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Award Number: W81XWH-11-2-0218

TITLE: Identification of New Drug Targets in Multi-Drug Resistant Bacterial Infections

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REPORT DATE: October 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

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1. REPORT DATE	**2. REPORT TYPE**	**3. DATES COVERED**
October 2012	Annual	26 September 2011 25 September 2012

4. TITLE AND SUBTITLE

Identification of New Drug Targets in Multi-Drug Resistant Bacterial Infections

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-11-2-0218

5c. PROGRAM ELEMENT NUMBER

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Hauptman Woodward Medical Research Institute, Inc.
Buffalo, NY 14203-1102
The Research Foundation for SUNY on behalf of University at Buffalo
Buffalo, NY 14260-7016

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Progress Report: We are combining two parallel efforts to explore new targets and pathways in A. baumannii. We hypothesize that two proteins involved in aromatic amino acid and metabolite biosynthesis are essential and are valid targets for development of inhibitors as potential antibiotics. We additionally hypothesize that a novel natural product is produced by A. baumannii that may play a role in pathogenesis or virulence. Goal 1. We aim to validate two protein targets that have been identified through genetic screening as essential targets in A. baumannii. These targets will be biochemically and structurally characterized, and will be screened in silico for potential fragment-based ligands that could bind to the enzyme active site. Conservation and prevalence of the targeted genes for goal 1 confirm essentiality and cloning, expression, purification, and preliminary crystallization was completed during year 1. Goal 2. We will characterize biochemically a novel natural product biosynthetic pathway to identify a new secondary metabolite and characterize the phenotype of a deletion strain that is unable to produce this compound. All genes for goal 2 have been cloned and catalytic proteins have been purified. Functional reconstitution of this pathway was initiated, identifying potential natural product building blocks. A structure of one protein has been determined.

15. SUBJECT TERMS
Acinetobacter baumannii, structural biology, genetic screening, biosynthetic pathways, essential gene identifcation

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	36	**19b. TELEPHONE NUMBER** *(include area code)*

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Introduction

A. baumannii is a gram-negative bacillus (GNB) known to cause health-care associated infections. Recently, community-acquired infections, infections in wounded U.S. service members, and infections in residents of long-term care facilities have been reported. The incidence of Acinetobacter infection in all venues is increasing worldwide. The changing epidemiology and incidence of infections due to Acinetobacter establishes it as a pathogen of increasing medical importance. Further, *A. baumannii* has acquired an alarming number of antimicrobial resistance genes. Resistance to all aminoglycosides, cephalosporins, and fluoroquinolones is common and resistance to carbapenems and beta-lactamase inhibitors is increasing. Safe reliable agents with predictable activity against *A. baumannii* are presently non-existent. Improved outcomes will require the development of new therapeutics. We aim to accomplish this is by identifying and examining two biosynthetic pathways as potential antimicrobial targets in *A. baumannii*. Our investigation of protein targets in *A. baumannii* includes two goals. The **first goal** focuses on validating chorismate synthase (CS) and prephenate dehydrogenase/3-phosphoshikimate-1-carboxyvinyl transferase (PD-PSCVT) as therapeutic targets in the *A. baumannii*. We will experimentally establish the prevalence and *in vivo* essentiality of these genes in multiple strains of *A. baumannii*. We will recombinantly express, purify, assay, and structurally characterize the CS and PD-PSCVT enzymes. Finally, we will evaluate the druggability of these proteins through structural and computational methods. The **second goal** will continue our efforts to characterize the natural product biosynthetic machinery of pathogenic GNB. We will examine a novel biosynthetic cluster that encodes non-ribosomal peptide synthetase (NRPS) enzymes that have been demonstrated to be involved in bacterial motility and to be upregulated in response to quorum signaling molecules. We will assay the synthetic enzymes to identify the substrate building blocks, identify the natural product through *in vitro* reconstitution and analysis of mutant strains, and assay the role of this pathway in bacterial growth and virulence.

Body

Acinetobacter baumannii is an emerging opportunistic pathogen that has been received significant attention recently due to the increasing incidence in a number of settings including long-term care facilities (*1, 2*) and US Military Medical Treatment Facilities (*3*). Many *A. baumannii* strains have acquired antibiotic resistance genes, further exacerbating the seriousness of this rise in infections (*4, 5*). The goal of this research project is to explore functionally and structurally two biosynthetic pathways from the *A. baumannii*. These pathways will be characterized functionally and examined for their essentiality in several models for infection. Additionally, this work will examine structures of the enzymes and identify potential small molecule probes that could bind to the target proteins to further explore their function and role in infection. This project represents a multi-disciplinary approach coupling genetic, molecular, biochemical, computational, and structural techniques.

The two goals of this project are to investigate several proteins from the biosynthetic pathways for chorismate and for a novel uncharacterized natural product. Targets from the first pathway were chosen because of two genes from this pathway were identified in a genetic screen for essential genes (*6*). *A. baumannii* strains harboring mutations in either the *aroA* (or *pd-pscvt*) or *aroC* (*cs*) genes show no growth defect on rich laboratory media. However, these strains exhibit a greater than five $\log_{10}$ reduction in growth in a rat subcutaneous infection model.

The second pathway was from an uncharacterized pathway for a non-ribosomally produced peptide. Similar peptide products have been observed to play roles in virulence. Additionally, the targeted pathway has been shown to play a role in *A. baumannii* type IV pilus-dependent motility and to be upregulated in response to the quorum signal N-(3-hydroxy)dodecanoyl homoserine lactone (*7*). While the precise relationship of motility to virulence has not been established in *A. baumannii* (*8, 9*), motility has been the subject of investigation in other gram negative pathogens such as *P. aeruginosa* (*10*).

Goal 1. Validation of Chorismate Biosynthesis as an Essential Target of *A. baumannii*

Our group has developed a novel and efficient approach to identify GNB genes and their protein products that are essential for growth and/or survival during infection, which is then validated via a rat soft-tissue infection model (*11*). This method has established that chorismate synthase (CS) and the bifunctional prephenate dehydrogenase 3-phosphoshikimate-1-carboxyvinyl transferase (PD-PSCVT) are essential proteins for *A. baumannii* infection (Figure 1) (*6*). Furthermore, PSCVT is a proven druggable target in plants (*12*), and the chorismate pathway is absent in humans. Therefore, our hypothesis is that these proteins are potential therapeutic targets in GNB. The object of this goal is to validate these proteins in the chorismate biosynthesis pathway as therapeutic targets.

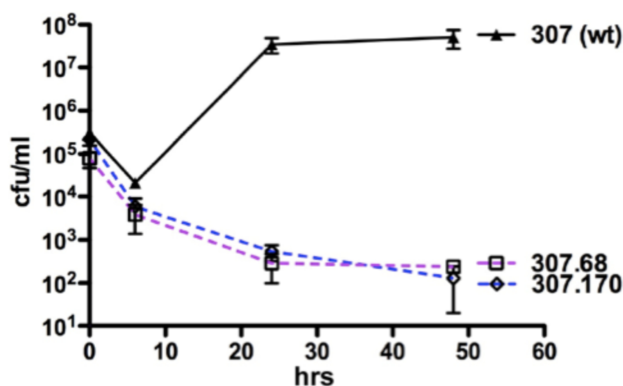

Figure 1. The mutant derivatives of the *A. baumannii* wild-type strain AB307-0294, AB307.68 and AB307.170, undergo significant and durable kill in the rat soft tissue infection model. AB307-0294 (wild-type), AB307.68 (*pd-pscvt*-minus), and AB307.170 (*cs*-minus). Data are Mean ± S.E.M. for n=3-4 for each time point.

PD-PSCVT and CS are part of the chorismate biosynthetic pathway, which is critical for the synthesis of the folate cofactors, bacterial siderophores, and

aromatic amino acids (Figure 2). These enzymes from *A. baumannii* have not been studied. Moreover, inactivation of multiple enzymes within this pathway will decrease the likelihood of the development of resistance, as was done with trimethoprim-sulfamethoxazole for the folate biosynthesis pathway. A similar metabolic pathway is present in other GNB exhibiting increasing occurrences of drug resistance (e.g., *Pseudomonas aeruginosa*, *Escherichia coli*, Enterobacter species, and *Klebsiella pneumoniae*), and our studies may translate to other GNB.

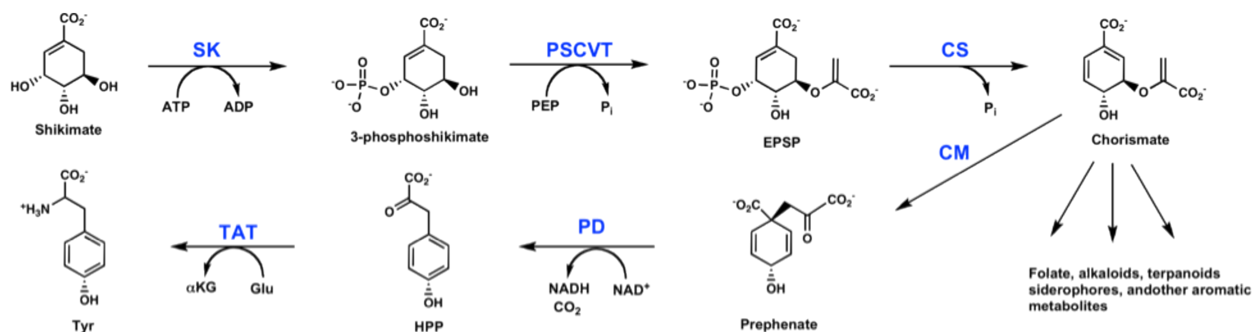

Figure 2. Chorismate biosynthesis. The biochemical steps in the synthesis of chorismate and tyrosine are shown to illustrate the enzymatic activities targeted in Goal 1. The PSCVT activity of PD-PSCVT couples 3-phosphoshikimate with pyruvate to produce 5-enoylpyruvyl-shikimate-3-phosphate (EPSP). Chorismate synthase (CS) is a lyase that removes the 3-phosphate to produce chorismate, a key building block for the formation of many aromatic compounds. The PD of PD-PSCVT removes a hydride from prephenate to form the aromatized product hydroxyphenylpyruvate (HPP), which then serves as the substrate for tyrosine aminotransferase (TAT), the enzyme that catalyzes the final step in tyrosine synthesis. In *A. baumannii*, PD and PSCVT are joined in a bifunctional protein.

Prevalence and conservation studies of CS and PD-PSCVT. An important consideration in validating a gene as an antimicrobial target is its level of conservation across strains. We have performed both a bioinformatic analysis and experimental verification of *cs* and *pd-pscvt* gene prevalence and sequence conservation. Thus far we have not observed any strains lacking either gene or displaying significant sequence variations (i.e., for *cs*: ≥99% identical at the nucleotide level, 100% identical at the amino acid level; for *pd-pscvt*: ≥98% identical at the nucleotide level, ≥99% identical at the amino acid level), supporting our hypothesis that these two genes are essential *in vivo* in numerous *A. baumannii* strains. Perhaps the most significant difference observed was in the PD-PSCVT protein, which exhibited differences in length due to a truncation/extension at the N-terminus, however, the active sites remain highly conserved. The *A. baumannii* strains formed two groups having either 748 or 756 amino acid residues in PD-PSCVT. It is unknown if this N-terminal truncation/extension has any functional significance. Table 1 summarizes the strains considered, including the geographical origin of each isolate, the occurrence of a multi-drug resistance (MDR) phenotype, and the tissue or body fluid the strain was isolated from, if known. The genomes of the majority of the strains have been both completely sequenced and annotated, or this process is currently underway. Three additional strains (AB932, AB1013, and ATCC 15308) lacking significant genomic sequence data are also under consideration, as two represent military isolates and the third has been a strain widely distributed for laboratory studies. For these strains, the respective genes were amplified by PCR from genomic DNA templates and then sequenced. A second related important consideration in the selection of antimicrobial targets is the determination of the lack of additional genes capable of overlapping function but potentially possessing sequence differences that may manifest themselves as a drug resistance mechanism. Thus far we have not identified the presence of any potential paralogous *cs* or *pd-pscvt* genes in any of the analyzed strains. While not conclusive, this observation suggests the absence of a related parallel biosynthetic pathway in *A. baumannii*.

Table 1. *A. baumannii* strains

Strain	Origin (Isolated/Sequenced)	Notes‡
1656-2	Kyungpook National University	MDR hospital isolate, South Korea
AB0057	WRAMC / Case Western Reserve Univ.	MDR bloodstream isolate from WRAMC (military)
AB307-0294	SUNY-Buffalo	Bloodstream isolate, ECMC, Buffalo
ACICU	Hospital S. Giovanni-Addolorata, Rome, Italy	MDR Hospital strain from an outbreak in Rome; cerebrospinal fluid isolate
ATCC 17978	ATCC	Clinical isolate, fatal meningitis (1951)
AYE	Le Kremlin-Bicêtre, France	Clinical isolate, pneumonia and a urinary tract infection
MDR-ZJ06	Zhejiang University, China	MDR bloodstream isolate, Hangzhou, China
SDF	Marseille, France	Isolated from body lice in France; presumably result of lice feeding on blood of infected human
TCDC-AB0715	Taipei, Taiwan	MDR clinical isolate, bloodstream
6013113	Birmingham, UK	Skin isolate, Human Microbiome Project reference strain
6013150	Birmingham, UK	Skin isolate, Human Microbiome Project reference strain
6014059	Birmingham, UK	Skin isolate, Human Microbiome Project reference strain
AB056	WRAMC / Case Western Reserve Univ.	MDR bloodstream isolate from WRAMC (military)
AB058	WRAMC / Case Western Reserve Univ.	MDR skin survey isolate from WRAMC (military)
AB059	WRAMC / Case Western Reserve Univ.	MDR urine isolate from WRAMC (military)
AB900	WRAMC / SUNY-Buffalo	Perineal isolate from WRAMC (military); obtained from Col. Craft
ATCC 19606	ATCC	Urine isolate (originally Schaub's strain 81)
AB932	WRAMC	Environmental isolate (military); obtained from Col. Craft
AB1013	WRAMC	Environmental isolate (military); obtained from Col. Craft
ATCC 15308	ATCC	Urine (originally named Schaub's strain Biol. 1)

‡MDR: multi-drug resistant strain; WRAMC: Walter Reed National Military Medical Center
Yellow: Publically released completed genomes (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi).
Green: Genomic sequencing in Whole Genome Shotgun (WGS) assembly stage (http://gelportal.gel.ym.edu.tw/services/genomes/microb3.php).

Establishing the in vivo essentiality of CS and PD-PSCVT in multiple strains of A. baumannii. CS and PD-PSCVT were identified in an initial screen for *in vivo* essentiality. Mutations in either gene in *A. baumannii* strain AB307-0294 compromise *A. baumannii* viability in the rat soft tissue infection model (Figure 1) (*6*). We are now testing if CS and PD-PSCVT are *in vivo* essential across a library of *A. baumannii* strains in order to provide confidence that this is a general feature of *A. baumannii* and not a strain-specific phenotype.

A directed knockout mutant of the *cs* (also known as *aroA*) gene in *A. baumannii* 307-0294 (AB307) has been prepared, and was used to confirm *in vivo* essentiality using our established rat soft tissue infection model. The construct used to prepare this directed knockout, pBS::*cs*::kan, was then used in the attempt to prepare directed knockouts in *A. baumannii* strains AB1013, AB932, and AB15308. Unexpectedly, we were unable to prepare directed knockout mutants within these strains.

We are developing insight into why this is. First, based on *cs* sequence generated from AB1013, AB932, and AB15308 we have learned that base changes have occurred that do not result in amino acid changes. Nonetheless, these changes may affect homologous recombination. Therefore for some AB strains, strain-specific constructs used for gene disruption will need to be generated. This has been completed for AB932 and AB15308. Secondly, and probably more importantly, the electroporation efficiency of AB1013, AB932, and AB15308 is significantly less than AB307-0294. This was established by the demonstration that the ability to develop random transposon mutants in AB1013, AB932, and AB15308 is less than AB307-0294. In an attempt to overcome the electroporation efficiency issue in a wild-type background we assessed electroporation efficiency under a variety of conditions in which resistance and voltage were

modified. The transposon EZ::TN<TMP> was used to select for successful electroporants. However, the conditions previously utilized could not be improved upon.

It is known that surface polysaccharides can affect transformation/electroporation efficiency. To confirm this in *A. baumannii,* we tested a mini-transposon that carries resistance to trimethoprim, EZ::TN<TMP>, to further confirm electroporation efficiency is greater in a capsule-minus and kanamycin-resistant background. Results from this experiment demonstrated that compared to the wild-type strain AB1013, the transposition frequency was increased 45-fold in its capsule minus-derivative. Therefore, we have considered using capsule deficient mutant derivatives. This is not unreasonable since CS and PD-PSCVT are metabolic pathway genes, hence we will be able to assess the effect of disrupting these genes in the absence of capsule (e.g. ascites with complement inactivated). However, this alternative will only be used if we exhaust all approached to generate the desired constructs in wild-type backgrounds.

Because of the difficulties we have had in creating site-directed constructs, we have discussed this issue with colleagues knowledgeable in bacterial genetics. Although we have always been aware of the restriction-modification issues that exist with the introduction a construct into *A. baumannii* from a non-*A. baumannii* background (e.g. *E. coli*), the quantitative effect has never been measured. We have now formally established the magnitude of this effect. pNLAC:*dho* (contained the gene for dihydroorotase) was purified from an *E. coli* (XL1-Blue) and *A. baumannii* (AB307-0294) backgrounds. Equal amounts of plasmid DNA were electroporated into AB307-0294. Perhaps not surprisingly, a 10-fold increase in transformants was observed when the plasmid construct purified from AB307-0294 was used. This defines that, at least for the AB307-0294 background, 90% of DNA that has not been methylated in the destination background is degraded. This is clearly a significant effect and has practical implications in the construction of gene knockouts. We will take advantage of this knowledge and use a “genetic trick” in our future experiment. Hopefully we will now be able to create constructs in a capsule-positive background. The approach is as follows:

Step 1: Clone the *cs* fragment that contains a kanamycin cassette into pNLAC-1.

Step 2: Electroporate this construct into the *A. baumannii* background in which the disruption will be made.

Step 3: Since pNLAC-1 has an *A. baumannii* origin of replication pNLAC: *cs*::*kan* can be purified from that background.

Step 4: Restrict pNLAC:*cs*::*kan* and purify the linear fragment containing *cs*::*kan*.

Step 5: Electroporate *cs*::*kan* back into the appropriate *A. baumannii* background and select for electroporants that are kanamycin resistant. These colonies are candidates for having site-specific disruptions of *cs*.

Since this approach is globally applicable to all *A. baumannii* strains, we have put all experiments designed to use a capsule-minus background for knockout construction on hold until the viability of this new approach is assessed. If successful, we will use a similar approach for preparing directed *pd-pscvt*-minus mutants. Additionally, if successful the development of this directed mutant protocol for generally applicable to all *A. baumannii* strains will be a significant contribution to *A. baumannii* genetics.

PD-PSCVT, a bifunctional enzyme

A. baumannii PD-PSCVT is bifunctional; the N-terminal PD domain catalyzes the removal of a hydride from prephenate to form 4-hydroxyphenyl pyruvate, the direct precursor of tyrosine. The C-terminal PSCVT domain catalyzes the transfer of the enolpyruvyl moiety of phosphoenolpyruvate (PEP) to the 5-hydroxy position of shikimate-3-phosphate (S3P)

(Figure 2), a requisite step in the synthesis of the amino acids Phe, Trp and Tyr, and other aromatic compounds (e.g. folate, alkaloids) (*13, 14*). Although PSCVT has been previously suggested as a drug target for antibacterials, most research in this field has concentrated on *Plasmodium falciparum* (antimalarials) and glyphosate resistant plants (*12, 15, 16*).

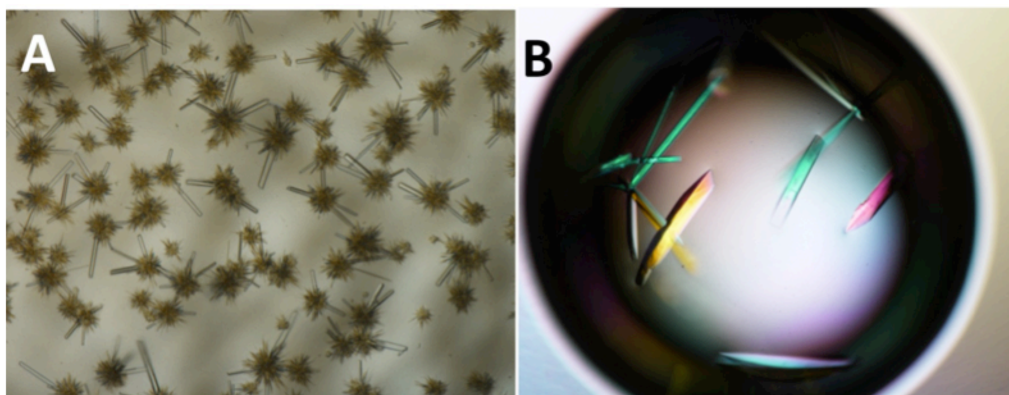

Figure 3. The effect of seeding on PD-PSCVT crystals. A. Crystals of PD-PSCVT obtained by vapor diffusion were not useful for X-ray diffraction experiments. B. Using crystals in A to seed crystallization experiments produced larger single crystals useful for data collection.

Crystallization of PD-PSCVT. In the past year, we have cloned, expressed, and purified PD-PSCVT. We have optimized the purification protocol and are able to produce 75 mg of pure PD-PSCVT from a 1L *E. coli* growth. The purified protein was submitted to our high-throughput crystallization screen and over 50 different crystallization 'hits" obtained. Initial crystals were readily obtained but not of diffraction quality (Figure 3A). Additional screening combined with crystal seeding was necessary to produce single crystals (Figure 3B). Seeding involved harvesting some of our previous crystallization drops (where there were small showers of crystals not suitable for diffraction), crushing the crystals and diluting the mixture to create a seed stock. This seed stock was added to a crystallization screen. This work was skillfully carried out by Hannah Brummer, an undergraduate summer intern in our laboratory. (Her work was supported with funds from the HWI Summer Internship Program.)

PD-PSCVT Crystal Data Collection. By adjusting the dilution of the crystal seed solution, we were able to produce larger single crystals of PD-PSCVT. Two crystals measuring (0.5 x 0.3 x 0.2 mm) were mounted at room temperature and data collected on our home X-ray source. In previous attempts to collect data at RT, the crystals were unstable, however, the crystals grown from seeding were stable throughout the data collection. Statistics of the data collection are reported in the Table 2.

Molecular Replacement of PD-PSCVT. A particularly challenging problem is the solution of the PD-PSCVT structure by molecular replacement. With the two different domains, our approach was to use search models derived from homologous structures of the individual PD and PSCVT domains. For PD, there were models from bacterial species *Streptococcus thermophilus* (unpublished, PDB code 3DZB), *Cornybacterium glutamicum ATCC 13032* (unpublished, PDB code 3KTD) and *Aquifex aeolicus* (*17*). For the PSCVT domain, a model was created from *Escherichia coli* (*18*) and *Coxiella burnetii* (unpublished, PDB code 3TR1) structures. The approach was to search for a single domain, fix the solution and then search for the other domain using PHASER (*19*). At this time, we are continuing to refine search models and optimize the search parameters, but have yet to find a verifiable solution. This is likely due to a significantly different protein conformation in the bifunctional PD-PSCVT that is not reflected in any single model. As a solution to this problem, we plan to grow selenomethionine substituted

protein for use in anomalous phasing. This *ab initio* method does not require a model for the solution of the structure.

Table 2. PD-PSCVT X-ray Data Collection Statistics

Data Collection Statistics	Crystal 1	Crystal 2
Wavelength (Å)	1.54	1.54
Space group*	P222, $P222_1$, $P2_12_12$, $P2_12_12_1$	P222, $P222_1$, $P2_12_12$, $P2_12_12_1$
Cell dimension		
a, b, c (Å)	47.329, 109.004, 148.732	47.275, 108.665, 148.247
α, β, γ (°)	90, 90, 90	90, 90, 90
Molecules/asymmetric unit	1	1
Resolution range (Å)**	50-3.2 (3.3-3.2)	50-2.9 (3.0-2.9)
No. of observations	342,347	420,053
No .of unique reflections	11,012	16,074
I/σ(I)**	18.0 (3.4)	19.8 (2.4)
Data Completeness (%)**	83.9 (57.1)	93.1 (75)
R_{merge}**	0.093 (.386)	0.095 (0.550)

*All possible space groups for this crystal class are listed as it was not possible to identify a single space group from the data alone. Molecular replacement solutions will be used to definitively identify the final space group.
**Numbers in parentheses refer to statistics for the highest resolution data shell.

Improving the quality of the crystals. It is also our goal to improve the diffraction of the crystals as the diffraction is anisotropic with respect to the crystallographic C-axis, indicating a structural problem in the crystal lattice. The disruption in the lattice could be the result of structural disorder due to the two-domain structure of PD-PSCVT. In the past year, we have cloned and expressed the stand-alone domains of PD and PSCVT from the PD-PSCVT construct. The rationale for expressing the individual domains is that they may individually crystallize better than the whole protein. Often the amino acid linker between two domains creates a high level of flexibility that is not amenable to the crystallization process. However, crystallization of the individual domains can eliminate the linker and associated flexibility to produce crystals with higher diffraction quality. Individual homologs of PD and PSCVT from other species have been crystallized and had structures solved to high resolution—providing support for our approach. Purification of these individual domains is underway.

SK and PD-PSCVT coupled assay. In the shikimate pathway, shikimate kinase (SK) converts shikimate to 3-phosphoshikimate, which is fused with phosphoenolpyruvate by PSCVT to create enolpyruvyl-shikimate 3-phosphate and inorganic phosphate. As described above, we have already expressed and purified the protein PD-PSCVT from *A. baumannii* and would like to assay the activity of this enzyme. However, the cost for the substrate, 3-phosphoshikimate is prohibitive and the supply of this necessary reagent limited. Although we can use a small amount for single assays, a larger supply will be necessary for co-crystallization experiments and ligand/drug screening.

In order to solve this problem, we cloned and expressed SK from *A. baumannii* for use in the synthesis of 3-phosphoshikimate. This technique has been used for the *Methanococcus jannaschii* SK (*20*). We applied for and received funding from the McCauley Foundation ($18,700) to clone, express, and purify SK from *A. baumannii*. We have been successful in obtaining over 80 mg of 95% pure SK for use. This effort will be further leveraged to efficiently produce the substrate for CS, 5-enoylpyruvyl-shikimate-3-phosphate (EPSP), which is produced enzymatically by PD-PSCVT. The commercial supply of EPSP is also limited and costly.

We have plans to develop a SK:PD-PSCVT coupled assay using the readout from malachite green, which detects the presence of inorganic phosphate with a simple color change. We have performed preliminary tests, however, SK requires ATP as a co-factor, which substantially increases the background by reacting with the malachite green. For the coupled reaction of SK and PD-PSCVT, we did see a 20% increase in signal over background, indicating that both the

SK and PD-PSCVT are active. We will continue to develop this assay for use in high throughput screening format. The goal is to couple the two reactions and then screen for inhibitors that disrupt the assay by inhibiting the activity of SK or PD-PSCVT. The target for active compounds can be determined in individual enzyme activity assays.

Chorismate Synthase

CS (EC 4.2.3.5) is the final enzyme in the seven-step bacterial chorismate biosynthesis pathway (*13*). It catalyzes the conversion of 5-enolpyruvylshikimate-3-phosphate (EPSP) to chorismate. CS has been identified as a rate-limiting enzyme in aromatic metabolite biosynthesis (*21*). CS possesses a distinctive protein fold (*22*), and the 1,4-*anti*-elimination of a phosphate and a proton from its substrate is a unique reaction that has not been observed in any other enzyme family (*23, 24*).

Cloning and Crystallization of CS

The full-length *cs* gene from AB307 was PCR amplified from genomic DNA template, and inserted into a customized expression vector, pET-Duet-SUMO, developed at HWI. The pET-Duet-SUMO expression vector is based on Novagen's pET-Duet-1 vector, but with a customized N-terminal fusion tag engineered into the first expression cassette. This fusion tag is His_6-Smt3, where the His_6 fragment allows for immobilized metal affinity chromatography (IMAC) purification of the overexpressed protein. The Smt3 fragment is the yeast ortholog of the human SUMO protein, and its presence increases expression levels and stability of overexpressed fusion proteins in *E. coli.* This fusion tag was designed to be cleaved post-IMAC purification using recombinant SUMO protease. This strategy yielded > 10 mg of purified CS per liter of *E. coli* culture, providing sufficient quantities for downstream experiments. Purified recombinant CS protein (14 mg/mL) was submitted to the automated high throughput HWI 1536 condition crystallization screen (*25*). Multiple screening conditions produced crystals (Figure 4).

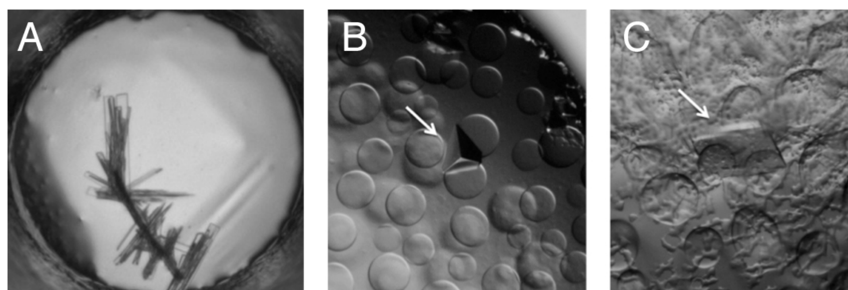

Figure 4. Example of CS crystallization. A) hits provided from the 1536 condition screen. Crystal conditions from the high-throughput screening were optimized in hanging drop vapor diffusion using 2 µl protein solution and 2 µl reservoir solution in the crystallization drop. White arrows highlight crystals growing within a background of B) phase separation and C) a mix of phase separation and precipitation. In both cases, the maximum dimension of the crystals is ~ 0.1 mm.

We are now optimizing the scaled-up conditions to improve reproducible growth and crystal size, as well as minimize the occurrence phase separation and precipitation. The protein contained in the phase separation fraction or as precipitate tends to form a sticky skin over the crystals, resulting in difficulty handling them in preparation for diffraction experiments. This material can also act as a sink for ligands added to the crystallization drop with the intention of soaking them into the CS crystals, and so it will be beneficial to find crystal growth conditions that minimize the occurrence of these non-crystalline materials. We are investigating the effect of changes in pH, chemical composition of the crystallization cocktails, temperature, and method of crystal growth (Figure 4).

Goal 2. Characterization of a Novel NRPS Biosynthetic Cluster

The Non-ribosomal peptide synthetases (NRPSs) are a family of large, modular enzymes that produce peptides with important activities (*26, 27*). NRPS products are diverse, incorporating as many as 100 different substrates amino acids and exhibiting additional chemical modifications including glycosylations, cyclizations, and halogenations. The products play important roles in microbial pathogenesis, including nutrient acquisition, intercellular communication, and exhibiting antibiotic activity. While novel NRPS produced natural products have been identified in soil and marine bacterial species, there has been limited effort to characterize the small NRPS clusters that are present in many human pathogens.

We identified an operon in *A. baumannii* that we wished to target to expand our investigation into NRPS synthesis of novel natural products. The operon encompasses eight genes that are cotranscribed (Table 3, blue). Upstream of the synthetic operon is a acyl-HSL synthase, an enzyme involved in synthesis of quorum signaling homoserine lactones. Immediately upstream of these genes encoding the synthetic operon at Orf3 is a homolog of the *P. aeruginosa* PhzR/LasR transcriptional regulators that are involved in quorum-sensor regulation (*28*). It is therefore highly likely that the ABBFA_003407 protein is involved in the quorum signal-dependent regulation of this operon (*7*).

Table 3. The targeted ABBFA_003406 NRPS operon of *A. baumannii*.

Gene	Function	AB307-0294	Status
1	Acyl-HSL Synthase (*abaI* or *cepI*)	ABBFA_003409	
2	Hypothetical	ABBFA_003408	
3	PhzR-type Regulator	ABBFA_003407	
4	NRPS (Self-standing Adenylation Domain)	ABBFA_003406	Cloned, Purified, Biochemically analyzed
5	Acyl-CoA dehydrogenase	ABBFA_003405	Cloned, expressed
6	NRPS (Self-standing Peptidyl Carrier Protein)	ABBFA_003404	Cloned, Purified, Biochemically Analyzed, Crystallized, Structure Determined
7	NRPS (Condensation-Adenylation-PCP-Thioesterase)	ABBFA_003403	Cloned, Purified, Biochemically Analyzed, Small Crystals
8	RND Transporter	ABBFA_003402	Cloned
9	Hypothetical	ABBFA_003401	Cloned
10	Bifunctional Dehydratase / esterase	ABBFA_003400	Cloned
11	Phosphopantetheinyl transferase (PPTase)	ABBFA_003399	Not targeted at this time

Genes are numbered consecutively. The proteins of the operon of interest are listed in blue. The upstream genes that are likely to be involved in regulation are included in black. The presumed function is listed based on sequence homology. Gene and Protein names from two strains are reported.

Within this NRPS operon are eight proteins. One, ABBFA_003402, is a RND transporter that is likely involved in efflux of the natural product. Another, ABBFA_003399, is a phosphopantetheinyl transferase (*29*), an enzyme that post-translationally modifies the acyl carrier protein domains of two of the NRPS proteins to convert from their inactive *apo* to the active *holo* state. The remaining six proteins therefore form the synthetic operon. We presented a preliminary synthetic pathway in the original application (Figure 5).

Our goals to understand this biosynthetic operon are two-fold. First, we aim to identify the natural product that is made by this cluster. Second, we wish to determine if this biosynthetic pathway plays a role in virulence of the *A. baumannii* pathogen. Even if the product is not strictly involved in virulence, we believe that it may offer insights into the fundamental issues of microbial physiology and potentially identify a novel signaling molecule or pathway that is related to bacterial motility.

Toward the first objective, we have worked on biochemical reconstitution of this pathway. During the past year, we have cloned the six genes that encode the synthetic proteins of the operon. ABBFA_003399, the PPTase, and ABBFA_003492, the transporter, were deemed to be low

priority because of the availability in the lab of an alternate promiscuous PPTase and of the lack of a role of the transporter in synthesis. The remaining proteins have all been expressed in *E. coli*. ABBFA_003400, ABBFA_003401, and ABBFA_003405 express solubly however they have not yet been purified to homogeneity.

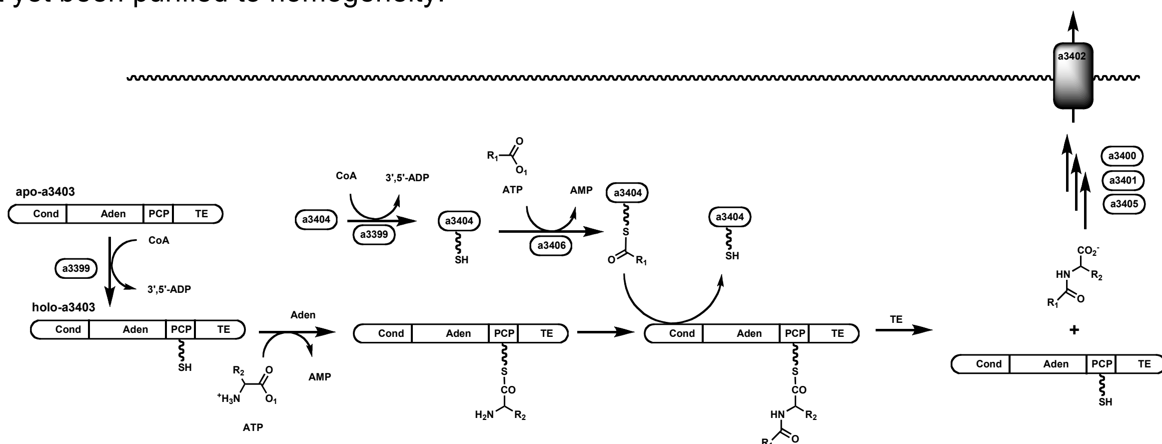

Figure 5. Biosynthetic steps of the NRPS cluster. The activities of a3399, a3402, a3403, a3404, and a3406 can be confidently assigned on the basis of sequence homology to well-characterized NRPS clusters.

Our initial effort therefore was to lay the groundwork for understanding this biosynthetic pathway by biochemically characterizing the three NRPS proteins that should form the peptide backbone of the this pathway. Identifying the building blocks of pathway will provide clues toward the natural product.

After cloning, expressing, and purifying ABBFA_003406 and ABBFA_003403, the two NRPS proteins harboring adenylation domains, we first determined the amino acid specificity of each protein. A standard assay that we have performed many times in the lab detects the reversible incorporation of radiolabeled pyrophosphate into ATP. We probed each protein with all 20 proteinogenic amino acids, as well as several other representative substrates to examine if a preferred non-amino acid might be used (Figure 6). Both proteins contain a conserved aspartic acid residue that interacts in crystal structures with the substrate amino group and has been used as a hallmark for amino acid-specific enzymes (*30*-*32*).

The results demonstrate that glycine is the preferred substrate of the ABBFA_003403 adenylation domain. As shown in the proposed biosynthetic pathway (Figure 6), this identifies the substrate for the second NRPS module suggesting R_2 is a hydrogen atom. The results with the self-standing adenylation domain ABBFA_003406 were more ambiguous. While lysine is the preferred substrate, the level of activity is lower than observed with the glycine-dependent module. Additionally the specific activity with the best substrate lysine is only twice as high as the next set of substrates, isoleucine, leucine, and valine. We are therefore not fully convinced that lysine is indeed the true substrate. We have tested the non-standard amino acids norleucine, ornithine, and D-lysine, which all show significantly weaker activity than with lysine. We have additionally demonstrated that pyrophosphate exchange activity can be observed in the presence of a complex mixture of potential substrates derived from rich media. We are exploring this result as an opportunity to use mass spectrometry to identify the compound that is preferentially loaded onto ABBFA_003404 in the presence of the 3406 adenylation domain.

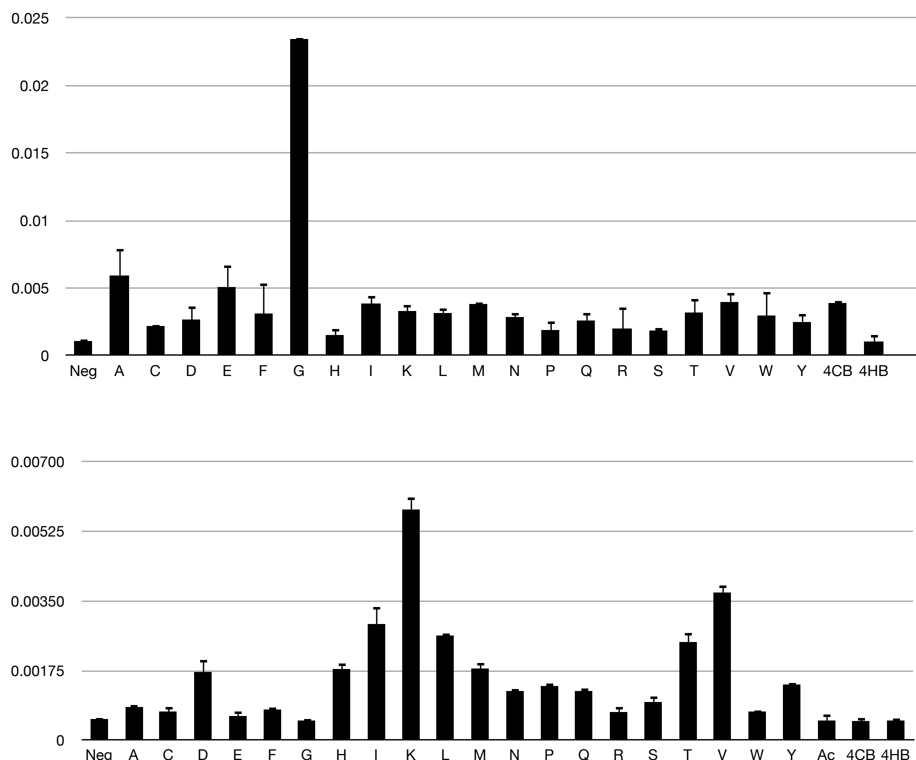

Figure 6. Substrate specificity for ABBFA_003403 (top) and ABBFA_003406 (bottom). Analysis of the adenylation domain for ABBFA_003403 was performed using the pyrophosphate exchange assay in the context of the full, four module protein. Y-axis represents μmol ^{32}P-ATP formed/min/mg. Amino acids are represented by one-letter code. Alternate substrates were also investigated: Ac is acetate, 4CB is 4-chlorobenzoate, and 4HB is 4-hydroxybenzoate.

As reported last month, we have identified from the literature (*7*) a report that implicates this natural product biosynthesis pathway in bacterial motility. We have contacted Dr. Philip Rather (Emory University) and have obtained two mutant strains that contain disruptions in the ABBFA_003405 and ABBFA_003403 genes. (More precisely, the gene names from the homologous ATCC 17978 strain are A1S_0115 and A1S_0113, respectively). These strains, along with the parental M2 isolate, will allow us to characterize the natural product profile to aid in the identification of the product of this synthetic cluster.

An additional goal is to understand more broadly the structural biology of NRPS-catalyzed reactions. Such analysis will aid in the engineering of NRPS systems to produce novel peptide products. More importantly, this will allow the prediction of NRPS products through bioinformatic and structural modeling of enzyme active sites. Towards this end, all three NRPS proteins have entered into crystallization trials for structural characterization. Small crystals have been identified for ABBFA_003403, the four domain NRPS protein. The identification of the substrate preferences of the two adenylation domains has allowed us to synthesize mechanism-based inhibitors that are used to trap the dynamic proteins in an active conformational state to improve the chance of crystallization. These inhibitors have proved to be critical in our prior structural studies of NRPS proteins (*31, 33*). In particular, the vinylsulfonamide containing inhibitors trap the incoming pantetheine thiol of the carrier protein domain forming a covalent link with the adenylate analog (*34*). Lysine- and glycine specific adenosine vinylsulfonamide inhibitors have been produced by Dr. Courtney Aldrich (University of Minnesota) and have entered into our crystallization trails. We have also crystallized the ABBFA_003404 peptidyl carrier protein.

These crystals diffract well and the structure of the PCP domain has been solved by molecular replacement.

Table 4. Diffraction and refinement statistics for ABBFA_003404

Diffraction Statistics	In-house	SSRL
Space Group	$P6_1$ or $P6_5$	$P6_1$ or $P6_5$
Cell Dimensions (Å)	a = b = 61.8, c = 76.9	a = b = 61.8, c = 76.8
Resolution	1.9 Å	31.2 – 1.35 Å
R_{sym}[a]	8.6 %	8.7 % (46 %)
$I/\sigma I$[a]	61.4	8.6 (5.6)
Completeness[a]	98.9 %	98.5 % (98.2 %)
Refinement		
R-factor[a]		15.4 % (17.4 %)
R-free[a]		16.5 % (18.6 %)
RMS Deviation from Ideal		
Bond Lengths		0.005 Å
Bond Angles		1.02°
Ramachandran Plot		
Favored		97.5 %
Outliers		0.00 %

[a]The value for the highest resolution shell is shown in parentheses for the synchrotron dataset.

Molecular replacement was not trivial require multiple iterations with many existing structures of acyl- and aryl-carrier proteins from fatty acid, polyketide, and NRPS synthetic pathways. The structure of the free-standing acyl/peptidyl carrier protein, 003404, was initially solved to a resolution of 1.9 Å using the in-house dataset. With two molecules in the asymmetric unit, the monomer is a simple four helix bundle common in previously determined PCP and ACP structures (Figure 7). The conserved serine (Ser40) that is the site of post-translational phosphopantetheinylation lies at the beginning of helix two. Along this helix, several residues form a hydrophobic surface patch composed of residues Ile41, Leu44, and Gly48 as well as the polar residue at Ser45. These residues make contacts with the adenylation domain in related structures and likely are conserved for the interaction with the adenylation domain of ABBFA_003406.

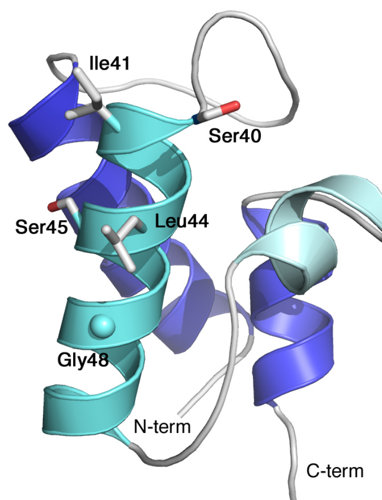

Figure 7. A ribbon trace of the peptidyl carrier protein, ABBFA_003404. The phosphopantetheinylation site at Ser40 is highlighted, along with residues along helix 2 that interact with adenylation domains in related structures.

Key Research Accomplishments

During the first year of our award, we have accomplished the following tasks that contribute towards goals of the project.

Goal 1. Preliminary data have established that chorismate synthase and prephenate dehydrogenase/3-phosphoshikimate-1-carboxyvinyl transferase are essential proteins for *A. baumannii* infection. Therefore our hypothesis is that these proteins are potential therapeutic targets. The object of this goal is to validate these proteins in the chorismate biosynthesis pathway as therapeutic targets.

- We have analyzed 20 strains of *A. baumannii* for prevalence and conservation of the genes encoding CS and PD-PSCVT. All strains show the presence of the genes are conserved at >99% similarity. The *aroC* gene, encoding CS, of three strains (AB932, AB1013, and ATCC15308) that had limited genomic sequence data were also sequenced and show only silent nucleotide changes compared to the *aroC* gene of *A. baumannii* strain AB307. The *aroA* gene, encoding PD-PSCVT, has been sequenced in strains AB932 and AB1013. Only silent nucleotide changes are seen in AB932 while two amino acid substitutions are present in the AB1013 strain.
- We have cloned the genes encoding CS and PD-PSCVT into a pET-Duet-SUMO vector for expression studies.
- The CS and PD-PSCVT proteins have been purified to homogeneity for structural and functional studies.
- The gene encoding the Shikimate Kinase (SK) protein was cloned into an expression vector. The SK protein was purified to homogeneity to be used in a biochemical assay to analyze CS and PD-PSCVT activity, and also to produce 3-phosphoshikimate, a costly reagent that will be useful for biochemical and structural studies.
- Crystals of the CS protein have been grown. Optimization experiments are underway to improve diffraction.
- Crystals of the PD-PSCVT protein have been grown. Current crystals diffract to 2.9Å and the space group and unit cell dimensions have been determined. A complete dataset has been collected and structure determination by molecular replacement is underway. Because of the bifunctional nature of the protein, this is non-trivial and experimental phasing techniques may be necessary.
- As an alternate approach to solving the structure of the bifunctional PD-PSCVT, the individual stand-alone domains PD and PSCVT have been cloned and expressed for crystallization experiments. The reduced flexibility of the individual domains provides the rationale for this approach.

Goal 2. Characterize a novel natural product synthetic pathway encoded by an *A. baumannii* Non-Ribosomal Peptide Synthetases pathway. Our hypothesis is that this cluster is responsible for the production of a novel compound that may impact growth or virulence of *A. baumannii*.

- We have cloned the genes encoding a3400, a3401, a3403, a3404, a3405, and a3406. All proteins express from pET-type expression plasmids.

- The proteins a3406 and a3403 have been purified to homogeneity. Both are in crystallization trials. Small crystals of a3403 have been identified. Optimization experiments are currently underway.
- The self-standing amino acyl carrier protein a3404 has been successfully cloned, expressed, purified, and crystallized. The protein structure has been solved at 1.4Å resolution. The structure factors and atomic coordinates have been deposited at the Protein Data Bank under accession code 4HKQ.
- The adenylating enzymes that serve as the "gate-keepers" for the natural product biosynthesis, catalyzing the incorporation of the amino acid substrates for the NRPS peptide assembly line, have been examined functionally. The a3406 protein shows optimal activity with the amino acid lysine, however modest activity is seen with valine and isoleucine. The adenylation domain of the four-domain a3403 protein shows clear preference for activity with glycine.
- Tailored, mechanism-based inhibitors lysine adenosine vinylsulfonamide and glycine adenosine vinylsulfonamide have been generated by our collaborator and are being pursued as tools for trapping the conformationally dynamic NRPS proteins.
- Biochemical evidence has demonstrated that the adenylation domain a3406 can load lysine onto the a3404 in a steady-state kinetic assay, providing evidence for the first step in the biosynthetic pathway. A molecular model of the functional interface between a3406 and a3404 has been generated which illustrates reasonable conservation with structurally characterized interfaces seen in other adenylation-PCP interactions from *E. coli* or *P. aeruginosa* NRPS proteins.
- We have acquired from Dr. Phil Rather (Emory University) two strains of *A. baumannii* in which directed knockouts of the ABBFA_003405 and ABBFA_003403 genes were generated as well as the parental strain. The metabolite profile of the mutant strains will be compared to that of the wild-type strain.

Reportable Outcomes

The following publication was supported by this award.

Umland, T. C., Schultz, L. W., MacDonald, U., Beanan J. M., Olson, R., and Russo, T. A. (2012) *In vivo*-Validated Essential Genes Identified in *Acinetobacter baumannii* by Using Human Ascites Overlap Poorly with Essential Genes Detected on Laboratory Media. mBio **3**, e00113-12. doi:10.1128/Bio.00113-12.

This publication is attached in the appendices and is also available online via open access at
http://mbio.asm.org/content/3/4/e00113-12.long

The following protein structure dataset, including atomic coordinates and structure factors, was deposited with the Protein Data Base (http://www.pdb.org) using funds from this award.

4HKQ, *Crystal Structure of Free-Standing Peptidyl Carrier Protein from Uncharacterized Acinetobacter baumannii Secondary Metabolic Pathway*.

The following grant application was submitted using work supported by this award as preliminary results.

A grant from the McCauley foundation was awarded ($18,700) to support the cloning and expression of shikimate kinase necessary for the biosynthesis of a substrate needed to develop assays to study the activity and inhibition of PSCVT.

Conclusions

A. baumannii remains a significant biomedical problem that is increasingly being recognized as a cause of nosocomial infections in a wide variety of health care settings (*35*). The prevalence of resistant strains of *A. baumannii (2, 36)* and the current lack of effective treatments suggests a pressing need for novel approaches to identify and validate new targets for antibiotic treatments. We have adopted two complementary approaches to address this problem that comprise the aims of this research project and together will identify, characterize, and validate novel antibiotic targets in *A. baumannii*.

In the first goal of this work, we are implementing an underappreciated strategy that will advance the traditional drug development pipeline. We have identified essential genes in a novel screening strategy in which we have used a selection media to identify essential genes that are necessary for growth under conditions that mimic the infection environment (*6*). The larger impact of this work is the demonstration that many existing screens for essential genes may not accurately reflect the true *in vivo* essentiality of the sampled genes. Indeed, of 18 genes recently identified and validated as being essential for growth and survival in a rat subcutaneous abscess model, including the genes encoding CS and PD-PSCVT, all are annotated as non-essential based on data obtained with rich laboratory media (*6*). In the second goal, we are making an investment in the understanding of the fundamental microbial physiology of this pathogen. Towards this end, we have initiated the investigation of a novel natural product biosynthetic pathway that has been implicated in bacterial motility. These genes encoding this pathway are upregulated in response to a bacterial quorum signal. We aim to identify this novel compound and understand its role in infection and virulence.

Specifically, we have significantly advanced our understanding of *A. baumannii* genetics and have developed new protocols for improving directed gene knockout efficiency that will be useful for this project but also to the general *A. baumannii* community. Generating these protocols positions us to establish *in vivo* essentiality of our targets across multiple *A. baumannii* strains.

Our goal of obtaining the structures of PD-PSCVT and CS has progressed as planned. We have cloned, expressed, purified and crystallized these targets. These results were prerequisite to proceed with the structure determination component of the project. Structures of these targets will be necessary for the fragment based screening and subsequent design of new drug lead compounds.

To accompany and validate the structural studies, we have also initiated activity assays for the screening of inhibitors for the discovery of new antimicrobials. A cost effective and efficient coupled assay is under development and will not only create a method for facile drug lead evaluation of PSCVT and CS but also will produce key reagents that are both expensive and in limited supply. Results of this work will be applicable to drug screening in other GNB.

We have made important progress in understanding the natural product produced by the identified non-ribosomal peptide synthetase cluster, having cloned and produced the synthetic enzymes and identified the molecular building blocks. We have additionally obtained two mutant strains that harbor genetic deletions in this operon and will allow our metabolic and phenotypic investigation of this pathway. The implication of this pathway in bacterial motility provides a phenotype that we can monitor in the further identification and characterization of this novel NRPS product.

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Appendices

We have attached the following publication that resulted from research supported by this award.

Umland, T. C., Schultz, L. W., MacDonald, U., Beanan J. M., Olson, R., and Russo, T. A. (2012) *In vivo*-Validated Essential Genes Identified in *Acinetobacter baumannii* by Using Human Ascites Overlap Poorly with Essential Genes Detected on Laboratory Media. mBio **3**, e00113-12. doi:10.1128/Bio.00113-12.

RESEARCH ARTICLE

In Vivo-Validated Essential Genes Identified in *Acinetobacter baumannii* by Using Human Ascites Overlap Poorly with Essential Genes Detected on Laboratory Media

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ABSTRACT **A critical feature of a potential antimicrobial target is the characteristic of being essential for growth and survival during host infection. For bacteria, genome-wide essentiality screens are usually performed on rich laboratory media. This study addressed whether genes detected in that manner were optimal for the identification of antimicrobial targets since the *in vivo* milieu is fundamentally different. Mutant derivatives of a clinical isolate of *Acinetobacter baumannii* were screened for growth on human ascites, an *ex vivo* medium that reflects the infection environment. A subset of 34 mutants with unique gene disruptions that demonstrated little to no growth on ascites underwent evaluation in a rat subcutaneous abscess model, establishing 18 (53%) of these genes as *in vivo* essential. The putative gene products all had annotated biological functions, represented unrecognized or underexploited antimicrobial targets, and could be grouped into five functional categories: metabolic, two-component signaling systems, DNA/RNA synthesis and regulation, protein transport, and structural. These *A. baumannii in vivo* essential genes overlapped poorly with the sets of essential genes from other Gram-negative bacteria catalogued in the Database of Essential Genes (DEG), including those of *Acinetobacter baylyi*, a closely related species. However, this finding was not due to the absence of orthologs. None of the 18 *in vivo* essential genes identified in this study, or their putative gene products, were targets of FDA-approved drugs or drugs in the developmental pipeline, indicating that a significant portion of the available target space within pathogenic Gram-negative bacteria is currently neglected.**

IMPORTANCE **The human pathogen *Acinetobacter baumannii* is of increasing clinical importance, and a growing proportion of isolates are multiantimicrobial-resistant, pan-antimicrobial-resistant, or extremely resistant strains. This scenario is reflective of the general problem of a critical lack of antimicrobials effective against antimicrobial-resistant Gram-negative bacteria, such as *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Enterobacter* sp., and *Escherichia coli*. This study identified a set of *A. baumannii* genes that are essential for growth and survival during infection and demonstrated the importance of using clinically relevant media and *in vivo* validation while screening for essential genes for the purpose of developing new antimicrobials. Furthermore, it established that if a gene is absent from the Database of Essential Genes, it should not be excluded as a potential antimicrobial target. Lastly, a new set of high-value potential antimicrobial targets for pathogenic Gram-negative bacteria has been identified.**

Received 11 April 2012 **Accepted** 23 July 2012 **Published** 21 August 2012

Citation Umland TC, et al. 2012. *In vivo*-validated essential genes identified in *Acinetobacter baumannii* by using human ascites overlap poorly with essential genes detected on laboratory media. mBio 3(4):e00113-12. doi:10.1128/mBio.00113-12.

Editor George Drusano, University of Florida—Albany

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The identification of bacterial essential genes (i.e., genes required for growth and/or survival) has been an important tool for dissecting biological pathways and functions, identifying evolutionary relationships, promoting synthetic biology, and predicting antimicrobial targets. An essential gene is often considered a member of the minimal gene set required for growth of a specific organism under optimal conditions (i.e., nutrient-rich media and absence of environmental stress) (1). This definition integrates with the practical consideration of using standard laboratory *in vitro* growth conditions. Despite this seemingly simple definition of "essential gene," in practice a number of experimental subtleties exist that may influence the identification of these genes (e.g., genetic variation across strains, random versus systematic mutagenesis protocols, growth condition differences, clonal versus mixed populations, and the working definition used to designate a gene as essential) (1–4). These complexities are exemplified by comparison of genome-wide essentiality screens conducted upon the same species using different methodologies. In the case of *Escherichia coli*, the Keio collection identified 303 essential genes (5), an earlier screen identified 620 essential genes (6), and Ecogene has reannotated the Keio collection as representing 289 essential genes by using more stringent criteria (7). Expanding the comparison across species to include additional Gram-negative bacteria (GNB) or more distantly related Gram-positive bacteria

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(GPB) further emphasizes the disparity between experimentally determined bacterial essential gene sets. For example, a comparison of *Helicobacter pylori* essential genes to *E. coli*, *Haemophilus influenzae*, or *Mycobacterium tuberculosis* indicated that 55% were essential in at least one of these species, but only 11% were essential in all considered species (8).

Gene essentiality is increasingly being viewed as contextual, with decreased nutrient levels, changes in carbon sources, or environmental stress (e.g., change of temperature) altering the set of genes required for growth (1, 9–11). A recent study testing yeast deletion mutants against a multitude of small molecules and environmental stresses concluded that up to 97% of its genes contribute to wild-type growth in the presence of one or more chemicals or environmental conditions (12), compared to ~20% of its genes annotated as essential under optimal laboratory growth conditions. A similar chemical genetics approach in *E. coli* identified 116 genes, unique from those present in the Keio collection, that were essential in rich laboratory media when stressed by a 324-chemical screen (13). These studies underscore the fact that microorganisms infrequently encounter ideal growth conditions except in the lab, and so they have evolved to grow and survive in multiple changing environments. For example, a pathogenic bacterium encounters a very different environment during infection of a host (nutrient poor, host defenses) than during growth on lab media (nutrient rich).

Whole-genome essentiality screening requires considerable resources, and bacterial species selected for such screens are largely important model systems (e.g., *E. coli*) or human pathogens. The Database of Essential Genes (DEG; version 6.8) contains genome-scale essentiality data for 17 unique bacterial species obtained from 20 published screens (14). For the majority of bacterial species, experimental data establishing gene essentiality are sparse, and so an annotation of "essential" for most bacterial genes is a prediction based on homology to experimentally established essential genes. Given the modest overlap of experimentally determined whole-genome essentiality screens (pairwise bacterial species comparisons typically exhibit 50 to 70% overlap, which rapidly decreases as more species are compared [8, 15–17]), confidence levels for *in silico* essentiality predictions may vary widely and in many cases have not been quantified. Bioinformatic selection strategies for target-based antimicrobial discovery rely heavily upon essentiality annotations (18–20), and so an evaluation of the accuracy of essential and nonessential gene predictions is of practical importance.

Essential genes hold the promise of being potential new drug targets. We are at risk for entering a postantibiotic era, due to the evolution of multidrug, extreme drug, and pan-drug (MDR, XDR, PDR) resistance in GNB. Identification of new drug targets will lead to the development of new antimicrobials, which are urgently needed. *Acinetobacter baumannii* is the poster child for this emerging threat (21), with both the incidence of infections and those due to XDR and PDR strains increasing (22–29). Treatment of infections due to *Acinetobacter* has become challenging, and the need to identify new antimicrobial targets is more pressing than ever. Unfortunately, as of 2009 there were virtually no new antimicrobial agents active against GNB in the pharmaceutical pipeline (21). A 2011 update found several antimicrobials in development that had activity against GNB, but none have reached phase 3 trials (30, 31).

Given the urgent need for new classes of antimicrobial therapeutics effective against *A. baumannii* and MDR, XDR, or PDR GNB in general, we employed a previously utilized experimental approach (32) for the identification of unrecognized or underexploited antimicrobial targets. This approach has the potential to effectively promote the development of new small-molecule antimicrobial therapeutics through novel targets and thereby circumvent current resistance mechanisms (33). This strategy was designed to efficiently identify *A. baumannii* genes essential for growth and survival during infection in a rat subcutaneous abscess model (i.e., *in vivo* essential genes) but not essential *in vitro* on rich laboratory media (34, 35). Here, we report 18 verified *in vivo* essential genes that form a set of putative antimicrobial targets, representing a largely new set of antimicrobial targets based upon evaluation against two drug target databases. These *A. baumannii* genes were compared to bacterial genes annotated as essential in DEG, revealing that 89% could not be readily predicted as *in vivo* essential. Data are presented that support the concept that the genes identified and catalogued as essential in DEG need to be interpreted within the context of their method of identification. Our review of the literature revealed that most screens were conducted using rich laboratory media, and none were performed using a clinically relevant medium, demonstrating a limitation of the data currently in DEG. This highlights that the cataloguing of essential genes is incomplete, and that although labor and cost intensive, experimental establishment of *in vivo* essential genes within an appropriate environmental context is needed. An understanding of this limitation within DEG is critical since it is often used as part of the decision process assessing the validity of potential drug targets. Consideration of potential drug targets within a clinically relevant context has enabled antimicrobial target space to be expanded, as well as enabled the refinement of bioinformatic methods used to prioritize antimicrobial targets.

RESULTS

Identification of *A. baumannii* genes essential for growth in human ascites *ex vivo*. *A. baumannii* strain 307-0294 (AB307-0294) was randomly mutagenized using the transposon EZ-Tn*5*<kan-2>. Mutants (2,934 total) were isolated on Mueller-Hinton (MH) kanamycin plates and subsequently gridded onto ascites plates containing kanamycin (40 μg/ml). Mutant derivatives (224 total; 7.6%) of AB307-0294 were identified that displayed significantly decreased or absent growth on ascites plates. Chromosomal sequencing using primers from the ends of EZ-Tn*5*<kan-2> was performed on all 224 mutants, enabling the location of the transposon insertion sites to be identified. Analysis of these data revealed a set of 90 unique genes where the loss of function due to transposon-mediated gene disruption resulted in the phenotype of near wild-type growth on rich laboratory (MH) medium plates but decreased or absent growth on ascites plates. This phenotype is likely to be predictive of AB307-0294 genes dispensable for *in vitro* growth under optimal conditions (rich laboratory medium, which does not reflect the *in vivo* environment) but essential for growth and survival during systemic infection of the human host (i.e., essential *in vivo*), since human ascites is similar to the exudative inflammatory fluid environment that extracellular bacterial pathogens encounter during human infection. This simple concept is the strength of this approach, which has not generally been used in screens for essential genes. Based upon a bioinformatic filter, 34 of the 90 *A. baumannii* genes were selected, primarily

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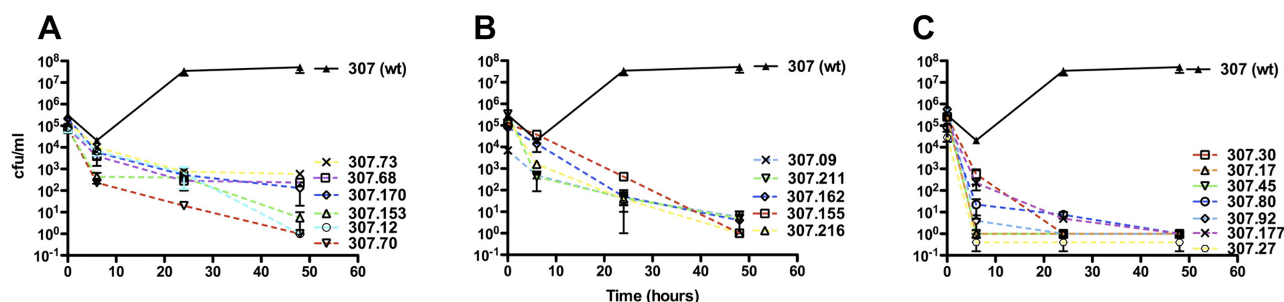

FIG 1 Growth/clearance of AB307-0294 wild type (wt) and mutant derivatives in the rat subcutaneous abscess infection model. (A) Comparison of wild type (AB307-0294) and mutants with disruptions in metabolic pathway and two-component signaling genes. AB307.12 (*carA*$^-$), AB307.68 (*aroA*$^-$), AB307.70 (*rstA*$^-$), AB307.73 (*aceE*$^-$), AB307.153 (*envZ*$^-$), AB307.170 (*aroC*$^-$). (B) Comparison of wild type and mutants with disruptions in DNA/RNA synthesis or regulation and protein transport genes. AB307.09 (*pyrC*$^-$), AB307.155 (*rpmA*$^-$), AB307.162 (*trmD*$^-$), AB307.211 (*secE*$^-$), AB307.216 (*relA*$^-$). (C) Comparison of wild type and mutants with disruptions in structural genes. AB307.17 (*ompF*$^-$), AB307.27 (*pbpG*$^-$), AB307.30 (*ptk*$^-$), AB307.45 (*epsA*$^-$), AB307.80 (*uppP*$^-$), AB307.92 (*spsC*$^-$), AB307.177 (*ostA*$^-$). Data are means ± SEM for 3 to 4 experiments for each time point.

based upon lack of homology to human genes, for verification of *in vivo* essentiality.

Fifty-three percent of AB307-0294 mutant derivatives with decreased growth in human ascites were essential in a rat subcutaneous abscess model. Thirty-four unique AB307-0294 mutant strains were tested in a well-established rat subcutaneous abscess model that has been validated for use with *A. baumannii* (36). Importantly, multiple host defense factors, including complement, professional phagocytes, and antimicrobial peptides, are present in this model. This critical step verifies *in vivo* essentiality via monitoring of the quantitative growth and survival of specific mutant strains over time. The use of an *in vivo* infection model (i.e., infection within a host) subjects the *A. baumannii* mutants to a range of conditions, including restricted nutrients and immune responses, difficult or impossible to completely replicate outside a host.

This *in vivo* assessment revealed that 18 of the 34 (53%) mutated genes were essential *in vivo* (Fig. 1; Table 1). The criterion used to classify a gene as *in vivo* essential was a $\geq 2 \log_{10}$ reduction from the original bacterial titer within 48 h postinfection (hpi). Impressively, 10 of the 18 mutant strains exhibiting a significant *in vivo* growth defect had a $\geq 5 \log_{10}$ reduction and/or complete kill within 48 hpi (Fig. 1). Furthermore, at 48 hpi, all of the mutant strains exhibited a $\geq 5 \log_{10}$ reduction compared to the corresponding titer of the wild-type AB307-0294 control. Mutations that resulted in bacteriostatic or mildly decreased growth over the test period were not classified as *in vivo* essential genes. All of the 18 *in vivo* essential genes had annotated biological functions and were grouped into five functional categories (metabolic, two-component signaling [TCS] systems, DNA/RNA synthesis and regulation, protein transport, and structural). These data demonstrate that the genes disrupted in mutants with decreased or no growth in human ascites are likely to be essential *in vivo*.

Genes identified to be essential for growth and/or survival under clinically relevant conditions are not essential in rich laboratory medium. Importantly, all 18 mutants grew in the rich

TABLE 1 AB307-0294 *in vivo* essential genes and gene products

Ab307-0294 gene locus	Gene	Mutation no.[a]	Annotation	UniProt no.	OMA no.	Polar effect exclusion
ABBFA_000154	*aceE*	73	Pyruvate dehydrogenase E1 component	B7GV82	ACIB300151	RT-PCR amplicon
ABBFA_000285	*envZ*	153	Osmolarity sensor protein EnvZ	B7GVK9	ACIB300278	RT-PCR amplicon
ABBFA_000350	*trmD*	162	tRNA (guanine-1)-methyltransferase	B7GVS0	ACIB300339	RT-PCR amplicon
ABBFA_000621	*ompF*	17	Outer membrane porin F precursor	B7GWT4	ACIB300599	Genome organization
ABBFA_000700	*uppP*	80	Undecaprenyl-diphosphatase UppP	B7GXE2	ACIB300672	RT-PCR amplicon
ABBFA_000738	*rpmA*	155	Ribosomal protein L27	B7GXH9	ACIB300709	RT-PCR amplicon
ABBFA_000787	*carA*	12	Carbamoyl-phosphate synthase small chain	B7GY14	ACIB300758	RT-PCR amplicon
ABBFA_001168	*aroA*	68	3-Phosphoshikimate 1-carboxyvinyltransferase	B7GZU8	ACIB301129	RT-PCR amplicon
ABBFA_001801	*aroC*	170	Chorismate synthase	B7H3A6	ACIB301758	Genome organization
ABBFA_001929	*ostA*	177	Organic solvent tolerance protein OstA	B7H3N4	ACIB301886	RT-PCR amplicon
ABBFA_002478	*pyrC*	9	Dihydroorotase, type II	B7GX48	ACIB302431	Complementation
ABBFA_002866	*rstA*	70	Transcriptional regulatory protein RstA	B7GZC5	ACIB302811	Downstream gene—identical signal path
ABBFA_002981	*relA*	216	GTP pyrophosphokinase	B7H017	ACIB302919	Genome organization
ABBFA_003254	*secE*	211	Preprotein translocase, SecE subunit	B7H1K4	ACIB303187	RT-PCR amplicon
ABBFA_003295	*pbpG*	27	Penicillin-binding protein 7/8 (PBP7/8)	B7H1P0	ACIB303223	Complementation
ABBFA_003451	*spsC*	92	Perosamine synthase	B7H2H8	ACIB303374	RT-PCR amplicon
ABBFA_003459	*epsA*	45	EPS I polysaccharide export outer membrane protein EpsA precursor	B7H2I6	ACIB303382	Complementation
ABBFA_003461	*ptk*	30	Tyrosine-protein kinase Ptk	B7H2I8	ACIB303384	Complementation

[a] Mutation number assigned to a specific AB307-0294 transposon mutant.

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TABLE 2 Growth characteristics of *A. baumannii in vivo* essential gene mutants

AB307-0294 gene	Function	Growth[a]		
		LB	Ascites	*In vivo*
aceE	Carbohydrate and amino acid metabolism	+2	+1	−2
envZ	Two-component system signaling	+2	0	−2
trmD	tRNA modification	+2	0	−2
ompF	Outer membrane porin	+2	−2	−2
uppP	Peptidoglycan synthesis	+2	−2	−2
rpmA	Ribosome assembly and translation	+2	0	−2
carA	Pyrimidine metabolism	+1	−2	−2
aroA	Chorismate and aromatic amino acid metabolism	+2	0	−2
aroC	Chorismate metabolism	+2	0	−2
ostA	Outer membrane LPS assembly	+2	−2	−2
pyrC	Pyrimidine metabolism	+2	0	−2
rstA	Two-component system signaling	+2	0	−2
relA	Nucleotide metabolism; stringent response	+2	0	−2
secE	Protein transport	+2	0	−2
pbpG	Peptidoglycan synthesis/modification	+2	−2	−2
spsC	LPS synthesis/modification	+2	−1	−2
epsA	Capsule transport, outer membrane	+2	−1	−2
ptk	Capsule synthesis	+2	−1	−2

[a] Growth of mutant strain compared to that of the wild type in LB-rich laboratory medium, human ascites, or *in vivo* in a rat subcutaneous abscess infection model. Growth scale: +2, wild type; +1, less than wild type; 0, neither growth nor kill; −1, modest kill; −2, significant kill.

Luria-Bertani (LB) laboratory medium, with all but the *carA*$^-$ mutant displaying growth at or near wild-type levels (Table 2). The optimal density at 600 nm (OD_{600}) of the *carA*$^-$ mutant culture was ~66% of the corresponding wild-type culture after 24 h. Although these mutant growth results on LB medium were expected, based on the design of our essentiality screen, they emphasize a fundamental difference from most other screens that define essentiality as a lack of growth in rich laboratory medium. In contrast, we further screened the set of mutants that survived in LB medium in human ascites. This point is critical since these genes, which have been shown to be essential for the growth and/or survival of *A. baumannii ex vivo* in human ascites and *in vivo* in a rat infection model, would not have been identified by a rich laboratory medium screen. Since the human host is a modified minimal medium (e.g., iron depleted) that contains host defense factors, the clinical relevance of essential genes identified on rich laboratory medium may be uncertain or misleading.

The correlation of experimentally identified AB307-0294 *in vivo* essential genes compared to those present in DEG was poor. The results of our AB307-0294 *in vivo* essentiality screen were compared to the bacterial genes annotated as essential within DEG (version 6.8) (14). DEG contains the results of 20 genome-wide essentiality screens across 17 unique bacterial species (see Table S1 and Fig. S1 in the supplemental material).

DEG was searched for orthologs to the 18 identified *A. baumannii in vivo* essential genes. The ortholog match criterion was selected to maximize the likelihood that the *A. baumannii* to DEG matches possessed identical or highly similar biological functions (37–39), a practice followed by others (40, 41). This is especially important for large superfamilies, where subfamilies may have distinct functional differences. DEG ortholog matches were then separated into GNB only; GNB only, excluding *A. baylyi*; non-GNB (i.e., GPB, mycobacteria, and mycoplasma); and all bacteria species (Fig. 2). The majority of *A. baumannii in vivo* essential

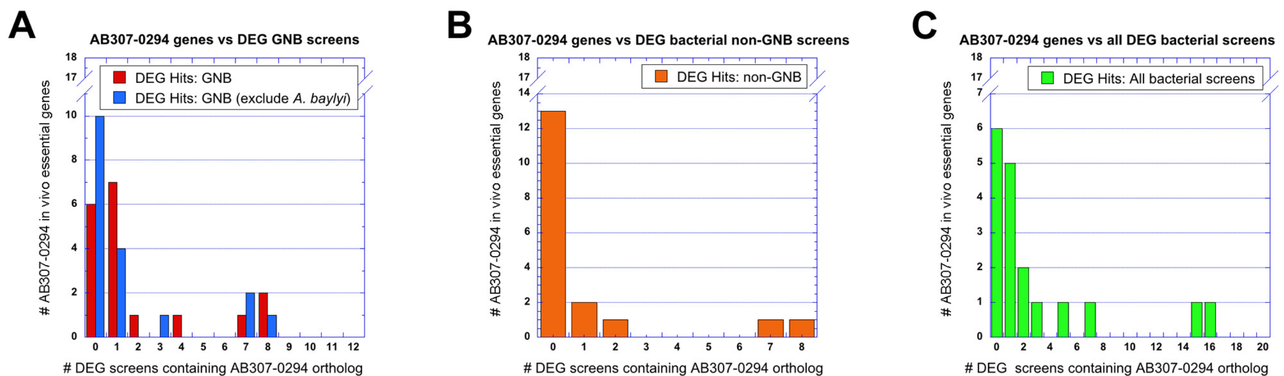

FIG 2 Histograms of the occurrence of orthologs to the 18 AB307-0294 *in vivo* essential genes identified in this study within the bacterial genome-wide essentiality screen data compiled in DEG. Columns at the left side of each histogram indicate AB307-0294 *in vivo* essential genes having few or no orthologs annotated as essential within DEG. (A) Comparison to all 12 GNB data sets within DEG (red columns) and to the same set excluding *A. baylyi* (blue columns); (B) comparison to the 8 non-GNB data sets (includes GPB, mycobacterium, and mycoplasma species) within DEG; (C) comparison to all 20 bacterial data sets present within DEG.

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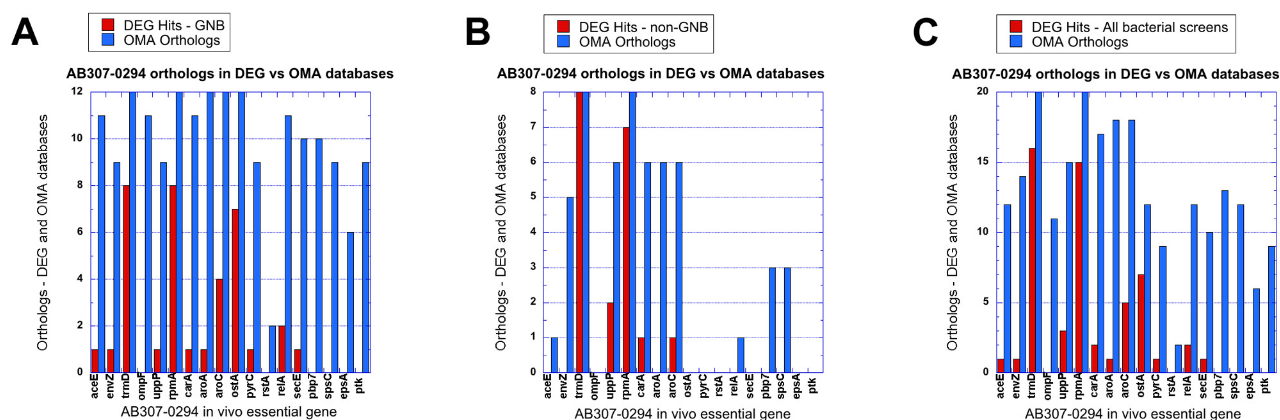

FIG 3 The occurrence of orthologs to each of the 18 AB307-0294 *in vivo* essential genes identified in this study within the bacterial genome-wide essentiality screen data compiled in DEG (red columns) and within the genomes of the same bacterial species (blue columns). (A) Comparison the 12 GNB data sets within DEG; (B) comparison to the 8 non-GNB bacterial data sets (includes GPB, mycobacterium, and mycoplasma species) within DEG; (C) comparison to all 20 bacterial data sets present within DEG.

genes/gene products had few or no bacterial orthologs annotated within DEG as essential. The distribution of DEG ortholog matches is skewed slightly toward a greater frequency when only the DEG GNB data are considered, with 13/18 (72%) genes with ≤1 ortholog and 6/18 (33%) with no matches (Fig. 2A). This observation may be expected, as generally the DEG GNB species are more closely related to *A. baumannii* than DEG's non-GNB species, for which 15/18 (83%) genes had ≤1 ortholog and 13/18 (72%) genes had no matches (Fig. 2B). Likewise, excluding consideration of the most closely related species, *A. baylyi*, shifts the distribution toward a lower occurrence of orthologs to *A. baumannii in vivo* essential genes within DEG's GNB set (14/18 [77%] with ≤1 ortholog and 10/18 [56%] with no matches) (Fig. 2A). Therefore, the fact that an ortholog is not listed as essential in DEG or that it has been identified as essential in only a few species by no means excludes it as being essential *in vivo*. This has obvious implications given that the data within DEG is typically heavily weighted during decisions on the validity of gene products being potential antimicrobial targets for a given genera or species.

It is noteworthy that even the *A. baumannii in vivo* essential genes that have many ortholog hits within DEG possess an important phenotypical difference from those matches. All bacterial essentiality screens within DEG, with the exception of the *A. baylyi* screen, were conducted on rich laboratory media. Hence, genes annotated as essential within those screens indicate that the respective gene disruption mutants were nonviable in rich media. However, all 18 of the *A. baumannii in vivo* essential genes identified in this study grew at or near wild-type levels in rich media (Table 2). Thus, all 18 *A. baumannii* genes form a set unique from the essential genes in DEG.

The poor correlation of experimentally identified AB307-0294 *in vivo* essential genes compared to those annotated in DEG was not due to the absence of orthologs. A potential reason why few *A. baumannii in vivo* essential gene orthologs were present in DEG may be that the 17 bacterial species annotated in DEG simply lack orthologous genes (i.e., a gene cannot be annotated as essential if that gene is not present in the genome of the wild-type organism). The OMA ortholog database was searched to confirm that for most of the 18 *A. baumannii* genes, orthologous genes were prevalent in the bacterial species contained in DEG (Fig. 3). Over 70% (13/18) of the *A. baumannii* genes have orthologs in >50% of the bacterial species annotated in DEG. Furthermore, only one of the *A. baumannii* genes, *rstA*, has an ortholog in <25% of the DEG bacterial species and so may be classified as unique to *Acinetobacter*. Orthologs are more frequently present in the DEG GNB species (Fig. 3A) versus the DEG non-GNB species (Fig. 3B), which is not unexpected. While the 18 *A. baumannii in vivo* essential genes are not universally conserved across bacteria, most are widely distributed across species, especially across other GNB. Thus, their general absence from being annotated as essential in DEG is not due to these genes being unique to *Acinetobacter*.

The growth environment used for the essentiality screen appears to be critical. The fact that AB307-0294 and mutant derivatives with disruptions in genes essential for growth and/or survival *in vivo* were identified using a screen with human ascites likely explains the poor correlation to genes annotated as essential in DEG. Nearly all of the screens used to identify genes in DEG were performed in rich laboratory medium. A single genome-wide essentiality screen within DEG was conducted against a defined minimal medium, which is a better representation of the nutrient levels available in the human growth environment. The organism screened belonged to the same genus (*Acinetobacter baylyi*), yet only seven of the 18 identified essential genes were in common (42) (Table 3). The Keio collection of *E. coli* genome-wide-directed single gene mutants, excluding mutants nonviable in rich media, has also been evaluated for growth characteristics in a defined minimal medium (10). This study detected four *E. coli* genes essential in minimal media in addition to the three *E. coli* genes essential on rich media that are orthologous to *A. baumannii in vivo* essential genes. The results of these two studies were compared to the 18 *A. baumannii in vivo* essential genes to determine if the *E. coli* and *A. baylyi* ortholog mutants' growth phenotypes in minimal media correlated to *in vivo* growth and survival phenotypes in *A. baumannii*. Only 3 of 18 of the *A. baumannii in vivo* essential genes unambiguously correlated to the minimal medium growth phenotype of mutations in orthologous genes in both *E. coli* and *A. baylyi*. While definitive conclusions cannot be drawn, these results suggest that minimal medium may be an improved screening environment compared to rich laboratory medium because it more closely represents the *in vivo* environment.

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TABLE 3 *A. baumannii in vivo* essential genes versus essentiality in minimal medium

AB307-0294 gene	Minimal medium study result[a]		Prediction *in vivo*[b]
	E. coli	*A. baylyi*	
aceE	N	N	NE
envZ	N	Y	?
trmD	NT	Y	EE
ompF	N	N	NE
uppP	N	N	NE
rpmA	NT	N	?
carA	Y	Y	E
aroA	Y	N	?
aroC	Y	Y	E
ostA	NT	N	?
pyrC	Y	Y	E
rstA	No ortho	N	NE
relA	N	Y	?
secE	No ortho	Y	?
pbpG	N	N	NE
spsC	No ortho	N	NE
epsA	N	N	NE
ptk	N	N	NE

[a] Keio Collection growth study of *E. coli* mutants in defined minimal MOPS (morpholinepropanesulfonic acid) medium (10) and *A. baylyi* essentiality study performed in defined minimal MA medium (42). N, gene not essential in minimal medium; Y, gene essential in minimal medium; No ortho, absence of orthologous gene; NT (*E. coli* only), not tested in minimal medium, as gene was essential in rich medium.
[b] Prediction that an AB307-0294 gene would be *in vivo* essential based upon minimal medium studies for *E. coli* and *A. baylyi*. E, predicted *in vivo* essential, no growth in minimal medium for both *E. coli* and *A. baylyi* ortholog mutants; EE, predicted essential under some condition (i.e., rich or minimal medium); NE, predicted as not *in vivo* essential, as growth occurs in minimal medium for both *E. coli* and *A. baylyi* ortholog mutants; ?, prediction of *in vivo* essentiality ambiguous due to different results in the *E. coli* and *A. baylyi* minimal medium screens.

However, it does not serve as an optimal surrogate for an *in vivo* growth environment for GNB. A partial explanation for this may be that *in vivo* conditions possess host defense factors that have bactericidal activity and that are not present in laboratory medium. In contrast, the use of human ascites likely enhanced our identification of genes essential for growth and/or survival *in vivo* because it contains active complement.

Further, a recent genome-wide chemical genetics *E. coli* study was designed to identify genes essential under specific chemical or environmental stresses (13). Few of the conditionally essential genes identified overlapped with the *A. baumannii in vivo* essential genes identified in our study, suggesting that extensive chemical genetics screens are not a replacement for *in vivo* studies.

The majority of the identified AB307-0294 *in vivo* essential genes are unrecognized or underexploited antimicrobial targets. Bioinformatic selection of putative antimicrobial drug targets typically requires that a gene or an ortholog has been annotated as essential (18, 40). The lack of significant numbers of orthologs annotated as essential in DEG for most of the identified *A. baumannii in vivo* essential genes suggested that few of these genes would have been selected as possible drug targets through a bioinformatic screen. In order to determine if orthologs to any of these genes have received serious attention as an antimicrobial target, both DrugBank and the Therapeutic Target Database (TTD) were searched (Table 4). This analysis revealed that none of the 18 *A. baumannii* genes identified in this study, or orthologs to these genes, were targets of FDA-approved drugs or drugs in the

TABLE 4 *A. baumannii in vivo* essential genes as antimicrobial targets

Gene	UR/UE[a]	Rank[b]
aceE	++	H
envZ	++	M
trmD	++	M
ompF	+++	L
uppP	+++	H
rpmA	++	M
carA	++	H
aroA	+	H
aroC	++	H
ostA	+++	L
pyrC	++	H
rstA	+++	L
relA	++	M
secE	+++	L
pbpG	+	H
spsC	+++	H
epsA	+++	M
ptk	+++	M

[a] UR/UE, unrecognized/underexploited drug target as evaluated by comparison to entries within DrugBank (56) and the Therapeutic Target Database (57). +++, no ortholog present; ++, ortholog entry present, listed inhibitor(s) classified as experimental (i.e., prior to preclinical studies) and nondrug like; +, no ortholog entry present but a related protein class is targeted by an approved drug or a chemical inhibitor in commercial use.
[b] Evaluation of potential as an antimicrobial drug target: H, high; M, medium; L, low.

developmental pipeline. Almost half lacked an entry in these drug target databases.

Two genes (*aroA* and *pbpG*) lacked an entry for an ortholog, but entries did exist for related protein families that were targets of drug or drug-like small molecules. In these two cases, the attention that these related proteins received as antimicrobial targets was due largely to spillover from more extensive efforts on nonbacterial targets. In plants, the gene product of *aroA*, 3-phosphoshikimate 1-carboxyvinyltransferase (PSCVT), is the target of the broad-spectrum herbicide glyphosate (Roundup). However, while plant PSCVTs are glyphosate sensitive (class I), bacterial PSCVT enzymes may belong to either class I or class II (glyphosate resistant). Additionally, in previous bacterial genome-wide essentiality screens, an *aroA* ortholog was detected as essential in only *Caulobacter crescentus* (Table 5). Supporting data exist indicating the *in vivo* importance of *aroA* in other pathogenic GNB. *Salmonella enterica aroA* mutants yield highly attenuated strains due to both the inability to produce aromatic metabolites and cell wall and outer membrane defects that reduce resistance to innate immune responses (43). The gene *pbpG* encodes penicillin-binding protein 7/8 (PBP-7/8), a low-molecular-weight (LMW) PBP. The high-molecular-weight (HMW) PBPs are heavily exploited antibacterial targets, but previous to the demonstration that *A. baumannii* PBP-7/8 was *in vivo* essential (35), LMW PBPs were not recognized as contributing to bacterial survival and hence were largely neglected as targets. *E. coli* LMW PBPs were recently reported to provide intrinsic resistance against β-lactams, further underscoring the previously unappreciated roles for these proteins during infection and antimicrobial treatment (44). A third gene, *pryC*, encoding dihydroorotase (DHOase), is noteworthy because the human and *Plasmodium* genes encoding this function have received attention as potential anticancer and antimalarial targets, respectively. However, the *Plasmodium* and human enzymes belong to a different DHOase

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TABLE 5 Ortholog distribution of AB307-0294 *in vivo* essential genes in bacterial species annotated in DEG[a]

	Gram-negative												Non-Gram-negative							
Ab307-0294 Gene	*A. baylyi*	*E. coli K-12*	*E.coli MG1655*	*E.coli combine*	*F. novicida*	*H. influenzae*	*H. pylori*	*P. aeruginosa*	*S.* Typhi	*S.* Typhimurium	*V. cholerae*	*C. crescentus*	*B. subtilis*	*M. tuberculosis*	*M. genitalium*	*M. pulmonis*	*S. aureus N315*	*S. aureus NCTC8325*	*S. pneumoniae*	*S. sanguinis*
aceE	Y	Y	Y	Y	Y	Y		Y	G+Y	Y	Y	Y		Y						
envZ	G+Y	Y	Y	Y				Y	Y	Y	Y	Y	Y	Y			Y	Y		Y
trmD	G+Y	G+Y	G+Y	G+Y	G+Y	Y	Y	Y	G+Y	G+Y	G+Y	Y	G+Y	G+Y	G+Y	G+Y	G+Y	G+Y	G+Y	G+Y
ompF	Y	Y	Y	Y	Y	Y		Y	Y	Y	Y	Y								
uppP	Y	Y	Y	Y				Y	Y	Y	Y	G+Y	Y	Y			G+Y	Y	G+Y	Y
rpmA	Y	G+Y	Y	G+Y	G+Y	Y	G+Y	G+Y	G+Y	Y	G+Y	G+Y	G+Y	G+Y	G+Y	G+Y	G+Y	G+Y	Y	G+Y
carA	G+Y	Y	Y	Y	Y		Y	Y	Y	Y	Y	Y	Y	G+Y			Y	Y	Y	Y
aroA	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	G+Y	Y	Y			Y	Y	Y	Y
aroC	G+Y	Y	Y	Y	Y	G+Y	G+Y	Y	Y	Y	Y	G+Y	Y	G+Y			Y	Y	Y	Y
ostA	Y	G+Y	G+Y	G+Y	Y	G+Y	Y	Y	G+Y	G+Y	G+Y	Y								
pyrC	G+Y	Y	Y	Y			Y	Y	Y	Y	Y									
rstA	Y							Y												
relA	G+Y	Y	Y	Y	Y	Y		Y	Y	Y	G+Y	Y								Y
secE	G+Y	Y	Y	Y	Y	Y		Y	Y	Y	Y									
pbpG	Y	Y	Y	Y		Y		Y	Y	Y	Y	Y	Y	Y						Y
spsC	Y	Y	Y	Y	Y		Y	Y	Y	Y			Y	Y					Y	
epsA	Y	Y	Y	Y					Y	Y										
ptk	Y	Y	Y	Y				Y	Y	Y	Y	Y								

[a] Green indicates the presence of an ortholog to an AB307-0294 *in vivo* essential gene annotated as essential in a bacterial species present within DEG version 6.8. Yellow indicates the presence of an ortholog of any type (essential or nonessential) to an AB307-0294 *in vivo* essential gene.

class than that found in the majority of bacteria, including exhibiting different active-site architectures.

Given our identification of these 18 *A. baumannii* genes as *in vivo* essential, we evaluated their likelihood of serving as potential anti-GNB targets by criteria commonly used in the bioinformatic selection of targets. This analysis revealed that many of the identified *A. baumannii* genes possess significant potential as therapeutic targets within GNB despite being previously overlooked (Table 4). Therefore, within the context of identifying essential genes for the purpose of developing new classes of antimicrobials for human infections, the growth medium/environment used for the essentiality screen is critical.

DISCUSSION

This study identified a unique set of *A. baumannii* genes representing high-value, unrecognized or underexploited, potential antimicrobial targets for pathogenic GNB. Further, this report established that if a gene is not in DEG, it should not be excluded from consideration as a potential drug target. Lastly, the data demonstrated the importance of using clinically relevant media and *in vivo* validation when screening for essential genes for the purpose of developing new classes of antimicrobials for human infections.

The strategy used for identifying *in vivo* essential genes within *A. baumannii* is not only efficient, it is also capable of identifying genes involved in multiple pathways and functions and enhances the understanding of pathogen biology. The putative products of three *in vivo* essential genes participate in metabolic pathways (Fig. 1A). The genes *aroA* and *aroC* encode 3-phosphoshikimate 1-carboxyvinyltransferase and chorismate synthase, respectively, which catalyze the final two steps of chorismate biosynthesis. This is an example of our screen identifying functionally related genes that participate in the same pathway but are not contained within the same operon. The *A. baumannii aroA* gene additionally encodes prephenate dehydrogenase (PD) activity, yielding an unusual fusion protein. Typically, these two activities are present as separate proteins in bacteria (45). PD is a member of the aromatic amino acid biosynthesis pathway, which uses a derivative of chorismate as its initial substrate. The third metabolic gene was identified as *aceE*, putatively encoding the pyruvate dehydrogenase E1 component. This protein participates in the conversion of pyruvate to acetyl coenzyme A (acetyl-CoA), which then feeds into the citric acid cycle and other metabolic pathways. Two genes participating in separate TCS systems, *envZ* and *rstA*, were identified as *in vivo* essential (Fig. 1A). The gene *envZ* putatively encodes the osmolarity sensor protein, a trans-inner membrane sensor protein that propagates its signal through a cytoplasmic histidine kinase domain. This protein participates in osmoregulation, responding to environmental stimuli. The *rstA* gene putatively encodes a response regulator protein, RstA, which propagates the signal from its corresponding sensor protein RstB by acting as a transcriptional regulator. The environmental stimulus that the RstA/RstB TCS system responds to is unknown. DNA/RNA synthesis and regulation forms a third group containing five *in vivo* essential genes (Fig. 1B). This group may be subdivided into tRNA modification [*trmD*, which putatively encodes tRNA (guanine-1)-methyltransferase], ribosome assembly and translation (*rmpA*, which putatively encodes ribosomal protein L27), pyrimidine me-

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tabolism (*carA*, which putatively encodes carbamoyl-phosphate synthase small chain; *pyrC*, which encodes a type II dihydroorotase), and pppGpp synthesis for signaling the stringent response that modulates transcription profiles and decreases tRNA and rRNA synthesis under stress conditions (*relA*, which putatively encodes GTP pyrophosphokinase). Both carbamoyl phosphate synthase small subunit and DHOase are key enzymes in the pyrimidine biosynthesis pathway. Identification of genes participating in the same metabolic pathway but distantly located within *A. baumannii*'s genome increased confidence in the screening results. One of the identified genes, *secE*, has a predicted protein transport function (Fig. 1B). It putatively encodes a component of the SecYEG translocon. This is an integral membrane protein complex spanning the inner membrane that actively translocates proteins into the periplasm. Seven of the identified *in vivo* essential genes possess annotated functions that are predicted to affect structural integrity: the outer membrane (*ompF*, which putatively encodes outer membrane porin F; *ostA*, which putatively encodes organic solvent tolerance protein), peptidoglycan synthesis and modification (*uppP*, which putatively encodes undecaprenyl-diphosphatase; *pbpG*, which putatively encodes PBP-7/8), capsule synthesis and transport (*ptk*, which putatively encodes tyrosine-protein kinase; *epsA*, which putatively encodes EPS I polysaccharide export outer membrane protein), and lipopolysaccharide synthesis and modification (*spsC*, which putatively encodes perosamine synthase) (Fig. 1C). Their structural role is reinforced by the results of quantitative growth studies of these mutants in human serum, demonstrating extreme sensitivity to the bactericidal activity of complement (34, 35) (data not shown). It was noteworthy that *pbpG* was identified in our screen (36). PBP-7/8 belongs to the LMW class of PBPs. LMW PBPs have been largely neglected because they were not recognized as essential using *in vitro* assays, unlike the HMW PBPs that are well-known targets of β-lactam antibiotics. These results strongly suggest that the mechanism of action of β-lactam antibiotics may be partially due to inactivation of LMW PBPs.

The strategy to identify *in vivo* essential genes in this study should be applicable to other bacterial species that can be cultured and subjected to gene disruption by random mutagenesis. Critical to the strategy is the approach of screening in a clinically relevant medium, in this case human ascites, which is an *ex vivo* medium reflective of inflammatory extracellular fluid. This simple innovation is critical for the efficiency of our approach. By selecting for transposon mutants on laboratory medium and then screening for essentiality on ascites plates, we are able to identify, in one step, genes (targets) that are both likely essential and expressed *in vivo*. The strength of this approach is that it is unbiased and highly efficient. We do not select the targets but allow the genetic screen, designed to identify the phenotype of *in vivo* essentially, to dictate the choices. This approach has a ~50% success rate for selecting genes later verified as essential in a rat abscess infection model. Target validation in an appropriate animal model(s) is a strong predictor of efficacy in humans (46) and is a critical step, since target essentiality *in vitro* or even *ex vivo* may not translate into the *in vivo* environment (47).

The results of this study also demonstrated that a set of *A. baumannii* genes that were initially identified on ascites plates and subsequently established to be *in vivo* essential is largely distinct from ortholog sets that are essential for growth in rich laboratory media. Broadly, this difference in essential gene sets can be attributed to differences in the growth environment used during the process of essential gene identification. While conceptually this difference is easy to understand, it often is not fully appreciated when interpreting results of essential gene screens or extrapolating from these results to computationally select antimicrobial drug targets or predict essential genes in other species. This concept is also applicable for the identification of virulence factors that may also serve as antimicrobial targets or vaccine candidates. DEG is a repository of publically released genome-wide essentiality screens and serves as a common source for bioinformatic methods requiring essentiality data. Overall, the data contained within DEG are of high quality when considered within the context of how they were generated. For all bacterial species in DEG, genome-wide screening was conducted *in vitro* using laboratory media, and all but one used rich media. Genes annotated as nonessential by such screens cannot be excluded *a priori* as having an essential role *in vivo*. These screens were not designed to replicate physiological conditions that may be encountered by a pathogen during infection. All 18 *A. baumannii in vivo* essential genes identified in this study carry the annotation of nonessential under *in vitro* conditions used in nearly all genome-wide screens published to date. It is important that the growth conditions used to identify essential genes should be concordant with the project's goal. The human host environment is deficient in selected requisite nutrients and possesses host defense factors. For example, certain purines, pyrimidines, and amino acids are variably present in various human body fluids, including plasma (48, 49). Based solely on concentration measurements, it is unclear whether their bioavailability is sufficient to support the growth of a corresponding auxotrophic bacterial strain. However, numerous biological studies have demonstrated that a pathogen's ability to synthesize or uptake certain noniron nutritional factors, such as purines, pyrimidines, and amino acids, is critical for virulence within the host (50). This study further supports the concept that human body fluids are a modified minimal medium beyond iron limitation. A number of the AB307-0294 mutants identified by the phenotype of being unable to grow in human ascites possessed gene disruptions in pyrimidine or amino acid metabolic pathways. Therefore, if the objective is to identify potential antimicrobial targets, it is important that essential genes are identified in a clinically relevant medium or environment. Likewise, the use of genome-wide chemical genetic screening in rich laboratory media may fail to detect a significant percentage of *in vivo* essential genes. Findings from this report demonstrate that the pool of potential antimicrobial targets with *A. baumannii* can be expanded and refined based on the use of a more clinically relevant definition of gene essentiality. This strategy is applicable to other human microbial pathogens.

The number of bacterial species with experimentally determined genome-wide essentiality is small (i.e., 20 total screens of 17 unique species in DEG version 6.8), and conducting such screens is resource intensive. This set of circumstances has naturally led to the extrapolation of the data within DEG to predict essential genes in other organisms. Cross-species prediction of gene essentiality has been recognized as difficult, and even ortholog conservation across multiple species is not necessarily a marker of essentiality (11). Analysis of the *A. baumannii* results against the bacterial entries in DEG was in agreement with the difficulty of inferring essentiality cross-species. The majority of *A. baumannii* genes verified as *in vivo* essential could not be readily predicted with a high confidence level based on comparison to DEG (Fig. 2; Table 5).

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Although differences in screening methods likely account for a significant amount of the variation between *A. baumannii in vivo* essential genes identified in this study and the essential genes catalogued within DEG, it has been shown that genes identified in one species in a defined environment are not necessarily essential in another species or genera under the same conditions (8).

Conversely, although not experimentally established in this report, the presence of a gene or an ortholog to that gene in DEG is not a guarantee of that gene being essential *in vivo*. Many of the elements of the type II fatty acid biosynthesis (FASII) pathway (i.e., *fab* gene cluster) in a number of bacteria have been annotated as essential, forming the basis of the development of antimicrobials targeting this pathway (51, 52). However, it has recently been demonstrated that this pathway may not be essential to GPB during infection. Specifically, the inhibited or genetically disrupted GPB FASII pathway can be compensated by fatty acids present in serum (47, 53). Thus, *in vitro* essentiality data obtained using rich laboratory medium failed to accurately reflect the genes required, and not required, for the promotion of infection in clinically relevant environments.

Several limitations should be noted regarding our strategy for identifying essential genes. First, it does not identify genes essential in rich laboratory media. However, it is likely that many of the genes that would be identified as essential on rich laboratory media will encode proteins that are already targeted by the present antimicrobial armamentarium, such as those involved with cell wall or protein synthesis. Second, genes identified as *in vivo* essential in one bacterial species (e.g., *A. baumannii*) are not certain to be *in vivo* essential in other genera or even species, but this is a problem present in all essentiality screening methods. Redundant or alternative metabolic or biosynthetic pathways may exist; therefore, conclusive proof of essentiality requires experimental confirmation under appropriate conditions. Additional research is required to determine if the level of conservation of the essentiality phenotype across species varies significantly between genes identified *in vitro* using rich laboratory media and those identified *in vivo*. Lastly, the *A. baumannii* genetic screen was designed to examine essentiality in extracellular inflammatory fluid. Bacterial infections occur in other physiological environments that ascites and the rat abscess model may mimic poorly. An interesting future research direction will be to test our results in one or more different infection models. It is likely that at least some variation will be observed for *in vivo* essentiality based on the site of infection and/or the animal species used. That said, we have previously tested several of our *A. baumannii* mutants in a rat pneumonia model, and in all cases significant decreases in bacterial titers were observed (35). It is also possible that bacterial genes found to be *in vivo* essential in a rat will not be *in vivo* essential in humans, or vice versa, due to specific pathogen-host interactions. For example, *Neisseria* species obtain iron from host transferrin and lactoferrin proteins. *Neisseria*'s receptors for these proteins have evolved to be specific for only these proteins from the human and closely related primates (54, 55). Pathogen-host specificity is a recognized issue for any animal model system and not solely a problem with our strategy.

The results presented here expand antimicrobial target space and increase awareness that the environment during infection may differ significantly from laboratory *in vitro* growth conditions. Furthermore, these results emphasize the need to incorporate appropriate *in vitro* screens and clinically relevant *in vivo* studies (i.e., animal infection models) early in the process of evaluating potential antimicrobial targets. Neglecting to do so may have significantly skewed past selection of targets. Antimicrobial drug development in general, and especially against GNB, is challenging for a variety of reasons. A successful antimicrobial drug must have characteristics beyond simple inhibition of the target's function. Features include permeability properties that enable target site access, resistance to degradation and efflux, favorable pharmacokinetic and pharmacodynamic properties, and acceptable toxicity. These and other necessary or desired features are often at odds with one another chemically, requiring multiple rounds of optimization (56). Expansion of target space allows for the exploration of different regions of chemical space, increasing the probability that appropriate lead molecules will be identified that can be developed into new classes of antimicrobials.

The identification of *in vivo* essential genes increases the understanding of the *in vivo* physiology of *A. baumannii* growth, which is an important initial step for target-based drug development strategies and may facilitate the understanding of the mechanism of action. A number of high-throughput screening (HTS) campaigns, designed to identify drug leads targeting essential genes as part of a target-based approach, have not, as of yet, come to fruition (46). However, one of the important points of this study is that many of these previous screens may not have been optimally designed with respect to target selection. Retrospective analyses of failed target-based HTS campaigns have identified problems in the HTS design, especially the use of chemical libraries of limited chemical diversity primarily designed for eukaryotic targets (33, 57). Multiple approaches may be used for the identification of lead compounds effective against specific targets, which can be subsequently validated using a combination of appropriate bacterial constructs in clinically relevant *in vitro* and *in vivo* models. Structurally enabled fragment-based lead discovery (FBLD) methods differ significantly in methodology and philosophy from HTS. Importantly, FBLD allows a much more efficient and larger sampling of chemical space while simultaneously allowing greater freedom for medicinal chemistry to engineer desired properties during the optimization of initial drug lead compounds towards therapeutics suitable for clinical testing (58, 59). Although the concept of an antimicrobial being active against multiple independent targets is appealing, particularly for durability, this is not a necessary requirement for an effective antimicrobial. Several strategies are compatible with leveraging our results toward drug development: single pharmacophore/multitarget compounds, hybrids of two pharmacophores, combinations of single-target inhibitors to avoid resistance development, and structure-based drug design to create multiple intramolecular drug–target interactions (60). Lastly, our results will allow the (re)examination of these genes. For example, it is possible that screening hits against several of the genes/gene products may have been identified in the past but never pursued because the targets were previously considered to be nonessential.

In summary, this study demonstrates the underappreciated concept that decisions on the potential validity of antimicrobial targets by investigators and pharmaceutical companies need to be made within the environmental context (e.g., rich laboratory medium versus infection model) of how a gene was identified as essential. The absence of a database that catalogues clinically relevant essential genes has impeded a fuller definition of the antimicrobial target space.

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MATERIALS AND METHODS

AB307-0294 is a clinical blood isolate representative of *A. baumannii*. *A. baumannii* strain 307-0294 (AB307-0294) (blood isolate from Buffalo, NY, sequence type [ST] 15, clonal group 1 based on Ecker et al. [61]) was selected as our model strain for these studies. We have previously used this strain in related studies (34–36). The genome of AB307-0294 has been fully sequenced, containing 3.76 Mbp and 3,531 predicted open reading frames (ORFs) (62). Compared with the five genomes in the public domain (ATCC 17978, 0057, AYE, ACICU, SDF) and three additional isolates (900 [perineal isolate], 853 [blood isolate from Iraq], and 979 [environmental isolate from Iraq]) sequenced by T. A. Russo, A. Campagnari, and S. Gill (unpublished data), available at the time of the initiation of these studies, AB307-0294 shared ~3 Mbp. AB307-0294 is relatively drug susceptible, enabling the performance of genetic manipulations. It is closely related to *A. baumannii* strains 0057 and AYE, with only approximately 3% of AB307-0294's genome differing from these strains, virtually all of which are two prophage clusters. Taken together, these data support AB307-0294 as being both clinically appropriate, representative of contemporary *A. baumannii* strains, and an ideal background to identify new or unrecognized antimicrobial targets of interest.

Identification of AB307-0294 genes essential for growth in human ascites *ex vivo*. The experimental approach was a modification of the method previously used to identify virulence factors in extraintestinal pathogenic *E. coli* (32) and was subsequently applied to *A. baumannii* (34, 35). Briefly, the EZ-Tn*5*<kan-2> transposome (EPICENTRE Biotechnologies) was electroporated into AB307-0294 to create random mutants. Mutagenized bacteria were selected on MH plates containing kanamycin (40 μg/ml), a nutrient-rich laboratory medium. Mutants were then gridded onto plates comprised solely from human exudative ascites, kanamycin (40 μg/ml), and agar (ascites plates). Human ascites was collected from deidentified patients who were undergoing therapeutic paracentesis for symptoms due to abdominal distension. These individuals were not being treated with antimicrobials and were not infected with human immunodeficiency, hepatitis B, or hepatitis C virus. The ascites was cultured to confirm sterility, divided into aliquots, and stored at −80°C. The procedures for obtaining human ascites were reviewed and approved by the Western New York Veterans Administration Institutional Review Board. Informed consent was used to obtain human blood for the preparation of serum (approval ID 00063). The Western New York Veterans Administration Institutional Review Board for the process of obtaining ascites waived informed consent (approval ID 00098). An expedited review was performed, because the ascites was collected from deidentified patients who were undergoing therapeutic paracentesis for symptoms due to abdominal distension. Mutants exhibiting no or minimal growth on ascites plates had their sites of transposon insertion identified by chromosomal sequencing using previously described methods (35), and each interrupted ORF was identified by comparison to the annotated AB307-0294 genomic sequence (GenBank no. CP001172). Note that saturation mutagenesis was not achieved. Depending on the method of estimation, the predicted number of mutants required to reach 95% saturation of the AB307-0294 genome would be 12,000 to 42,000 (63). Further, transposon-mediated mutagenesis is more likely to interrupt longer genes, and so our data set may be biased against short genes. However, given the objective of evaluating the growth phenotype on ascites plates as predictive of *in vivo* essentiality rather than identifying all *in vivo* essential genes, this lack of saturation mutagenesis was acceptable.

Quantitative *in vitro* growth analyses. Mutant derivatives of AB307-0294 that demonstrated decreased or absent growth on ascites plates underwent quantitative growth assessment in rich laboratory medium (LB) and human ascites as described (35, 36).

Exclusion of polar effects in mutant derivatives of AB307-0294. Potential transposon-mediated polar effects on downstream genes were evaluated. For 10 of the mutants, reverse transcription-PCR (RT-PCR) was performed on the gene immediately downstream that was in the same transcriptional orientation, and an amplicon of the expected size was successfully generated (data not shown). For these RT-PCR studies, RNA was isolated from each mutant strain using the RNeasy Protect minikit (Qiagen Inc., Valencia, CA). Samples were treated with DNase I twice to completely digest contaminating chromosomal DNA. Absence of contaminating DNA was confirmed by testing all RNA samples prior to RT-PCR analysis using the appropriate primer pair and GoTaq Green master mix (Promega, Madison, WI). The Qiagen OneStep RT-PCR kit was used for subsequent RT-PCR analyses of all samples. Primers were designed to amplify 100 to 500 bp of the RNA transcript being assessed. Primers are listed in Table S2 in the supplemental material. A positive control that amplified the RNA transcript from the single-stranded binding protein (SSB) was run concurrently with all samples tested.

Four of the AB307-0294 mutants were complemented *in trans* using the *A. baumannii* cloning vector pNLAC1 with restoration of the phenotype of being able to grow and survive in human ascites (34, 35) (data not shown for *pyrC*). For one mutant derivative, a polar effect was excluded because the downstream gene was part of the same multisubunit two-component regulatory system. In three of the mutants, the downstream gene was in a reversed transcriptional orientation, excluding polar effects. Results are summarized in Table 1.

Assessment of AB307-0294 mutant derivatives with decreased growth in human ascites in a rat subcutaneous abscess model. The University at Buffalo and Veterans Administration Institutional Animal Care Committees approved the rat subcutaneous abscess model. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, and all efforts were made to minimize suffering. Mutants exhibiting significantly decreased or absent growth in human ascites were evaluated in an established rat soft-tissue infection model for *in vivo* growth/survival (36). Briefly, a subcutaneous space is created through the injection of 30 ml of air into the back of anesthetized Long-Evans rats (200 to 225 g), followed by the injection of 1 ml of 1% croton oil in a filter-sterilized vegetable oil vehicle. The space was allowed to mature into an encapsulated, fluid-filled (8- to 12-ml) pouch over 6 to 8 days. Neutrophils would have migrated into the abscess in response to appropriate chemotactic signals. The abscess' subcutaneous location enabled multiple injections and samplings to be performed over time. AB307-0294 or mutant derivatives were injected alone into the abscess of an anesthetized animal, resulting in an estimated starting abscess concentration of 1×10^5 CFU/ml. Within 1 min after the bacteria were injected into the abscess, 0.5 ml of abscess fluid was removed to measure the actual starting bacterial titer. Fluid aliquots (0.5 ml) were subsequently obtained from anesthetized animals 3, 6, 24, and 48 h after the initial bacterial challenge, and bacterial titers were enumerated. *In vivo* data are presented as means ± standard errors of the means (SEM). To normalize *in vivo* data, $\log_{10}$ transformed values were utilized, the area under each curve was calculated, and these areas were compared using two-tailed unpaired *t* tests (Prism 4 for Macintosh, GraphPad Software Inc.).

Prioritization of genes identified by *in vitro* ascites screen for *in vivo* evaluation. Genes were analyzed for conservation across five additional *A. baumannii* strains possessing sequenced genomes (AB0057, ACICU, ATCC 17978, AYE, SDF) at the initiation of this study and for the lack of close human homologues using BLAST (64). The identified genes were conserved across *A. baumannii* strains but exhibited a wide range of similarity to human genes. This filtering process was performed to reduce the number of mutant strains and animals used, with the current study focusing on those potential targets with little to no homology to human genes.

Comparison of *A. baumannii in vivo* essential genes to DEG. Putative protein sequences were generated from the DNA sequences of the 18 AB307-0294 genes experimentally established to be essential *in vivo*. These proteins were compared to bacterial genome-wide essentiality screen data available in the Database of Essential Genes, version 6.8 (14), by BLASTp (64). Significant DEG BLASTp hits (E values of $<$1e−10 and coverage of $>$75% of the AB307-0294 protein) were then evaluated using the OMA

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Browser to determine orthologs (i.e., OMA ortholog and close ortholog groups) to *A. baumannii* targets (65).

Comparison to known drug targets. Similarly, two drug target databases (Therapeutic Targets Database [TTD] and DrugBank) (66, 67) were searched using BLASTp for proteins similar to *A. baumannii* targets, and significant matches were confirmed as orthologs with the OMA browser. The putative protein products derived from the *A. baumannii* genes experimentally established *in vivo* as being essential were evaluated for their potential as antimicrobial targets. Criteria were similar in concept to other published methods (18, 40) modified for GNB. In addition to the absolute criterion that the genes were verified as *in vivo* essential in *A. baumannii*, other factors considered included conservation across *A. baumannii* strains and multiple clinically important GNB, a knowledge of the biological role to aid understanding inhibitor/drug lead mechanisms of action, a defined druggable site on the protein, the availability of an activity assay, the feasibility of biochemical and biophysical experimental study, and the lack of closely related proteins in humans.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00113-12/-/DCSupplemental.

Figure S1, TIF file, 2.5 MB.
Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.1 MB.

ACKNOWLEDGMENTS

This work was support in part by a Telemedicine and Advance Technical Research Center (TATRC) cooperative agreement W23RYX1055N607, an interdisciplinary grant from the University at Buffalo (L.W.S., T.A.R., and T.C.U.), and a VA Merit Review grant from the Department of Veterans Affairs (T.A.R.).

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