<td>2</td>
<td>939.4</td>
<td>939.39</td>
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<tr>
<td>3</td>
<td>62.1</td>
<td>461.7</td>
<td>2</td>
<td>921.4</td>
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<td>656.79</td>
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<tr>
<td>5</td>
<td>69.4</td>
<td>708.9</td>
<td>2</td>
<td>1,417.6</td>
<td>1,417.57</td>
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<td>6</td>
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<td>7</td>
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<td>1</td>
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<td>9</td>
<td>111.7</td>
<td>931.39</td>
<td>2</td>
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<td>1,860.82</td>
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<tr>
<td>10</td>
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<td>1,843.77</td>
<td>2</td>
<td>1,842.78</td>
<td>1,842.81</td>
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<tr>
<td>11</td>
<td>119.8</td>
<td>1,392.59</td>
<td>2</td>
<td>2,783.18</td>
<td>2,783.23</td>
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<tr>
<td>12</td>
<td>124.2</td>
<td>1,392.59</td>
<td>2</td>
<td>2,783.18</td>
<td>2,783.23</td>
</tr>
<tr>
<td>13</td>
<td>126.1</td>
<td>1,383.58</td>
<td>2</td>
<td>2,765.16</td>
<td>2,765.24</td>
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<tr>
<td>14</td>
<td>129.3</td>
<td>1,383.58</td>
<td>2</td>
<td>2,765.16</td>
<td>2,765.24</td>
</tr>
</tbody>
</table>

*GM, GlcNAc-MurNAc; anh, 1,6-anhydro-. Associated stem peptides defined within parentheses: A, alanine; Q, glutamine; E, glutamic acid; m, meso-diaminopimelic acid.

**Corresponds to the labeled peaks in Figure S3.

**Muropeptides in bold type are the products of L,D-transpeptidase activity.

**Calculated as the integration of each assigned RP-HPLC peak relative to the total integration of all assigned peaks for that sample, ± standard deviation (SD) (n = 3 technical replicates).

***The muramoyl residue carrying the stem peptide not identified.

**The muramoyl residue with the 1,6-anhydro group not identified.

The growth rate of cells overexpressing ADC-7 was not altered relative to the wild type (see Fig. S1 in the supplemental material). To determine if this level of induced blaADC expression was similar to that seen in clinical isolates, three clinical isolates containing ISAba-blaADC (MU1956, MU1984, PR322) were tested and displayed 2.5- to 4.9-fold higher blaADC expression relative to the induced ATCC 17978 Tn7-blaADC strain (Table 1), indicating that our artificial overexpression strain underestimated the expression levels in blaADC-overexpressing clinical isolates. To verify the β-lactamase transcriptional overexpression, the nitrocefin cleavage rates of uninduced and induced cells were compared (Fig. S2A). The 16.6-fold increase in blaADC correlated to an ~10-fold increase in nitrocefin cleavage, verifying that the transcriptional overexpression results in increased β-lactamase activity.

Next, the impact of ADC-7 overexpression on peptidoglycan (PG) composition was determined. We purified peptidoglycan sacculi from an exponential-phase A. baumannii ATCC 17978 Tn7-blaADC strain grown in the presence of IPTG and from 17978 containing an empty Tn7 transposon which served as a control. We digested the isolated sacculi with mutanolysin to generate a pool of soluble muropeptides and then analyzed these muropeptide libraries first by reverse-phase high-performance liquid chromatography (RP-HPLC) and then by liquid chromatography–quadrupole time-of-flight mass spectrometry (LC-Q-TOF-MS) for compositional analysis (Table 2 and Fig. S3). The most apparent global change in peptidoglycan composition was an increase in the degree of cross-linking, with the ADC-7-overexpressing cells containing muropeptide dimers and trimers at approximately 3.8% higher relative abundance than the control (fractions 4 to 14 in Table 2; compare 69.2 ± 1.8% to 65.4 ± 1.7%, respectively [t(4) = −2.6584, P = 0.06]). However, this result was not statistically significant. In addition, we observed a corresponding approximately 3.6% reduction in the relative abundance of muropeptide monomers (fractions 1 to 3) in the ADC-7-overexpressing strain (15.3 ±
0.9%) relative to the control (18.9 ± 0.2%) [t(4) = −6.763, P = 0.002]. This finding aligns with our companion study of the OXA-23 β-lactamase overexpression in A. baumannii 17978, which also resulted in an increase in the relative abundance of peptidoglycan cross-linking (39). However, in contrast to overexpression of OXA-23, we did not observe any evidence of increased amidase activity in the ADC-7-overexpressing A. baumannii. Instead, we observed the appearance of a series of cross-linked muropeptides with tripeptide stems joined by a 3,3-cross-link which we could not detect in the control (fraction 7 in Table 2). The presence of these new muropeptides in the ADC-7-overexpressing sample would be consistent with an increase in L,D-transpeptidase activity, which forms cross-links between the meso-diaminopimelic acid (mDAP) residues at position three of muropeptide stems (54). The presence of 3,3-cross-linked tripeptide species was not correlated with a concomitant global increase in muropeptide tetra- or tri-peptides. Instead, we observed an approximately 1.7% decrease in the relative abundance of monomeric muropeptide tripeptides in the ADC-7-overexpressing strain (4.33 ± 0.23%) relative to the control [6.08 ± 0.08%; t(4) = −12.447, P = 0.0002; Table 2]. As tetrapeptide muropeptides are the substrate for L,D-transpeptidases, this finding suggests that the compositional changes we observed are consistent with increased L,D-transpeptidase activity.

To further elucidate the potential mechanism of ADC-7 overexpression on peptidoglycan composition changes, we determined if ADC-7 could function as a D,D- or L,D-carboxypeptidase (Fig. S4). Purified ADC-7 enzyme was incubated with synthetic peptidoglycan pentapeptide or tetra-peptide stem mimetics for 48 h, and the liberation of the terminal D-Ala was measured. ADC-7 cleaved between the D-Ala–D-Ala of the pentapeptide substrate, but not between the L-Lys–D-Ala of the tetrapeptide substrate, indicating that ADC-7 displays weak D,D-carboxypeptidase function. This suggests that ADC-7 may be spatially or temporally associated with LdtJ, the recently discovered L,D-transpeptidase responsible for the formation of 3,3-cross-links in A. baumannii (55–57).

Identification and characterization of canB as conditionally essential during ADC-7 overexpression in A. baumannii. To determine the effects of $\text{bla}_{\text{ADC-7}}$ overexpression in the ATCC 17978 $\Delta$canB::kanR deletion mutant, this strain was electroporated with chromosomal DNA containing Tn7-$\text{bla}_{\text{ADC-7}}$ (apraR), and transformants were selected directly on apramycin plates with and without IPTG to calculate the percent survival of transformed cells (Table 3).
The \textit{canB} deletion resulted in a 19.9-fold reduction in survival in cells overexpressing \textit{bla}_{ADC-7}. It is important to note that a low level of sodium bicarbonate (5 mM) was required in the plates to allow growth of the \textit{canB}::\textit{kanR} mutant and to detect changes in viability with \textit{\beta}-lactamase overexpression, as no bicarbonate supplementation resulted in no viable colonies.

The conditionally essential phenotype was successfully complemented compared to the strain carrying the vector control on 5 mM NaHCO$_3$ supplementation (3.33% survival for vector versus 102% for \textit{canB} complement relative to control), suggesting that the CanB protein is crucial for viability in \textit{A. baumannii} overexpressing ADC-7. Further, the \textit{canB} complemented strain was able to grow on LB agar without NaHCO$_3$ supplementation, supporting that \textit{canB} is essential in \textit{A. baumannii}. Additionally, the lethal combination of ADC-7 overexpression and \textit{\Delta canB::kanR} was fully rescued by the addition of 40 mM sodium bicarbonate (data not shown), supporting that CanB function was necessary to counteract the collateral effects of ADC-7 overexpression.

\textbf{CanB is conditionally essential in strains overexpressing the OXA-23 or TEM-1 \textit{\beta}-lactamases.} Recent studies from our lab showed that overexpression of the class D \textit{\beta}-lactamase OXA-23 in \textit{A. baumannii} caused collateral changes to PG that resulted in certain genes becoming conditionally essential (39). This led us to ask if \textit{canB} was conditionally essential when other classes of serine \textit{\beta}-lactamases were overexpressed (Table 3). To first confirm overexpression of the \textit{\beta}-lactamases, we evaluated the ampicillin and imipenem susceptibility changes in ATCC 17978 strains containing an IPTG-inducible \textit{bla}_{OXA-23} or \textit{bla}_{TEM-1} gene (ATCC 17978 \textit{Tn7-bla}_{OXA-23} or ATCC 17978 \textit{Tn7-bla}_{TEM-1}). Upon OXA-23 overexpression, cells displayed a \(\geq\)4- and \(\geq\)64-fold increase in ampicillin and imipenem MIC, respectively, whereas TEM-1 overexpression only resulted in a \(\geq\)4-fold increase in the ampicillin MIC, similar to ADC-7 overexpression (Table 1). Next, we measured the survival of \textit{\Delta canB::kanR} in ATCC 17978 \textit{Tn7-bla}_{OXA-23} and ATCC 17978 \textit{Tn7-bla}_{TEM-1} strains with and without IPTG. ATCC 17978 \textit{\Delta canB::kanR} displayed a 64.5-fold reduction in survival with OXA-23 overexpression and a 10.4-fold reduction in survival with TEM-1 overexpression (Table 3). Similar to ADC-7 overexpression, the \textit{canB} conditionally essential phenotype was successfully complemented compared to the strain carrying the vector control on 5 mM NaHCO$_3$ supplementation (11.5% and 2.22% survival for vector versus 85.5% and 105% for \textit{canB} complement for OXA-23 and TEM-1, respectively). This suggested that CanB function was crucial for cells to counteract the effects of the overexpression of different classes of \textit{\beta}-lactamases and

\begin{table}
\centering
\caption{Percent survival of a \textit{\Delta canB} mutant under \textit{\beta}-lactamase (ADC-7, OXA-23, TEM-1)-overexpressing conditions on plates supplemented with 5 mM NaHCO$_3$.}
\begin{tabular}{lcc}
\hline
ATCC 17978 strain & Survival (+IPTG/control) (%) & Fold change relative to \textit{\Delta canB} \\
\hline
\textit{\Delta canB} & 99.2 $\pm$ 5.54 & – \\
\textit{Tn7-bla}_{ADC-7} \textit{\Delta canB} & 5.08 $\pm$ 1.78 & 19.9 \\
\textit{Tn7-bla}_{OXA-23} \textit{\Delta canB} & 1.55 $\pm$ 0.96 & 64.5 \\
\textit{Tn7-bla}_{TEM-1} \textit{\Delta canB} & 9.57 $\pm$ 3.41 & 10.4 \\
\textit{Tn7-bla}_{ADC-2,S88A} \textit{\Delta canB} & 5.16 $\pm$ 2.35 & 19.4 \\
\hline
\end{tabular}
\end{table}
that there was a common mechanism responsible for conditional essentiality in cells depleted of carbonic anhydrase.

Lastly, we determined if canB was essential in a clinical isolate. For this purpose, an AB5075 derivative cured of p1AB5075 to confer kanamycin susceptibility was used (AB5075.R7) (58). In this strain, the OXA-23 β-lactamase is naturally overexpressed due to an ISAba1 insertion. In fact, the blaOXA-23 gene was expressed at a level 4.0 times higher in AB5075.R7 than in the induced ATCC 17978 Tn7-blaOXA-23 strain, further supporting that our artificial overexpression strains underestimate the transcriptional changes associated with ISAba1 insertion (Table 1). Next, the ΔcanB::kanR mutation and a control kanR insertion in phosphoenolpyruvate carboxylase (ppc) was introduced by transformation into AB5075.R7. Spot dilutions of the ΔcanB::kanR and the control insertion were plated on increasing concentrations of NaHCO3 (Fig. 2). We observed that the ΔcanB::kanR mutation resulted in an ~10,000-fold reduction in viability compared to the control ppc gene disruption, which was incrementally rescued by NaHCO3 supplementation. Furthermore, disruption of the blaOXA-23 gene resulted in an 86.6-fold increase in viability of the ΔcanB::kanR mutant, confirming that canB essentiality in AB5075.R7 was dependent on OXA-23 overexpression.

CanB synthetic lethality does not require β-lactamase activity. Our previous work demonstrated that the conditional essentiality of various genes during blaOXA-23 overexpression did not require OXA-23 β-lactamase activity (39). Therefore, we determined if ADC-7 β-lactamase activity was required for canB conditional essentiality. An IPTG-inducible blaADC-7 mutant was constructed with the catalytic serine mutated to an alanine (ADC-7-S88A). Induction of ADC-7-S88A overexpression did not result in ampicillin resistance (Table 1) or an increase in nitrocefin cleavage activity (Fig. S2B). Next, we evaluated the viability of the ΔcanB::kanR mutant under noninducing and inducing conditions in both the wild-type ADC-7 and catalytically inactive ADC-7-S88A overexpression strains by directly plating transformants from electroporations on apramycin plates with and without IPTG to select for transformants with the Tn7-blaADC-7-S88A and measuring the CFU (Table 3). ΔcanB::kanR did not display significant differences in IPTG sensitivity between the wild-type or catalytically inactive ADC-7 overexpression strains. These results suggest that ADC-7 β-lactamase activity was not required for the canB synthetic lethality, and it was likely due to indirect effects from ADC-7 overexpression.

Decreased carbonic anhydrase activity alters antimicrobial susceptibility. Due to the role of carbonic anhydrase in providing bicarbonate ions important to many biosynthetic processes (44, 59), including the first step of UTP synthesis required for UDP-N-acetylglucosamine and PG (60, 61), we hypothesized that canB mutation would result in increased antimicrobial susceptibility specifically to inhibitors targeting enzymes involved in PG biosynthesis, but not other antibiotic classes. In particular, we evaluated the susceptibility of ATCC 17978 canB::EZ-Tn5kan to the PG synthesis inhibitors D-cycloserine, bacitracin, and fosfomycin and control antibiotics rifampin and ciprofloxacin (Table 4).
D-cycloserine is a cyclic analogue of D-alanine, which inhibits two crucial enzymes involved in the cytosolic building of the peptidoglycan peptide stem, alanine racemase (Alr) (62) and D-alanine:D-alanine ligase (Ddl) (63). Bacitracin inhibits the final dephosphorylation step in the phospholipid carrier cycle, which interferes with the muropeptide transfer to the growing cell wall (64). Fosfomycin inhibits MurA, which is the first step in bacterial cell wall biosynthesis, catalyzing the formation of the peptidoglycan precursor UDP-N-acetylmuramic acid (UDP-MurNAc) (65). Rifampin inhibits DNA-dependent RNA polymerase by directly blocking the exit channel for the elongating RNA and terminating RNA synthesis (66). Ciprofloxacin inhibits DNA gyrase and topoisomerase enzymes required for DNA replication, transcription, repair, and recombination (67). For this analysis, the canB::EZ-Tn5kan mutant was used instead of the DcanB::kanR deletion because the insertion mutant retained low-level carbonic anhydrase activity and grew in LB medium without sodium bicarbonate, though at a much slower rate than the WT. In contrast, the deletion mutant required bicarbonate supplementation, which has been shown to inhibit bacterial growth alone (68) and directly influence antibiotic susceptibility through proton motive force dissipation (69). We also verified that bicarbonate alone altered antibiotic susceptibility (data not shown); therefore, bicarbonate was not used in these experiments. The ATCC 17978 canB::EZ-Tn5kan mutant displayed a 6-fold reduction in the MIC for fosfomycin compared to the ATCC 17978 WT (Table 4). Although not considered significant, the mutant showed a consistent 2-fold MIC reduction to D-cycloserine and bacitracin. Surprisingly, the canB::EZ-Tn5kan mutant exhibited an 8-fold increase in rifampin sensitivity. However, a significant change in the ciprofloxacin MIC between the canB::EZ-Tn5kan mutant and the wild type was not observed. Furthermore, the changes in antimicrobial susceptibility in the canB::EZ-Tn5kan mutant were successfully complemented by a plasmid constitutively expressing canB (pQF1266.canB) compared to a vector control (Table 4). These data suggest that CanB plays an important role in PG biosynthesis and possibly RNA synthesis, but not in DNA replication.

**Carbonic anhydrase inhibition leads to cell death.** The above-described data indicate that canB was essential in *A. baumannii*; therefore, chemical inhibition of CanB may lead to cell death. To test this, we evaluated a known carbonic anhydrase inhibitor, ethoxzolamide (EZA), for effects on cell growth (Table 5) (70–73). We initially tested

### Table 4: MICs of antibiotics against ATCC 17978 WT, canB::EZ-Tn5kan transposon insertion, and canB complemented strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (µg/mL) for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fosfomycin</td>
</tr>
<tr>
<td>17978 WT</td>
<td>256</td>
</tr>
<tr>
<td>17978 canB::EZ-Tn5kan</td>
<td>32</td>
</tr>
<tr>
<td>17978 canB::EZ-Tn5kan pQF1266-hyg vector</td>
<td>64</td>
</tr>
<tr>
<td>17978 canB::EZ-Tn5kan pQF1266-hyg canB</td>
<td>256</td>
</tr>
</tbody>
</table>

*The MICs reported are the medians of n ≥ 3 replicates.*

### Table 5: MICs of the carbonic anhydrase inhibitor ethoxzolamide (EZA) against ATCC 17978 and AB5075.R7 WT, efflux deficient, canB::EZ-Tn5kan mutant, and canB complemented strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>EZA MIC (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17978 WT</td>
<td>&gt;16</td>
</tr>
<tr>
<td>17978 canB::EZ-Tn5kan</td>
<td>&gt;16</td>
</tr>
<tr>
<td>17978 ΔadeB Δadel</td>
<td>&gt;16</td>
</tr>
<tr>
<td>17978 ΔadeB Δadel canB::EZ-Tn5kan</td>
<td>4</td>
</tr>
<tr>
<td>17978 ΔadeB Δadel canB::EZ-Tn5kan pQF1266 vector</td>
<td>2</td>
</tr>
<tr>
<td>17978 ΔadeB Δadel canB::EZ-Tn5kan pQF1266.canB</td>
<td>&gt;16</td>
</tr>
<tr>
<td>AB5075.R7</td>
<td>&gt;16</td>
</tr>
<tr>
<td>AB5075.R7 ade::T26</td>
<td>&gt;16</td>
</tr>
<tr>
<td>AB5075.R7 ade::T26 canB::EZ-Tn5kan</td>
<td>4</td>
</tr>
<tr>
<td>AB5075.R7 ade::T26 canB::EZ-Tn5kan pQF1266 vector</td>
<td>4</td>
</tr>
<tr>
<td>AB5075.R7 ade::T26 canB::EZ-Tn5kan pQF1266.canB</td>
<td>&gt;16</td>
</tr>
</tbody>
</table>
EZA against wild-type ATCC 17978, but no growth inhibition was observed. Next, the ATCC 17978 canB::EZ-Tn5kan mutant was tested, as the reduced carbonic anhydrase activity in the mutant was predicted to render it more susceptible to EZA inhibition. In MIC assays, the canB::EZ-Tn5kan mutant did not show a difference, but we did consistently observe a slight reduction in the optical density at 600 nm (OD₆₀₀) of the mutant compared to the WT across all concentrations tested (data not shown). Next, we tested whether EZA was actively being effluxed from the cell, thus preventing our ability to detect its inhibitory activity, as A. baumannii expresses a plethora of efflux pumps capable of transporting structurally varied molecules, including antimicrobial compounds, out of the cell (74). An ATCC 17978 strain with insertions in adeB and adeJ (17978 ΔadeB ΔadeJ), where both major resistance-nodulation cell division (RND) family efflux pumps AdeABC and AdeIJK were inactivated, was used for this purpose (75). The antimicrobial activity of EZA against 17978 ΔadeB ΔadeJ canB::EZ-Tn5kan was then tested, and the canB::EZ-Tn5kan mutant exhibited a >4-fold reduction in MIC to EZA compared to the 17978 ΔadeB ΔadeJ parent. Importantly, this increase in EZA susceptibility was complemented by the canB gene in trans compared to a pQF1266 vector control (Table 5). In addition, a similar result was observed in the clinical isolate AB5075.R7, with only adeJ disrupted and containing the canB::EZ-Tn5kan insertion (Table 5). These results indicate that EZA is an inhibitor of the A. baumannii CanB enzyme but is readily effluxed. Furthermore, CFU plating of MICs revealed that EZA had a bactericidal effect on 17978 ΔadeB ΔadeJ canB::EZ-Tn5kan and AB5075.R7 canB::EZ-Tn5kan (data not shown).

With EZA inhibitory effects established against A. baumannii CanB, we tested if EZA displayed synergistic activity with fosfomycin (Table 6 and Fig. S5), as we predicted that both would inhibit PG synthesis, and two antimicrobials affecting independent steps in the same pathway may have combined antimicrobial effects, similar to the combination of folic acid biosynthesis inhibitors trimethoprim and sulfamethoxazole (76). A checkerboard assay of 17978 ΔadeB ΔadeJ canB::EZ-Tn5kan mutant with EZA and fosfomycin displayed a fractional inhibitory concentration (FIC) of 0.3125, whereas EZA plus the control antibiotic ciprofloxacin did not show any combined activity (FIC, 1). As further confirmation of fosfomycin (FOF)-EZA synergism, we performed a time course killing assay with a dimethyl sulfoxide (DMSO) control, subinhibitory FOF or subinhibitory EZA alone, and subinhibitory FOF-EZA combination with the 17978 ΔadeB ΔadeJ canB::EZ-Tn5kan mutant. We observed that the 0.25 MIC of FOF plus the 0.25 MIC of EZA resulted in significantly greater killing than either drug alone at 2, 4, and 24 h. These data further support the link between carbonic anhydrase activity and peptidoglycan synthesis in A. baumannii.

### Identification of suppressors that bypass CanB essentiality

During our inducible β-lactamase overexpression assays using the ΔcanB::kanR mutant, we observed rare surviving colonies on IPTG plates. These suppressor mutations arose at a frequency of 2.1 × 10⁻⁷. Two independent suppressors were characterized by whole-genome sequencing, which revealed different mutations in the same gene, a 12-bp deletion from 138 to 149/1,359 nucleotides (nt), and an M245K substitution in A1S_2287, which is predicted to encode a histidine kinase with 29% amino acid identity to the E. coli BaeS protein. To verify that inactivation of this gene was responsible for the suppressor phenotype, we introduced
an A1S_2287::T26 mutation (T26 insertion at position 275/1,359 nt) into 17978 ΔcanB::kanR and evaluated the viability (Fig. 3). Disruption of A1S_2287 resulted in a 180-fold increase in viability compared to the ΔcanB::kanR mutant, confirming that A1S_2287 disruption suppresses the ΔcanB::kanR lethality.

**DISCUSSION**

This work has characterized the collateral effects of ADC-7 overexpression in *A. baumannii*. One notable change imposed by ADC-7 overexpression was on peptidoglycan composition, where there was an increase in the overall cross-linking and the appearance of a series of 3,3-cross-link-containing muropeptides consistent with increased L,D-transpeptidase activity (Table 2). Additionally, our wild-type peptidoglycan compositional analysis is similar to those reported for stationary ATCC 17978 cells (55, 77). However, in these publications, an increase in 3,3-cross-linked peptides compared to exponential cells was also correlated with a significant increase in tetrapeptide monomers, whereas we did not observe a correlated increase in the overall abundance of muropeptide tetrapeptides associated with ADC-7 overexpression. Because we measured weak D,D-carboxypeptidase activity for ADC-7 (Fig. S4), we hypothesize that ADC-7 may be responsible for generating the tetrapeptide substrate and be spatially or temporally associated with L,D-transpeptidase activity of LdtJ in the periplasmic space to account for the increase in 3,3-cross-links without an increase in the tetrapeptide pool. However, it remains a possibility that LdtJ activity is enhanced by ADC-7 overexpression by another mechanism, and further study of this phenomenon is necessary for a complete understanding of the observed compositional changes.

An additional goal of this study was to identify conditionally essential genes associated with ADC-7 overexpression in *A. baumannii*. This resulted in the identification of carbonic anhydrase (CanB). A transposon insertion in the distal 3’ end of the canB gene (canB::EZ-Tn5kan) was conditionally essential in a strain overexpressing the blaADC-7 gene (Fig. 1). The canB::EZ-Tn5kan mutation alone conferred a growth defect in the absence of blaADC-7 overexpression, and follow-up studies indicated that a complete deletion of the canB gene was lethal. This indicated that the canB::EZ-Tn5kan insertion resulted in a partial loss of CanB function, and this combined with blaADC-7 overexpression was conditionally lethal. In a canB deletion mutant, CanB-dependent downstream functions were partially bypassed by using a low level of sodium bicarbonate, and under these conditions, blaADC-7 overexpression also resulted in conditional lethality (Table 3). Remarkably, decreased carbonic anhydrase activity also enhanced killing when the OXA-23 and TEM-1 β-lactamases were overexpressed (Table 3). This suggests a common cellular mechanism by which
β-lactamase overexpression confers increased killing when carbonic anhydrase activity is reduced. Moreover, in a clinical isolate derivative, AB5075.R7, canB was also essential, and this essentiality was dependent on blaoxa-23 overexpression (Fig. 2). Finally, we showed that canB conditional essentiality was maintained when a catalytically inactive ADC-7 mutant (ADC-7-S88A) was overexpressed (Table 3), indicating that that β-lactamase activity was not responsible for the synthetic lethality observed. The active and inactive ADC-7 enzymes may interact with peptidoglycan in a similar manner and alter its composition by sterically blocking the activity of peptidoglycan-modifying enzymes or by recruiting them via protein-protein interactions. Additional work is required to test this possibility.

Since canB was found to be conditionally essential in A. baumannii overexpressing three different classes of serine β-lactamases, we were also interested to determine if the conditionally essential genes during OXA-23 overexpression in our previous work (zipA, A1S_1185, and A1S_0408) would show a similar phenotype in the ADC-7-overexpressing strain (39). We tested these mutants in our ADC-7-overexpressing strain and found all these genes to be disposable for viability (98.9%, 116%, and 114% survival, respectively). This is not surprising, as the peptidoglycan changes associated with OXA-23 or ADC-7 overexpression in A. baumannii are different; OXA-23 indirectly resulted in increased amidase activity (39), whereas ADC-7 indirectly resulted in increased L,D-transpeptidase activity.

Since bicarbonate plays a central role in many metabolic processes, such as amino acid, nucleotide precursor, fatty acid, and PG biosynthesis, we hypothesized that A. baumannii cells with reduced carbonic anhydrase function would result in changes to antimicrobial susceptibility to inhibitors of the above-mentioned metabolic pathways. We showed that the A. baumannii canB::EZ-Tnkan mutant had increased susceptibility to inhibitors of the early stages in PG synthesis (fosfomycin, d-cycloserine, and bacitracin) and the transcription antibiotic rifampin, but not to the DNA gyrase/topoisomerase antibiotic ciprofloxacin (Table 4). While the significant increase in sensitivity to rifampin was unexpected, bicarbonate has been shown to play a role in pyrimidine biosynthesis (78, 79). This may be due to the role of bicarbonate in pyrimidine biosynthesis, which produces nucleotide precursors required for RNA synthesis. However, rifampin is a large and charged molecule, which could better gain cell entry due to altered cell wall composition and, by extension, changes in the cell envelope imposed by the canB mutation.

Our data show that canB was essential for ATCC 17978 and AB5075 viability and conditionally essential upon ADC-7, OXA-23, and TEM-1 overexpression, which suggests that the CanB enzyme may serve as a novel anti-Acinetobacter drug target. As a first step, we evaluated the susceptibility of A. baumannii to the known carbonic anhydrase inhibitor ethoxzolamide (EZA). Unfortunately, wild-type strains were not susceptible to EZA. However, efflux-deficient A. baumannii strains with reduced carbonic anhydrase activity were sensitive (Table 5). EZA also displayed synergistic activity with fosfomycin (Table 6 and Fig. S5), suggesting that CanB inhibitors combined with PG synthesis inhibitors could yield effective combination therapies and possibly restore activity against MDR A. baumannii strains. For example, a recent study showed that Acinetobacter calcoaceticus-A. baumannii complex strains resistant to both imipenem and fosfomycin independently displayed synergistic killing due to PG recycling dysregulation (80). Interestingly, several publications have shown the potency of carbonic anhydrase inhibitors in other bacterial pathogens as well as development of improved EZA compounds (43, 48–50, 52, 81–84).

While the EZA-mediated inhibition of CanB displayed in this study may serve as a promising foundation for a novel therapeutic option against MDR A. baumannii, this compound suffers from several liabilities that preclude it from being used directly in the clinic. First, EZA did not show activity in A. baumannii wild-type strains due, in part, to efflux by the AdeIJK RND efflux pump. Therefore, further optimization of EZA to minimize efflux would be required. Additionally, EZA was initially discovered for its activity against human carbonic anhydrase enzymes, whose dysfunction is involved in eye disease. Thus, antimicrobial development would also have to consider limiting the activity.
against human orthologs of CanB. Furthermore, the frequency of resistance to EZA was measured at $2.1 \times 10^{-7}$, which suggests EZA treatment would lead to rapid resistance development and likely clinical failure. However, it should be noted that while the A15_2287 mutation in AB5075.R7 did suppress canB deletion plus OXA-23 overexpression lethality, these double mutant cells had significant defects in growth (data not shown). This suggests that A15_2287 mutation, if it occurs with EZA treatment, would likely result in reduced fitness and/or virulence. Another possible EZA resistance mutation that can occur is disruption of $\beta$-lactamase overexpression. Despite the liabilities associated with EZA for A. baumannii treatment, there are benefits from this study. For example, the strains described in this study can be utilized in future high-throughput screens to identify more potent CanB inhibitors.

In conclusion, we propose that reduced carbonic anhydrase activity in combination with $\beta$-lactamase overexpression results in lethality by the following mechanism. Carbonic anhydrase is required for the synthesis of bicarbonate, a precursor required for production of UTP, which is then utilized for PG synthesis. Based on our results, we hypothesize that a decrease in carbonic anhydrase activity leads to reduced PG synthesis. In combination with ADC-7 overexpression, which alters PG composition, cells exhibit a lethal dysregulation of PG synthesis. Moreover, the above-described finding suggests that genes required for various steps in PG synthesis and the generation of PG precursors may also become essential when the ADC-7 $\beta$-lactamase is overexpressed, thus revealing a number of potential new targets for the development of new antimicrobials against MDR A. baumannii.

MATERIALS AND METHODS

**Bacterial strains, growth conditions, and antibiotics.** The bacterial strains, plasmids, and primers used in this study are listed in Tables S1, S2, and S3, respectively. Acinetobacter baumannii ATCC 17978 and AB5075 were from lab stocks. Escherichia coli EC100D was used for plasmid propagation (Epitentre, Madison, WI). A. baumannii and E. coli were cultured in Luria-Bertani broth (LB). Where indicated, the medium was supplemented with kanamycin (Kan; 30 $\mu$g mL$^{-1}$), tetracycline (Tc; 5 $\mu$g mL$^{-1}$), apramycin (Apra; 40 $\mu$g mL$^{-1}$), or hygromycin (Hyg; 150 $\mu$g mL$^{-1}$) for plasmid maintenance or transposon selection (Sigma-Aldrich, St. Louis, MO).

**Electroporation of A. baumannii.** Electroporations were conducted with cultures of A. baumannii grown at 37°C with shaking to the late log phase. Cells were pelleted by centrifugation and washed three times with sterile molecular-grade water. Cells were mixed with either genomic DNA (gDNA) or plasmid DNA in 0.2-cm cuvettes and electroporated at 2.50 kV. Transformants were recovered in 1 mL LB for 30 min at 37°C without shaking, followed by 1 h at 37°C with shaking. Transformants were then selected for by plating on the appropriate antibiotic. For canB mutants, 35 mM sodium bicarbonate was supplemented into media at all steps to ensure growth and reduce suppressor mutation rates.

**Construction of an A. baumannii 17978 strain with an inducible $\beta$-lactamase gene.** A mini-Tn7 transposon system was used to generate an IPTG-inducible copy of the blaOXA-23 gene or the blaTEM-1 gene in a single copy as described in reference 39. Briefly, the mini-Tn7-containing tac promoter (ptac)-controlled expression plasmids, pTn7-blaoxaS7, and pTn7-blatem1, were constructed by amplifying a DNA fragment beginning 30 bp upstream from the predicted start codon and ending 20 bp downstream from the predicted stop codon by PCR using chromosomal DNA from A. baumannii strain AB5075 or the pWH1266 plasmid as the template, respectively (Phusion Hot Start polymerase; Thermo Fisher Scientific, Waltham, MA). The fragments were purified, ligated into pUC18-mini-Tn7-LAC-Apra (BS), transformed into E. coli TransforMax EC100D competent cells, and selected on LB agar with apramycin. Plasmids containing the $\beta$-lactamase gene insertion were identified by size shift and verified by sequencing.

To incorporate the IPTG-inducible bla gene into the attTn7 locus within the ATCC 17978 chromosome, electroprotected ATCC 17978 was coelectroporated with pTn7-blaoxaS7 or pTn7-blatem1, and the pTNS2 helper plasmid (85) and transformants were selected on LB agar with apramycin, resulting in the strains ATCC 17978 Tn7-blaoxaS7 and ATCC 17978 Tn7-blatem1, respectively. Successful mini-Tn7 integrants were confirmed by PCR using primers outside the Tn7 insertion site near glmS.

A catalytically inactive ADC-7 variant (SB88A) was generated using a QuickChange II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). Briefly, pTn7-blaoxacS7 was amplified using mutagenesis primers according to the manufacturer's instructions. The parental plasmid was digested with DpnI, and the remaining mutagenized plasmid was transformed into TransforMax EC100D electocompetent E. coli. Transformants were selected for using apramycin, and the transformant was confirmed by sequencing. The mutagenized mini-Tn7-containing plasmid was then electroporated together with pTNS2 into ATCC 17978 and confirmed as described above.

**Total bacterial RNA purification.** Total A. baumannii RNA was purified as previously described (86). Briefly, cultures of A. baumannii ATCC 17978 control and ATCC 17978 Tn7-blaoxaS7, were grown in LB with or without 2 mM isopropyl-$\beta$-D-thiogalactopyranoside (iPTG; Sigma-Aldrich) at 37°C with shaking to an OD$_{600}$ of ~0.8. The cells were harvested from cultures by centrifugation, and RNA was isolated using
a MasterPure RNA purification kit (Epicentre) according to the manufacturer’s protocol. Contaminating DNA was removed by treatment using a TURBO DNA-free kit (Ambion, Austin, TX) according to the manufacturer’s protocol. The RNA concentration was quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific). To confirm that samples were not contaminated with DNA following DNase treatment, PCR of RNA samples was performed.

Reverse transcription-quantitative PCR (qRT-PCR). Total RNA (1 µg) was used to prepare cDNA using the iScript cDNA synthesis kit (Bio-Rad) with random primers as described by the manufacturer. Reaction mixtures lacking reverse transcriptase served as a control for the presence of contaminating DNA. cDNA reactions and controls were then diluted 1:10 with sterile water and used as a template for qRT-PCR. The primers used for qRT-PCR are listed in Table S3. Data were generated using cDNA prepared from three independent RNA isolations, and qRT-PCRs were performed in technical triplicates to ensure accuracy. Fold changes in gene expression relative to the control strain (ATCC 17978) and a control gene (16S rRNA) were determined using the 2^(-∆∆Ct) method (87).

Peptidoglycan isolation. Peptidoglycan sacculi were isolated from cultures of A. baumannii ATCC 17978 empty Tn control and ATCC 17978 Tn7-βlactamase grown in 200 mL LB with 2 mM IPTG at 37°C with shaking to an OD600 of ~0.8 as described (88). Briefly, frozen cell pellets were suspended in 20 mL of cold (4°C) 20 mM sodium phosphate buffer, pH 7.5 (Sigma). The suspension was added dropwise to an equal volume of boiling 8% sodium dodecyl sulfate (Sigma) in 20 mM sodium phosphate buffer, pH 7.5. The samples were boiled with stirring for 3 h and allowed to cool overnight. The SDS was removed by repeated ultracentrifugation at 45,000 g for 1 h at 37°C with gentle rocking. Samples were then boiled in 4% SDS for 3 h with stirring and allowed to cool overnight. The removal of SDS was done by repeated ultracentrifugation at 45,000 x g for 30 min and washing with 20 mM sodium phosphate buffer, pH 7.5, four times. Washed sacculi were suspended in 1 mL 20 mM sodium phosphate buffer, pH 6.0, and incubated with 100 µg/mL amylyase (Sigma), 10 µg/mL DNase I (Sigma), 50 µg/mL RNase A (Sigma), and 20 mM MgCl2 (Sigma) for 1 h at 37°C. Next, 200 µL of 0.1% pronase (Roche, Basel, Switzerland) was added and incubated for an additional 18 h at 37°C with gentle rocking. Samples were then boiled in 4% SDS for 3 h with stirring and allowed to cool overnight. The mobile phase gradient was as follows: A, 50 mM sodium phosphate, pH 4.3; and B, 15% (vol/vol) aqueous methanol in 50 mM sodium phosphate, pH 5.1. The samples were applied to the column in 100% A at a flow rate of 0.5 mL/min. The elution conditions were 100% A for 10 min, followed by a linear gradient to 100% B over 110 min.

Peptidoglycan compositional analysis. Lyophilized peptidoglycan sacculi were resuspended in 50 mM sodium phosphate, pH 6.5, at a concentration of 10 mg/mL. The suspensions were sonicated using a microprobe for 20 s at 30% amplitude. The resulting homogenized peptidoglycan suspensions were digested with 100 µg/mL mutanolysin for 18 h at 37°C. The reaction was stopped by heating to 90°C for 10 min, insoluble debris were removed by centrifugation (21,000 g for 30 min), and soluble muramidase was assayed in the supernatant. The resulting soluble muramopentapeptide libraries were separated by reverse-phase high-pressure liquid chromatography (RP-HPLC) on a 4.6 mm by 250 mm Gemini C5 (5-µm) analytical column (Phenomenex, Inc., Torrance, CA). The mobile phases were as follows: A, 50 mM sodium phosphate, pH 4.3; and B, 15% (vol/vol) aqueous methanol in 50 mM sodium phosphate, pH 5.1. The samples were applied to the column in 100% A at a flow rate of 0.5 mL/min. The elution conditions were 100% A for 10 min, followed by a linear gradient to 100% B over 110 min. Detection was achieved by monitoring absorbance at 205 nm. Fractions of interest were collected for identification by liquid chromatography-mass spectrometry (LC-MS).

LC-MS analyses were performed on an Agilent 1200 HPLC liquid chromatograph interfaced with an Agilent UHPLC 6440 Q-ToF mass spectrometer at the Mass Spectrometry Facility of the Advanced Analysis Centre, University of Guelph. A C5 column (Agilent AdvantageBio Peptide Map, 50 mm by 2.1 mm by 2.7 µm) was used for chromatographic separation with the following solvents: 0.1% formic acid for A; 0.1% formic acid in acetonitrile for B. The mobile phase gradient was as follows: initial conditions, 2% B for 2 min increasing to 15% B in 13 min and then to 50% B for an additional 10 min followed by column wash at 98% B and 10-min reequilibration.

ADC-7 protein purification. ADC-7 β-lactamase was purified as previously described (89). E. coli BL21(DE3) containing a pET28(+)-βlactamase construct was grown in superoptimal broth (SOB) containing 50 µg/mL kanamycin at 37°C in shaker flasks to achieve an OD600 of 0.8. IPTG (isopropyl-β-D-thiogalactopyranoside) was added to the culture to a final concentration of 0.2 mM, and the culture was grown for 3 more hours. The cells were centrifuged and frozen overnight at -20°C. Cells were lysed using a QIAexpress nickel-nitrilotriacetic acid (Ni-NTA) fast-start kit, followed by nickel column purification of the His-tagged protein according to the manufacturer’s protocol (Qiagen, Inc., Valencia, CA). To remove the His tag, the eluted protein was incubated with thrombin (Novagen, Madison, WI) overnight at 4°C (1.6 units per mg protein). The cleaved protein was separated from the 6×His tag peptides by size exclusion chromatography using a HiLoad 16/60 Superdex 75 column (GE Healthcare Life Science).

In vitro o,o-carboxypeptidase activity assay. o,o-carboxypeptidase activity of purified ADC-7 was determined as described (90). Briefly, 1 mM synthetic peptidoglycan carboxypeptidase substrates-pentapeptide (Nac,Nc-diacyetyl-Lys-o-Ala-o-Ala, Sigma) or tetrapeptide (L-Ala-o-Glu-o-Lys-o-Ala, BioMatik) were incubated with 10 µg ADC-7 in 50 mM Tris, pH 8.5 (60 µL volume), at 37°C for up to 48 h. Reactions were stopped by boiling at 100°C for 20 min. To each reaction, 10 µL 10 mg/mL a-diansidine dichloride and 140 µL enzyme-coenzyme mix (80 µL 50 mM Tris-Cl, pH 8.5, 40 µL 0.3 mg/mL flavin adenine dinucleotide (FAD), 20 µL 10 µg/mL horseradish peroxidase, 4 µL 5 mg/mL a-aminooxy acid oxidase) were added and incubated in the dark at 37°C for 30 min. The colorimetric assay was stopped by adding 40 µL 0.005 N HCl. Free a-alanine concentrations were determined by absorbance readings at 450 nm compared to a a-alanine standard curve.

Nitrocefin cleavage assay. Purified enzyme and whole-cell β-lactamase activity was determined using a β-lactamase activity assay kit (Sigma, MAK221). For purified ADC-7, enzyme was diluted in the
provided β-lactamase assay buffer to a final volume of 48 μL in triplicate. Nitrocefin (0.8 mM final concentration) was added and the absorbance at 490 nm monitored every minute for 1 h at room temperature. To verify β-lactamase overexpression in whole cells, ATCC 17978 Tn7-blacoc7 cells were resuspended at an OD600 of 0.025 in 2 mL LB ± 5 mM IPTG and grown with shaking for 2 h at 37°C. Cells were then pelleted and resuspended in 5 μL of β-lactamase assay buffer per mg of sample. Samples were subjected to three rapid freeze-thaw cycles to lyse cells and then centrifuged for 20 min at maximum speed at 4°C. The supernatant was transferred to a fresh tube, and dilutions were prepared and processed as described above in triplicate.

MIC. MICs were determined using a microtiter broth dilution method (91). Briefly, 10 μL of an exponential-phase culture diluted to ~3 × 10^6 CFU/mL was added to individual wells of a 96-well flat-bottom plate containing 88 μL of LB and 2 μL of the antimicrobial compound (ranging from 0 to 512 μg/mL). Mixtures were incubated at 37°C for up to 40 h, and the OD600 was read using a BioTek spectrophotometer. The MIC was defined as the lowest concentration of compound with an OD600 reading equivalent to that of the no-bacteria-added control. All MIC tests were performed in at least triplicate, and the median MIC was reported. Compounds tested include ethoxzolamide (EZA), fosfomycin (FOF), α-cyclodextrine (DC), bacitracin (BAC), rifampin (RIF), and ciprofloxacin (CIP). All compounds were purchased from Sigma-Aldrich.

Fractional inhibitory concentration (FIC). The dilution method used for MIC determination was also used to evaluate the FIC of EZA with FOF. A series of 2-fold dilutions were prepared for both EZA and FOF and then mixed to obtain a matrix of combinations. Combinations were inoculated with ~3 × 10^6 CFU/mL and incubated for 40 h at 37°C. The OD600 was read using a BioTek spectrophotometer. The MIC was defined as the lowest concentration of compound with an OD600 reading equivalent to that of the no-bacteria-added control. The FIC was calculated as follows: FIC (MIC of the combination of EZA/ EZA MIC alone) = (MIC of the combination of FOF/FOF MIC alone). The FIC was interpreted as follows: (i) a synergistic effect when the FIC was <0.5; (ii) an additive or indifferent effect when the FIC was >0.5 and <1, and (iii) an antagonistic effect when the FIC was >1. All FICs were performed in triplicate.

Antibiotic time-kill curve. The concentrations of the drugs used in the following experiments represent fractions of the MICs: 16 μg/mL of 2 FOFO (0.25×MIC) and 2 μg/mL of EZA (0.25×MIC). A. baumannii cells were grown to exponential-phase culture and then diluted to ~1 × 10^6 CFU/mL, and the appropriate single antibiotic or combination was added in triplicate. These were then incubated at 37°C with shaking and samples were taken at 2-h intervals for up to 6 h and 24 h. Cell densities for each sample were estimated from colony counts by dilution in phosphate-buffered saline and plated on LB agar.

Random transposon library generation and screening for conditionally essential genes. To create an insertional library, A. baumannii ATCC 17978 Tn7-blacoc7, was mutagenized using the EZ-Tn5kan kit (Lucigen, Middleton, WI) according to the manufacturer’s instructions. Briefly, ATCC 17978 Tn7-blacoc7 was grown to an OD600 of 0.8 in 2 mL LB and pelleted by centrifugation. Cells were washed 3 times and resuspended in 120 μL nuclease-free sterile water. Cells (70 μL) were electroporated with 1 μL EZ-Tn5kan <Kan-2> Tnp, plated on LB agar with kanamycin, and incubated overnight at 37°C.

To screen for insertions that become conditionally essential upon ADC-7 overexpression, individual A. baumannii colonies containing random transposon insertions were patched onto LB agar containing 30 μg/mL Kan and 5 mM IPTG and incubated at 37°C overnight. Patches that failed to grow on IPTG were cultured, and genomic DNA was isolated as described below. Genomic DNAs from mutants were subjected to a partial Ssau31 digestion. Fragments in the 2- to 5-kb range were gel purified and ligated to pACYC184 (92) and digested with BamHI. The resulting ligation product was electroporated into competent EC100D EC cells and plated into LB agar plates containing chloramphenicol (25 μg/mL) and kanamycin (20 μg/mL). Plasmids were sequenced with the FP1 and RP1 primers, which read outward from the transposon, and the chromosomal region disrupted by the transposon was determined by BLAST analysis.

Genomic DNA (gDNA) isolation. Cultures used for gDNA preparations were grown with shaking at 37°C overnight in LB with the appropriate antibiotics or 35 mM NaHCO₃ for canB mutants. Cells (1 mL) were pelleted through centrifugation and then resuspended in Tris-EDTA. Cells were lysed by incubation for 1 h at 37°C with 0.5% SDS and 400 μg/mL proteinase K. Sodium chloride was added to a final concentration of 0.7 M, and DNA was extracted twice using equal volumes of phenol-chloroform and isoamyl alcohol. DNA was isolated as described above in the protocol. Samples were sequenced with the FP1 and RP1 primers, which read from the transposon, and the chromosomal region disrupted by the transposon was determined by BLAST analysis.

Characterization of canB mutation during β-lactamase overexpression. To assess the IPTG sensitivity of conditional essential mutants with ADC-7, OXA-23, and TEM-1 β-lactamases, gDNA from the IPTG-inducible β-lactamase strains was freshly electroporated into the A. baumannii ATCC 17978 canB: EZ-Tn5kan or ΔcanB::kanR strains, 100 μL was recovery plated on LB plus Apra agar with or without 5 mM IPTG and supplemented with increasing amounts of NaHCO₃ (ranging from 0 to 40 mM), and the mixture was incubated for 24 h at 37°C. CFU were enumerated from each plate, and the percent IPTG sensitivity was calculated by dividing the number of CFU on the IPTG plate by the number of CFU on the control plate (CFUiptG/CFUcontrol × 100).

To evaluate the complementation of the IPTG sensitivity phenotype, the same process as described above was followed. IPTG-inducible β-lactamase strain gDNA was freshly electroporated into ATCC
17978 ΔcanB::kanR pQF1266-hyg vector and ATCC 17978 ΔcanB::kanR pQF1266-hyg canB complementation plasmid. 10 μL was recovered plated on LB plus Hyg agar with and without 5 mM IPTG and supplemented with increasing amounts of NaHCO₃ (ranging from 0 to 40 mM), and incubated for 24 h at 37°C, and the percent IPTG sensitivity calculated.

To evaluate canB essentiality in A. baumannii clinical isolates, electrocompetent cells were electroporated with ΔcanB::kanR QDN as described above and selected on kanamycin with 35 mM sodium bicarbonate. Well-isolated transformants were picked and resuspended into 50 μL LB, serial diluted, and spotted onto kanamycin with increasing amounts of sodium bicarbonate.

**Frequency of canB suppressor mutations and whole-genome sequencing.** A. baumannii 17978 ΔcanB::kanR was grown to an OD₆₀₀ of 0.4 in LB supplemented with 40 mM sodium bicarbonate. Cells were washed twice in fresh LB to remove residual bicarbonate and then plated on LB agar and grown at 37°C for 24 h. Dilutions were plated on LB agar with 40 mM sodium bicarbonate to determine the total CFU plated. Suppressor mutation frequency was calculated as CFU on LB agar/total CFU plated. Genomic DNA from two independent suppressor mutants and their corresponding input strain was isolated as described above and sent to SeqCenter LLC for library preparation, sequencing on the NextSeq 2000 platform at 2 × 151 bp depth, and alignment/variant calling to ATCC17978-mmf (GenBank accession no. CP012004).

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1, PDF file, 0.5 MB**

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