MAJOR ARTICLE

A Global Virulence Regulator in *Acinetobacter baumannii* and Its Control of the Phenylacetic Acid Catabolic Pathway

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Background. *Acinetobacter baumannii* is one of the most notorious hospital-acquired pathogens, and novel treatment strategies are desperately required. Two-component regulatory systems represent potential therapeutic targets as they mediate microorganism adaptation to changing environments, often control virulence, and are specific to bacteria. Here we describe the first global virulence regulator in *A. baumannii*.

Methods and Results. Using transcriptional profiling and functional assays of a deletion mutant in the *A. baumannii* sensor kinase gene, A1S_0574 (termed as *gacS*), we show that this sensor kinase regulates key virulence characteristics, including pili synthesis, biofilms, and motility, resulting in virulence attenuation in a mammalian septicemia model. Notably, we also identified that GacS regulates an operon novel to *A. baumannii* (*paa* operon), which is responsible for the metabolism of aromatic compounds. Deletion of *paaE* (A1S_1340) confirmed the role of this operon in *A. baumannii* virulence. Finally, we identified the cognate response regulator (A1S_0236) for GacS and confirmed their interaction. A1S_0236 was shown to regulate 75% of the GacS transcriptome and the same virulence phenotypes. Overexpression of A1S_0236 restored virulence in the *gacS* mutant.

Conclusions. Our study characterizes a global virulence regulator, which may provide an alternate therapeutic target, in one of the most troublesome hospital-acquired pathogens.

Keywords. *A. baumannii*; 2-component system; sensor kinase; response regulator; GacS; GacA; virulence; transcriptome; *paa*; phenylacetate.

Acinetobacter baumannii is a gram-negative human pathogen associated with a wide range of hospital-acquired infections, particularly pneumonia and bloodstream infection but also skin, soft-tissue, and bone infections in injured military personnel [1, 2]. *A. baumannii* strains are notorious for their level of resistance to our current armamentarium of antibiotics, with infections caused by extensively drug-resistant bacteria already described in healthcare institutions [3].

Given the paucity of new antimicrobials in the pipeline with activity against gram-negative bacteria such as *A. baumannii*, the discovery of drug targets that affect bacterial virulence, persistence, or adaptation has become attractive [4]. Two-component regulatory systems (TCS) consist of a pair of regulatory molecules (sensor kinase and response regulator), which mediate the adaptation of microorganisms to changing environments. Sensor histidine kinases exert their control through transfer of a phosphate group to their cognate response regulators, which either recognize and bind to specific DNA sequences to regulate transcription [5] or physically bind target proteins [6]. Commonly, genes encoding both components are co-located in the genome; however, orphan sensor kinases and response regulators have also been described [7, 8]. These systems often act as global virulence regulators, and given their absence in mammalian cells, inhibitors of such systems are considered to be a promising strategy to pathogen control [9].

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Despite *A. baumannii* emerging as a worldwide problem, relatively few virulence factors have been described [10]. Similarly, little is known about the role of *A. baumannii* TCS in gene regulation and virulence. Thus far, 3 TCS have been described in *A. baumannii*, including the BfmSR TCS, which regulates the *csu* operon important for pili assembly and biofilm formation [11], PmrAB, whose increased expression was associated with polymyxin resistance [12], and AdeRS, which regulates the expression of the AdeABC efflux pump important in antibiotic resistance [13, 14]. Given the lack of knowledge of TCS as virulence regulators in *A. baumannii,* the potential for them to be novel antivirulence targets, and the desperate need for alternative strategies against pandrug-resistant gram-negative bacteria, we characterized the function of the first global virulence regulator in *A. baumannii.*

METHODS

Strains and Growth Conditions

A. baumannii ATCC 17978 was used as the wild-type *A. baumannii* strain for all experiments. *A. baumannii* strains were grown in Luria Bertani (LB) or Heart Infusion (HI) broth/agar at 37°C. The wild-type *Candida albicans* DAY286 strain was used for relevant experiments and was grown overnight in yeast peptone dextrose (YPD; Difco) broth/agar at 30°C, or on HI agar at 37°C. Carbenicillin (Cb) 100 µg/mL, kanamycin (Km) 50 µg/mL, tetracycline (Tc) 5 µg/mL, and amphotericin B (AmB) 32 µg/mL were added for selection as needed. The secretion of polyphenols into bacterial culture supernatants was performed using the Gibbs assay as described elsewhere [15].

Genetic Manipulation

In-frame gene deletions were performed as described elsewhere with modifications [16]. In brief, 1250 to 1500 bp fragments corresponding to the regions up and downstream of the respective genes and a kanamycin resistance cassette were cloned into pCR2.1-TOPO (Invitrogen). The subsequent plasmids were then used as templates for the amplification of a linear cassette for the transformation of *A. baumannii* ATCC 17978 to generate 17978*ΔgacS*, 17978*ΔgacA*, 17978*ΔpaaA*, 17978*ΔpaaE*. Polymerase chain reaction (PCR), sequencing, and Southern hybridisation were used to confirm the allelic exchange. Complementation using pWH1266 was performed as described elsewhere to generate pWH1266-*gacS* and pWH1266-*gacA* [11].

RNA Sequencing

A. baumannii cultures were grown to late-log phase (OD_{600} 1.75) in HI broth shaking (200 rpm) at 37°C before RNA extraction. Two independent experiments were performed. Details of RNA sequencing and quantitative real-time (qRT)-PCR are described in Supplementary material.

Bacterial 2-hybrid Analysis

The genes encoding GacS without the transmembrane regions and the full length GacA were cloned separately into vectors pKT25 and pUT18C, respectively, and the resultant constructs were used to cotransform *Escherichia coli* BTH101 as described elsewhere [17]. Strains expressing a fusion protein and carrying the corresponding empty vector were used as negative controls. Blue colonies on LB agar plates containing selective antibiotics, X-gal, and IPTG (Fisher Scientific) signified protein-protein interactions [17].

Virulence Assays

A mouse septicemia model was employed as described elsewhere with modification [18]. In brief, bacterial suspensions mixed with 10% porcine mucin (Sigma) were injected (intraperitoneal) into female BALB/c mice (16–20 grams). Animals were monitored every 3 hours for signs of infection (n = 10 per strain) and were killed appropriately. Survival data were compared using the log-rank test. Organ bacterial density (spleen, liver, and kidney) was assessed from 3 animals per strain at 12 hours postinfection. PCR was used to confirm the presence of the shuttle vector in colonies of the complemented mutant strains. Bacterial loads were compared using the *t* test with statistical significance defined as $P < .05$. All animal experiments were performed in accordance with the Animal Care Committee of Monash University. For further details and methods for in vitro co-cultures with *C. albicans*, motility assays, biofilm, and growth in human serum, see Supplementary material.

Microscopy

A. baumannii pili were examined by transmission electron microscopy following the method described by Tomaras and colleagues [11]. Confocal laser scanning microscopy was used to examine the structural difference between mature biofilms formed by the wild-type (WT), mutant, and complemented strains (details described in Supplementary material).

RESULTS

Organization and Predicted Function of the *A. baumannii* GacS-like Sensor Kinase

Previously, our group demonstrated that, compared to a wild-type clinical strain of *A. baumannii*, a transposon mutant with a disrupted *gacS*-like sensor kinase gene (termed as *gacS*) was significantly attenuated in its ability to kill the unicellular eukaryote and most common human fungal pathogen, *Candida albicans* [19]. Based on previous literature [20, 21], we hypothesized that bacteria have evolutionary conserved virulence mechanisms toward a range of eukaryotic species including

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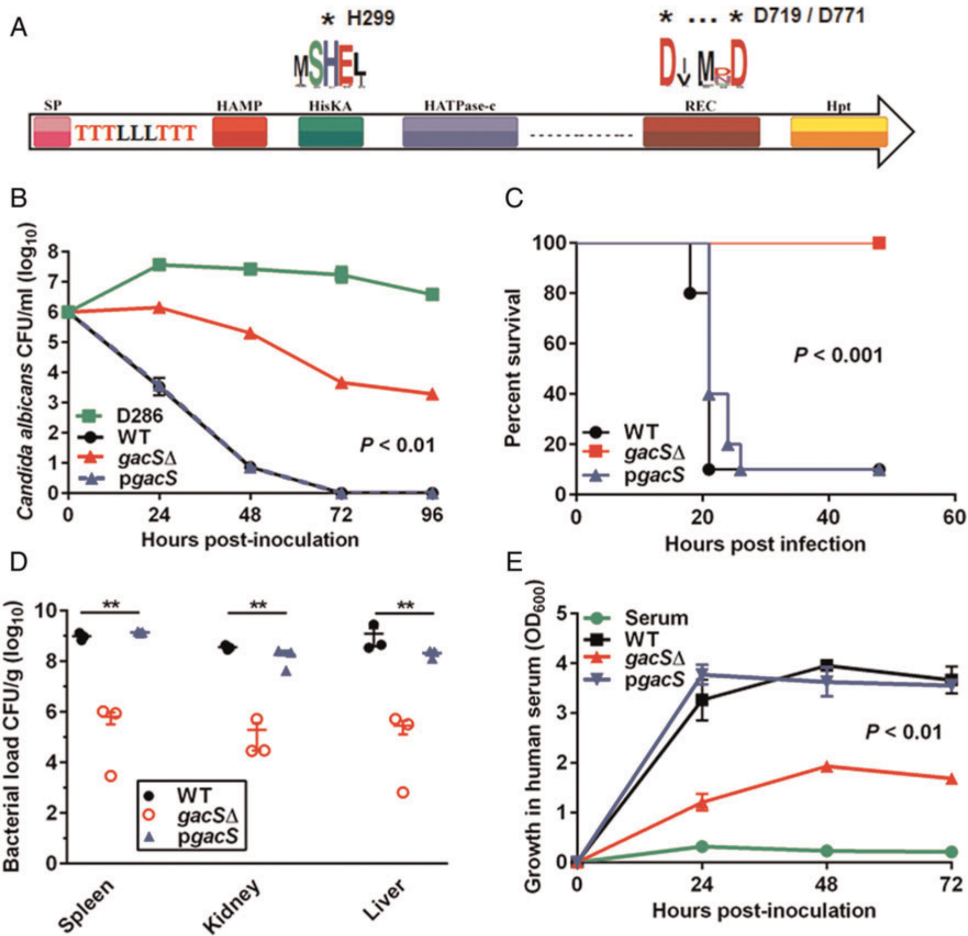

Figure 1. *gacS* is important for *Acinetobacter baumannii* virulence toward different eukaryotic species. *A*, *in silico* characterization of the *A. baumannii* GacS hybrid sensor kinase. The predicted protein structure of the ATCC 17978 GacS protein exhibits conserved Histidine (H299) and aspartic acid (D719/771) residues (asterisk). Two transmembrane regions (represented by T) were also identified between amino acids 21–41 and 173–192, respectively. In addition, domains similar to the signal peptide (SP, amino acids 1–37), amphipathic histidine kinase, adenylyl cyclase, methyl-accepting protein, and phosphatase (HAMP 196–250), histidine kinase (HisKa, 289–355), ATPase (HATPase-c 402–523), response regulator (REC, 663–786) and phosphotransfer (Hpt, 873–924) were also characterized. *B*, An *A. baumannii gacS* deletion mutant was attenuated in killing the unicellular eukaryote *Candida albicans* (*P* value shown is the comparison between WT and *gacS* mutant, 2-way ANOVA) and (*C*) mice using a murine septicaemia model (*P* value signifies difference between WT and *gacS* mutant, Log rank test). *D*, Organ bacterial density studies performed at 12 hours postinfection showed that the *gacS* deletion mutant was capable of systemic dissemination but was at significantly lower levels than WT and complemented strains (mean and standard errors are shown, $^{**}P \leq .01$, *t* test). *E*, Survival of the *gacS* mutant in 50% human serum was significantly decreased in comparison to WT and complemented strains (4 independent experiments, *P* value shown is the comparison between WT and the *gacS* mutant, 2-way ANOVA). Transformation of mutant bacteria using pWH1266 without an insert had no effect (data not shown). WT, wild-type ATCC 17978 strain; *gacS* delta, *gacS* deletion mutant; p*gacS*, pWH1266-*gacS*; D286, *C. albicans* DAY286 strain. Abbreviations: ANOVA, analysis of variance; WT, wild-type.

mammals, and that virulence toward *Candida* could predict virulence toward mammals. We therefore characterized the *gacS* regulatory gene in more detail. In *A. baumannii*, the *gacS* gene (ORF A1S_0574) is 2808 bp in length and has 43% nucleotide identity to its homologue in *P. aeruginosa* PAO1. A neighboring response regulator was not found and *gacS* homologs were identified in all other *Acinetobacter* species and strains sequenced thus far (www.cns.fr/agc/mage). It encodes a predicted protein of approximately 107 kDa, and—similarly to other hybrid sensor kinases—the *A. baumannii* GacS contains both histidine kinase (HisKA–aminoacids 289 to 355) and response regulator (REC – aminoacids 663 to 786) domains (Figure 1*A*). The protein contains the necessary conserved histidine (H299) residue important for autophosphorylation, 2 aspartate residues (D719 and 771, important for phosphate and Mg2+ binding, respectively) necessary for the formation of the acidic pocket required for phosphotransfer, and 2 transmembrane domains (aminoacids 21 to 41 and 173 to 192) (Figure 1*A*). These features support the ability of GacS to act as a functional hybrid system.

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GacS Controls *A. baumannii* Virulence Toward Eukaryotic Species

To characterize the role of the GacS hybrid system in *A. baumannii* virulence, we first made an in-frame gene deletion of *gacS* in *A. baumannii* ATCC 17978 (17978Δ*gacS*), and a complemented strain (pWH1266-*gacS*). As shown in Figure 1*B*, we confirmed our previous findings that GacS was important for *A. baumannii* virulence toward *C. albicans* [19]. To test our hypothesis that *A. baumannii* virulence toward *C. albicans* is predictive of virulence toward a mammal, we infected mice with *A. baumannii* ATCC 17978, the *gacS* mutant and complemented strain (Figure 1*C*). Using a mouse septicemia model, we showed that the *gacS* mutant was avirulent at an inoculum of 5×10^4 CFU/mL. Virulence was restored to wild-type levels with complementation (Figure 1*C*). Importantly, in vitro growth of the *gacS* mutant was similar to that of its parental and complemented strains (data not shown). To determine if the attenuation in virulence was due to the inability of the *gacS* mutant to proliferate and disseminate systemically, organ bacterial density studies at 12 hours postinfection were performed. The *gacS* mutant was able to disseminate from the primary site of infection and infect multiple organs (kidney, liver, and spleen); however, the bacterial load was 10^3 to 10^5-fold lower ($P < .01$) than that observed for the wild-type and complemented strains (Figure 1*D*). These murine data were also supported by growth assays in human serum, whereby the *gacS* deletion mutant was susceptible to the killing effects of serum, with growth similar to wild-type levels restored in the complemented bacteria (Figure 1*E*).

GacS Acts as a Global Virulence Regulator in *A. baumannii*

The organization of GacS as a hybrid TCS system and its important role in *A. baumannii* virulence led us to question the regulatory activity exerted by GacS. Transcriptomic profiling showed that 674 genes were significantly affected by deletion of *gacS* (233 and 441, up- and down-regulated, respectively; Supplementary Table 1), and they were dispersed across a wide variety of functions (Figure 2). Importantly, the regulation of genes responsible for *A. baumannii* biofilms, pili formation, and motility were observed; virulence attributes that are likely relevant for *A. baumannii* to persist and cause infection in healthcare environments.

The first of these were components of the *csu* operon, *csuB* (A1S_2216), and *csuC* (A1S_2215), which were significantly repressed 4.5- and 4-fold, respectively, in the *gacS* mutant. The *csu* operon is composed of 6 genes (*csuA/BABCDE*) and encodes a chaperone-usher pili assembly system that is important for pili synthesis, initial bacterial attachment, and biofilm formation on abioitc surfaces [22]. The qRT–PCR confirmed that GacS regulates the *csu* operon, with restoration of *csuC* expression in the *gacS* complement strain (Figure 3*A*). These data were supported by TEM analysis, which showed that pili were significantly shorter in the *gacS* mutant (Figure 3*B*). We then assessed the ability of the *gacS* mutant to form biofilms on plastic. As seen in Figure 3*C* and 3*D*, the mutant produced a thinner biofilm composed of a more sparse distribution of cells. Apart from the *csu* operon, other genes regulated by GacS that may be associated with biofilm formation are shown in Supplementary Table 1.

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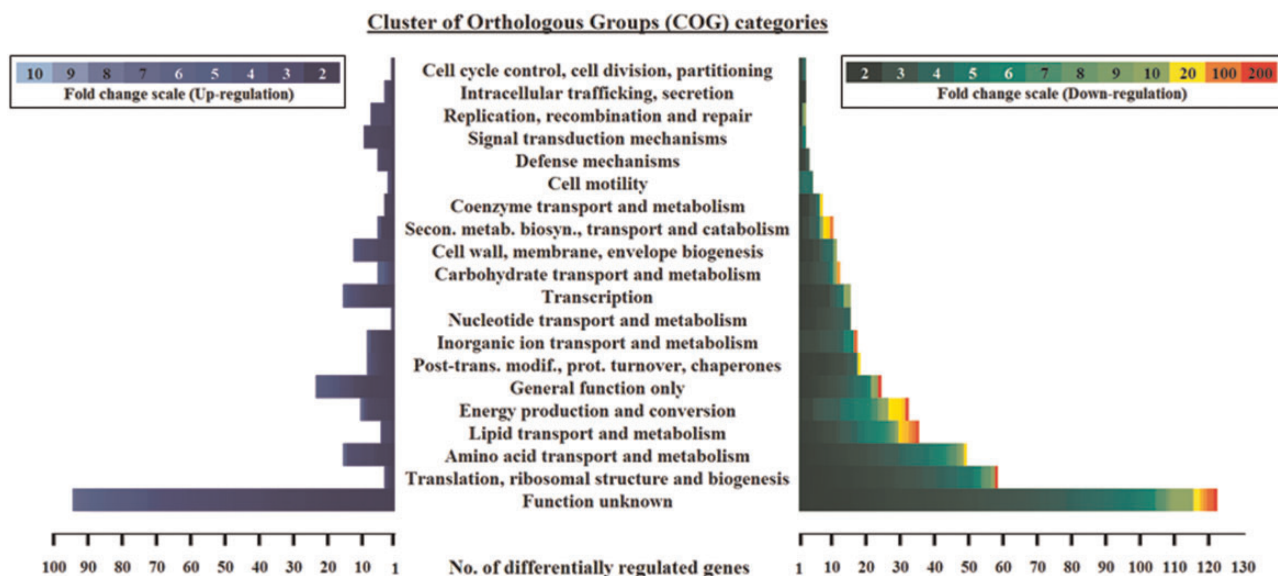

Figure 2. Heatmap showing differences in gene expression between the *gacS* deletion mutant and the wild-type strain. The differentially expressed genes were organized per COG category.

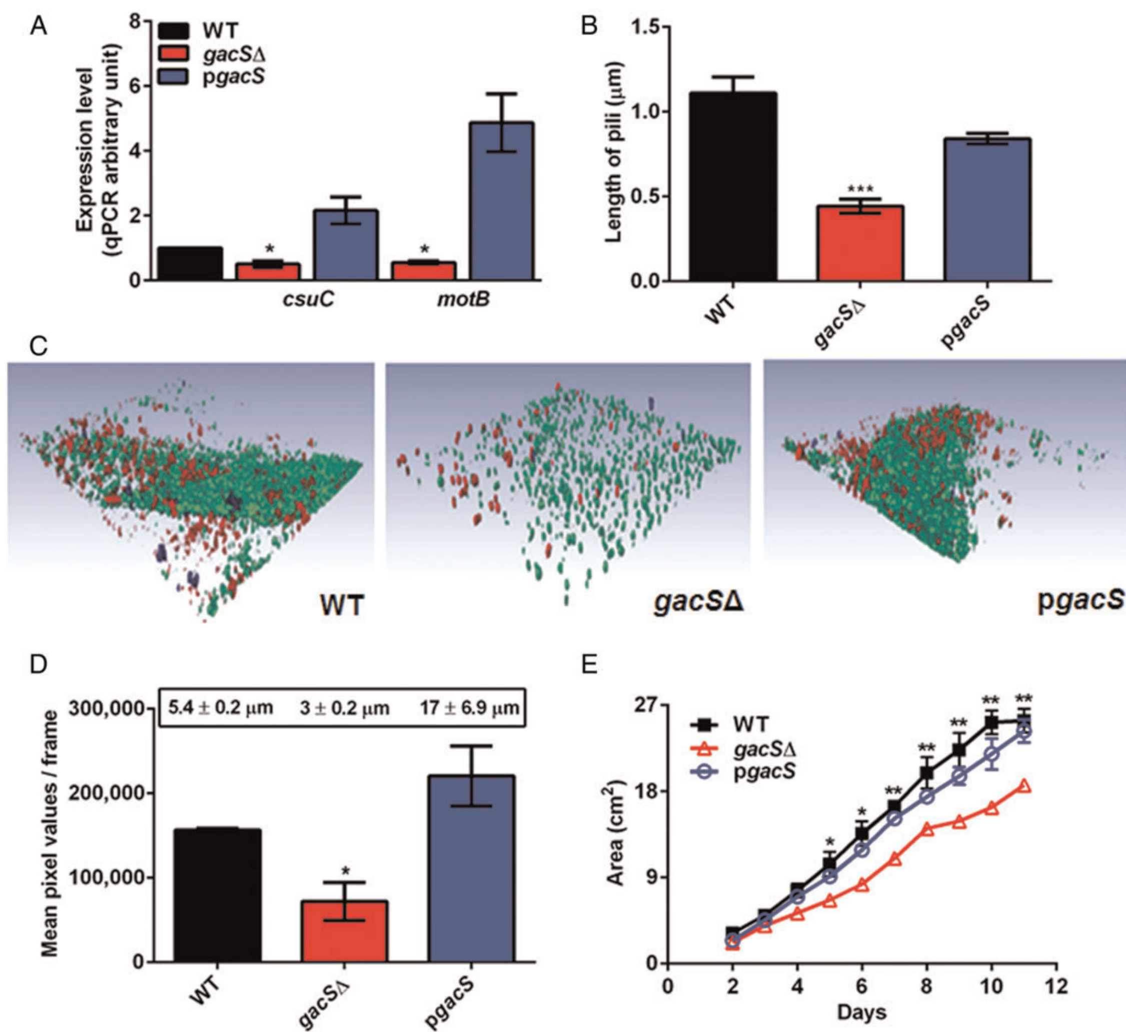

Figure 3. *Acinetobacter baumannii* GacS regulates pili synthesis, biofilms, and motility. *A*, *csuC* and *motB* gene expression was reduced in the *gacS* deletion mutant compared with WT and complemented strains. Results are presented relative to the parental strain ATCC 17978, which was normalized to 1 and are expressed as mean ± SEM for at least 3 biological replicates. $^{*}P \leq .05$, *t* test. *B*, Pili length were reduced in the *gacS* deletion mutant (67% ± 10% shorter than WT; $^{***}P < .001$, *t* test) and (*C*) 3-dimensional biofilm structures were more sparse after 24 hours growth at 37°C. Live and dead cells were labeled in gray and black, respectively (see methods section) and were used to determine (*D*) the mean biofilm thickness per strain in μm (values above columns, mean for 3 biological replicates. $P = .001$, *t* test) and cell density per frame by pixel quantification (columns ± SEM for 3 biological replicates. $^{*}P \leq .05$, *t* test). *E*, Twitching motility was impaired in the *gacS* deletion mutant, which was restored to WT levels in the complemented strain. The results are expressed as mean ± SEM for at least 3 biological replicates. $^{*}P < .05$ and $^{**}P < .01$, *t* test. WT, wild-type ATCC 17978 strain; *gacS* delta, *gacS* deletion mutant; p*gacS*, pWH1266-*gacS*. Abbreviations: SEM, standard error of the mean; WT, wild-type.

Despite *A. baumannii* deriving its genus name from being nonmotile (*akineto*), recent evidence shows that under certain conditions it exhibits motility [23, 24]. We observed that the expression of 2 genes belonging to the COG category "Cell motility" were significantly repressed in the *gacS* mutant (A1S_0643, 4.48–fold and A1S_1507, 4.55–fold). A1S_0643 encodes for a hypothetical protein, whereas A1S_1507 is a Type I pilus gene that has recently been found to be up-regulated in a hypermotile *A. baumannii* strain [24]. Additionally, the *ompA/motB* gene (A1S_1193), which is known to contribute to motility and virulence in other gram-negative bacteria [25], was also significantly down-regulated (2.27-fold) in the *gacS* mutant; qRT-PCR confirmed that GacS regulates *motB* (Figure 3*A*). We also observed a significant up-regulation of *algZ* (A1S_0260, 2.18-fold), which encodes a transcriptional regulator that is known to inhibit flagellum biosynthesis in nonmotile *P. aeruginosa* [26]. These gene expression data were corroborated with motility studies showing that the *gacS* mutant was significantly impaired in twitching motility (Figure 3*E*). Other *A. baumannii* genes that were regulated by GacS and

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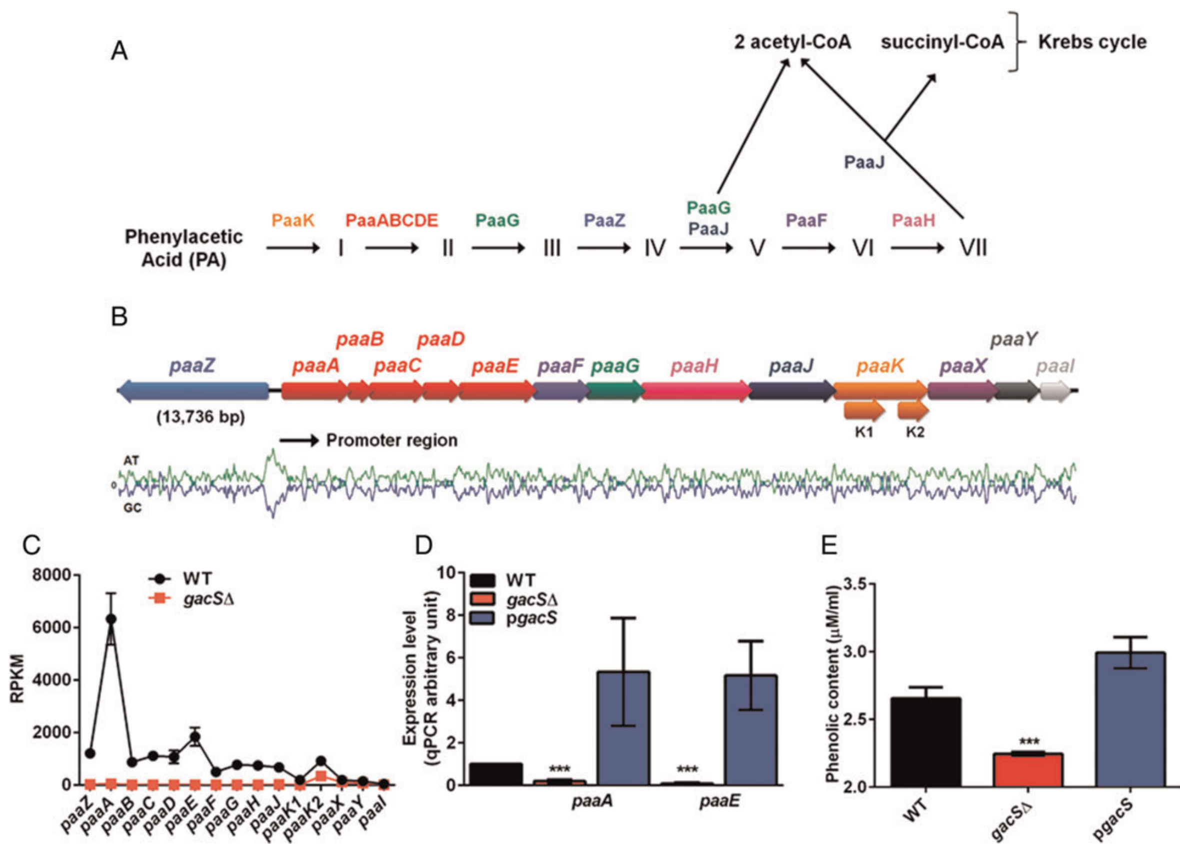

Figure 4. GacS regulates the *Acinetobacter baumannii* phenylacetate catabolic pathway. *A*, Reactions and intermediates of the pathway are based on *Escherichia coli* [27]. Enzymes: PaaK, phenylacetate-CoA ligase (AMP forming); PaaA-E, ring 1,2-phenylacetyl-CoA epoxidase (NADPH); PaaG, ring 1,2-epoxyphenylacetyl-CoA isomerase (oxepin-CoA forming); PaaZ, oxepin-CoA hydrolase (NADP+); PaaJ, 3-oxoadipyl-CoA; PaaF, 2,3-dehydroadipyl-CoA hydratase; PaaH, 3-hydroxyadipyl-CoA dehydrogenase (NAD+). Subproducts: I, phenylacetyl-CoA; II, ring 1,2-epoxyphenylacetyl-CoA; III, 2-oxepin-2(3H)-ylideneacetyl-CoA; IV, 3-oxo-5,6-dehydrosuberyl-CoA; V, 2,3-dehydroadipyl-CoA; VI, 3-hydroxyadipyl-CoA; VII, 3-oxoadipyl-CoA. *B*, Organization of the gene cluster responsible for phenylacetate degradation in *A. baumannii* ATCC 17978. The entire cluster is composed of 15 coding sequences and a single promoter region (arrow, identified by in silico analysis of GC%). The total length of the *paa* gene cluster was determined to be 13 736 bp. *C*, The normalized number of sequencing reads mapped to the genes (RPKM - reads per kilobase per million mapped reads) in the PAA metabolism operon was significantly reduced in the *gacS* deletion mutant compared to WT (gray and black lines, respectively). *D*, GacS regulates the *paaA* and *paaE* genes. Results are presented relative to WT which was normalized to 1. The results are expressed as mean ± SEM for at least 3 biological replicates. ****P* < .001, *t* test. *E*, The Gibbs assay was employed to assess the ability of the strains to degrade phenylacetates (PA) and secrete phenolic metabolites into culture supernatants [15]. The *gacS* deletion mutant secreted significantly less phenolic metabolites compared to WT and the complemented strain. The results are expressed as mean ± SEM for 4 biological replicates. ****P* < .001, *t* test. WT, wild-type ATCC 17978 strain; *gacS* delta, *gacS* deletion mutant; p*gacS*, pWH1266-*gacS*. Abbreviations: RPKM, reads per kilobase per million mapped reads; SEM, standard error of the mean; WT, wild-type.

have putative virulence function are shown in Supplementary Table 1.

GacS Controls the Expression of a Novel Pathway in *A. baumannii*; the Phenylacetic Acid (PAA) Catabolic Pathway

The PAA catabolic pathway encoded by the *paa* operon is a pivotal route in the catabolism of several aromatic compounds that converge and are directed to the Krebs cycle (Figure 4*A*). Phenylacetate-metabolizing bacteria were identified >50 years ago [28], but the degradation pathway was characterized only recently [27]. The ability to metabolize aromatic compounds confers to a number of bacterial species a role in remediation of environmental pollutants. In pathogens, the early intermediates of this metabolic pathway are thought to contribute to virulence [27]. The *A. baumannii paa* operon is shown in Figure 4*B*, and alignment analyses showed that its structure and ORF sequences are similar to *E. coli paa* genes [27]. Deletion of *gacS* resulted in repression of the entire *paa* operon (A1S_1336 – A1S_1349, 35.9–201.4-fold). Furthermore, half of the genes had reads per kilobase per million mapped reads (RPKM) values <10 (Figure 4*C*), which suggests a loss of function [29]; qRT-PCR confirmed that *A. baumannii* GacS regulates the *paa* operon (Figure 4*D*).

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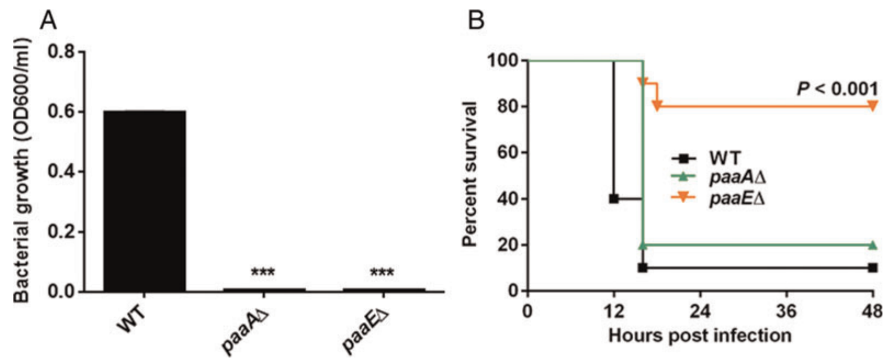

Figure 5. The functional significance of the *paa* operon in *Acinetobacter baumannii*. *A*, *paaA* and *paaE* deletion mutants were unable to grow in 10 mM L-Phenylalanine as a sole carbon source after 48 hours at 37°C. Growth was measured by determining the optical density (see Material and Methods). Columns represent the mean growth ± SEM for 3 independent experiments (****P* < .001, *t* test). *B*, An *A. baumannii paaE* deletion mutant was attenuated in vivo (*P* value signifies difference between WT and *paaE* mutant, Log rank test). WT, wild-type ATCC 17978 strain; *paaA* delta, *paaA* deletion mutant; *paaE* delta, *paaE* deletion mutant. Abbreviations: SEM, standard error of the mean; WT, wild-type.

To assess the functional significance of these findings we first tested the ability of our strains to degrade phenylacetates (PA) using the Gibbs assay, which measures the amount of phenolic metabolites secreted in culture supernatants [15]. The *gacS* deletion mutant exhibited a reduction in the amount of phenolic content compared to wild-type ($P < .01$), which was restored in the complemented strain (Figure 4*E*). To characterize further the role of the *paa* operon in the virulence of *A. baumannii*, we created a deletion mutant of both *paaA* and *paaE*. These genes were selected as they encode proteins early in the PAA catabolic pathway. Functional assessment of the mutants showed that both were unable to use L-phenylalanine as a sole carbon source, confirming loss of function of the PAA pathway (Figure 5*A*). We then tested the mutants in our mouse septicaemia model and showed that the *paaE* deletion mutant, but not the *paaA* mutant, was significantly attenuated (Figure 5*B*). Multiple attempts at complementation were unsuccessful; however, a further independent mutant was created and showed the same phenotype (data not shown).

A. baumannii GacS Functions With an Orphan Response Regulator

In other gram-negative pathogens, GacS interacts with a response regulator known as GacA [30]. Given the importance of GacS in *A. baumannii*, we performed an in silico analysis to identify the *gacA* homologue. Alignment analyses using the *P. aeruginosa* PAO1 *gacA* sequence showed that ORF A1S_0236 from *A. baumannii* ATCC 17978 encoded a predicted response regulator that shared 57% amino acid identity (75% similarity). ORF A1S_0236 is 636 bp in length and the predicted protein contains both REC (aminoacids 5 to 119) and LuxR_C_like (helix-turn-helix, aminoacids 149 to 205) domains, which are characteristic of response regulators. We termed this ORF the *A. baumannii gacA*.

To determine whether *A. baumannii* GacA is a response regulator for GacS, we first performed a functional analysis by overexpressing GacA in the *gacS* mutant and assessing for virulence restoration. It is established that excess production of a cognate response regulator enables bacterial cells to overcome the effect of a mutation in the sensor kinase [31]. GacA overexpression restored virulence of the *gacS* mutant to wild-type levels in the *Candida–Acinetobacter* coculture and mouse septicaemia models (Figure 6*A* and 6*B*). Overexpression of an unrelated response regulator (*adeR*) had no effect on the *gacS* deletion mutant (data not shown). These data were then supported by a bacterial 2-hybrid analysis, which showed interaction between GacS and GacA (Figure 6*C*).

We then created a *gacA* deletion mutant and complemented strain and showed that similar to the *gacS* mutant, the *gacA* deletion mutant was significantly impaired in pili formation, motility and biofilm structure, and was attenuated toward *C. albicans* and in the mouse septicaemia model, all of which were restored by complementation with the wild-type *gacA* gene (Supplementary Figure 1). Furthermore, the GacA-regulated transcriptome showed that 75% of the GacA-regulated genes were also regulated, in the same direction, by GacS (Figure 6*D* and Supplementary Table 1), including the key virulence-associated genes and operons; the *csu* operon (A1S_2213–17), the Type I pili gene (A1S_1507), *motB* (A1S_1193), the *paa* cluster (A1S_1335–49), an arginine metabolism associated operon (*ast,* A1S_3128–31), as well as upregulation of *algZ* (A1S_0260).

DISCUSSION

Sensor kinases play an essential role in bacterial biology and pathogenesis. The ability to sense and respond to environmental stimuli is a survival strategy that links the control of gene

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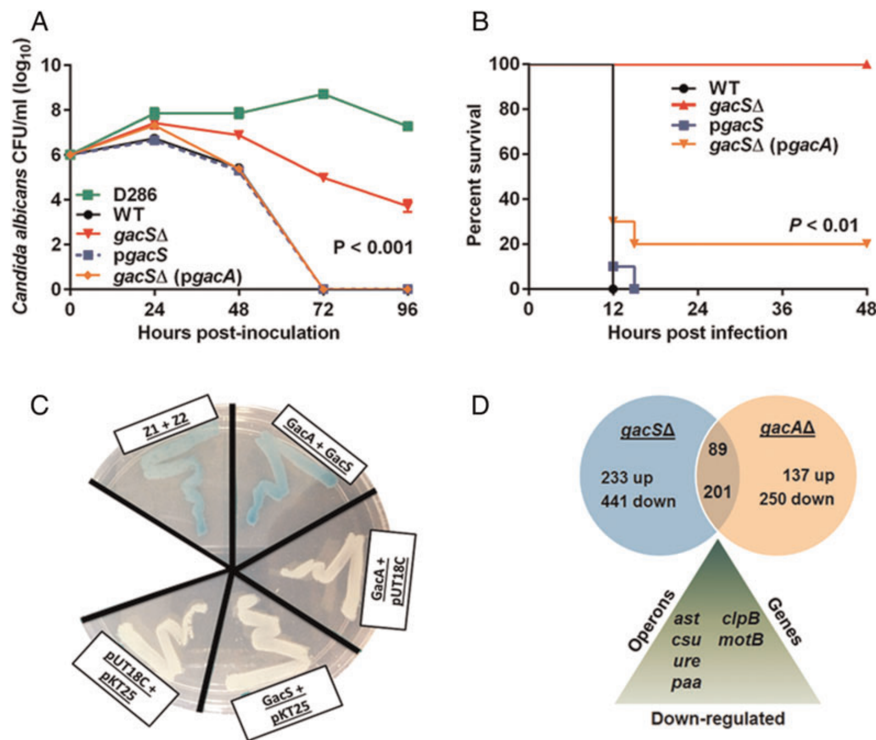

Figure 6. GacS and GacA form a 2-component regulatory system. *A*, Overexpression of GacA in an *Acinetobacter baumannii gacS* deletion mutant restored its virulence toward the unicellular eukaryote *Candida albicans* (3 biological replicates, $P < .001$, *t* test analysis of the time-points) and (*B*) mice (*P* value signifies difference between *gacS* delta and *gacS* delta (p*gacA*), Log rank test). *C*, Bacterial 2-hybrid analysis confirmed the interaction between GacS and GacA. The dark gray color (equivalent to blue color change) signifies activation of β-galactosidase indicative of protein-protein interaction. Z1 + Z2 were used as positive controls and represent vectors pKT25-Zip and pUT18C-Zip, respectively. Gac constructs were pKT25-*gacA* and pUT18C-*gacS*. pKT25 and pUT18C empty vectors were used as negative controls together or combined with one of the Gac constructs. *D*, A Venn diagram of all genes differentially expressed ≥2-fold (FDR < 0.01) in both *gacS* and *gacA* deletion mutants relative to the parental strain. Important virulence-associated operons and genes that were down-regulated in both *gacS* and *gacA* mutants are shown in the green triangle. WT, wild-type ATCC 17978 strain; *gacS* delta, *gacS* deletion mutant; p*gacS*, pWH1266-*gacS*; D286, *C. albicans* DAY286 strain; p*gacA*, pWH1266-*gacA*.

expression to the appearance of specific phenotypes. In this study we have shown that the *A. baumannii* GacS sensor kinase acts as a global virulence regulator. It controls the expression of previously characterized and novel *A. baumannii* virulence genes and is important for pathogenesis in mammals. In addition, we showed that *A. baumannii* GacS regulates gene expression and virulence through a response regulator (A1S_0236) we have termed GacA. Finally, we identified that the PAA catabolic pathway is regulated by GacSA and is a functional system for the metabolism of aromatic compounds and a novel virulence-associated pathway in *A. baumannii*.

Little is known about TCS in *A. baumannii* and the way they control the biology of this troublesome pathogen. Currently described TCS include the aforementioned BfmSR [11], PmrAB [12], and AdeSR [13, 14]; however, to our knowledge, this study is the first characterization of a global virulence regulator in *A. baumannii*. The *A. baumannii* GacSA regulates a plethora of virulence functions, including the control of pili synthesis, motility, biofilms, resistance against human serum, and metabolism of aromatic compounds, all of which contribute to virulence within a mammalian infection model. We confirmed that *A. baumannii* GacSA is a 2-component system using both protein-protein interaction and functional assays; indeed, 75% of the GacA-regulated transcriptome correlated with that of GacS. The fact that not all Gac-regulated genes overlapped provides evidence that alternative interactions or cross-talk with other regulatory proteins exists, a finding that is not unexpected with TCS [32, 33].

Notably, our study uncovered a novel virulence pathway in *A. baumannii*, the *paa* cluster. The *paa* genes encode proteins responsible for the metabolism of aromatic compounds and have been linked to virulence in other human pathogens [15, 34]. Previous studies have also associated the intermediate products generated during the catabolism of aromatic compounds with severe lung infections in cystic fibrosis or immunocompromised patients [35]. The genes from the *paa* operon were the most down-regulated genes in our *gacSA* mutants, and we hypothesized that they participate in *A. baumannii* virulence. Previously, mutagenesis of both *paaA* and *paaE* genes in *B. cenocepacia* rendered the mutant bacteria unable to survive in a rat

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pulmonary infection model [36] and led to attenuation in a *Caenorhabditis elegans* model [15]. Indeed, a recent study suggests that the initial steps of phenylacetate degradation may generate compounds toxic to the host including ring-1,2-epoxide and its phenolic breakdown product 2-hydroxyphenylacetate [27]. Apart from showing that GacS regulates the *paa* operon, we showed that deletion of *gacS* had a functional effect on the metabolism of phenylacetates (Figure 4*E*). Furthermore, deletion of *paaE*, but not *paaA*, attenuated *A. baumannii* virulence in the mammalian septicemia model (Figure 5*B*). Interestingly, the *paaA-D* genes are conserved in all bacteria possessing this degradation pathway, although a *paaE* homologue appears to be lacking from some ancestral bacterial groups, suggesting that *paaE* may be necessary for the in vivo formation of the toxic epoxides required for virulence. This hypothesis needs further investigation.

The ability to produce biofilms is one of the most important virulence factors in *A. baumannii* as it contributes to the pathogen's ability to persist in the host and the environment, evade immune responses, and resist antibiotic treatment [37, 38]. The *csu* operon was previously shown to be essential for *A. baumannii* pili synthesis, with a role in attachment to and biofilm formation on abiotic surfaces [22]. *A. baumannii* are known to produce long (Csu-dependent) and short (Csu-independent) pili [39, 40]. TEM analysis of the *gacS* mutant revealed cells with short pili (Figure 3*B*), which are hypothesized to be synthesized by the *papADC* operon (A1S_1510-08) [41–43] whose expression was unaffected by GacSA (data not shown). It was previously established that the *csu* operon was regulated by BfmSR [11]; however, our data now show that the GacSA TCS also has a regulatory role on pili synthesis. Moreover, we have previously shown that the *csu* operon is only found in pathogenic species of *Acinetobacter* [44], highlighting the potential differences in regulatory function GacSA may have in different species, which all have this TCS. We also identified other repressed genes with a putative role in biofilm formation, including a Type I pili (A1S_1507) and the *motB* gene, both of which need further confirmation of their role in *A. baumannii* virulence.

Given the dwindling pipeline of new antimicrobials, especially for gram-negative bacteria such as *A. baumannii*, novel strategies are desperately needed. TCS provide a potentially exciting drug target as they are not found on mammalian cells and play a critical role in the virulence of some of the most problematic human pathogens.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

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