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AbuO, a TolC-like outer membrane protein of *Acinetobacter baumannii* is involved in antimicrobial and oxidative stress resistance

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ABSTRACT

Although *Acinetobacter baumannii* is well accepted as a nosocomial pathogen, only a few of the outer membrane proteins (OMPs) have been functionally characterized. In this study, we demonstrate the biological functions of AbuO, a homolog of TolC from *Escherichia coli*. Inactivation of *abuO* led to increased sensitivity to high osmolarity and oxidative stress challenge. The Δ*abuO* displayed increased susceptibility to antibiotics such as amikacin, carbenicillin, ceftriaxone, meropenem, streptomycin, tigecycline and hospital-based disinfectants such as benzalkonium chloride and chlorhexidine. The RT-PCR analysis indicated increased expression of efflux pumps [RND efflux pump *acrD*, 8 - fold; SMR-type *emrE* homolog, 12-fold; MFS-type *ampG* homolog, 2.7-fold] and two component response regulators [*baeR*, 4.67-fold; *ompR*, 10.43-fold] in Δ*abuO* together with down regulation of *rstA* (4.22-fold) and pilin chaperone (9-fold). The isogenic mutant displayed lower virulence ability in a nematode model ($P<0.01$). Experimental evidence for the binding of MerR-type transcriptional regulator, SoxR to radiolabelled *abuO* promoter suggests regulation of *abuO* by SoxR in *A. baumannii*.

INTRODUCTION

Outer membrane proteins (OMP) are known to have a pivotal role in bacterial physiology, such as adherence, invasion, and serum resistance, maintenance of cell structure, binding a variety of substances, including passive and active transport [1]. The archetypical OMP, TolC has been coined to be a multifunctional protein due to its involvement in cell membrane integrity, acid tolerance, expulsion of metabolites, export of siderophores that are required in iron acquisition, export of plasmid and chromosomal encoded toxins such as hemolysin, colicin V and microcins, and virulence as evident from studies in *Enterobacter*, *Borrelia*, *Salmonella*, *Vibrio*, *Legionella*, *Francisella and E. coli* [2-10]. In *E. coli* TolC is promiscuous as it supports the functioning of multidrug resistance efflux pumps such as AcrD, AcrEF and MdtABC (resistance nodulation cell division super family-RND) [11-13], EmrAB and EmrKY (major facilitator super family-MFS), and MacAB (ATP-binding cassette super family-ABC) [14-17]. Though the functions of TolC homologs in many Gram-negative bacteria such as *E. coli*, *Vibrio vulnificus*, *Stenotrophomonas maltophilia*, *Enterobacter cloacae*, *Yersinia pestis* [18, 5, 19-21] have been elucidated, however its biological functions in an important human pathogen *Acinetobacter baumannii* has remained enigmatic so far.

The multidrug resistant (MDR) *Acinetobacter* kills up to 50% of infected patients despite treating with last resort of drugs, and resistance rates of such strains continue to escalate globally [22, 23]. Significant increase in *A.baumannii* strains that are resistant to carbapenems, cephalosporins, aminoglycosides and fluoroquinolones with diverse antibiotic resistome have been reported from hospitals in USA and other countries [24-27]. In our previous study, we demonstrated the role of antibiotic resistance genes and efflux pumps in mediating antimicrobial resistance in *A. baumannii* isolates from Ohio, USA [28, 29]. To date, three OMPs have been

implicated in carbapenem resistance when their expression is reduced; CarO, Omp 33–36 and OprD homolog [30-32].

In continuation with our efforts on understanding the origin/network of multidrug resistance in *Acinetobacter*, in this study, using genetic and molecular approaches we demonstrated the role of putative OMP (homolog of TolC, designated as AbuO) in bacterial stress physiology in general and antimicrobial resistance in particular for the first time in *A. baumannii.*

MATERIALS AND METHODS

Bacterial strains and Media

A. baumannii AYE was purchased from American type culture collection (ATCC BAA1710). Bacterial cultures were grown in Luria-Bertani (LB) broth or agar (Difco, Becton-Dickinson, Sparks, MD) with 400 µg/ml hygromycin for mutant and 200 µg/ml zeocin for complemented strains. Restriction digestion, ligation, transformation, and agarose gel electrophoresis were done according to standard protocols. Plasmid and Genomic DNA were prepared using a Gene Aid kits according to the manufacturer's protocol. DNA products were sequenced to confirm their authenticity (Applied Biosystems). Primers used in the present study were custom-synthesized (Eurofins MWG operons, Germany).

Construction of Δ*abuO* in *A. baumannii* AYE

A 693 bp internal fragment was amplified using Δ*abuO*-F/Δ*abuO*-R primers (Table ST1), cloned into pUC4K derived suicide vector harboring hygromycin cassette. The obtained plasmid pUC-*abuO* was transformed into *A.baumannii* AYE to construct Δ*abuO*. The gene disruption was

confirmed by Southern hybridization and PCR analysis. The zeocin cassette was amplified from pCR Blunt II-TOPO vector (Life Technologies), using zeo-NT and zeo-CT primers (Table ST1) and cloned into shuttle vector pWH1266. Further, the intact *abuO* along with its promoter was amplified with FL*abuO*-F and FL*abuO*-R (Table ST1), cloned into modified pWH1266 vector. The resulting construct was transformed into Δ*abuO* and selected on LB agar plates supplemented with 200 µg/ml zeocin to obtain the transcomplemented strain Δ*abuO*Ω*abuO*. Mutant and complemented strains were characterized; their phenotypes compared with WT (*A. baumannii*, AYE).

Bacterial phenotypic assays

The growth profiles of WT, Δ*abuO* and Δ*abuO*Ω*abuO* were monitored in LB at different pH (5.0, 6.0, 7.0, 8.0, 10.0 and 12.0) for 18 hours at 37°C shaking using Bioscreen C automated growth analysis system (Labsystems, Helsinki, Finland) at OD_{600nm}. The growth inhibition assay was performed as before with slight modifications using ciprofloxacin (0.005 µg/ml), ethidium bromide (EtBr; 4 µg/ml) and chlorhexidine (1.6 µg/ml) [33]. The impact of *abuO* inactivation on motility behavior and biofilm formation was examined as mentioned before [34]. Studies to decipher the impact of oxidative stress inducing agent hydrogen peroxide (H_2O_2) and nitrosative stress inducing agents (sodium nitroprusside (SNP) and acidified nitrite) on WT, Δ*abuO* and Δ*abuO*Ω*abuO* was examined as mentioned before [33]. The WT, Δ*abuO* and Δ*abuO*Ω*abuO* were exposed to hostile stress conditions at different concentrations such as bile salt deoxycholate, sodium chloride (NaCl), EtBr, acridine orange, acriflavine, rhodamine, safranine, ampicillin, neomycin, ciprofloxacin, chloramphenicol, tetracycline, benzalkonium chloride, chlorhexidine and triclosan, survival capability determined as mentioned before [33].

Drug susceptibility, efflux assay and OMP preparations

Antibiotic susceptibility and minimum inhibitory concentration (MIC) were examined using commercial discs and E-strips (Hi Media, Bombay, India), data was analysed according to the interpretation criteria recommended by CLSI [35]. Accumulation assays using fluorescent substrates EtBr/ ciprofloxacin and purification of OMPs from WT, Δ*abuO* and Δ*abuO*Ω*abuO* was done as described before [33].

RNA isolation and real-time reverse transcription PCR (RT-PCR)

Total RNA was extracted from log-phase cultures using RNeasy Mini Kit according to manufacturer's instructions. Aliquots of 500 ng of DNase I treated total RNA served as template for complementary DNA (cDNA) synthesis using superscript III reverse transcriptase (Invitrogen). Gene expression levels were monitored by real time RT-PCR using universal SYBR green super mix (Bio-Rad) in an iCycler thermal cycler (Bio-Rad) and the melting curve analysis were carried out to confirm amplification of a single product. Total RNA was isolated from three independently grown cultures, and real-time RT-PCR experiments were performed six times with *rpoB* as an endogenous control.

***Caenorhabditis elegans* killing assay**

Bacterial virulence assays were performed using nematode model, *C. elegans* strain Bristol N2 as before with slight modifications [36]. To examine the ability of WT, Δ*abuO*, Δ*abuO*Ω*abuO* and *E. coli* OP50 strains to kill *C. elegans*, bacterial lawns of *A. baumannii* and *E. coli* control strain were prepared on nematode growth (NG) media and incubated at 37°C for 6h. The plates were kept at room temperature for 1hr and then seeded with L4-stage worms (25 to 30 per plate).

Further the seeded plates were incubated at 25°C and examined for live worms under a stereomicroscope (Leica MS5) after every 24 hours. When the worm did not react to touch it was considered dead. At least five replicates repeated three times were performed for each selected strain.

Gene cloning, expression, purification and electrophoretic mobility shift assays (EMSA)

The genome of *A. baumannii* AYE strain reveals the presence of ~ 214 signal transducing proteins (Accession No: CU459141.1). The MerR type DNA-binding HTH-type transcriptional regulator ABAYE2390 (*soxR*; 453bp, 150aa and 17.01 Kda) was amplified using gene specific primers, which had NdeI and BamHI sites of the pET28C vector to generate an N-terminal His_6-SoxR fusion protein. The ability of SoxR to bind *abuO* promoter was deciphered by EMSA as mentioned previously [33]. To confirm that the interaction between SoxR and the promoter region of *abuO* was specific, experiments with competitive (specific: 10 fold excess of cold promoter and non-specific: poly dI-dC) and non-competitive inhibitor (BSA) were also performed.

Bioinformatic analysis and Statistical analysis

The NCBI Internet server was used to perform homology searches, similarity and identity analysis, conserved domain architecture analysis. All data are presented as means ± the standard error of the mean. Statistical analysis was performed on crude data by using Student t test. P values of <0.05 were considered significant.

RESULTS

Bioinformatic analysis of *A.baumannii* TolC like protein, AbuO.

The *abuO* is composed of 1347 bp which encodes a 448aa long, type I secreted OMP AbuO (Genbank YP_001715271.1), with a predicted signal peptide sequence cleavage site at N-terminal region between Ala_{19} and Leu_{20}. Analysis established that AbuO is predicted to localize to the outer membrane and contains duplicate domains that belongs to the outer membrane efflux protein family. The sequence alignment of AbuO with homologs from different bacteria exhibited conservation at amino acid level for example to *E.coli* TolC protein: P02930 (27.8% identity & 47.9% similarity), *Salmonella enterica* subsp. *enterica* serovar Enteritidis: AAC43973.1 (27% identity & 46.6 % similarity) *Pseudomonas aeruginosa*: AGV57551.1 (25.8% identity & 44.7 % similarity) and *Vibrio cholerae*: Q9K2Y1.1 (27.9 % identity & 49.1 % similarity) (Figure 1).

Secondary structure prediction indicated that AbuO consists of three domains, β-barrel or channel domain (with four β-strands β1, β2, S4 and S5), α-helical barrel or tunnel domain (comprised of long H3, H7 and shorter α-helices H2, H4, H6 and H8) and mixed α/β domains or equatorial domain (small β strand and α-helical structures S3 and S6, and H1, H5 and H9) similar like that of *E. coli* outer membrane protein TolC (Figure 1). The MiST2 database www.mistdb.com contains genome sequences of ~ 157 *Acinetobacter* strains (142 draft and 15 complete), size ranging from 2.9Mbp to 5.0Mbp [37, 38], with the presence of putative AbuO. Multiple alignment of these putative homologs from sequenced *A. baumannii* genomes exhibited 99% identity with amino acid alterations at position 178 (asn to ser; α3 domain) and 218 (thr to ser; α4 domain) when compared to AbuO (data not shown). Overall, *in silico* analysis established AbuO to be a TolC like protein highly conserved in *A.baumannii*.

Novel contributions of AbuO, an OMP in stress response in *A. baumannii*

Analysis of growth profiles indicated that *abuO* mutant exhibited slower growth at various pH values compared to WT strain (Figure 2). When the cultures were grown in LB plates with different agar concentrations, WT cells migrated all over, while Δ*abuO* cells exhibited affected motile behavior (Figure S1-A). On the other side *in vitro* biofilm forming ability of Δ*abuO* was only ~ 0.8-fold ± 0.173 lesser compared to WT strain (Figure S1-B). Thus it indicated that *abuO* has no direct role to play in influencing the motility and biofilm forming phenotypes of the pathogen. When tested with varied concentrations of sodium deoxycholate (bile salt), survival ability of Δ*abuO* was marginally affected compared to WT (Figure 3-A). The ability of cells to grow in the presence of varied concentrations of NaCl was tested interestingly at 0.75M NaCl, percentage of survival for WT was ~ 2.15-fold ±0.024 higher compared to Δ*abuO* regardless of the inoculum size (Figure 3-B).

The *abuO* mutant exhibited >4-fold ± 0.05 stunted growth compared to WT in LB when tested in presence of varied concentrations of H_2O_2 respectively (Figure S1-C). On performing the oxidative survival assay, the *abuO* mutant exhibited 4.5-fold ±0.058 reduced survival compared to WT when treated with 3.1576 mM of H_2O_2 (Figure 3-C). Role of *abuO* in nitrosative stress response was elucidated by comparing the growth profiles and survival of WT, Δ*abuO* and Δ*abuO*Ω*abuO* in LB broth at different concentrations of SNP (Figure S1-D) or acidified nitrite (Figure S1-E), and apparently no significant change was observed. Overall results strongly suggest the involvement of AbuO, an OMP in protecting against high osmotic and oxidative stress challenges in *A. baumannii*.

Role of AbuO in conferring broad spectrum antimicrobial resistance in *A. baumannii*

Analysis of MIC values for Δ*abuO* displayed increased susceptibility to amikacin, carbenicillin, ceftriaxone, meropenem, streptomycin, tigecycline when compared to WT (Table 1). The survival of WT, Δ*abuO* and Δ*abuO*Ω*abuO* was monitored in presence of antibiotics representing different classes for *e.g.* ampicillin (Figure 4-A), neomycin (Figure 4-B), ciprofloxacin (Figure 4-C), chloramphenicol (Figure 4-D) and tetracycline (Figure 4-E). The total CFU of WT at 256 µg/ml of neomycin, 512 µg/ml of ampicillin, 16 µg/ml of tetracycline was 1.4-fold ± 0.018, 1.7-fold ± 0.089 and 1.25-fold ± 0.056 higher than Δ*abuO* cells respectively. Overall results convincingly suggested AbuO to be a novel MDR determinant in *A. baumannii*.

The *abuO* mutant cells exhibited reduced survival when exposed to different concentrations of efflux pump substrates such as EtBr (Figure S2-A), acridine orange (Figure S2-B), acriflavine (Figure S2-C), rhodamine (Figure S2-D) and safranin (Figure S2-E). Growth inhibition assay using CCCP with such substrates for *e.g.* EtBr; 4µg/ml (Figure S2-F) or antibiotic ciprofloxacin; 0.005 µg/ml (Figure S2-G) indicated stunted growth by mutant reflecting the loss of drug extrusion capacity in the isogenic mutant. Results so far corroborate AbuO to be an OMP mediating MDR *via* active efflux.

Further whole cell EtBr accumulations assays were performed to authenticate the observation. As the mutant lacks AbuO in its functional form, the fluorescence intensity was higher in *abuO* mutant relative to WT (Figure 5-A, B). Addition of CCCP further increased the fluorescence signal in mutant as the inhibitor dissipated the proton electrochemical gradient diminishing active efflux. The study with ciprofloxacin yielded a similar conclusion on loss of efflux capability by Δ*abuO* (Figure 5-C, D). Alterations in OMP profile of mutant with expression of additional bands indicates pathogen's alternative strategy to combat MDR stress

232 (data not shown). Hence, we summarize inactivation of *abuO* does distort active efflux capability
233 in *A. baumannii.*

234 Survival assays of WT, Δ*abuO* and Δ*abuO*Ω*abuO* using different concentrations of
235 benzalkonium chloride (Figure 6-A), chlorhexidine (Figure 6-B) and triclosan (Figure 6-C) and
236 growth inhibition assay (Figure S2-H) confirmed the ability of AbuO in conferring disinfectant
237 resistance in *A. baumannii*. Results in this section demonstrate that AbuO; an OMP confers
238 broad spectrum antimicrobial resistance *via* active efflux in *A. baumannii.*
239
240 **Mutation in *abuO* impacts expression of various cellular genes in *A. baumannii***
241 Compared to WT strain, the expression of RND-type (for e.g. *acrD*: 8-fold), ABC-type (*macB*:
242 18-fold) and SMR-type (*emrE*: 12-fold) efflux pumps were increased in Δ*abuO* in *A. baumannii*.
243 Altered expression of OMPs like OmpA, CarO and CsuA, together with ~ 9-fold decreased
244 expression of pilin chaperone and ~ 12-fold increased expression of *pilT* suggests possible
245 involvement of *abuO* in influencing motility and membrane permeability in *A. baumannii*.
246 Altered expression of signal transducing proteins *baeS*, *baeR* and *ompR* with down regulation of
247 *rstA* pinpoints the crucial role of *abuO* in maintaining the cellular physiology in *A. baumannii*
248 (Table 2). Complementation of *abuO* mutation almost restored expression of all the tested genes
249 (P values <0.0001), implying the overall broader role of *abuO* in *A. baumannii*.
250
251 **Role of *abuO* in virulence in *A. baumannii***
252 The *Caenorhabditis elegans* - *A. baumannii* infection model was employed to determine the
253 involvement of *abuO* in virulence [39]. The WT and Δ*abuO* strains were examined for their
254 abilities to kill *C. elegans*. The wild type strain displayed 10% and 20% killing at 96 and 120 h

255 respectively. However, the Δ*abuO* and Δ*abuO*Ω*abuO* strains killed only 5% and 8% of the
256 worms after 96 h (P<0.01) respectively. The *E.coli* strain OP50 was used as negative control.
257 Thus, our findings demonstrate that the *abuO* mutant kills *C. elegans* slowly than WT strain.
258
259 **Studies on the regulation of *abuO* by SoxR in *A. baumannii***
260 We assessed the promoter region of *abuO* and analysis revealed the presence of a conserved
261 putative SoxR binding site in the promoter (Figure S3-A). The SoxR; ABAYE2390, is a 453 bp
262 gene that encodes a polypeptide of 150aa (17.01kDa). To define the possible interaction of SoxR
263 with the promoter of *abuO*, we tested whether SoxR directly interacts with the promoter region
264 of *abuO*. We carried out gel shift assays using the ^{32}P-labeled *abuO* promoter fragment and
265 purified SoxR protein. Protein-DNA complexes formed upon incubation of SoxR with 300 bp
266 radiolabelled *abuO* promoter in reaction buffer, resolved on 5% PAGE revealed a clear
267 retardation which was directly proportional to the protein concentration (Figure S3-B). No
268 binding with absence of DNA-protein complexes in autorad on using different controls such as
269 competitive (specific: 10 fold excess of cold promoter and non-specific: poly dI-dC) and non-
270 competitive inhibitor (bovine serum albumin, BSA) in independent experiments clearly
271 demonstrated the specific DNA binding ability of SoxR to promoter region of *abuO* in *A.*
272 *baumannii*.
273
274 **DISCUSSION**
275 In this study we have shown the unprecedented involvement of AbuO, a TolC homolog in stress
276 physiology and antimicrobial resistance in *A. baumannii*. The expression of OMP in *V. cholerae*
277 and *Sinorhizobium meliloti* has been associated with susceptibility to osmotic stress [40, 41].

Bile is a substrate of AcrAB-TolC in *S.* enterica*, E. coli* and *V. cholerae* [42-44]. In the presence of osmotic/bile challenges, *abuO* mutant exhibited >1.5 – 2.0 fold lower survival capabilities than WT, and its level of growth in physiological pH was found affected, therefore, we conclude that AbuO helps bacteria survive environmental challenges such as high osmolarity and bile.

The *abuO* mutant was sensitive to oxidative stress, our observations are in agreement with the established role of TolC in *S. meliloti* and *S. enterica* [41, 45]. AbuO may possibly help in efflux of reactive oxygen species and help bacterial survival inside humans. However, a study pertaining to this hypothesis is highly warranted. The TolC-like protein HgdD of the cyanobacterium *Anabaena* sp. PCC 7120 is reported to be involved in secondary metabolite export and antibiotic resistance [46]. Our results demonstrated that inactivation of *abuO* rendered cells sensitive to various antibiotics. Subsequent assays pinpoint the crucial role of AbuO in active efflux in *A. baumannii* with broad substrate specificity. Resistance to quaternary ammonium compounds was dependent on the expression of OprR in *P. aeruginosa* [47]. Outer membrane changes in *Pseudomonas stutzeri* led to resistance to chlorhexidine diacetate and cetylpyridinium chloride [48]. In conjunction, we found AbuO also had a role in conferring biocide resistance phenotype in *A. baumannii*.

Besides, the *abuO* mutant displayed lower virulence capability suggesting that in addition to its role in multidrug efflux, this novel OMP may be involved in secretion of a toxin or virulence factor required for the pathogenesis in *A. baumannii*. Altered protein interaction/signaling events prevailing in the mutant may be different from the WT, due to which virulence defect is not fully restored upon complementation; however experiments are strictly warranted to explain the hypothesis. Alteration in expression of cellular genes in *abuO* mutant indicates a broader regulatory role of AbuO in *A. baumannii*; detailed studies may help elucidate

the interacting partners/cascade. The *abuO* mutants were constructed in different Indian clinical isolates and functionally characterized (data not shown); data analysis authenticated its conserved functions. Therefore AbuO indeed appears to be an intrinsic broad spectrum antimicrobial resistance determinant in *A. baumannii*.

CONCLUSIONS

Overall, this study reporting the wide physiological functions of AbuO in mediating stress response and antimicrobial resistance in *A. baumannii* for the very first time has brought us one step ahead in our efforts to understand the origin of multidrug resistance in *Acinetobacter*.

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TRANSPERANCY DECLARATION

The authors have declared that no competing interests exist.

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485 **Tables**

486 **Table 1**

487 **Determination of MIC for wild type, Δ*abuO* and Δ*abuO*Ω*abuO* strains in *A. baumannii***

Antibiotics	WT	Δ*abuO*	Fold change [a]	Δ*abuO*Ω*abuO*
Amikacin	256	64	4	128
Amoxicillin	>240	120	>2	>240
Ampicillin	>1024	512	>2	>256
Carbenicillin	>512	128	>4	>256
Cefepime	256	256	1	256
Ceftazidime	256	256	1	256
Ceftriaxone	256	64	4	128
Ciprofloxacin	60	30	2	60
Chloramphenicol	512	256	2	>256
Clindamycin	5	2.5	2	5
Colistin	0.01	0.01	1	0.01
Co-trimoxazole	240	240	1	240
Doripenem	24	16	1.5	24
Ertapenem	>32	32	>1	32
Gentamicin	128	128	1	128
Kanamycin	240	240	1	240
Meropenem	6	2	3	4
Nalidixic acid	>240	120	>2	>240
Neomycin	256	>128	2	256
Ofloxacin	8	4	2	8
Rifampicin	4	2	2	4
Sparfloxacin	1	1	1	1
Streptomycin	10	2.5	4	10
Tetracycline	16	16	1	16
Ticarcillin	10	10	1	10
Tigecycline	2	0.75	2.6	2
Vancomycin	>8	4	>2	>8

488

489 **Table 2**

490 **Real time PCR analysis performed in wild type and *abuO* mutant strains**

Gene Identifier	Annotation or description	Average fold change
Transport		
ABAYE1822	*adeB*; RND protein; K18146 multidrug efflux pump	-2.11 ±0.38
ABAYE1823	*adeC*; outer membrane protein; K18147 outer membrane protein	-6.50 ±1.94
ABAYE0747	RND protein (AdeB-like); K18138 multidrug efflux pump	-5.24 ±0.69
ABAYE0746	outer membrane protein AdeC-like; K18139 outer membrane protein	-2.17 ±0.51
ABAYE1796	multidrug resistance efflux pump	5.30 ±1.37
ABAYE3381	*norM*; multidrug ABC transporter; K03327 multidrug resistance protein, MATE family	1.37 ±0.07
ABAYE3248	*macB*; macrolide ABC transporter ATP-binding/membrane protein	18.59 ±2.27
ABAYE0728	MFS family transporter – *ampG* homolog	2.70 ±0.32
ABAYE1181	multidrug resistance efflux protein; K03297 small multidrug resistance family protein	12.13 ±1.93
ABAYE3515	hypothetical protein; K03298 drug/metabolite transporter, DME family	3.65 ±0.56
ABAYE0008	RND type efflux pump involved in aminoglycoside resistance (AdeT)	2.36 ±0.77
ABAYE0010	*adeT*; RND type efflux pump involved in aminoglycoside resistance	2.62 ±0.89
ABAYE0827	multidrug efflux protein	8.92 ±1.21

ABAYE0640	Outer membrane protein OmpA-like	16.87 ±2.40
ABAYE0924	porin protein associated with imipenem resistance	18.74 ±0.53
Motility		
ABAYE1319	protein CsuA/B; secreted protein related to type I pili	6.96 ± 1.45
ABAYE1857	pilin chaperone; K07346 fimbrial chaperone protein	- 9.85 ±2.19
ABAYE0304	fimbrial protein	6.32 ± 1.71
ABAYE2918	*pilT*; twitching motility protein	11.99 ± 2.28
Signaling systems		
ABAYE0599	*baeS*; kinase sensor component of a two component signal transduction system	14.51 ±0.83
ABAYE0600	*baeR*; OmpR family transcriptional regulator	4.67 ±0.50
ABAYE0259	*ompR*; osmolarity response regulator	10.43 ±0.76
ABAYE3064	*rstA* response regulator	-4.22 ±0.54

491

492 **Table footnotes**

493 **Table 1**

494 **Determination of MIC for WT, Δ*abuO* and Δ*abuO*Ω*abuO* strains in *A. baumannii***

495 E-strips were used to determine the precise MIC for different group of antibiotics such as
496 amikacin, amoxicillin, ampicillin, carbenicillin, cefepime, ceftazidime, ceftriaxone,
497 ciprofloxacin, chloramphenicol, clindamycin, colistin, co-trimoxazole doripenem, ertapenem,
498 gentamicin, kanamycin, meropenem, nalidixic acid, neomycin, ofloxacin, rifampicin,
499 sparfloxacin, streptomycin, tetracycline, ticarcillin, tigecycline and vancomycin following the
500 CLSI guidelines. Complementation restored the MIC values. Units for MIC values are µg/ml.

501 [a] Fold change is the ratio of MICs for WT and Δ*abuO*.

502

503 **Table 2**

504 **Real time PCR analysis performed in wild type and *abuO* mutant strains.**

505 The gene expression was normalized to endogenous control (*rpoB*) and the average fold change
506 was reported relative to the wild-type from at least six independent experiments along with
507 standard deviations.

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Figure legends

Figure 1

Multiple sequence alignment of *abuO* and its homologs

Sequence alignments were made in CLUSTAL Omega (https://www.ebi.ac.uk/Tools/msa/clustalo) and formatting using the ESPript server (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi). The accession number of TolC homologs from different bacteria are given in brackets respectively; *Escherichia coli* (P02930), *A.baumannii* (ABAYE3514) *Yersinia pestis* KIM10+ (AAM87064.1), *Enterobacter aerogenes* (CAD13188.1), *Salmonella enterica subsp. enterica serovar Enteritidis* (AAC43973.1), *Vibrio cholerae* (Q9K2Y1.1), *Erwinia amylovora* (CBA19383.1), *Pseudomonas aeruginosa* PAO581 (AGV57551.1). The predicted secondary structural elements of *A.baumannii* AbuO are shown on the lines above the sequence alignment using PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/). The arrows indicate β-sheet, the coils indicate α-helices, TT indicates β turns and η indicates 3_{10} helices. Residues strictly conserved have a black background and is indicated by bold letters; residues conserved between groups are boxed.

Figure 2

Bacterial growth curves: Impact of inactivation of *abuO* in *A. baumannii*

Effect on bacterial growth was monitored for WT, Δ*abuO* and Δ*abuO*Ω*abuO* in LB medium at varied pH. The patterns of representative pH {5.0 (A), 6.0 (B), 7.0 (C), 8.0 (D) and 10.0 (E); $P < 0.01$} are shown here. At 540 min the mutant exhibited 1.21-fold ± 0.057 (at pH 5.0), 1.32-fold ± 0.032 (at pH 6.0), 1.37-fold ± 0.043 (at pH 7.0), 1.74-fold ± 0.027 (at pH 8.0) and 1.57-fold ± 0.067 (at pH 10.0) slower growth compared to WT. The other tested pH conditions 3.0 and 4.0

539 was toxic to both the cultures. Complementation restored the growth defect. The data presented
540 is the means of triplicate measurements performed three times.
541
542 **Figure 3**
543 **Stress challenge assays: Effect of loss of OMP AbuO in *A. baumannii***
544 A. The ability of WT to survive in the presence of varied deoxycholate concentrations (16, 64,
545 256, 1024 and 4096 µg/ml) was compared with Δ*abuO* ($P < 0.01$).
546 B. The survival ability of WT tested under varied NaCl concentrations (0.075, 0.15, 0.25, 0.5
547 and 0.75M) and compared with Δ*abuO* ($P=0.004$).
548 C. The survival ability of *abuO* mutant was analysed in presence of varied H_2O_2 concentrations
549 (0.07894, 0.7894, 1.5788, 2.3682 and 3.1576 mM) ($P= 0.0023$). Asterisk indicate significant
550 difference in mutant with respect to WT (* $P < 0.01$)**.**
551
552 **Figure 4**
553 **Contributions of AbuO in antibiotic resistance in *A. baumannii***
554 The survival ability of WT in the presence of A) ampicillin [$P = 0.001$], B) neomycin [$P =$
555 0.001], C) ciprofloxacin [$P= 0.003$], D) chloramphenicol [$P = 0.018$] and E) tetracycline [$P =$
556 0.003] at different concentrations (0.5, 4, 16, 64, 256 and 1024 µg/ml) were compared to Δ*abuO*.
557 All data presented here is the mean of independent measurements performed three times.
558 Asterisk indicate significant difference in mutant with respect to WT (* $P < 0.01$)**.**
559
560
561

562 **Figure 5**

563 **Flourimetric accumulation assay**

564 When treated with varied concentrations of fluorescent substrates in independent experiments in
565 the presence of 0.4% glucose at 37°C, the fluorescence intensity was relatively lower in WT
566 compared to Δ*abuO*. At 40.1 min the WT exhibited (1.21- fold - 0.01 µg/ml; 0.85- fold - 0.05
567 µg/ml; 1.04- fold - 0.5 µg/ml; 1.07- fold – 1.0 µg/ml; 1.04- fold – 2.0 µg/ml; 1.21- fold – 4.0
568 µg/ml; 1.04- fold – 6.0 µg/ml; 1.06- fold – 8.0 µg/ml) lower EtBr accumulation when compared
569 to mutant respectively (A, B). At 50.1 min, the WT exhibited (1.0- fold - 0.01 µg/ml; 1.02- fold -
570 0.05 µg/ml; 1.44- fold - 0.5 µg/ml; 1.67- fold – 1.0 µg/ml; 1.72- fold – 2.0 µg/ml; 1.43- fold – 4.0
571 µg/ml; 1.98- fold – 6.0 µg/ml; 1.35- fold – 8.0 µg/ml) lower ciprofloxacin accumulation
572 compared to mutant respectively (C, D). The fluorescence was monitored in spectrofluorometer
573 (Hitachi) at 37°C.

574

575 **Figure 6**

576 **Biocide challenge assays: Result of loss of functional AbuO in *A. baumannii***

577 The survival ability of WT in the presence of A) benzalkonium chloride (at 6.4 µg/ml was 1.8-
578 fold ±0.091; P = 0.005), B) chlorhexidine (at 3.2 µg/ml was 1.2-fold ±0.045; P = 0.001) and C)
579 triclosan (at 0.001 µg/ml was 1.28-fold ±0.034; P = 0.001) was higher when compared to Δ*abuO*
580 and complementation restored the phenotype. All data presented here is the mean of independent
581 measurements performed three times. Asterisk indicate significant difference in mutant with
582 respect to WT (* P < 0.01)**.**

583

584

Figure 1

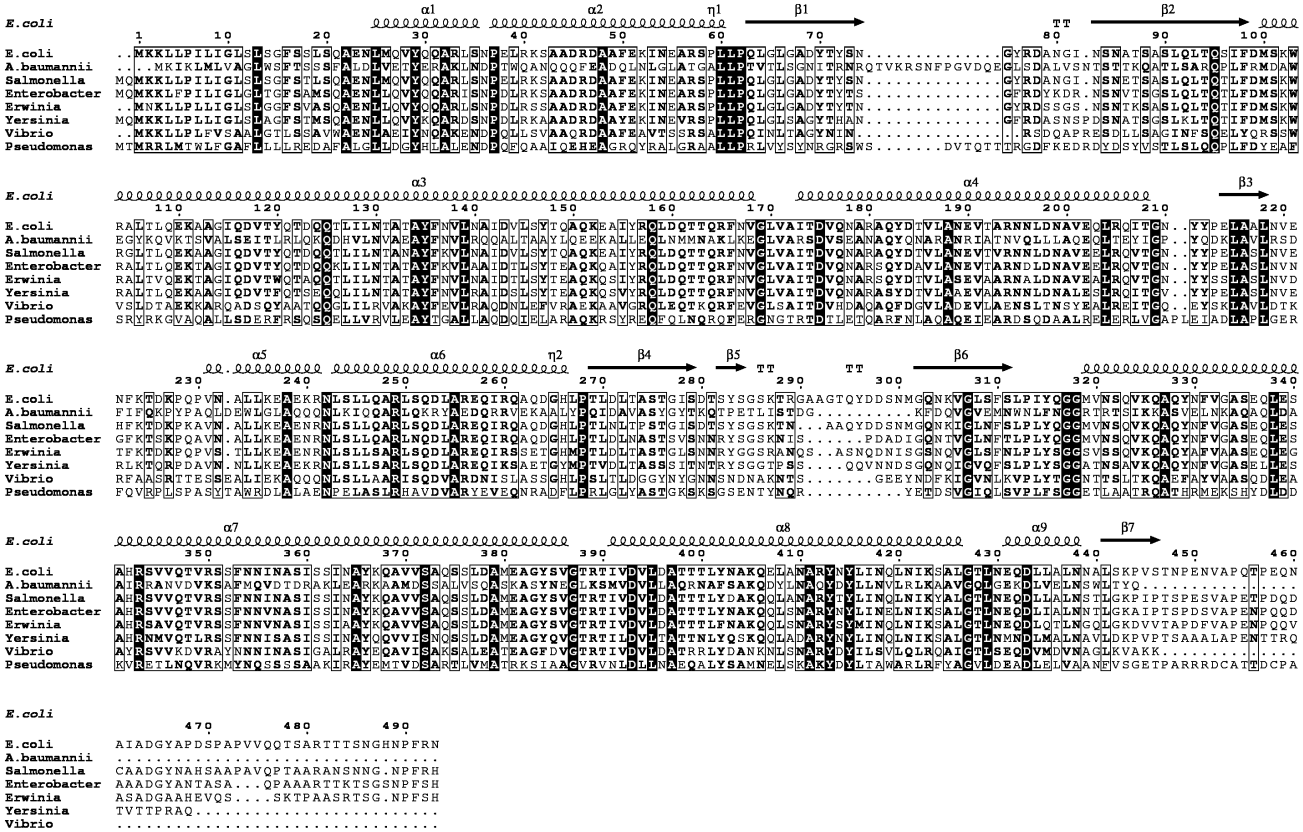

Figure 2

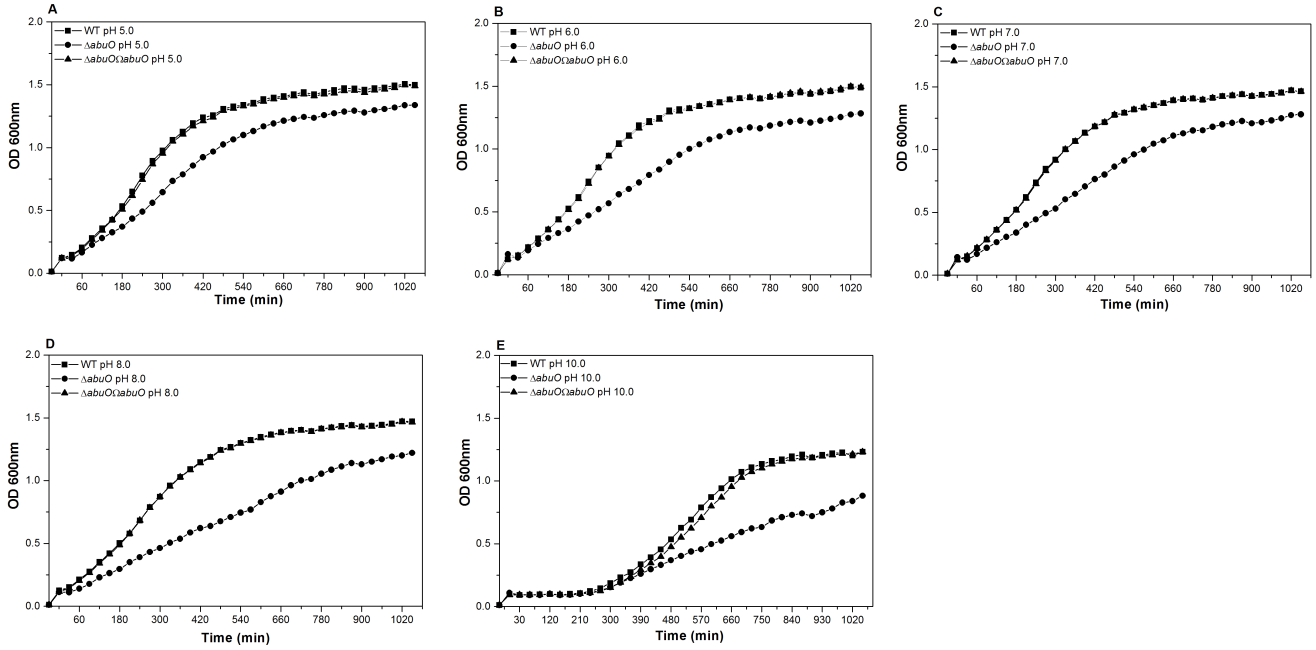

Figure 3

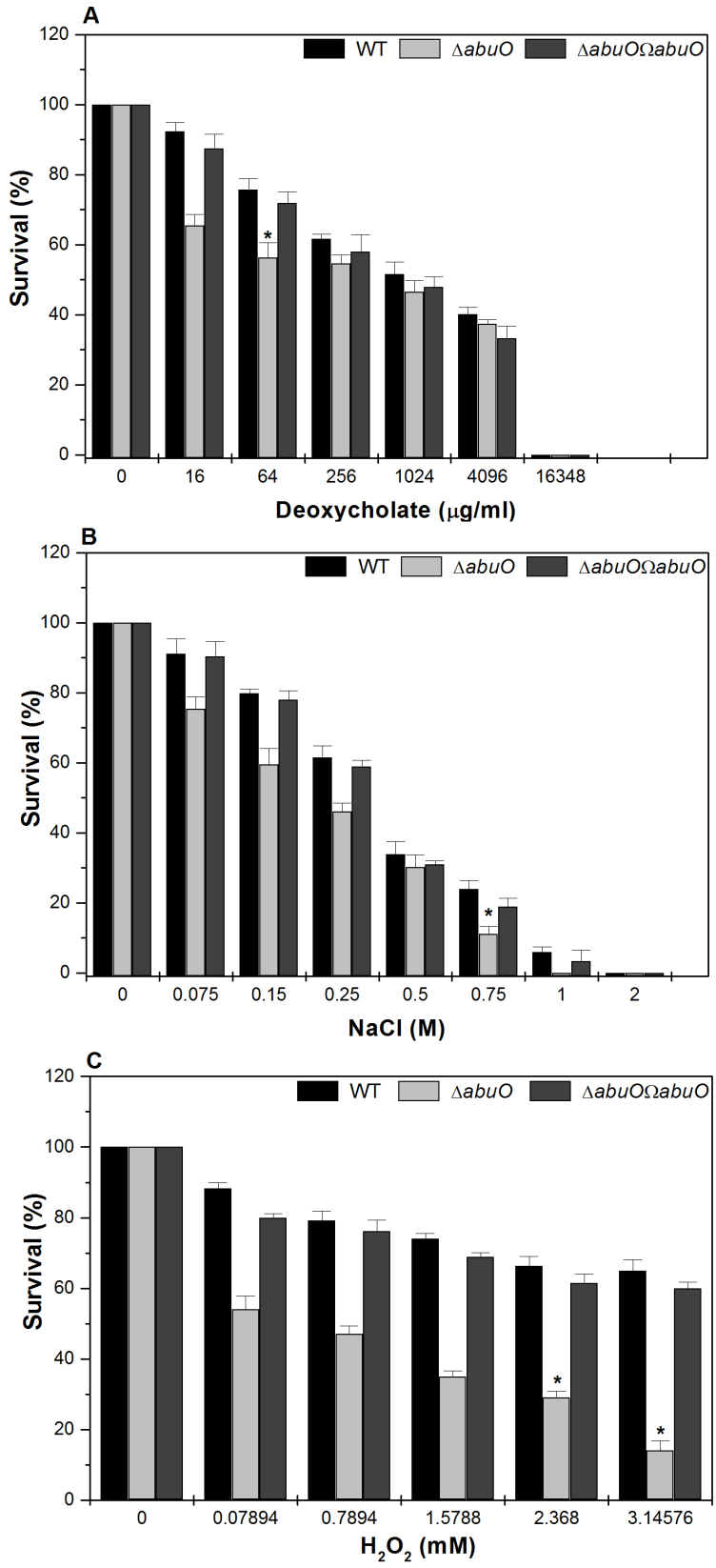

Figure 4

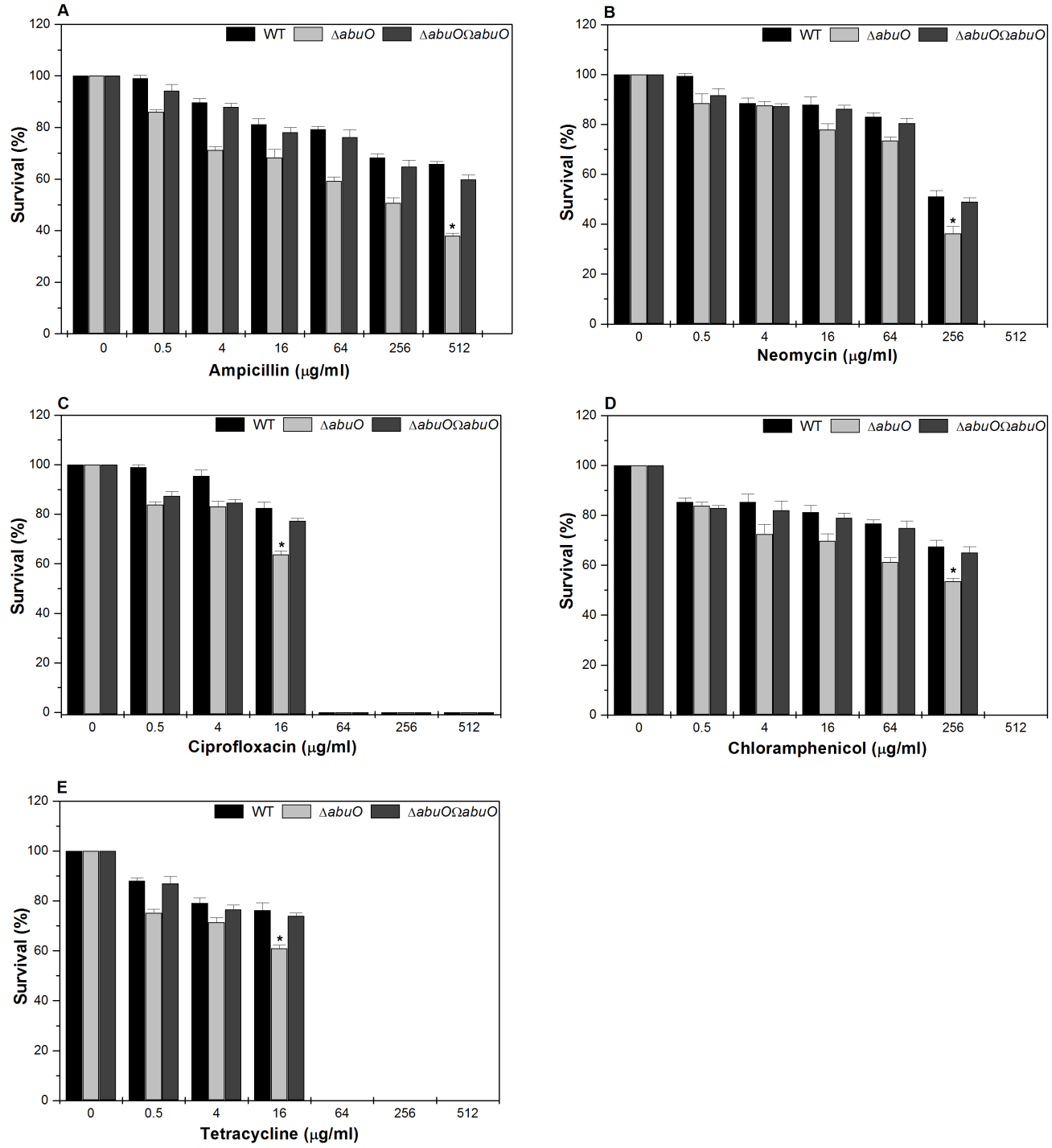

Figure 5

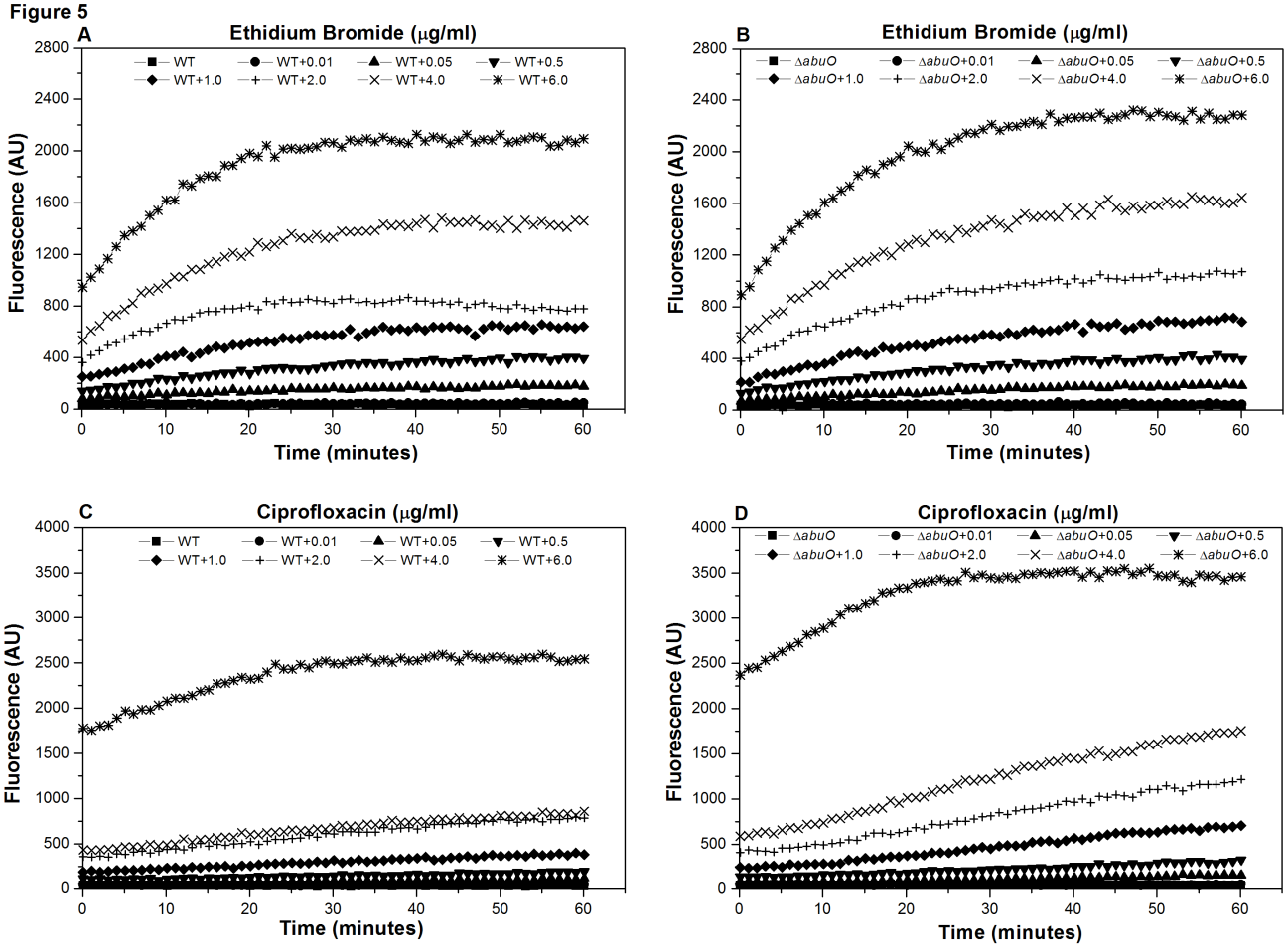

Figure 6