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Identification of a Novel LysR Family Transcriptional Regulator Controlling Acquisition of Sulfur Sources in *Acinetobacter baumannii*

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Keywords

Acinetobacter baumannii · Transcriptional regulation · LysR · Sulfur uptake · Cysteine biosynthesis · Transcriptomics

Abstract

L-cysteine biosynthesis from inorganic sulfur represents a major mechanism by which reduced sulfur is incorporated into organic compounds. Cysteine biosynthesis and regulation is well characterized in *Escherichia coli*. However, the regulation of sulfur metabolism in *Acinetobacter baumannii* is only partly understood, with the LysR-type regulator, GigC known to control some aspects of sulfur reduction. In this study, we have used transcriptomics and bioinformatic analyses to characterize a novel LysR-type transcriptional regulator encoded by ABUW_1016 (*cbl*), in a highly multidrug resistant and virulent isolate of *A. baumannii*. We have shown that Cbl is involved in controlling expression of the genes required for uptake and reduction of various sulfur sources in *A. baumannii*. Collectively, we have identified the global regulon of Cbl and proposed a model of cysteine biosynthesis and its regulation by Cbl and GigC in *A. baumannii*.

Introduction

Acinetobacter baumannii is a Gram-negative opportunistic pathogen primarily associated with hospital-acquired infections. It has been designated as a "red alert" human pathogen in hospital settings due to its propensity to acquire extensive antibiotic resistance and its ability to survive for prolonged time periods in hospital environments enabling nosocomial transmission [Dijkshoorn et al., 2007; Cerqueira, and Peleg, 2011; Howard et al., 2012]. Carbapenem-resistant *A. baumannii* has been included in the critical group of pathogens that pose a high threat to global health by the WHO and CDC, thus making it a high priority for research and development of new antibiotic treatments [WHO, 2017; CDC, 2019]. Despite the urgency to develop new antimicrobial treatments, we do not yet completely understand the virulence determinants and their regulatory mechanisms that enable *A. baumannii* to colonize, disseminate, and persist in hospital environments.

Sulfur is an essential element that is required for the growth and survival of organisms across all domains of life. The sulfur assimilation pathway is highly conserved in *Gammaproteobacteria* and is essential for the

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biosynthesis of L-cysteine. L-cysteine is central to many cellular processes as it serves as a primary source of reduced sulfur in several biomolecules including lipoic acid, L-methionine, molybdopterin, thiamine, coenzyme A, iron-sulfur clusters and also in glutathione synthesis [Kredich, 2005; Kessler, 2006; Aguilar-Barajas et al., 2011].

Cysteine biosynthesis and regulation has been well studied in the Gram-negative organisms *Escherichia coli* and *Salmonella enterica* [Kredich, 2005]. Sulfate and thiosulfate transport in *E. coli* and *S. enterica* are mediated by proteins encoded in the gene cluster *cysPTWA* and the unlinked gene *sbp*, where *sbp* and *cysP* encode for sulfate and thiosulfate periplasmic binding proteins, respectively. The *cysT* and *cysW* genes encode for membrane proteins that function as a transporter for sulfate, thiosulfate, and other related anions, and the *cysA* gene encodes for an ATPase that provides energy to drive transport [Kredich, 2005; Kessler, 2006; Aguilar-Barajas et al., 2011]. Once taken up by the organism, reduction of sulfate occurs via the ATP sulfurylase subunits encoded by *cysD* and *cysN* to yield adenosine 5′-phosphosulfate (APS) [Kredich, 2005]. APS kinase encoded by *cysC* then phosphorylates APS to convert it to phosphoadenosine 5′-phosphosulfate (PAPS), which is reduced to sulfite by PAPS sulfotransferase encoded by *cysH* [Kredich, 2005]. The sulfite is further reduced to sulfide, and this reaction is catalyzed by NADPH-sulfite reductase subunits encoded by *cysI* and *cysJ*.

In *E. coli, cysC* is linked with *cysDN* to form a single *cysDNC* cluster, and similarly, *cysH* is linked with *cysIJ* to form a *cysHIJ* cluster. In the final step, sulfide is combined with O-acetyl serine (OAS) to yield the end product cysteine in the presence of two distinct OAS (thiol)-lyase isozymes encoded by *cysK* and *cysM* [Kredich, 2005]. However, when there is sulfide present in the environment, cysteine biosynthesis is a two-step process involving conversion of L-serine to OAS, which then reacts with sulfide to yield cysteine. In that case, serine transacetylase encoded by *cysE* is required for the acetylation of L-serine by acetyl-CoA to produce OAS, which acts as an immediate precursor for L-cysteine biosynthesis [Kredich, 2005].

The major steps involved in sulfur assimilation in *A. baumannii* follow the same pattern as that in *E. coli*. However, *A. baumannii* and many other non-enteric bacteria lack the APS kinase encoding gene, *cysC*; in that case, an additional enzyme APS reductase encoded by *cysH* reduces APS to adenosine 5′-phosphate and sulfite; thus, PAPS is not an intermediate in this pathway [Bick et al., 2000; Kredich, 2005]. Also, *A. baumannii* lacks a *cysJ* homolog and therefore only the NADPH-sulfite reductase subunit *cysI* may be involved in the reduction of sulfite to sulfide, which then combines with OAS to yield L-cysteine.

The genes required for sulfate assimilation and organic sulfur utilization are collectively known as the cysteine regulon, which are coordinately controlled by the LysR-type transcriptional regulator (LTTR) CysB in *E. coli* [Sirko et al., 1990; Kredich, 2005; Aguilar-Barajas et al., 2011]. CysB appears to have a dual regulatory role, one as an activator of the cysteine regulon and the other as a negative regulator of its own expression. Maximal expression of the cysteine regulon requires not only the CysB regulator but also the inducers OAS or N-acetyl-L-serine (a derivative of OAS) for transcriptional activation, and limitation of sulfur in the environment. Sulfur limitation is a requirement because in the presence of cysteine, there is a feedback inhibition of serine transacetylase (CysE), the enzyme that is required for OAS synthesis, and thus, cysteine can act as a negative regulator of the cysteine regulon [Ostrowski, and Kredich, 1991; Kredich, 2005].

In the absence of cysteine or inorganic sulfur, *E. coli* can also utilize taurine (2-aminoethanesulfonic acid) and other aliphatic sulfonates as a sulfur source [van der Ploeg et al., 2001; Kredich, 2005]. The proteins required for taurine and alkanesulfonate utilization are encoded by the *tauABCD* and *ssuEADCB* operon, respectively. Collectively, *tauABC* and *ssuABC* encode for the ABC-type transporters which are required for the uptake of taurine and alkanesulfonates, while *tauD* encodes for an alpha-ketoglutarate-dependent dioxygenase, and *ssuD* and *ssuE* encode for a two-component FMNH2-dependent sulfonate monooxygenase, involved in desulfonation and their conversion to sulfite [van der Ploeg et al., 1997; van der Ploeg et al., 2001; Kredich, 2005]. Expression of *tau* and *ssu* genes requires both CysB and a second LTTR Cbl. CysB is required not only because it controls expression of *cbl* but also for the direct activation of *tauABCD* expression, while *cbl* binding to the promoter region is sufficient for the transcription initiation of *ssu* operon [Iwanicka-Nowicka and Hryniewicz, 1995; van der Ploeg et al., 1997; Kredich, 2005].

A. baumannii lacks a clear CysB homolog, and the genetic organization of putative genes involved in the cysteine biosynthetic pathway is different to that of *E. coli*. The ABUW_3161 gene encodes a LTTR, which has been named *gigC,* as it was identified in a genome-wide screen for virulence determinants in a *Galleria mellonella* study [Gebhardt et al., 2020]. Recently, GigC has been shown to

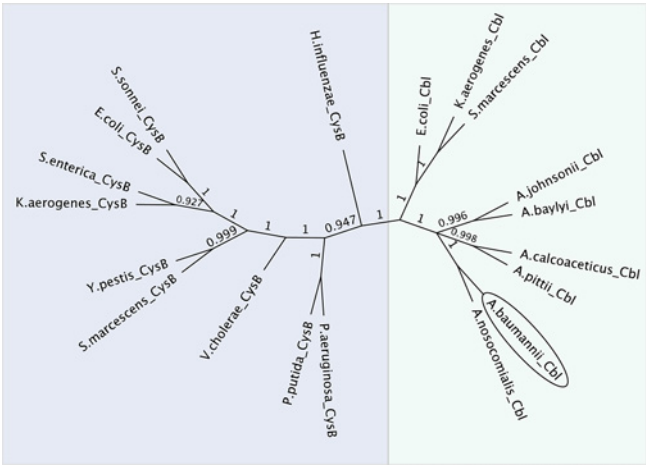

Fig. 1. Tree showing phylogenetic relationships of LTTRs, CysB, and Cbl in *Gammaproteobacteria*. The tree was generated using MrBayes 3.2.6 [Huelsenbeck, and Ronquist, 2001] from the protein sequences aligned with Clustal Omega. Branch labels are the posterior probability values generated by MrBayes. The CysB and Cbl clusters in the phylogenetic tree are shaded in blue and green, respectively.

regulate several genes involved in cysteine biosynthesis, but it only shares 23% sequence identity to CysB in *E. coli* [Gebhardt et al., 2020]. GigC acts as an activator for *cysI* and *cysDN* expression, and a repressor for *cysH* and *cysQ* expression [Gebhardt et al., 2020]. *A. baumannii* AB5075_UW also encodes another LTTR (ABUW_1016) which shares 45% sequence identity to CysB and 51% to Cbl in *E. coli*. Here, we report the genome-wide transcriptional profile of an ABUW_1016 transposon-inactivated mutant. ABUW_1016 appears to be a global regulator controlling acquisition and reduction of sulfur sources in *A. baumannii* and is important for virulence in an insect infection model.

Results and Discussion

ABUW_1016 Encodes Cbl in A. baumannii

Phylogenetic analysis performed on 19 Gammaproteobacterial LTTR proteins demonstrated that CysB and Cbl form two distinct clusters and ABUW_1016 clusters together with the Cbl orthologs (Fig. 1); hence, we have named ABUW_1016 as Cbl. It was observed that *E. coli, Klebsiella aerogenes*, and *Serratia marcescens* encode both the CysB and Cbl regulators, while most other *Gammaproteobacteria* only have a copy of CysB, and the *Acinetobacter* genus only possess a copy of Cbl. In *E. coli*, *cysB* and *cbl* are monocistronic, and while *A. baumannii* lacks a clear CysB ortholog, *cbl* is located immediately upstream of the gene cluster involved in sulfate/thiosulfate transport and thus is likely to be encoded on a single transcript (Fig. 2, 3).

Transcriptional Response Overview

RNA sequencing transcriptomics was performed on the *A. baumannii* parental strain, AB5075_UW and a transposon-inactivated *cbl* mutant. These strains were grown in Mueller-Hinton (MH) media, a defined rich media commonly used to grow *Acinetobacter* strains. Genes showing significantly altered expression in the mutant were defined as those with $\log_2$ fold change ≥1.5 and an adjusted p value ≤0.01 relative to the parental strain. Collectively, transcriptomic analysis revealed that a total of 211 genes show differential expression in the isogenic *cbl* mutant compared to the parental strain AB5075_UW, of which 170 genes display decreased expression and 41 genes display increased expression (Fig. 4; online suppl. Table S1; see www.karger.com/doi/10.1159/000529038 for all online suppl. material).

Functional class analysis of differentially expressed genes identified enrichment of genes in various clusters of orthologous (COG) categories including amino acid transport and metabolism, inorganic ion transport and metabolism, energy production, cellular processes and signaling, and genes with unknown function (Fig. 5). Generally, genes in these categories showed a significant reduction in expression when *cbl* was inactivated. These included genes involved in uptake and reduction of various sulfur sources and genes in the cluster ABUW_0066-0071, which encode for the enzymes required for L-tyrosine degradation and its conversion to fumarate. Furthermore, various enzymes involved in Fe-S cluster assembly and several ferredoxins had higher expression in the *cbl* mutant.

Additionally, genes and operons involved in cellular processes and signaling also displayed significantly altered expression in the *cbl* knockout. These included an operon encoding a chaperone-usher pili assembly system composed of 6 genes (*csuA/BABCDE*), which showed a significant downregulation with $\log_2$-fold change between 6.3 and 7.5. Other genes in these categories encode for various fimbriae and pili assembly proteins which mostly had reduced expression, while ABUW_2053 encoding a pili assembly chaperone showed increased expression in the *cbl* mutant (Fig. 4).

Cbl, a LysR family regulator, regulates expression of various genes involved in sulfur acquisition and reduction. Cbl in *A. baumannii* is a LTTR which appears to be a

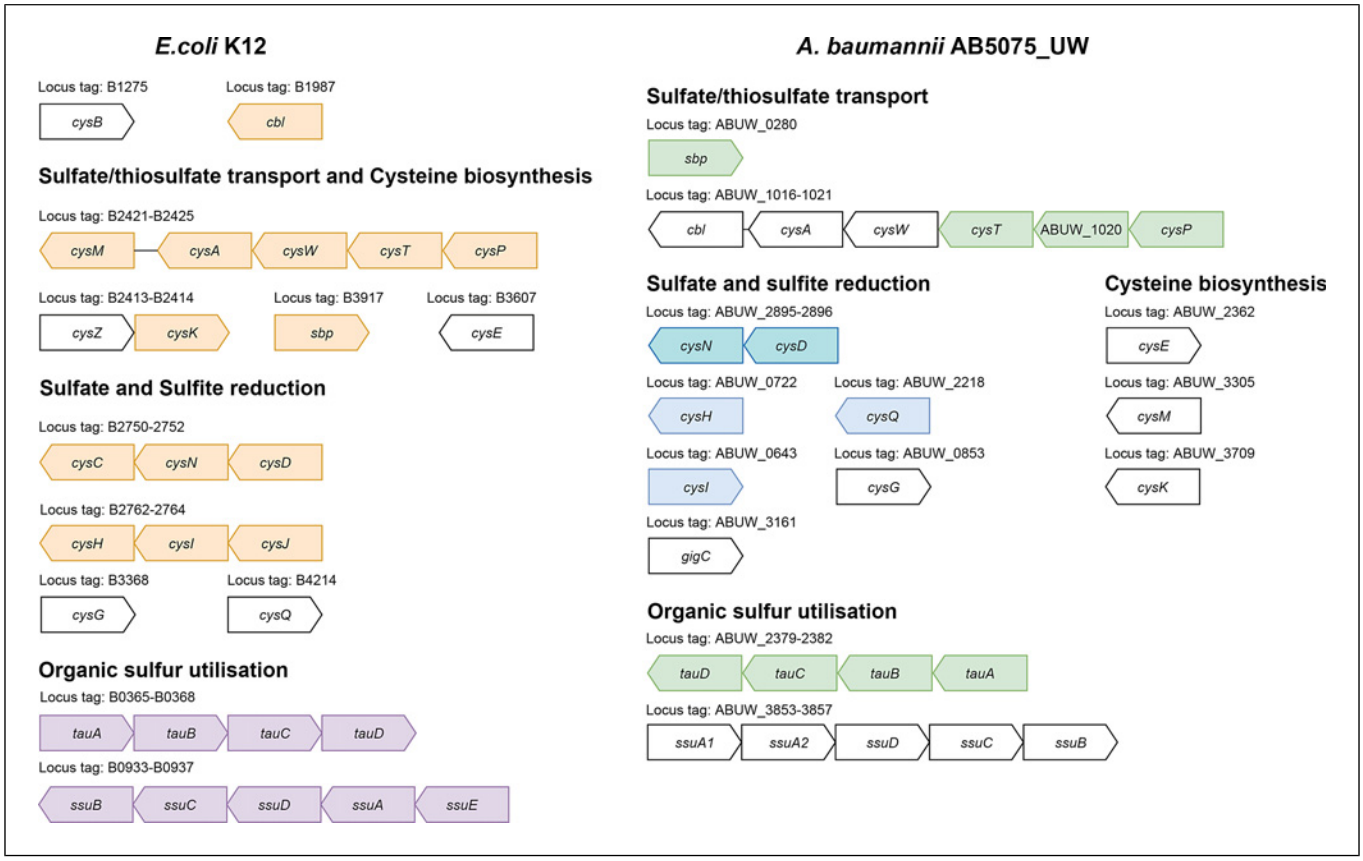

Fig. 2. Genetic organization of the genes involved in sulfur assimilation and cysteine biosynthesis in *E. coli* K12 (left panel) and *A. baumannii* AB5075_UW (right panel). The genes are scattered throughout the genomes. In *E. coli*, the genes that are under control by CysB are highlighted in orange and the genes that are shaded in purple are involved in taurine and alkanesulfonate utilization which requires both Cbl and CysB for activation. In *A. baumannii*, the genes that appear to be under control of Cbl (our study) are shaded in green and the genes that are shaded in light blue are under control of GigC [Gebhardt et al., 2020]. The genes shaded in turquoise indicate that these genes might be under regulation of both Cbl and GigC in *A. baumannii*, while it is not clear what regulates expression of the genes that are not shaded in both organisms.

global regulator of sulfur acquisition and reduction. The *cysP* (ABUW_1021), *cysT* (ABUW_1019), and ABUW_1020 genes in the ABUW_1017-1021 cluster show significant reduction in expression in the *cbl* mutant compared to the parental strain. ABUW_1020 encodes for an alpha/beta hydrolase fold protein and is part of the *cysPTWA* cluster in AB5075_UW, unlike in *E. coli* (Fig. 2). Interestingly, no changes in expression were observed with *cysW* and *cysA* genes, potentially suggesting that there is a second promoter within the cluster that drives the expression of these genes. ABUW_0280, which encodes the sulfate binding protein Sbp, also showed reduced expression ($\log_2$-fold change −3.02) in the *cbl* knockout strain. Similarly, the *cysD* and *cysN* genes encoding ATP sulfurylase had significant reduction in expression ($\log_2$-fold change between 3.15 and 4.15) when *cbl* was inactivated. This suggests *cbl* is required for the regulation of genes involved in sulfate and thiosulfate uptake and reduction in *A. baumannii*. However, no significant change in expression of the genes required for conversion of APS to sulfide (*cysH* and *cysI*) or of the genes required for biosynthesis of cysteine from sulfide (*cysK* and *cysM*) was observed in the *cbl* mutant under the chosen growth condition.

As mentioned previously, GigC was recently found to be involved in the regulation of *cysD, cysN, cysI, cysH,* and *cysQ* genes, all of which except *cysQ* are required for the multistep reduction of sulfate to sulfide [Gebhardt et al., 2020]. The *cysQ* gene encodes for a putative monophosphatase that helps control the level of PAPS and protects the organism against its toxicity; however, it is not a part of the cysteine regulon [Neuwald et al., 1992;

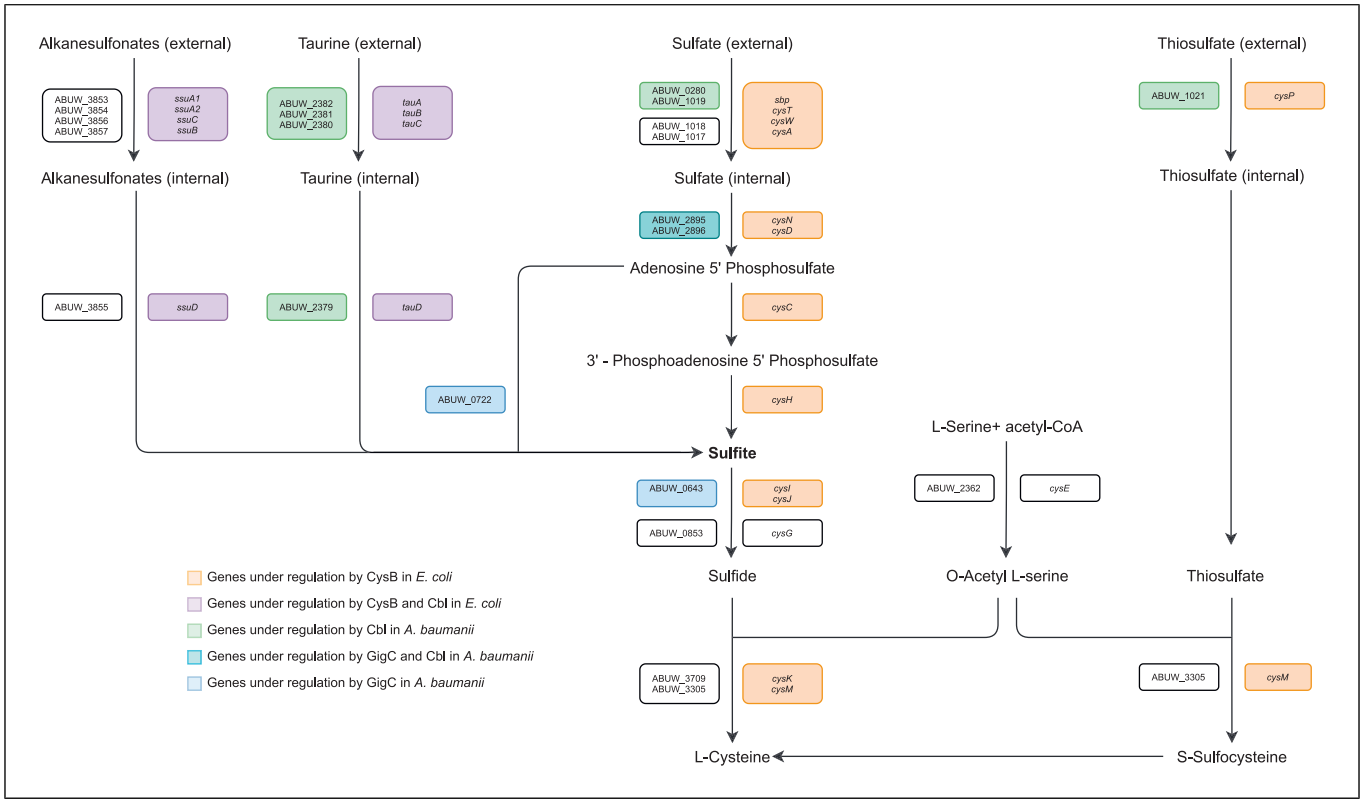

Fig. 3. L-cysteine biosynthesis in *E. coli* and *A. baumannii* from sulfate, thiosulfate, taurine, and alkanesulfonates. ABUW locus tags indicate the putative homologs of the genes required for cysteine biosynthesis identified in *A. baumannii* AB5075_UW. ABC transporters encoded by *cysPTWA*, *sbp*, *tauABC*, *ssuA1A2BC* are involved in the uptake of various sulfur sources in *A. baumannii*. Following the uptake, sulfate reduction in *A. baumannii* occurs via phosphorylation by CysDN, APS reduction to sulfite by CysH. Taurine and alkanesulfonates get desulfonated after transport by TauD and SsuD yielding sulfite. Sulfite reduction to sulfide occurs with the help of CysI, which then combines with OAS to yield the end product L-cysteine. Thiosulfate gets combined with OAS directly after transport to yield S-sulfocysteine, which then gets converted to L-cysteine by an as yet unknown mechanism. The color shading represents the mode of transcription of the cysteine regulon in *E. coli* and *A. baumannii* as shown in the key.

Kredich, 2005]. The CysQ ortholog in *A. baumannii* might have similar functions to that in *E. coli*. The fact that the expression of *cysDN* is impacted in both *cbl* and *gigC* knockouts indicates that they might have partially overlapping regulatory networks in *A. baumannii*.

The genes involved in taurine uptake and metabolism also showed significantly reduced expression in the *cbl* mutant, with *tauA* and *tauB* showing $\log_2$-fold changes of more than 4, suggesting Cbl also regulates taurine utilization in *A. baumannii* similar to that in *E. coli*. *A. baumannii* possesses the *ssuA1A2DCB* genes required for alkanesulfonate utilization (Fig. 3), but no change in expression of any gene in this operon was observed in the *cbl* mutant. AB5075_UW encodes for two *ssuA* paralogs located adjacent to each other (Fig. 2) unlike in *E. coli*, which are probably required to interact with different alkanesulfonate substrates. In *E. coli,* desulfonation of alkanesulfonate requires a FMNH2-dependent monooxygenase-type enzyme (SsuD) and a NAD(P)H-dependent FMN reductase (SsuE). However, the *ssu* gene cluster in *A. baumannii* does not include a *ssuE* gene and thus SsuD may interact with a different FMN reductase encoded elsewhere in the genome. Interestingly, a TetR family transcriptional regulator encoded by ABUW_3858 is located directly downstream of the *ssuA1A2BCD* operon. It is possible that ABUW_3858 acts as a regulator of alkanesulfonate utilization, and it could also be involved in regulating expression of the rest of the genes in the cysteine biosynthetic pathway that are not under control by Cbl or GigC in *A. baumannii*.

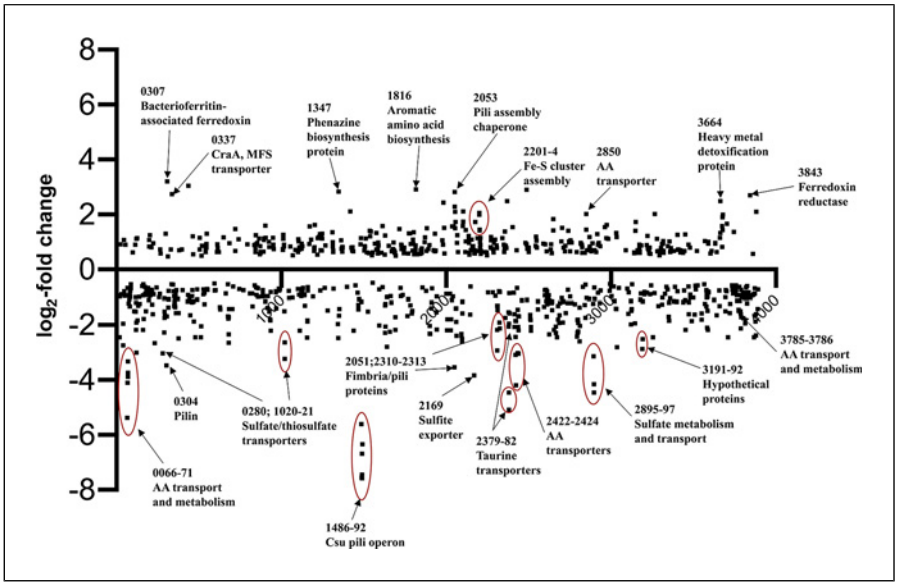

Fig. 4. Global transcriptional response of an isogenic *cbl* mutant compared to the parental AB5075_UW strain. Each dot represents the differential expression levels (log_2-fold change) of ORFs in AB5075_UW genome. Only selected genes with an adjusted *p* value of less than 0.01 relative to the parental strain are plotted against the gene locus tag numbers. Genes of interest are highlighted and labeled.

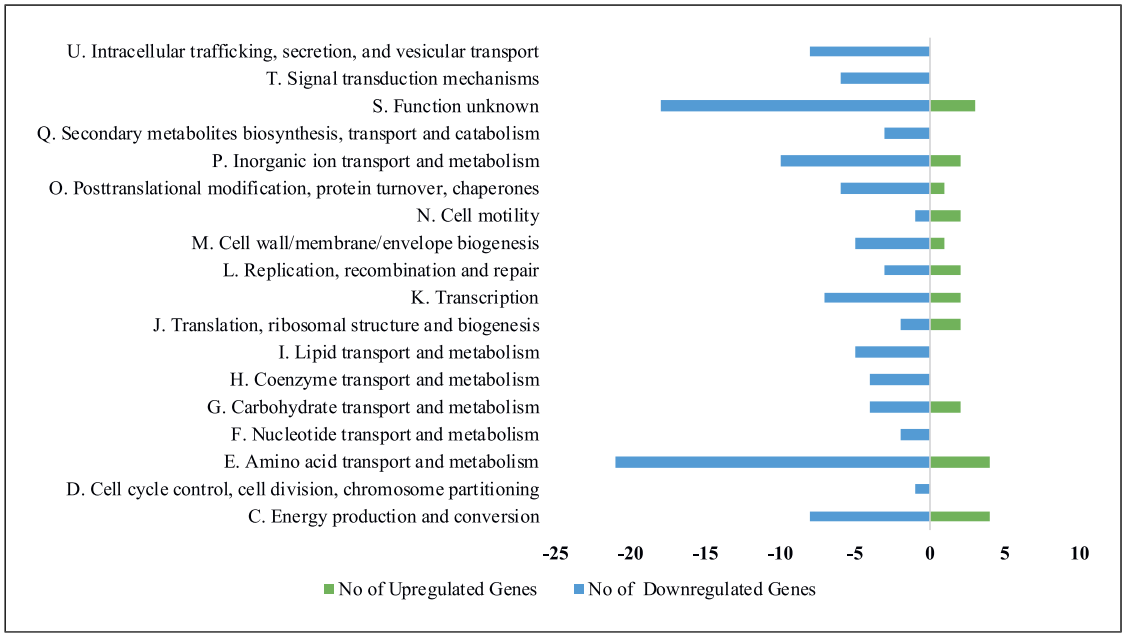

Fig. 5. COG enrichment of genes that show differential expressions in the *cbl* mutant compared to the parental AB5075_UW strain. Differentially expressed genes belong to various functional classes, the most highly represented being amino acid transport and metabolism.

Taken together, the majority of the genes involved in the assimilatory sulfate reduction pathway appear to be under control by LTTRs encoded by *cbl* and *gigC* in *A. baumannii* (Fig. 3). However, it is not yet clear what regulates expression of the genes encoding serine transacetylase (*cysE*) and cysteine synthase (*cysK* and *cysM*). AB5075_UW also contains a serine transacetylase paralog, encoded by ABUW_2415, which shares 46% sequence identity to CysE (ABUW_2362). However, unlike *cysE*, ABUW_2415 had reduced expression

(log_2-fold change of −1.6) in the *cbl* inactivated strain, but the role of ABUW_2415 in cysteine biosynthesis is not clear.

Cbl *Inactivation Impacts Expression of Amino Acid Biosynthesis and Transport Genes*

A range of amino acid transport and metabolism genes were significantly differentially expressed in the *cbl* mutant compared to the parental strain. The ABUW_0066-0071 gene cluster involved in aromatic amino acid transport and metabolism had significantly decreased expression (log_2-fold change of between 3.3 and 5.3) in the *cbl* mutant. The genes in this cluster are involved in conversion of L-tyrosine to fumarate, which then enters the TCA cycle. The ABC transporter genes ABUW_2422-2424 encoding a glutamate/aspartate transporter and ABUW_3785-3789 encoding D-amino acid biosynthesis and transport also showed significantly decreased transcript abundance in *cbl* inactivated mutant. In contrast, ABUW_1816 encoding an enzyme in the shikimate pathway for aromatic amino acid biosynthesis showed increased expression (log_2-fold change of 2.91) in the *cbl* mutant. The altered expression of these genes in the *cbl* knockout mutant might be due to disruptions in amino acid homeostasis caused by the effects of Cbl on cysteine biosynthesis.

The *A. baumannii* major facilitator superfamily (MFS) drug transporter CraA (ABUW_0337) had significantly increased expression in the *cbl* mutant (log_2-fold change of 2.75). An MFS family efflux pump, encoded by *ydeD*, has previously been shown to extrude L-cysteine and O-acetyl-L-serine, and may also export other intermediates in the cysteine biosynthesis pathway in *E. coli* [Daßler et al., 2000]. Similar to that of other MFS transporters, *craA* also possesses a broad substrate specificity profile ranging from various antibiotics to cationic substrates [Foong et al., 2019]. However, the natural physiological substrate of *craA* is still unknown. The upregulation of *craA* in the *cbl* mutant suggests a possible physiological role in the secretion of toxic levels of metabolites and precursors during sulfur assimilation or cysteine biosynthesis in *A. baumannii*.

Cbl Might Be Required for Virulence in A. baumannii

Expression of several pili assembly genes was significantly altered in the *cbl* inactivated mutant. The csu pili operon, which is known to be important for cell adherence, and biofilm formation [Moon et al., 2017] had the most significant reduction in expression. In addition, various other fimbriae and pili assembly genes also showed an altered expression in the absence of *cbl*, potentially suggesting a role for *cbl* in pathogenicity of *A. baumannii*.

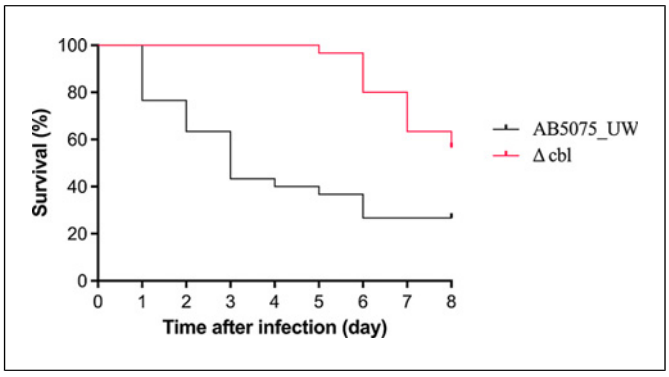

Fig. 6. Kaplan-Meier survival curve of *G. mellonella* injected with 1 x 10^7 AB5075_UW parental and transposon-inactivated *cbl* mutant strains. Survival of larvae was enumerated every 24h post-infection for 10 days. Larvae injected with the parental strain showed a significantly lower survival rate compared to those injected with the *cbl* mutant strain ($p < 0.001$).

We investigated the role of *cbl* in virulence of *A. baumannii* by using the *G. mellonella* (the greater wax moth) infection model, which has previously been shown to be an effective model system to study in vivo pathogenesis for *A. baumannii* and other bacterial pathogens [Dinh et al., 2021]. The *G. mellonella* infection assay was performed on the parental AB5075_UW and the transposon-inactivated *cbl* mutant strain. The *cbl* mutant demonstrated reduced virulence compared to the parental strain, which killed all larvae within 6 days post-infection while *cbl* mutant killed the first larvae only after 5 days post-infection (Fig. 6). We also assessed the growth of the *cbl* mutant strain in MH media and observed that the inactivation of *cbl* in AB5075_UW leads to a slight growth defect (online suppl. Fig. S1). The requirement of *cbl* for active growth of the organism and also for virulence in an insect model is likely due to the strain inability to utilize sulfur and/or synthesize cysteine. A study by Gebhardt and others previously outlined the importance of several genes in sulfur assimilation and cysteine biosynthesis pathway for the growth of *A. baumannii* in the *G. mellonella* larvae [Gebhardt et al., 2015]. This also included *gigC*, which is required for virulence in both the insect and murine infection models in addition to its role in the regulation of several genes involved in cysteine biosynthesis [Gebhardt et al., 2015, 2020]. This further emphasizes the role of sulfur assimilation/cysteine metabolism in the pathogenicity and virulence of *A. baumannii*.

Conclusion

In conclusion, we report the characterization of a novel LTTR, Cbl. RNA-Seq transcriptomics was used to investigate the global response of *cbl* inactivation in a highly multidrug resistant and virulent strain of *A. baumannii,* AB5075_UW. Based on the transcriptomic analysis, we have shown that *cbl* acts as a global regulator controlling expression of the genes involved in acquisition and reduction of various sulfur sources. Combining the findings from our study and Gebhardt et al. [Gebhardt et al., 2020], we have put together a proposed model of cysteine biosynthesis and their transcription in *A. baumannii* compared to that of *E. coli* (Fig. 3). Taken together, Cbl and GigC represent the major regulatory networks required for sulfur uptake and reduction in *A. baumannii*. However, an unknown third regulator might be involved in the expression of rest of the genes in the cysteine biosynthetic pathway. This study also demonstrated the importance of *cbl* in *A. baumannii* virulence in an insect infection model. This highlights the importance of sulfur metabolism and regulation in the virulence of *A. baumannii,* which is probably required for the successful colonization of the host. The cysteine regulon might therefore represent a potential therapeutic target to combat this problematic pathogen.

Materials and Methods

Bacterial Strains, Growth Media, and Conditions

Bacterial strains used in this study include *A. baumannii* AB5075_UW and a T26 mutant of *cbl* (ABUW_1016-125::T26). The transposon mutant strain was obtained from the Manoil laboratory (University of Washington) three-allele collection [Gallagher et al., 2015]. Bacterial strains were routinely cultured in MH media at 37°C at 200 rpm unless otherwise stated. Disruption of the *cbl* mutant was confirmed by using gene-specific primers flanking the gene of interest. Genome sequencing of the ABUW_1016-125::T26 mutant showed no genetic changes relative to the parental strain outside of ABUW_1016 disruption.

Bacterial Growth Assays

Overnight cultures of the parental AB5075_UW and *cbl* inactivated mutant were diluted in MH broth (1:100) and grown continuously for 20 h in the PHERAstar FS (BMG Labtech) at 37°C with shaking at 200 rpm, with optical density being measured every 6 min at 600 nm.

RNA Sequencing and Analysis

Parental AB5075-UW and the *cbl* inactivated mutant strain were grown overnight. The cells were diluted to 1:100 dilution from overnight cultures and grown to mid-exponential phase cultures (15 mL, OD600–0.6) at 37°C, with shaking at 200 rpm. Cell pellets were harvested at 5,000×g, 15 min at 4°C, and lysed using QIAzol reagent (Qiagen). Total RNA was isolated using the miRNeasy Mini Kit (Qiagen), and DNA digestion was performed on the total RNA using DNaseI (TURBO DNA-free™ Kit, Invitrogen). Ribosomal RNA was depleted using a Ribo-Zero Magnetic Kit (bacteria) (Illumina, Inc., USA), and the cDNA library was generated using the TruSeq® Stranded Total RNA Sample Preparation Kit (Illumina, Inc., USA) by the sequencing provider at the Ramaciotti Centre (Sydney, Australia). The samples were then sequenced on an Illumina NextSeq 500 platform. The quality of raw sequenced reads was examined by using the FastQC tool [Babraham Bioinformatics, London, UK]. The sequence reads from six RNA-Seq samples (three biological replicates per strain) were aligned to the *A. baumannii* AB5075_UW reference genome (GenBank accession: CP008706.1) and counted using EDGE-PRO (Estimated Degree of Gene Expression in Prokaryotic Genomes) [Magoc et al., 2013]. Differential gene expression between the parental and mutant samples was analyzed using the DESeq2 R package [Love et al., 2014]. A negative binomial model was used to test the significance of differential expression between the parental and the mutant cells. The complete data set has been deposited in the gene expression omnibus database (accession number: GSE183337). Functional enrichment analyses were conducted by assigning COG group categories to AB5075-UW genes using eggNOG mapper [Huerta-Cepas et al., 2017].

Insect Model Infection Experiments

The *G. mellonella* infection assay was performed as previously described [Frei et al., 2021]. Briefly, larvae (200–230 mg) were injected with 1×10^7 *A. baumannii* parental and *cbl* inactivated strains. Following injection, the larvae were incubated at 37°C and monitored every 24 h for 10 days. Larval performance was assessed according to the *G. mellonella* Health Index Scoring System [Loh et al., 2013]. The experiments were performed in 6 replicates (5 larvae/replicate), with 30 larvae per strain in total.

Statement of Ethics

This study protocol was reviewed and approved by the Institutional Biosafety Committee, approval number 5201401141.

Conflict of Interest Statement

The authors have no conflict of interests to declare.

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Author Contributions

This work was conceptualized by Ian T. Paulsen, Karl A. Hassan, and Alaska Pokhrel. All experimental work was

conducted by Alaska Pokhrel except for the insect model infection experiment, which was performed by Hue Dinh. All of the data analyses were performed by Alaska Pokhrel. The manuscript was drafted by Alaska Pokhrel, with contributions and guidance from Ian T. Paulsen, Liping Li, Hue Dinh, Karl A. Hassan, and Amy K. Cain.

Data Availability Statement

The RNA sequencing data that support the findings of this study have been deposited in the gene expression omnibus database, accession number: GSE183337. Further inquiries can be directed to the corresponding author.

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