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.**

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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

(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

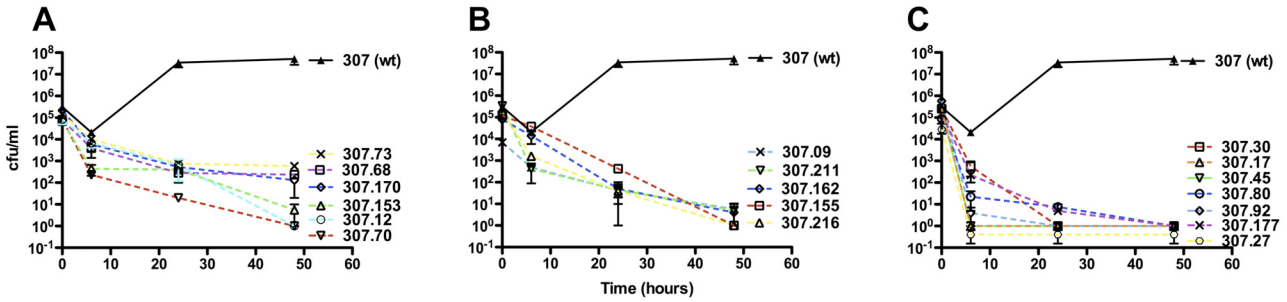


FIG 1 Growth/clearance of AB307-0294 wild type (wt) and mutant derivatives in the rat subcutaneous abscess infection model. (A) Comparison of wild type (AB307-0294) and mutants with disruptions in metabolic pathway and two-component signaling genes. AB307.12 (*carA*$^-$), AB307.68 (*aroA*$^-$), AB307.70 (*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 ≥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 ≥5 $\log_{10}$ reduction and/or complete kill within 48 hpi (Fig. 1). Furthermore, at 48 hpi, all of the mutant strains exhibited a ≥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.

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

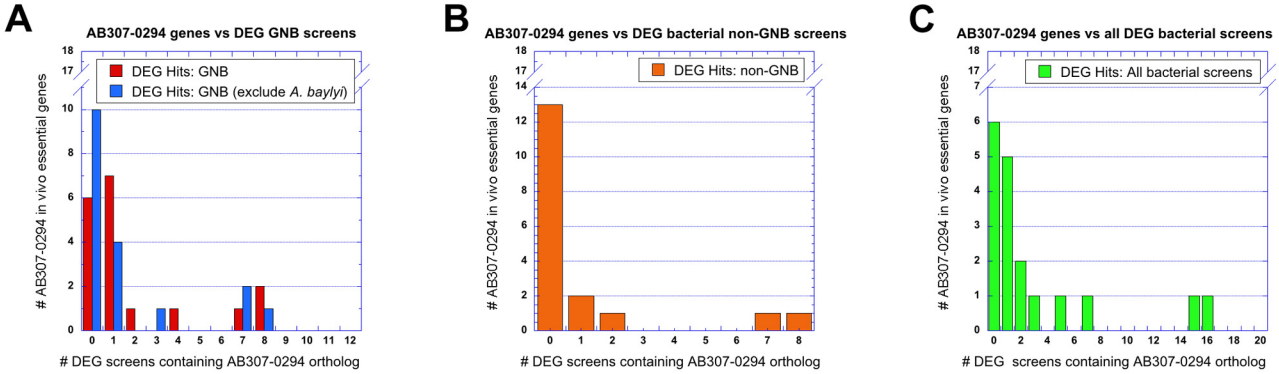


FIG 2 Histograms of the occurrence of orthologs to the 18 AB307-0294 *in vivo* essential genes identified in this study within the bacterial genome-wide essentiality screen data compiled in DEG. Columns at the left side of each histogram indicate AB307-0294 *in vivo* essential genes having few or no 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.

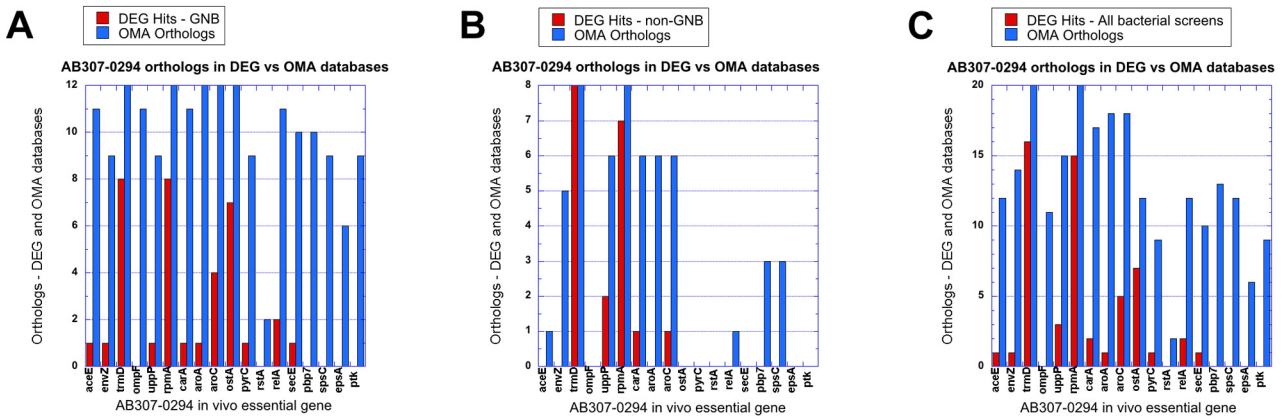


FIG 3 The occurrence of orthologs to each of the 18 AB307-0294 *in vivo* essential genes identified in this study within the bacterial genome-wide essentiality screen data compiled in DEG (red columns) and within the genomes of the same bacterial species (blue columns). (A) Comparison the 12 GNB data 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.

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.

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.

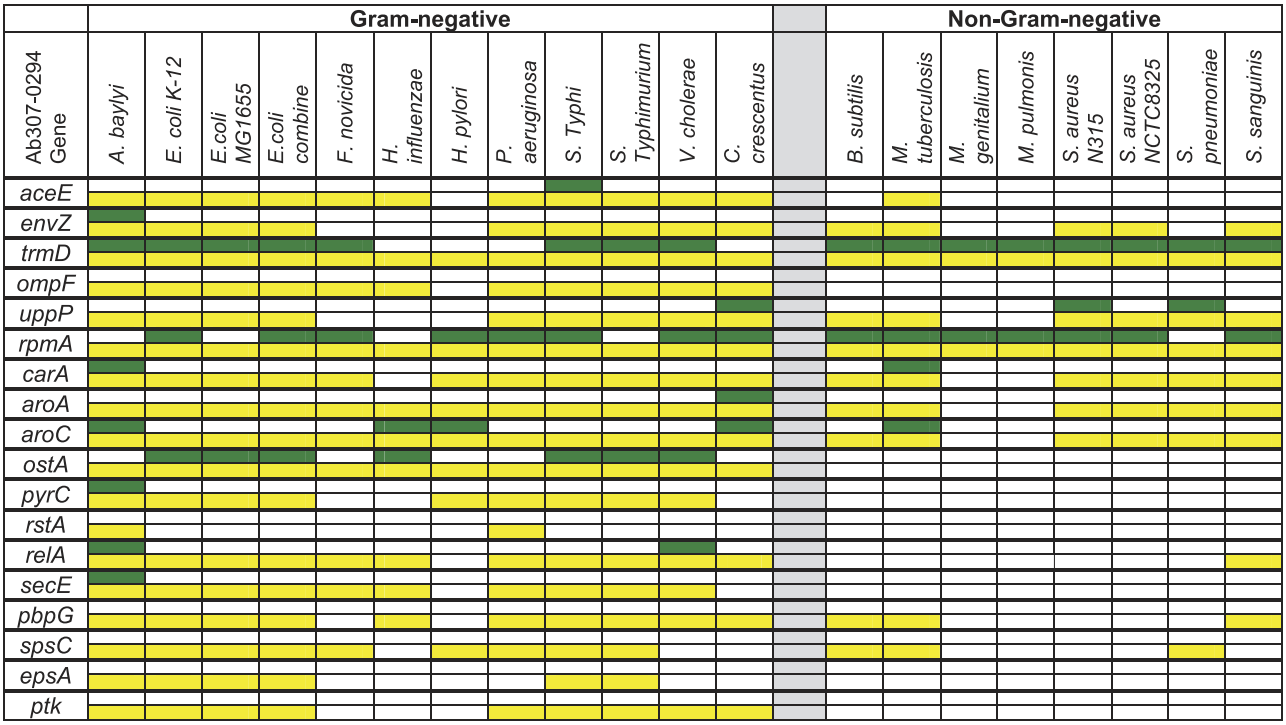
However, it does not serve as an optimal surrogate for an *in vivo* growth environment for GNB. A partial explanation for this may be that *in vivo* conditions possess host defense factors that have bactericidal activity and that are not present in laboratory medium. In contrast, the use of human ascites 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 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

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



[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-

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).

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.

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

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.

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