Characterization and Inhibition of 1-Deoxy-D-Xylulose 5-Phosphate Reductoisomerase: A Promising Drug Target in *Acinetobacter baumannii* and *Klebsiella pneumoniae*

Haley S. Ball,* Misgina B. Girma, Mosufa Zainab, Iswarduth Soojhawon, Robin D. Couch,* and Schroeder M. Noble*

**ABSTRACT:** The ESKAPE pathogens comprise a group of multidrug-resistant bacteria that are the leading cause of nosocomial infections worldwide. The prevalence of antibiotic resistant strains and the relative ease by which bacteria acquire resistance genes highlight the continual need for the development of novel antibiotics against new drug targets. The methylerythritol phosphate (MEP) pathway is an attractive target for the development of new antibiotics. The MEP pathway governs the synthesis of isoprenoids, which are key lipid precursors for vital cell components such as ubiquinone and bacterial hopanoids. Additionally, the MEP pathway is entirely distinct from the corresponding mammalian pathway, the mevalonic acid (MVA) pathway, making the first committed enzyme of the MEP pathway, 1-deoxy-D-xylulose 5-phosphate reductoisomerase (IspC), an attractive target for antibiotic development. To facilitate drug development against two of the ESKAPE pathogens, *Acinetobacter baumannii* and *Klebsiella pneumoniae*, we cloned, expressed, purified, and characterized IspC from these two Gram-negative bacteria. Enzyme inhibition assays using IspC from these two pathogens, and compounds fosmidomycin and FR900098, indicate IC₅₀ values ranging from 19.5–45.5 nM. Antimicrobial susceptibility tests with these inhibitors reveal that *A. baumannii* is susceptible to FR900098, whereas *K. pneumoniae* is susceptible to both compounds. Finally, to facilitate structure-based drug design of inhibitors targeting *A. baumannii* IspC, we determined the 2.5 Å crystal structure of IspC from *A. baumannii* in complex with inhibitor FR900098, and cofactors NADPH and magnesium.

**KEYWORDS:** drug discovery, antibiotics, multidrug-resistance, bacteria, *Acinetobacter baumannii*, *Klebsiella pneumoniae*
**K. pneumoniae** is a bacterium that belongs to a large family of Gram-negative bacteria known as *Enterobacteriaceae*. **K. pneumoniae** is a naturally occurring member of human body flora that typically colonizes the intestines, where it does not cause disease. Like *A. baumannii* infections, most *K. pneumoniae* infections occur in healthcare settings, where patients are exposed via ventilators, catheters, or bodily wounds. Consequently, *K. pneumoniae* is a common cause of bacterial pneumonia and has been identified as the second most common source of urinary tract infections (UTIs) following *Escherichia coli*. *K. pneumoniae* infections are largely attributed to the surface pili of the bacteria, which aids in its adherence to the respiratory and urinary epithelium.

Additionally, Klebsiella species are the most antibiotic resistant of all identified *Enterobacteriaceae*. In 1983, *K. pneumoniae* was first shown to exhibit resistance to various β-lactam antibiotics, via production of extended-spectrum β-lactamases (ESBLs). As indicated in the 2019 CDC AR Threats Report, ESBL-producing *Enterobacteriaceae* caused an estimated 197,400 infections and 9,100 deaths in hospitalized patients in 2017. Typically, carbapenems are regarded as the antibiotic class of choice for treatment of ESBL-producing infections; however, in addition to β-lactam antibiotic resistance, many ESBL-producing *K. pneumoniae* have also acquired resistance to quinolones, aminoglycosides, and carbapenems, further complicating treatment. Per the CDC 2019 AR Threats Report, carbapenem-resistant *Enterobacteriaceae* caused an estimated 13,100 infections and 1,100 deaths in hospitalized patients in 2017.

In February 2017, the World Health Organization published its first ever list of antibiotic-resistant “priority pathogens”, which included both carbapenem-resistant *A. baumannii* and *K. pneumoniae* and ESBL-producing *K. pneumoniae* as “Priority 1: Critical” pathogens. The rapid increase of MDR Gram-negative pathogens, such as *A. baumannii* and *K. pneumoniae*, has led to increased use of the “last-resort drug” colistin, despite its serious nephrotoxicity and neurotoxicity issues. With the elevated usage of colistin to treat MDR Gram-negative infections, the emergence of colistin-resistant pathogens now poses a dire health threat and an urgent need for developing a novel class of antibiotics. Underscoring this need, in September 2016, a patient died in Reno, Nevada due to *K. pneumoniae* induced sepsis. The specific strain of *K. pneumoniae* isolated from her infection was found to be resistant to all 26 antibiotics available in the United States, including colistin.

Increasing antimicrobial resistance, in conjunction with the shrinking arsenal of effective antibiotics to treat MDR *A. baumannii* and *K. pneumoniae* infections, have prompted an urgent need for developing new antibiotics with novel targets. The methylenicrythitol phosphate (MPP) pathway of isoprenoid biosynthesis (Figure 1) is an attractive target for the development of novel antimicrobial drugs. Isoprenoids comprise a large and diverse group of over 30,000 known products with vital biological functions, such as electron transport and peptidoglycan biosynthesis in bacteria. Bacteria synthesize isoprenoids via the methylenicrythitol phosphate (MPP) pathway, whereas mammals synthesize isoprenoids via the mevalonic acid (MVA) pathway. The MEP pathway is entirely distinct from the MVA pathway making it an attractive target for antibiotic development.

Two potent phosphonate inhibitors of the first committed MEP pathway enzyme, IspC, include fosmidomycin (a) and its acetyl derivative FR900098 (b) (Figure 2), which are naturally produced by the filamentous bacteria *Streptomyces lavendulae* and *Streptomyces rubelomurinus*, respectively. Fosmidomycin, in conjunction with clindamycin, piperaquine, or azithromycin has shown promise in the treatment of malaria. In addition to demonstrating growth inhibition of a causative agent of malaria, *Plasmodium falciparum*, fosmidomycin and/or FR900098 have also shown in vitro growth inhibition of the bacteria *Yersinia pestis*, *Escherichia coli*, *K. pneumoniae*, and *Francisella tularensis*.

Herein, we describe the cloning, expression, purification, and kinetic characterization of the first committed MEP pathway enzyme, IspC, from *A. baumannii* (AbIspC) and *K. pneumoniae*
(KpIspC). Additionally, we characterize enzyme and bacterial growth inhibition using two known IspC inhibitors, fosmidomycin and FR900098. Furthermore, to facilitate structure-based drug design of inhibitors targeting AbIspC, we determined the 2.5 Å crystal structure of AbIspC in complex with inhibitor, FR900098, and cofactors NADPH and magnesium.

**RESULTS AND DISCUSSION**

**MEP Pathway as an Antimicrobial Target for A. baumannii and K. pneumoniae.** To evaluate the effectiveness of fosmidomycin and FR900098 in inhibiting the growth of *K. pneumoniae* and *A. baumannii*, we performed antimicrobial susceptibility tests. The determined minimum inhibitory concentrations (MICs) for fosmidomycin and FR900098 for each strain of *A. baumannii* and *K. pneumoniae* are summarized in Table 1. Each strain is indicated as being either resistant (R), or susceptible (S), to each compound.

| Strains   | Fosmidomycin MIC (μg/mL) | FR900098 MIC (μg/mL) | Rifampicin MIC (μg/mL) |
|-----------|--------------------------|-----------------------|------------------------|
| Ab5075    | >512 (R)                 | 256 (S)               | 4 (S)                  |
| Ab5711    | >512 (R)                 | >512 (R)              | 4 (S)                  |
| Ab19606   | >512 (R)                 | 128 (S)               | 2 (S)                  |
| KpBAA-1705| 128 (S)                  | 256 (S)               | 64 (S)                 |
| KpNSC-277 | 64 (S)                   | 256 (S)               | 64 (S)                 |

“Susceptibility to compounds is represented as “S” and resistance as “R”. Each experiment was performed in triplicate.

Ab5075, which was isolated from a patient at Walter Reed Army Medical Center (WRAMC), is a highly virulent MDR strain, and Ab5711 is a substantial biofilm-forming MDR clinical strain. Virulence has been linked to antibiotic resistance in multiple animal infection models, and biofilm-forming pathogens generally exhibit decreased susceptibility to antibacterial agents. Accordingly, higher MIC values were expected for Ab5075 and Ab5711. However, Ab19606 is a less virulent and antibiotic susceptible strain obtained from ATCC; therefore, lower MIC values were expected.

KpBAA-1705, obtained from ATCC, is a MDR carbapenem-resistant *K. pneumoniae* strain, and KpNSC-277 is a MDR carbapenem-sensitive clinical strain. Because the MEP pathway is present in many Gram-negative bacteria, Fosmidomycin and FR900098 were generally expected to have inhibition effects on the whole cells.

As depicted in Table 1, all three *A. baumannii* strains (Ab5075, Ab5711, and Ab19606) were resistant to fosmidomycin at the tested drug concentration range (1−512 μg/mL). However, *A. baumannii* strains Ab5075 and Ab19606 were susceptible to FR900098 with MICs of 256 and 128 μg/mL, respectively; strain Ab5711 was resistant to FR900098. Conversely, the *K. pneumoniae* strains (KpBAA-1705 and KpNSC-277) were more susceptible to fosmidomycin than the *A. baumannii* strains, with MICs of 128 and 64 μg/mL, respectively. Both *K. pneumoniae* strains were susceptible to FR900098 with an MIC of 256 μg/mL.

To enter bacterial cells and inhibit the MEP pathway, fosmidomycin is dependent on the glycerol-3-phosphate transporter (GlpT). Some pathogens which are lacking the GlpT transporter, such as *Mycobacterium tuberculosis*, are impermeable to fosmidomycin. However, lipophilic phosphonate prodrugs have proven effective MEP pathway inhibitors against bacteria lacking the GlpT transporter.

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**Figure 3.** Substrate dependent catalytic activity of *A. baumannii* and *K. pneumoniae* IspC. Shown are Michaelis–Menten plots of AbIspC reaction velocity as a function of (A) DXP concentration and (B) NADPH concentration. Also shown are the Michaelis–Menten plots of KpIspC reaction velocity as a function of (C) DXP concentration and (D) NADPH concentration. KM values were obtained using GraphPad Prism 5.0. Least-squares best fit of the data to the Michaelis–Menten equation produces the kinetic parameters depicted here and listed in Table 2. The R² value for each plot is indicated. All assays were performed in duplicate. The error bars indicate the standard deviation for each data point.
and to provide a framework for developing more potent
AbIspC and KpIspC, respectively. All assays were performed in duplicate. The standard errors for the KM
homogeneity via an N-terminal histidine tag. In parallel, the recombinant AbIspC enzyme was a
codon plus RIL cells for protein expression. The resulting
transformed into chemically competent
Inc., Piscataway, NJ), cloned into a pMCSG28 vector, and
gene was fully synthesized (GenScript USA
IspC Enzymes.

This study

K. pneumoniae and Strains
K. pneumoniae

K. pneumoniae

K. pneumoniae

Figure 4. Cation specificity of A. baumannii and K. pneumoniae IspC.

Asp IspC were performed with fixed concentrations of NADPH (150 μM), DXP (400 μM), and divalent cation (25 mM). AbIspC and KpIspC preferentially use Mg2+ for catalysis.35,57,73 Like many other IspC enzymes,35,57,73 evaluation of enzyme activity in the presence of various divalent cations reveals that recombinant AbIspC and KpIspC preferentially use Mg2+ for catalysis.35,57,73

Next, we determined the half-maximal inhibitory (IC50) concentrations of the phosphate inhibitors fosmidomycin and FR900098, with AbIspC and KpIspC (Figure 5). While AbIspC was more potently inhibited by FR900098 than fosmidomycin, KpIspC was inhibited by fosmidomycin and FR900098 at similar potencies. The improved potency of FR900098 over fosmidomycin against AbIspC agrees with the non-linear regression fitting of enzyme velocity versus substrate concentration was used to determine the apparent kinetic constants (Figure 3). The KM for DXP was obtained using assays performed with a saturating concentration of NADPH (150 μM), whereas the KM for NADPH was obtained using assays performed with 400 μM DXP. Generally, the recombinant A. baumannii IspC and K. pneumoniae IspC have KMapp, DXP, and KMapp, NADPH values that are comparable to those reported for homologous enzymes from other organisms (Table 2). The apparent specificity constant (Kcatapp, DXP/KMapp) of both A. baumannii and K. pneumoniae IspC are approximately 2-fold higher than that reported for Yersinia pestis IspC, approximately 30-fold lower than that reported for the E. coli enzyme, and approximately 2-fold lower than those reported for the Francisella tularensis and Mycobacterium tuberculosis enzymes (Table 2).

IspC requires a divalent cation—generally Mg2+ or Mn2+—for catalysis.35,57,73 Like many other IspC enzymes,35,57,73 evaluation of enzyme activity in the presence of various divalent cations reveals that recombinant AbIspC and KpIspC preferentially use Mg2+ for catalysis.35,57,73

Table 2. IspC Apparent Kinetic Parameters

|        | AblIspC | KpIspC | EblIspC | FblIspC | MblIspC | YblIspC |
|--------|---------|--------|---------|---------|---------|---------|
| KMapp (DXP) (μM) | 127.6 ± 7.2 | 155.5 ± 10.8 | 81 ± 250 | 103.7 ± 12.1 | 47 | 221.5 ± 34.3 |
| KMapp (NADPH) (μM) | 19.53 ± 0.53 | 14.51 ± 0.83 | 0.5 ± 18 | 13.3 ± 1.5 | 29.7 | 12.7 ± 1.5 |
| kcatapp (s−1) | 1.92 ± 0.03 | 2.21 ± 0.06 | 33 | 2.0 ± 0.09 | 1.2 | 1.7 |
| kcatapp (s−1) | 1.64 ± 0.01 | 1.96 ± 0.03 | 1.3 ± 0.04 | 1.0 |
| IC50app (DXP) (M−1 min−1) | 9.0 × 10−6 ± 5.3 × 10−6 | 8.5 × 10−6 ± 6.5 × 10−6 | 2.4 × 10−7 | 1.2 × 10−7 | 1.5 × 10−6 | 4.6 × 10−7 |
| IC50app (NADPH) (nM) | 46.8 (38.2–57.2) | 20.2 (15.9–25.6) | 35 | 247 | 80 | 710 |
| IC50app (DXP) (nM) | 23.9 (21.4–26.7) | 23.1 (18.3–23.7) | 35 | 230 | 160 | 231 |

ref | This study | This study

*A BLAST search with the E. coli K-12 GlpT protein sequence (accession no. P08194) identifies a homologous transport protein in the K. pneumoniae KpBA-1705 proteome (accession no. EMR29960; 93.06% identity). A BLAST search with the E. coli K-12 GlpT protein sequence and the A. baumannii Ab5075 proteome did not identify a GlpT transporter. The BLAST searches were performed with the National Center for Biotechnology Information (NCBI) BLAST (blastp) suite. Sequenced proteomes for KpNSC-277, Ab5711, and Ab19606 were not available for searching. A. baumannii resistance to fosmidomycin may therefore be due to poor cellular uptake. Additionally, previous studies have shown that uptake of FR900098 is only partially dependent on GlpT, which may explain the growth inhibition of A. baumannii strains Ab5075 and Ab19606.48 Furthermore, resistance of Ab5711 to fosmidomycin and FR900098 could be due to its substantial biofilm formation characteristics in vitro.64 Fosmidomycin and FR900098 are hydrophilic compounds which may not be able to effectively penetrate the A. baumannii and K. pneumoniae cell. Alternatively, or in combination, resistance to Fosmidomycin and FR900098 may be due to efflux. However, additional studies are warranted to validate the mechanism of cellular uptake and/or efflux in A. baumannii and K. pneumoniae species conclusively. Because fosmidomycin and/or FR900098 clearly inhibited A. baumannii and K. pneumoniae growth, we cloned and characterized both the A. baumannii and K. pneumoniae IspC enzyme to further assess IspC as an antibiotic target for A. baumannii and K. pneumoniae and to provide a framework for developing more potent derivatives.

Characterization of A. baumannii and K. pneumoniae IspC Enzymes. To enable the enzymatic characterization of A. baumannii IspC, the A. baumannii ispC gene was PCR amplified from genomic DNA, cloned into a pBG1861 vector, and transformed into chemically competent E. coli BL21(DE3) codon plus RIL cells for protein expression. The resulting recombinant AbIspC enzyme was affinity purified to near homogeneity via an N-terminal histidine tag. In parallel, the K. pneumoniae ispC gene was fully synthesized (GenScript USA Inc., Piscataway, NJ), cloned into a pMCSG28 vector, and transformed into chemically competent E. coli BL21(DE3) codon plus RIL cells. The resulting recombinant KpIspC enzyme was affinity purified to near homogeneity via a C-terminal histidine tag.

The catalytic activity of each purified recombinant enzyme was determined by a spectrophotometric assay monitoring the substrate dependent oxidation of NADPH. Non-linear regression fitting of enzyme velocity versus substrate concentration was used to determine the apparent kinetic constants (Figure 3). The KM for DXP was obtained using assays performed with a saturating concentration of NADPH (150 μM), whereas the KM for NADPH was obtained using assays performed with 400 μM DXP. Generally, the recombinant A. baumannii IspC and K. pneumoniae IspC have KMapp, DXP, and KMapp, NADPH values that are comparable to those reported for homologous enzymes from other organisms (Table 2). The apparent specificity constant (Kcatapp, DXP/KMapp) of both A. baumannii and K. pneumoniae IspC are approximately 2-fold higher than that reported for Yersinia pestis IspC, approximately 30-fold lower than that reported for the E. coli enzyme, and approximately 2-fold lower than those reported for the Francisella tularensis and Mycobacterium tuberculosis enzymes (Table 2).

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Asp IspC were performed with fixed concentrations of NADPH (150 μM), DXP (400 μM), and divalent cation (25 mM). AbIspC and KpIspC preferentially use Mg2+ for catalysis.35,57,73
MIC values wherein all three *A. baumannii* strains were resistant to fosmidomycin at 1–512 μg/mL. It is noteworthy that, in general, fosmidomycin and FR900098 are more potent against AbIspC and KpIspC than the homologous recombinant enzymes from *F. tularensis*, *M. tuberculosis*, and *Y. pestis* (Table 2), thus highlighting their promise as targets for therapeutics against *A. baumannii* and *K. pneumoniae*.

**Overall Structure of AbIspC in Complex with FR900098, NADPH, and Mg^{2+}**. The structure of AbIspC in complex with FR900098, NADPH, and Mg^{2+} was refined at 2.5 Å resolution with *R*_free and *R*_work values of 24% and 23%, respectively (Figure 6). AbIspC crystallized in space group *P*2_1_2_1 and the unit cell parameters were *a* = 66.572, *b* = 118.251, *c* = 53.911, and *α* = *β* = *γ* = 90. The refinement and statistics are summarized in Table 3. AbIspC crystallized as a monomer in the asymmetric unit, with the homodimer formed by crystallographic symmetry. The biological assembly of IspC is known to be a homodimer.78 The overall structure of the AbIspC monomer (Figure 6A) is similar to those that have been previously reported, in which each monomer consists of three domains: a central catalytic domain, an N-terminal NADPH-binding domain, and a C-terminal α-helical domain.79,80 The AbIspC N-terminal NADPH binding domain is comprised of a seven-stranded parallel β-sheet and six α-helices joined by a Rossmann or adenosine diphosphate (ADP)-binding βαβ fold which binds NADPH in a classical manner.81 The C-terminal α-helical domain is comprised of a four-helix bundle. The central catalytic domain, which binds the divalent magnesium ion and FR900098, is located within a deep cleft at the center of the monomer, and is covered by an active site...
loop (Figure 7) which is known to close over the active site upon substrate binding.80,82 The four-stranded β-sheet which sits below the catalytic domain contains one parallel and two antiparallel alignments and is known to comprise part of the dimer interaction with the second IspC monomer.79

Active Site and Active Site Loop. FR900098 is bound to the active site via hydrogen bonding interactions with the oxygens of its phosphonate and hydroxamate groups, coordination with the divalent magnesium ion, and hydrophobic interactions with the propyl backbone of FR900098 (Figure 6B). The phosphonate oxygens of FR900098 form hydrogen bonds with Asn235, two water molecules, and highly conserved residues, Ser230, Ser194, and Lys236.74,79 Both the side chain and backbone nitrogen of Ser194 are hydrogen bonded to the phosphate oxygen. The divalent magnesium cation is coordinated to highly conserved residues Asp159, Glu161, and Glu239 and the carbonyl and hydroxyl oxygens of the FR900098 hydroxamate group in a distorted trigonal bipyramidal geometry.74,79 The metal interactions are similar to those observed in other quaternary IspC complexes with FR900098, NADPH, and a divalent cation. In the quaternary structure of IspC from *M. tuberculosis* in complex with FR900098, NADPH, and Mn²⁺, only five oxygen atoms coordinate each metal ion, with an approximate octahedral geometry.83 Similarly, in the quaternary structure of IspC from *Plasmodium falciparum* in complex with FR900098, Mg²⁺, and NADPH, the Mg²⁺ ion binds to three protein ligands and two inhibitor atoms, resulting in a distorted trigonal bipyramidal geometry.84 The Ser160 side chain and its backbone nitrogen also form hydrogen bonds with the hydroxyl oxygen of the hydroxamate group. The active site loop is closed over the FR900098 and Mg²⁺ binding site, and the propyl backbone of FR900098 interacts with the adjacent Trp220 and Met222 (of the active site loop) at approximate distances of 4.138 and 4.150 Å, respectively.

Closed Conformation of AbIspC in Complex with FR900098, NADPH, and Mg²⁺. The primary difference between the quaternary AbIspC structure (AbIspC in complex with FR900098, NADPH, and Mg²⁺) and the apo AbIspC structure (deposited by the Seattle Structural Genomics Center for Infectious Disease, PDB ID: 4ZN6) is the overall conformation of the enzyme, wherein the apo structure adopts a more open conformation and the quaternary structure adopts a closed conformation. This can be observed by superimposing
In summary, we have shown that A. baumannii and *K. pneumoniae* IspC are valid targets for the development of novel antibiotics. Although all *A. baumannii* strains were resistant to fosmidomycin at 512 µg/mL and Ab5711 was resistant to both inhibitors at 512 µg/mL, *A. baumannii* strains Ab5075 and Ab19606 were susceptible to FR900098 at MICs of 256 and 128 µg/mL, respectively. A. baumannii resistance to fosmidomycin may be due to lack of GlpT uptake and/or impermeability. Ab5711’s resistance to both fosmidomycin and FR900098 could likewise be attributed to impermeability due to the substantial biofilm formation characteristics of Ab5711. Conversely, *K. pneumoniae* was more susceptible to fosmidomycin than FR900098. Strains KpBAA-1705 and KpNSC-277 were susceptible to fosmidomycin at MICs of 128 and 64 µg/mL, respectively, whereas, both strains were susceptible to FR900098 at an MIC of 256 µg/mL. As mentioned, fosmidomycin uptake is GlpT-dependent, whereas FR900098 uptake is partially GlpT-dependent. *K. pneumoniae*’s improved susceptibility to fosmidomycin over FR900098 may be attributed to uptake via GlpT. Nonetheless, Fosmidomycin and FR900098 are highly hydrophilic compounds; therefore, resistance may be generally attributed to impermeability regardless of GlpT uptake. Future studies are warranted to assess the method of uptake, or efflux, of these compounds in *A. baumannii* and *K. pneumoniae*.

Unlike the bacterial growth inhibition assays, both *A. baumannii* and *K. pneumoniae* IspC were potently inhibited by fosmidomycin and FR900098 *in vitro*. Additionally, both *A. baumannii* and *K. pneumoniae* IspC were generally more susceptible to the inhibitors than other homologous enzymes. The *in vitro* potency of fosmidomycin and FR900098 against *A. baumannii* and *K. pneumoniae* IspC highlights their promise as targets for therapeutics against *A. baumannii* and *K. pneumoniae*. We resolved the quaternary structure of AbIspC in complex with FR900098, NADPH, and Mg\(^{2+}\). This structure can guide additional structure–based drug design, which may yield derivatives with more favorable bacterial growth inhibition. As mentioned previously, where cellular penetration has posed a challenge for bacterial growth inhibition, lipophilic phosphonate prodrugs have demonstrated potential for effective bacterial growth inhibition. Accordingly, lipophilic prodrugs of fosmidomycin and FR900098 analogs likely represent the best prospects for *A. baumannii* and *K. pneumoniae*, as well as other ESKAPE pathogens.

**CONCLUSION**

In summary, we have shown that *A. baumannii* and *K. pneumoniae* IspC are valid targets for the development of novel antibiotics. Although all *A. baumannii* strains were resistant to fosmidomycin at 512 µg/mL and Ab5711 was resistant to both inhibitors at 512 µg/mL, *A. baumannii* strains Ab5075 and Ab19606 were susceptible to FR900098 at MICs of 256 and 128 µg/mL, respectively. A. baumannii resistance to fosmidomycin may be due to lack of GlpT uptake and/or impermeability. Ab5711’s resistance to both fosmidomycin and FR900098 could likewise be attributed to impermeability due to the substantial biofilm formation characteristics of Ab5711. Conversely, *K. pneumoniae* was more susceptible to fosmidomycin than FR900098. Strains KpBAA-1705 and KpNSC-277 were susceptible to fosmidomycin at MICs of 128 and 64 µg/mL, respectively, whereas, both strains were susceptible to FR900098 at an MIC of 256 µg/mL. As mentioned, fosmidomycin uptake is GlpT-dependent, whereas FR900098 uptake is partially GlpT-dependent. *K. pneumoniae*’s improved susceptibility to fosmidomycin over FR900098 may be attributed to uptake via GlpT. Nonetheless, Fosmidomycin and FR900098 are highly hydrophilic compounds; therefore, resistance may be generally attributed to impermeability regardless of GlpT uptake. Future studies are warranted to assess the method of uptake, or efflux, of these compounds in *A. baumannii* and *K. pneumoniae*.

**METHODS**

**Bacterial Strains and Growth Conditions.** Clinical isolates of *A. baumannii* strains Ab5075 and Ab5711 and *K. pneumoniae* strain KpNSC-277 were obtained from the Multidrug-resistant organism Repository and Surveillance Network (MRSN) at Walter Reed Army Institute of Research. Ab5075 is the most virulent and multidrug-resistant strain. Ab5711 is also an MDR strain and is known to form substantial biofilms *in vitro*. *A. baumannii* strain Ab19606, *K. pneumoniae* strain KpBAA-1705, and *Escherichia coli* strain Ec25922 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The bacterial isolates were cultured on blood agar plates (Tryptic Soy Agar with 5% sheep blood) at 37 °C for 16–18 h prior to antimicrobial susceptibility tests. Recombinant proteins were expressed in *Escherichia coli* BL21 CodonPlus (DE3)-RIL cells obtained from Stratagene (La Jolla, CA). *E. coli* was cultured at 37 °C in Luria–Bertani (LB) media supplemented with 100 µg/mL ampicillin and 50 µg/mL chloramphenicol with constant shaking at 250 rpm. Agar (1.5 wt %/vol) was added to prepare solid media.
Antimicrobial Susceptibility Assays. The minimum inhibitory concentration (MIC) of each compound was evaluated using the broth microdilution method according to the guidelines described in the Clinical and Laboratory Standards Institute (CLSI).

Fosmidomycin and FR900098 were purchased from Sigma-Aldrich (St. Louis, MO). Inhibitors stock solution were prepared in phosphate buffered saline (PBS) and stored at −80 °C. The compounds were 2-fold diluted in cation-adjusted Mueller-Hinton Broth (CAMHB) in 96-well round-bottom, polystyrene microtiter plates to final concentrations ranging from 1 to 512 μg/mL. 100 μL of the drug containing broth was dispensed into the wells of the 96-well plate in triplicate. Wells containing broth only served as growth and sterility controls. Prior to antimicrobial susceptibility testing, A. baumannii strains were cultured on blood agar plates at 37 °C for 16–18 h, and K. pneumoniae strains were cultured in CAMHB media at 37 °C for 5–6 h.

Fresh colonies and suspension cultures in PBS were adjusted spectrophotometrically to an optical density of 0.1, at wavelength of 600 nm, to yield approximately 1 × 10⁸ colony-forming units (CFU)/mL. The bacterial suspension was further diluted in CAMHB, and 5 μL of the suspension was inoculated into respective wells to yield a starting inoculum of around 5 × 10⁴ CFU/mL. The 96-well plates were incubated at 37 °C for 18 to 20 h. The MIC was recorded as the lowest concentration of antimicrobial agent that inhibits the visible growth of the bacterial isolate after 18–20 h incubation.

E. coli strain ATCC 25922 was used as the quality control strain with ampicillin as the antibiotic. A minimum of three replicates were performed for each compound.

Cloning, Expression, and Purification of A. baumannii and K. pneumoniae IspC. The A. baumannii and K. pneumoniae IspC genes (ispC) were identified in the complete genome sequence using primary sequence homology with orthologs from other organisms. Sequence alignment was performed with the National Center for Biotechnology Information (NCBI) BLAST (blastp) suite with the following NCBI reference sequences: E. coli (U00906.2, AAC73284.1), F. tularensis (AJ749949.2, CAG46207.1), M. tuberculosis (NC_000962.3, NP_217386.2), Y. pestis (NC_001431.1, NP_002346091.1), A. baumannii (NZ_AFCCZ00000002.1, EJP43779.1), and K. pneumoniae (KK036887.1, EWF09955.1).

The A. baumannii IspC clone was generously provided by the Seattle Structural Genomics Center for Infectious Disease (SSGCID). To prepare the clone, the A. baumannii ispC gene was PCR amplified from genomic DNA and cloned into a pBG1861 vector to yield pAbIspC, facilitating the expression of A. baumannii IspC with an N-terminal His₆-tag. The K. pneumoniae ispC gene was fully synthesized (GenScript USA Inc., Piscataway, NJ) and cloned into a pMCSG28 vector to yield pKpIspC, facilitating the expression of K. pneumoniae IspC with a C-terminal His₆-tag.

Each expression plasmid (pAbIspC and pKpIspC) was separately transformed into chemically competent E. coli BL21 CodonPlus (DE3)-RIL cells for protein expression. To express the His-tagged protein, a 10 mL overnight seed culture was added to 1 L of LB media supplemented with 100 μg/mL ampicillin and 50 μg/mL chloramphenicol and then incubated with shaking at 37 °C and 250 rpm. At an OD₆₀₀ of 0.8, protein expression was induced with addition of isopropyl b-D-thiogalactopyranoside (IPTG) to 0.5 mM and the culture was further incubated with shaking at 37 °C and 250 rpm for an additional 18 h. Cells were harvested via centrifugation (4648g, 20 min, 4 °C) and stored at −80 °C. Protein was subsequently isolated and purified from the cells via chemical lysis and affinity chromatography.

Cells were lysed with lysis buffer A (100 mM Tris pH 8.0, 0.032% lysozyme, 3 mL per gram cell pellet), followed by lysis buffer B (0.1 M CaCl₂, 0.1 M MgCl₂, 0.1 M NaCl, 0.02% DNase, 0.3 mL per gram cell pellet). Clarified cell lystate was collected after centrifugation (48,000g, 20 min, 4 °C) and passed through a TALON immobilized metal affinity column (Clontech Laboratories, Mountain View, CA).

The column was washed with 20 column volumes of equilibrium buffer (50 mM HEPES pH 7.5, 300 mM NaCl), 10 column volumes of wash buffer A (50 mM HEPES pH 7.5, 300 mM NaCl, 10 mM imidazole), and 15 column volumes of wash buffer B (100 mM HEPES pH 7.5, 600 mM NaCl, 20 mM imidazole). The protein was eluted with 5 column volumes of elution buffer (150 mM imidazole pH 7.0, 300 mM NaCl). Buffer was exchanged with protein storage buffer (100 mM Tris pH 7.5, 1 mM NaCl, 5 mM DTT) during concentration by ultrafiltration. Protein concentration was determined using Advanced Protein Assay Reagent (Cytoskeleton, Denver CO) with γ-globulins (Sigma-Aldrich) as the standard and by measuring the absorbance at 280 nm (A₂₈₀).

Purified protein was visualized by Coomassie stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The yield of AbIspC averages 20 mg per 1 L shake flask, whereas the yield of KpIspC averages 10 mg per 1 L shake flask.

Crystallization. Crystallization experiments were set up with AbIspC according to the conditions published by the Seattle Structural Genomics Center for Infectious Disease (SSGCID) in the RCSB Protein Data Bank (PDB ID: 4ZN6). Crystals were obtained via sitting drop vapor diffusion at 16 °C from drops containing 100 mM sodium citrate: HCl pH 5.60, 250 mM ammonium sulfate, and 24% PEG 4000. Crystals were transferred to 100 mM sodium citrate: HCl pH 5.60, 250 mM ammonium chloride, and 24% PEG 4000 at 16 °C.

Subsequently, the crystals were soaked with 50 mM magnesium chloride, 1 mM NADPH, and 50 mM FR900098.

Data Collection and Processing. The crystals were cryoprotected in mother liquor containing 10% ethylene glycol, followed by mother liquor containing 20% ethylene glycol, and then flash frozen in a N₂ gas stream. Data was collected at 100 K to 2.4 Å resolution using a Bruker Microstar Rotating Anode X-ray generator with a Pt 135 CCD detector in-house at the Walter Reed Army Institute of Research X-ray Crystallography Center with integration and scaling using Proteum (Bruker).

Molecular Replacement and Refinement. The structure was determined by molecular replacement using the AbIspC structure (PDB ID 4ZN6) as a search model for phasing. Molecular replacement was performed using the program Phaser within the PHENIX suite. The NADPH, FR900098, and Mg²⁺ molecules were built within the mFo-Fc difference density map using Coot and Phenix. Refinement of the AbIspC: NADPH:: FR900098: Mg-tetra complex was performed using the phenix.refine within the Phenix software suite and Coot.

Enzyme Assays. Data was analyzed using GraphPad Prism 5.0 for Windows (GraphPad Software Inc., San Diego, CA). A.
Diego, CA). IC50 values were determined by nonlinear version 5.00 for Windows (GraphPad Software Inc., San City, UT) to the assay mixture, as described previously.87 The oxidation of NADPH was monitored at 340 nm using an Agilent 8453 UV–vis spectrophotometer equipped with a temperature regulated cuvette holder. All assays were performed in duplicate.

To determine the apparent $K_M$ for DXP, 120 μL assay solutions contained 100 mM Tris pH 7.8, 25 mM MgCl2, 150 μM NADPH, 0.18 μM IspC, and variable concentrations of DXP. The assay solution was incubated at 37 °C for 10 min, prior to addition of DXP, to facilitate the association of NADPH with the enzyme. To determine the apparent $K_M$ for NADPH, assays were performed with a fixed DXP concentration (400 μM) and variable concentrations of NADPH. The kinetic constants were determined by nonlinear regression to the Michaelis–Menten equation using GraphPad PRISM version 5.00 for Windows (GraphPad Software Inc, San Diego, CA). IC50 values were determined by nonlinear regression to a dose–response curve: $Y = \frac{(100)}{(1 + 10^\left((\logIC_{50}-X)\right))}$ using GraphPad PRISM version 5.00 for Windows.

To determine cation specificity, assays were performed with either MgCl2, CoCl2, CuCl2, MnCl2, or NiCl2 at 25 mM final concentration. Half-maximal inhibition ($IC_{50}$) of enzyme activity by fosmidomycin and FR900098 was determined using a plot of fractional enzyme activity as a function of inhibitor concentration using GraphPad Prism 5.0 for Windows. As both inhibitors are slow, tight binding inhibitors,10 fosmidomycin and FR900098 were preincubated with the enzyme at 37 °C for 10 min prior to the addition of the substrate, DXP.

## ASSOCIATED CONTENT

### Accession Codes

7504

## AUTHOR INFORMATION

### Corresponding Authors

Haley S. Ball — Department of Chemistry and Biochemistry, George Mason University, Manassas, Virginia 20109, United States of America; Wound Infections Department, Bacterial Diseases Branch, Center for Infectious Diseases Research, Walter Reed Army Institute of Research, Silver Spring, Maryland 20910, United States of America; orcid.org/0000-0002-8840-3861; Email: hball3@gmu.edu

Robin D. Couch — Department of Chemistry and Biochemistry, George Mason University, Manassas, Virginia 20109, United States of America; Email: rcouch@gmu.edu

Schroeder M. Noble — Wound Infections Department, Bacterial Diseases Branch, Center for Infectious Diseases Research, Walter Reed Army Institute of Research, Silver Spring, Maryland 20910, United States of America; Email: schroeder.m.noble.civ@mail.mil

### Authors

Misgina B. Girma — Department of Chemistry and Biochemistry, George Mason University, Manassas, Virginia 20109, United States of America; orcid.org/0000-0002-5510-0514

Mosuľa Zainab — Department of Chemistry and Biochemistry, George Mason University, Manassas, Virginia 20109, United States of America

Iswarduth Soojhawon — Wound Infections Department, Bacterial Diseases Branch, Center for Infectious Diseases Research, Walter Reed Army Institute of Research, Silver Spring, Maryland 20910, United States of America

Complete contact information is available at: https://pubs.acs.org/10.1021/acsinfecdis.1c00132

### Notes

The authors declare no competing financial interest. This work has been reviewed by the Walter Reed Army Institute of Research (WRAIR). There is no objection to its presentation and/or publication. The opinions and assertions contained herein are the private views of the authors and are not to be construed as official or reflecting the views of the Department of Defense, the Department of the Army, or any other agency of the U.S. Government.

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### ABBREVIATIONS USED

FR, FR900098

Fos, Fosmidomycin

Ab, Acinetobacter baumannii

Kp, Klebsiella pneumoniae

### REFERENCES

(1) Santajit, S.; Indrawattana, N. Mechanisms of Antimicrobial Resistance in ESKAPE Pathogens. BioMed Res. Int. 2016, 2016, 1.

(2) Lindberg, R. B.; Wetzler, T. F.; Newton, A.; Howard, J. M.; Davis, J. H.; Strawitz, J. The Bacterial Flora of the Blood Stream in the Korean Battle Casualty. *Ann. Surg.* 1955, 141 (3), 366–368.

(3) Tong, M. J. Septic Complications of War Wounds. *JAMA*, J. Am. Med. Assoc. 1972, 219 (8), 1044–1047.

(4) Petersen, K.; Riddle, M. S.; Danko, J. R.; Blazes, D. L.; Hayden, R.; Tasker, S. A.; Dunne, J. R. Trauma-Related Infections in Battlefield Casualties from Iraq. *Ann. Surg.* 2007, 245 (5), 803–811.

(5) Calhoun, J. H.; Murray, C. K.; Martin, M. M. Multidrug-Resistant Organisms in Military Wounds from Iraq and Afghanistan. *Clin. Orthop. Relat. Res.* 2008, 466 (6), 1356–1362.

(6) Gootz, T. D.; Marra, A. Acinetobacter Baumannii: An Emerging Multidrug-Resistant Threat. *Expert Rev. Anti-Infect. Ther.* 2008, 6 (3), 309–325.
Bacteria. Infections Associated with the Three Most Common Uropathogenic
BMC Infect. Dis. 2006, 58 (8), 2700–2706.

(29) WHO | WHO publishes list of bacteria for which new antibiotics are urgently needed https://www.who.int/news/item/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed (accessed 2017 –11 –30).

(30) Beringer, P. The Clinical Use of Colistin in Patients with Cystic Fibrosis. Curr. Opin. Pulm. Med. 2001, 7 (6), 434–440.

(31) Landman, D.; Georgescu, C.; Martin, D. A.; Quale, J. Polymyxins Revisited. Clin. Microbiol. Rev. 2008, 21 (3), 449–465.

(32) Chen, L. Notes from the Field: Pan-Resistant New Delhi Metallo-Beta-Lactamase-Producing Klebsiella Pneumoniae — Washoe County, Nevada, 2016. MMWR Morb Mortal Wkly Rep 2017, 66, 33.

(33) Lange, B. M.; Rujan, T.; Martin, W.; Croteau, R. Isoprenoid Biosynthesis: The Evolution of Two Ancient and Distinct Pathways across Genomes. Proc. Natl. Acad. Sci. U. S. A. 2000, 97 (24), 13172–13177.

(34) Lombard, J.; Moreira, D. Origins and Early Evolution of the Mevalonate Pathway of Isoprenoid Biosynthesis in the Three Domains of Life. Mol. Biol. Evol. 2011, 28 (1), 87–99.

(35) Jawaid, S.; Seidle, H.; Zhou, W.; Abdrahman, H.; Abadeer, M.; Hix, J. H.; van Hoek, M. L.; Couch, R. D. Kinetic Characterization and Phosphorilation of the Francisella Tularensis 1-Deoxy-D-Xyulose 5-Phosphate Reductoisomerase (MEP Synthase). PLoS One 2009, 4 (12), e8288.

(36) Heuston, S.; Begley, M.; Gahan, C. G. M.; Hill, C. Isoprenoid Biosynthesis in Bacterial Pathogens. Microbiology 2012, 158 (6), 1389–1401.

(37) Rohdich, F.; Bacher, A.; Eisenreich, W. Isoprenoid Biosynthetic Pathways as Anti-Infective Drug Targets. Biochem. Soc. Trans. 2005, 33 (4), 785–791.

(38) Singh, N.; Chevé, G.; Avery, M. A.; McCurdy, C. R. Targeting the Methyl erythritol phosphate (MEP) Pathway for Novel Antimicrobial, Antitubercular and Herbicidal Drug Discovery: Inhibition of 1-Deoxy-D-Xyulose-5-Phosphate Reductoisomerase (DXR) Enzyme. Curr. Pharm. Des. 2007, 13 (11), 1161–1177.

(39) Lange, B. M.; Wildung, M. R.; McCaskill, D.; Croteau, R. A Family of Transketolasomes That Directs Isoprenoid Biosynthesis via a Mevalonate-Independent Pathway. Proc. Natl. Acad. Sci. U. S. A. 1998, 95 (5), 2100–2104.

(40) Koppisch, A. T.; Fox, D. T.; Blagg, B. S. J.; Poulter, C. D. E.; Coli, M. E. P. Synthase: Steady-State Kinetic Analysis and Substrate Binding. Biochemistry 2002, 41 (1), 236–243.

(41) Altincicek, B.; Kollas, A. K.; Sanderbrand, S.; Wiesner, J.; Hintz, M.; Beck, E.; Jomaa, H. GcpE Is Involved in the 2-C-Methyl-D-erythritol 4-Phosphate Pathway of Isoprenoid Biosynthesis in Escherichia Coli. J. Bacteriol. 2001, 183 (8), 2411–2416.

(42) Cunningham, F. X.; Lafond, T. P.; Gantt, E. Evidence of a Role for LytB in the Nonmevalonate Pathway of Isoprenoid Biosynthesis. J. Bacteriol. 2000, 182 (20), 5841–5848.

(43) Altincicek, B.; Duin, E. C.; Reichenberg, A.; Hedderich, R.; Kollas, A.-K.; Hintz, M.; Wagner, S.; Wiesner, J.; Beck, E.; Jomaa, H. LytB Protein Catalyzes the Terminal Step of the 2-C-Methyl-D-erythritol 4-Phosphate Pathway of Isoprenoid Biosynthesis. FEMS Lett. 2002, 532 (3), 437–440.

(44) McAteer, S.; Coulson, A.; McLennan, N.; Masters, M. The LytB Gene of Escherichia Coli Is Essential and Specifies a Product Needed for Isoprenoid Biosynthesis. J. Bacteriol. 2001, 183 (4), 7403–7407.

(45) Rohdich, F.; Hecht, S.; Gärtnert, K.; Adam, P.; Krieger, C.; Amslinger, S.; Arigoni, D.; Bacher, A.; Eisenreich, W. Studies on the Nonmevalonate Terpene Biosynthetic Pathway: Metabolic Role of
(78) Henriksson, L. M.; Unge, T.; Carlsson, J.; Aqvist, J.; Mowbray, S. L.; Jones, T. A. Structures of Mycobacterium Tuberculosis 1-Deoxy-D-Xylulose-5-Phosphate Reductoisomerase Provide New Insights into Catalysis. *J. Biol. Chem.* **2007**, **282** (27), 19905−19916.

(79) Yajima, S.; Hara, K.; Iino, D.; Sasaki, Y.; Kuzuyama, T.; Ohsawa, K.; Seto, H. Structure of 1-Deoxy-D-Xylulose 5-Phosphate Reductoisomerase in a Quaternary Complex with a Magnesium Ion, NADPH and the Antimalarial Drug Fosmidomycin. *Acta Crystallogr., Sect. F: Struct. Biol. Cryst. Commun.* **2007**, **63** (6), 466−470.

(80) Mac Sweeney, A.; Lange, R.; Fernandes, R. P. M.; Schulz, H.; Dale, G. E.; Douangamath, A.; Proteau, P. J.; Oefner, C. The Crystal Structure of E.Coli 1-Deoxy-D-Xylulose-5-Phosphate Reductoisomerase in a Ternary Complex with the Antimalarial Compound Fosmidomycin and NADPH Reveals a Tight-Binding Closed Enzyme Conformation. *J. Mol. Biol.* **2005**, **345** (1), 115−127.

(81) Hanukoglu, I. Proteopedia: Rossmann Fold: A Beta-Alpha-Beta Fold at Dinucleotide Binding Sites. *Biochem. Mol. Biol. Educ.* **2015**, **43** (3), 206−209.

(82) Steinbacher, S.; Kaiser, J.; Eisenreich, W.; Huber, R.; Bacher, A.; Rohdich, F. Structural Basis of Fosmidomycin Action Revealed by the Complex with 2-C-Methyl-d-Erythritol 4-Phosphate Synthase (IspC) Implications for the Catalytic Mechanism and Anti-Malaria Drug Development. *J. Biol. Chem.* **2003**, **278** (20), 18401−18407.

(83) Björkelid, C.; Bergfors, T.; Unge, T.; Mowbray, S. L.; Jones, T. A. Structural Studies on Mycobacterium Tuberculosis DXR in Complex with the Antibiotic FR-900098. *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **2012**, **68** (2), 134−143.

(84) Umeda, T.; Tanaka, N.; Kusakabe, Y.; Nakashima, M.; Kitade, Y.; Nakamura, K. T. Molecular Basis of Fosmidomycin’s Action on the Human Malaria Parasite Plasmodium Falciparum. *Sci. Rep.* **2011**, **1**, DOI: 10.1038/srep00009.

(85) CLSI. *Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard—Eighth ed.* Clinical and Laboratory Standards Institute, Wayne, PA, 2009; Vol. 8th ed. M07−A8.

(86) Wiegand, I.; Hilpert, K.; Hancock, R. E. W. Agar and Broth Dilution Methods to Determine the Minimal Inhibitory Concentration (MIC) of Antimicrobial Substances. *Nat. Protoc.* **2008**, **3** (2), 163−175.

(87) Takahashi, S.; Kuzuyama, T.; Watanabe, H.; Seto, H. A 1-Deoxy-D-Xylulose 5-Phosphate Reductoisomerase Catalyzing the Formation of 2-C-Methyl-d-Erythritol 4-Phosphate in an Alternative Nonmevalonate Pathway for Terpenoid Biosynthesis. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, **95** (17), 9879−9884.