Water occupancy in the *Acinetobacter baumannii* F-ATP synthase *c*-ring and its implications as a novel inhibitor target

Alexander Krah[1,*], Vandana Grover[2], Tuck Choy Fong[2], Peter J. Bond[1,3,**] and Gerhard Grüber[1,2,4,***]

[1]Bioinformatics Institute (BII), Agency for Science, Technology, and Research (A*STAR), 30 Biopolis Street, #07-01 Matrix, Singapore 138671

[2]School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, Singapore 637551

[3]Department of Biological Sciences, National University of Singapore, 16 Science Drive 4, Singapore 117558

[4]Lead contact

[*]Correspondence: kraha@bii.a-star.edu.sg

[**]Correspondence: peterjb@bii.a-star.edu.sg

[***]Correspondence: ggrueber@ntu.edu.sg

SUMMARY

The *Acinetobacter baumannii* F_1F_O-ATP synthase is essential for the opportunistic human pathogen. Its membrane-embedded F_O-domain consists of the *c*-ring and subunit *a*. The *c*-ring translocates protons via a conserved carboxylate across the membrane via two half-channels in subunit *a* and its revolution enables the F_1-domain to carry out ATP formation. Here, we used molecular dynamics simulations, free-energy calculations, and *in vivo* mutational experiments to assess the likely existence of water molecules in the binding site of the *A. baumannii c*-ring. We first predicted its binding site structure in the ion-locked conformation, and extrapolated the presence of two water molecules in the ion-binding site. Based on our predictions, amino acid point mutations confirmed the critical role of key residues involved in the water binding-site upon ATP synthesis ability and cell growth. We discuss the implications of our findings in the context of rational drug design to target the *A. baumannii* F_O-domain.

KEYWORDS

ATP synthase; MD simulations; *c*-ring; *Acinetobacter baumannii*

INTRODUCTION

F_1F_O-ATP synthases are molecular motors which synthesize ATP. The catalytic event is carried out in the F_1-domain by the asymmetric $\alpha_3\beta_3$ catalytic hexamer[1], which changes its conformation in each dimer when the central stalk γ rotates[2]. This rotation is driven by the *c*-ring, a multi-*c*-subunit-protein assembly in the membrane-embedded F_O domain (Figure 1a). In synthesis direction, a *c*-ring rotary motion[3] is driven by a sodium-[4] or in most cases a proton gradient[5,6]; the selectivity of the *c*-ring is defined by its ion/proton-binding site composition[7], and the ion/proton is bound and released by an essential, conserved carboxylate residue[8] via two solvated half-channels in subunit *a*[9], which is also part of the F_O-domain. To reach the conserved carboxylate *c* subunit residue, which is located at the approximate midpoint across the bilayer membrane, the proton is proposed to pass the inlet half-channel via polar and charged residues and water wires, following a Grotthuss-type mechanism[10,11]. After protonation, the protonated carboxylate residue of the *c*-ring rotates within the lipid membrane to release the proton into the exit half-channel. Water-mediated proton transfer to the proton acceptor of the exit half-channel has been proposed[10,11]. *Vice versa*, the *c*-ring can rotate in the opposite direction, transporting protons to the other side of the membrane bilayer, driven by ATP hydrolysis in the F_1 domain[12].

Recently, the cryo-electron microscopy (cryo-EM) structure of the F_1F_O-ATP synthase from *Acinetobacter baumannii*, which is one of the most common multidrug-resistant pathogens, has been resolved[13]. As a strictly aerobic, nonfermenting bacterium[14], *A. baumannii* relies on oxidative phosphorylation to form ATP, making the F_1F_O-ATP synthase essential for its survival[15]. The cryo-EM structure reveals the F_1-domain subunits α_3:β_3:γ:ε, the proton-translocating F_O-domain subunits *a*:c_{10}, and the peripheral stalk subunits δ:b_2 ([13], Figure 1a). It also highlights an *A. baumannii* specific extension at the N-terminus of subunit *a*, leaving the location of the proton entrance open[13]. Unfortunately, the resolution does not allow water

molecule density to be assigned to the two half-channels or the proton-binding site, including at the carboxylate residue *c*D60.

Molecular dynamics (MD) simulations have been demonstrated to be a powerful tool for predicting the presence of water molecules in the *c*-ring proton-binding site of the *Escherichia coli* [16] and *Bacillus* PS3 F-ATP synthases[17]. Here, we used MD simulations and free energy calculations of the *c*-ring from *A. baumannii*, revealing that its *c*-ring likely harbours two water molecules in the proton-binding site in its ion-locked conformation. Experimentally mutating residues, predicted to be involved in water-binding, drastically inhibits ATP synthesis of the *A. baumannii* F-ATP synthase and affects growth. Since (i) the *c*-ring turbine and the subunit *a*/*c*-interface have been identified as anti-tuberculosis (anti-TB)[18] and anti-non-tuberculous mycobacteria (anti-NTM)[19] drug targets, and (ii) *A. baumannii* depends on oxidative phosphorylation, such detailed structural- and mechanistic information of its *c*-ring opens the door for targeting its aqueous cavity with novel compounds.

RESULTS AND DISCUSSION

Extended simulations elucidate the proton-binding site of the *c*-ring from *A. baumannii*

Previously, structural data for several *c*-rings have been reported, while MD simulations have clarified specific features that were not observed due to certain experimental limitations[16,17]. Examples include distinguishing between a water and hydronium ion[20] in the proton binding site from *Bacillus pseudofirmus* OF4[21], the presence of a water molecule in the Na^+ coupled *c*-ring from *I. tartaricus*[22] and the proton coupled *c*-ring from *Bacillus PS3*[17] where the ExxG and ExxP sequences respectively contribute to the interaction of water in the ion/proton-binding site. Analogously, we aimed to study the ion-binding site of the *A. baumannii c*-ring in its ion-locked conformation. According to amino acid sequence alignment it harbours a GxxDxxP-

79 motif (Figure 1b), leading us to hypothesise that at least one water molecule resides in the ion-
80 binding site.

81 To clarify the ion-binding site of *A. baumannii*, we first applied conventional MD
82 simulations. In two different sets of simulations with slightly different treatment of the non-
83 bonded interactions (see STAR★Methods) we found that after 3 μs of unbiased simulation, 8-
84 9 of the 10 ion-binding sites in the *c*-ring became spontaneously occupied with two water
85 molecules (Figure 2a)). In each occupied site, the key-aspartate *c*D60 donates a hydrogen bond
86 to Wat1:O. Wat1 simultaneously donates a hydrogen bond to *c*L58:O and Wat2. Wat2 accepts
87 hydrogen bonds from Wat1 and *c*V62:N, and donates hydrogen bonds to *c*T23:Oγ1 and *c*L59:O.
88 In addition, *c*T23:Oγ, which could potentially coordinate / accept a hydrogen bond of the key-
89 glutamate, donates a hydrogen bond to G19:O. However, during the simulations another
90 coordination variant was occasionally observed, whereby D60:Oδ2 binds to T23:Oγ (Figure
91 S2). We speculate that this configuration represents a local energetic minimum and extended
92 timescale may be required for the migration of additional water molecules to the binding sites,
93 as this configuration is less frequently observed compared to the state harbouring two water
94 molecules. In summary, we predict that two water molecules likely bind in the *A. baumannii c*-
95 ring proton-binding site, where Wat1 accepts the proton of *c*D60 and is further stabilized by
96 Wat 2 (Figure 2c), thereby providing an energetically favourable environment for the
97 protonated *c*D60 when the *c*-ring rotates within the lipid bilayer. We further observe that Wat1
98 is bound more stably to D60:Oδ2 and that Wat2 which is coordinated by V62:N exhibits higher
99 on/off events, while Wat1 is bound relatively stable (Figure S3).

100 The predicted ion-binding site is significantly different from the one solved by cryo-EM[13]
101 (Figure 2b)), as shown in Figure 2c). In the cryo-EM structure, most of the *c*-subunits are found
102 in an open conformation (*c*D60 is facing towards the bulk), which tends to be observed in a
103 solvated environment[23–25], but not in the membrane environment form when the key-

carboxylate binds a proton[20,26]. In addition, no water molecules are observed in the ion-binding site of the *c*-ring. The cryo-EM structure may represent a trapped frozen state not reflective of the biological membrane-bound environment. In the more physiologically realistic modelled membrane state, we observed spontaneous closure of *c*D60 to the ion-locked conformation, which is stabilized by water molecules at the site.

Free energy calculations confirm the solvated structure of the proton-binding site

To assess the energetic stability of the water molecules found in the *c*-ring site from *A. baumannii*, we calculated their free energy using three different approaches (see STAR★Methods). First, we applied an approach previously used to clarify the occupancy in the ion-binding site of the *c*-ring from *E. coli*, annihilating two water molecules simultaneously[16]. In a second approach we annihilated first Wat1 and then Wat2, while in a third we annihilated first Wat2 and then Wat1. All three approaches revealed that the presence of the pair of water molecules energetically favourable in their respective binding site and both setups show similar results (please refer to the STAR★Methods part for differences in setup 1 and setup 2) (Table 1). We further calculated the solvation free energy of a water molecule in the bulk, denoting -6.5 ± 0.03 kcal/mol (setup 1) and -6.5 ± 0.01 kcal/mol (setup 2), which is in good agreement with previously published results[27].

Therefore, the free energy data are consistent with the conventional MD simulations, and we postulate that in the proton-binding site from *A. baumannii* two water molecules are harboured. It should be mentioned that the results of the different water annihilation sequences are slightly different, but all three approaches show a favourable binding free energy for two water molecules occupying the site. The differences may occur from an artificial binding site composition during the desolvation process, which may not be resolved accurately in the simulation time. We also observe sites which only harbour one water molecule (Figure 2a). To

129 test whether the presence of one water molecule is also favourable, we carried out free energy
130 calculations. These calculations show binding free energies of +0.8 ± 0.6 / +0.6 ± 0.3 kcal/mol
131 for the presence of water. Thus, our results indicate that binding one water molecule is
132 energetically unfavourable and is likely a structural transition state of the ion-binding site on
133 the pathway to complete solvation; this hypothetical state is shown in Supplementary Figure
134 S4.
135
136 **Biochemical characterization of the critical residues *c*T23, *c*L59, and *c*D60A**
137 To assess the importance of the *c*-subunit residues including *c*T23, proposed to
138 coordinate/accept a hydrogen bond with a water molecule in the binding site (Figure 2c), and
139 *c*L59, in proximity to *c*D60 and *c*T23, in proton translocation and finally ATP synthesis, we
140 cloned the *atp*-operon encoding the entire *Ab*F_1F_O-ATP synthase for the WT or its mutants
141 (*c*T23A, *c*L59A, and *c*D60A), produced the enzyme in *E. coli* DK8 cells and generated IMVs
142 to measure ATP synthesis of the WT and its variants, driven by the electron donor NADH.
143 Similar amounts of WT and variant proteins were obtained as determined by the BCA assay
144 and visualized in the 12% SDS-PAGE, showing a uniform intensity of the 55 kDa band across
145 the samples, corresponding to the subunit α and β[13,28] (Figure 3a)) as confirmed by LC-MS/MS
146 (Supplementary Table 1). This signifies comparable expression of the variants relative to the
147 WT *Ab*F_1F_O-ATP synthase. The F-ATP synthase-deficient *E. coli* DK8 IMVs showed no
148 expression of the *Ab*F_1F_O-ATP synthase, reflected by the absence of the characteristic 55 kDa
149 band (Figure 3a)). When compared to the IMVs containing WT *Ab*F_1F_O ATP synthase (2.83 ±
150 0.13 nmol·min^{-1}(mg protein)$^{-1}$), the IMVs harbouring *Ab*F-ATP synthase variants *c*T23A,
151 *c*L59A, and *c*D60A revealed a drastically reduced ATP synthesis activity of 0.03 ± 0.01
152 nmol·min^{-1}(mg protein)$^{-1}$, 0.27 ± 0.21 nmol·min^{-1}(mg protein)$^{-1}$, and 0.08 ± 0.03
153 nmol·min^{-1}(mg protein)$^{-1}$, respectively (Figure 3b)). These data underline that the *A.*

154 *baumannii c*-subunit residues T23, L59, and D60 are critical for proper proton transport and
155 ATP synthesis.

156 To further explore whether the significant reduction in ATP synthesis for the mutant *Ab*F_1F_O-
157 ATP synthase variants *c*T23A and *c*D60A is in congruence with growth kinetics, a minimal
158 media growth curve experiment was set up, wherein succinate was the sole carbon source. This
159 limited the ATP synthesis of the oxidative phosphorylation pathway and focused on the
160 significance of the essential energy-producing machinery, the F_1F_O ATP synthase. *E. coli* DK8
161 cells harbouring the WT*Ab*F_1F_O ATP synthase exhibited the most growth in succinate minimal
162 media (0.24 ± 0.09 h^{-1}) (Figure 3c)), whereas the F-ATP synthase-deficient *E. coli* DK8 cells
163 as a negative control showed no growth (0.04 ± 0.0005 h^{-1}) in minimal media, confirming the
164 reliability of this assay. In trend with the ATP synthesis results (Figure 3b)), the mutant *Ab*F_1F_O-
165 ATP synthase mutants *c*T23A and *c*D60A showed severely reduced growth rates of 0.02 ±
166 0.002 h^{-1} and 0.03 ± 0.009 h^{-1}, respectively, in succinate minimal media (Figure 3c)).
167
168 **Variations in *c*-ring residues harbouring water molecules and its therapeutic implications**
169 It has previously been claimed that a P/GxxE or ExxP/G motif[17] is required to harbour a
170 water molecule in the ion/proton-binding site of the *c*-rings from *E. coli*[16], *I. tartaricus*[22] or
171 *Bacillus PS3*[17]. The predicted *A. baumannii* proton-binding site harbours two water molecules,
172 held in place by the amino acids *c*T23, along with *c*L58, *c*L59, *c*D60, and *c*V62, which belong
173 to the related GxxDxxP sequence of the *c*-ring in this pathogen (Figure 1b). In comparison, the
174 *c*-subunit from *B. pseudofirmus* OF4 contains a PxxExxP motif, but only one water molecule
175 is found in its ion-binding site[21], indicating that the reduced side chain length of aspartate
176 compared to glutamate may in part provide space for a second water molecule. As shown for
177 the *Mycobacterium phlei c*-ring[29], which like the related protein from *Mycobacterium*
178 *tuberculosis* contains a GxxExxY sequence, two water molecules have been found in the X-ray

179 structure. These comparisons highlight the common existence of one or two water molecules at
180 the ion/proton-binding site, whose number may in part reflect variations in the P/GxxE/DxxP/Y
181 sequence. Importantly, this study underscores the importance of the *A. baumannii* residues *c*T23
182 and *c*L59 in harbouring the water molecules and governing proton-translocation during ATP
183 synthesis. These residues also differ compared to their counterparts, including the
184 mycobacterial residues *c*A24 and *c*V60 and human mitochondrial *c* ones, *c*S87 and *c*S123
185 (Figure 1b), which may indicate fine-tuned structural alterations of the ion-binding site for
186 adaptation to various specific membrane-compositions and physiological conditions. The
187 amino acid alignment also demonstrates the significant variation of amino acid composition of
188 *c*-subunits among bacteria and human mitochondria (Figure 1b) and underscores the similarity
189 of the *A. baumannii* strains, while different to others. This is in line with the target specificity
190 of the anti-TB and anti-NTM inhibitors bedaquiline (BDQ)[18], TBAJ-876[30,31] or TBAJ-5307[19],
191 binding preferentially with high affinity to the *c*-ring and *a*/*c*-interface of the mycobacterial F_O-
192 domain[32] and lower affinity to the human F-ATP synthase[33,34], and highlights the relevance of
193 the *c*-ring and its proton-translocating partner subunit *a* as an attractive drug target. Even slight
194 changes, either in the sequence or the chemical structure of the drug can cause remarkably
195 altered binding affinities of the compounds[35,36]. Here, we show for the *A. baumannii* F-ATP
196 synthase *c*-ring that two water molecules are harboured in the proton-binding site by residues
197 essential for proton transport, ATP synthesis and viability (Figure 3). These residues differ from
198 the human host mitochondrial counterpart, making this epitope a suitable *A. baumannii* target
199 for inhibitors to bind, with reduced tendency for off-target toxicity. Furthermore, the water-
200 filled proton-binding site would allow compounds with aqueous solubility to bind, further
201 preventing the toxicity effects described for the anti-TB- and anti-NTM drug BDQ[33].
202
203

Limitations of the study

First, it should be noted that our approach only clarifies the ion-binding site in its ion-locked conformation when facing the membrane environment. The *c*-ring's proton binding site structure at the *a*/*c*-interface is expected to be different, as the two half channels[9] are solvating the essential aspartate (D60), which causes an outward-facing conformation of the proton translocating carboxylate side chain[23–25]. Also, the role of the extended α-helix in subunit *a*, which faces an additional *c*-subunit, and the potential influence of this extension on the key carboxylate in this *c*-subunit, are yet to be revealed.

Second, longer simulations are required to observe all 10 *c*-ring binding sites to be occupied by two water molecules. To derive the underlying energetics features of the presence of two water molecules, we carried out free energy calculations. Based on these energetics, we could extrapolate the possibility of the presence of water molecules. However, there are some discrepancies in the free energy calculations. These differences may arise from the relatively short simulation time of 1 ns per window. Increased sampling may lead to a free energy estimate with increased accuracy; thus, longer sampling may be applied in future projects. However, as all free energy calculations clearly support the presence of two water molecules, the structural feature of the presence of two water molecules derived from our free energy calculations is reliable.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Gerhard Grüber (ggrueber@ntu.edu.sg).

Materials availability

Plasmids generated in this study will be made available upon request to the lead contact.

Data and code availability

- All data reported in this paper will be shared by the lead contact upon request
- This paper does not report any original code.
- Any additional data required to reanalyze the data reported in this paper is available from the lead contact upon request.

ACKNOWLEDGMENTS

AK and PJB were supported by BII core funds. This research was supported by the National Research Foundation (NRF) Singapore, NRF Competitive Research Programme (CRP), Grant Award Number NRF-CRP27-2021-0002 to G.G.

AUTHOR CONTRIBUTIONS

Conceptualization: A.K. and G.G.; Investigation: A.K., V.G. and T.C.F.; Formal analysis: A.K. V.G., T.C.F., P.J.B. and G.G.; Visualization: A.K. and V.G.; Writing-original draft: A.K. and G.G.; Writing - review and editing: A.K., V.G., T.C.F., P.J.B. and G.G.; Supervision: P.J.B. and G.G.; Funding acquisition: P.J.B. and G.G.

DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTARY INFORMATION

Document S1: Figures S1-S4 and Table S1

Table 1: Binding free energy calculations for different setups. For details of the differences of setup 1 and setup 2, refer to the STAR★Methods section. In row 1 the order of desolvation in the ion binding site is indicated. All data contain the appropriate entropic correction. Averages and errors (standard deviation) were calculated from the collective data of three independent runs.

Setup / Desolvation of	**Setup 1**	**Setup 2**
Wat1 and Wat2 simultaneously	-2.1 ± 0.3	-1.8 ± 0.2
Wat1 then Wat2		
Wat1	-0.6 ± 0.1	-0.2 ± 0.3
Wat2	-2.1 ± 0.2	-1.6 ± 0.3
Wat2 then Wat1		
Wat2	-0.6 ± 0.3	-0.5 ± 0.1
Wat1	-0.3 ± 0.3	-0.4 ± 0.2

STAR★Methods

KEY RESOURCES TABLE (to be kept either here or in a separate word file)

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
E. coli TOP10	Invitrogen by ThermoFisher Scientific, United States	C404050
E. coli DK8 strain	Klionsky et al.[51]	N.A.
Chemicals, peptides, and recombinant proteins		
DpnI Restriction Enzyme	New England Biolabs, United States	R0176L
Isopropyl β-d-1-thiogalactopyranoside (IPTG)	Bio Basic Asia Pacific, Singapore	IB0168
Pierce Protease Inhibitor Tablets, EDTA-free	ThermoFisher Scientific, United States	A32965
Carbenicillin Disodium Salt	Sigma-Aldrich, United States	C1389
Tryptone Powder	Bio Basic Asia Pacific, Singapore	TG217(G211)
Yeast Extract	Bio Basic Asia Pacific, Singapore	G0961
Luria Bertani Powder	Bio Basic Asia Pacific, Singapore	SD7002G
Critical commercial assays		
KAPA HiFi HotStart PCR Kit	Roche, Switzerland	KK2501
GeneJet Miniprep Kit	ThermoFisher Scientific, United States	K0503
Pierce BCA Protein Assay Kit	ThermoFisher Scientific, United States	23227
CellTiter-Glo® Luminescent Cell Viability Assay	Promega, United States	G7571
Oligonucleotides		
Oligonucleotides used in this study are listed in the Method Details	This study	N.A.
Software and algorithms		
CHARMM-GUI Membrane Builder	Lee et al.[38]	https://www.charmm-gui.org
GROMACS 2018	Abrahams et al.[46]	https://www.gromacs.org
VMD 1.9	Humphrey et al.[49]	https://www.ks.uiuc.edu/Research/vmd/
UCSF Chimera	Pettersen et al.[50]	https://www.rbvi.ucsf.edu/chimera/

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

E. coli TOP10 strain

Electrocompetent *E. coli* TOP10 cells (Invitrogen by ThermoFisher Scientific, United States) were used for recombinant plasmid propagation to facilitate their isolation and sequencing to

confirm the single-point substitutions. This strain was grown in Luria-Bertani (LB) medium (Bio Basic Asia Pacific, Singapore) supplemented with 50 μg/ml of carbenicillin (Sigma-Aldrich, United States) at 37 °C, 180 rpm (Infors HT Minitron, Switzerland).

***E. coli* DK8 strain**

Electrocompetent *E. coli* DK8 cells (*1100 Δ(uncB-uncC) ilv::Tn10*) were procured from Klionsky et al.[51] for upscaled production of the mutant F_1F_O-ATP synthase protein. This strain was grown in 2xYT medium (Luria-Bertani powder (Bio Basic Asia Pacific, Singapore), tryptone powder (Bio Basic Asia Pacific, Singapore), and yeast extract (Bio Basic Asia Pacific, Singapore)) supplemented with 50 μg/ml of carbenicillin (Sigma-Aldrich, United States) at 37 °C, 180 rpm (Infors HT Minitron, Switzerland).

METHOD DETAILS

Conventional molecular dynamics simulations

We simulated the isolated *c*-ring of *A. baumannii* (PDB-ID: 7P2Y)[13] after inserting the protein into a POPC (1-palmitoyl-2-oleoyl-glycero-3-phosphocholine) model membrane, using the CHARMM-GUI[37] membrane builder module[38]. As a central plug in the *c*-ring has been found previously[39], we also modelled this plug as described elsewhere[17]. No water molecules were initially modelled in the proton-binding site. The molecular system is shown in Supplementary Figure S1. The CHARMM36m[40], CHARMM36[41] and TIP3P water model[42] were used for protein, lipids and water, respectively. We prepared two different setups, calculating van der Waals interactions up to 12 Å with a switching function of either 8 Å (setup 1) or 10 Å (setup 2), as these parameter settings are recommended for membrane systems[41] or standard CHARMM configurations[40], respectively. Electrostatic interactions were calculated using PME with a 12 Å real space cut-off. Temperature and pressure were kept constant at 300

K and 1 bar, using the v-rescale thermostat[43] and Parinello Rahman barostat[44], respectively. The LINCS algorithm[45] was used to constrain bonds involving hydrogen atoms. For both setups an equilibration protocol of 20 ns with position restraints applied to protein backbone atoms was caried out, followed by a production run of 3 μs. We carried out one simulation for both setups. All simulations were carried out with the program suite GROMACS (v. 2018)[46].

Free energy calculations

First, we calculated the solvation free energy of a water molecule and compared this to previously derived results[27], followed by thermodynamic integration (TI) calculations of water molecules harboured in the ion binding site. These calculations were carried out via three different strategies: (i) two water molecules in the ion-binding site were annihilated simultaneously[16]; (ii) the first water (Wat1) was annihilated, followed by the second water (Wat2); or (iii) Wat2 was first annihilated before Wat1 was annihilated. 20 windows were used for each free energy calculation. 1 ns simulations were carried out for each window, discarding the first 100 ps as equilibration time. A Beutler soft-core potential[47] was applied. The results were derived by the bar method[48] and a thermodynamic cycle, as described previously[17]. An entropic correction to Wat1 and Wat2 was applied in all three free energy calculations strategies:

For water molecules annihilated in sequence, one by one:

$$\Delta G_{b1}^{0} = \Delta G_{site1} - \Delta G_{bulk} - k_B T ln C^0 V_{site1} \quad (1)$$

$$\Delta G_{b2}^{0} = \Delta G_{site2} - \Delta G_{bulk} - k_B T ln C^0 V_{site2} \quad (2)$$

with the binding free energy then derived by:

$$\Delta G_{b}^{0} = \Delta G_{b1}^{0} + \Delta G_{b2}^{0} \quad (3)$$

For both water molecules annihilated simultaneously:

$$\Delta G_{b}^{0} = \Delta G_{site} - 2 \times \Delta G_{bulk} - k_B T ln C^0 V_{site1} - k_B T ln C^0 V_{site2} \quad (4)$$

Here, C^0 is the standard volume of 1 M water, equal to 1/1661 Å^3. V_{site1} and V_{site2} are the accessible volumes of Wat1 and Wat2 during the simulations. We measured these volumes with the VMD[49] volmap tool and analyzed them using UCSF Chimera[50].

Experimental generation of *A. baumannii* F_1F_O-ATP synthase *c* subunit variants

The single-point substitutions of the *A. baumannii* F_1F_O-ATP synthase *c* subunit were generated via polymerase chain reaction (PCR) site-directed mutagenesis with KAPA HiFi HotStart PCR Kit (Roche, Switzerland) to generate the *c* subunit variants *c*T23A, *c*L59A, and *c*D60A, respectively. The pTrc99A-*Ab*F_1F_O-ATP synthase plasmid [28] was used as a template for PCR amplification. The following primers (Integrated DNA Technologies, United States) were used:

*c*T23A Forward	5’ - CTT TGG GTG CTG CGA TTG GTT TTG GTC TTT TGG GTG GT - 3’
*c*T23A Reverse	5’ - AAA ACC AAT CGC AGC ACC CAA AGC ACC GAA AGC GAT CAA GAT A - 3’
*c*L59A Forward	5’ - GGG TCT TGC CGA TGC TGT GCC TAT GAT CGG TGT TGG TAT TGG - 3’
*c*L59A Reverse	5’ - AGC ATC GGC AAG ACC CGC GAT TAA GAA CAT ACG AGT TTG AAG TTG TGG - 3’
*c*D60A Forward	5’ - GTC TTC TTG CTG CTG TGC CTA TGA TCG GTG TTG GTA TTG G – 3’
*c*D60A Reverse	5’ - GCA CAG CAG CAA GAA GAC CCG CGA TTA AGA ACA TAC GAG TTT GAA G – 3’

The PCR products underwent DpnI digestion (New England Biolabs, United States) followed by transformation into electrocompetent *E. coli* TOP10 cells for plasmid amplification. The

plasmids were then isolated and purified using the GeneJet Miniprep Kit (ThermoFisher Scientific, United States) according to the manufacturer's protocol. The mutations were confirmed by Sanger DNA sequencing (Bio Basic Asia Pacific, Singapore).

Generation of heterologous *Ab*F_1F_O-ATP synthase inverted membrane vesicles

The F_1F_O-ATP synthase deficient *E. coli* DK8 strain[51] without or with the pTrc99A-*Ab*F_1F_O-ATP synthase and the respective subunit-*c* single-point mutations *c*T23A, *c*L59A, and *c*D60A constructs were first cultured in 100 ml of 2xYT media, supplemented with 50 μg/ml of carbenicillin (Sigma-Aldrich, United States) and grown overnight at 37 °C, 180 rpm (Infors HT Minitron, Switzerland). The starter cultures were then reseeded into 2 L 2xYT media with carbenicillin at an initial optical density at 600 nm (OD_{600}) of 0.05. The reseeded cultures were grown at 37 °C, 180 rpm and then induced with Isopropyl β-d-1-thiogalactopyranoside (IPTG) (Bio Basic Asia Pacific, Singapore) at OD_{600} 0.6. The cultures were then cooled to 18 °C with 180 rpm shaking until an OD_{600} of 1.0 and afterwards centrifuged (ThermoFisher Scientific, United States) at 4,000 × *g*, 4 °C for 15 minutes. The harvested cells were snap-frozen and stored at -80 °C. Preparation of inverted membrane vesicles (IMVs) was performed as described in Saw et al.[28], to purify membrane fractions for the ATP synthesis rate assay. In this procedure, approximately 4 g (wet weight) harvested cells of the F_1F_O-ATP synthase deficient *E. coli* DK8 strain[51] without or with the pTrc99A-*Ab*F_1F_O-ATP synthase and the respective subunit-*c* single-point mutations *c*T23A, *c*L59A, and *c*D60A constructs were resuspended in 50 ml IMV preparation buffer (50 mM 4-Morpholinepropanesulfonic acid (MOPS), 2 mM $MgCl_2$, pH 7.5) containing EDTA-free Pierce protease inhibitor tablet (one tablet per 50 ml IMV preparation buffer) (ThermoFisher Scientific, United States). All subsequent steps were performed on ice. The cells were lysed with two passages at 11,000 kPa through the PandaPLUS 2000 homogeniser (GEA Niro Soavi, Italy) at 4 °C. The lysed cell suspension was centrifuged

(Eppendorf, Germany) at 10,000 × *g*, 4 °C for 10 minutes, and the resulting supernatant was ultra-centrifuged (Hitachi, Japan) at 27,000 × *g*, 4 °C for 40 minutes. The resulting supernatant containing the membrane fraction was then ultra-centrifuged (Hitachi, Japan) at 160,000 × *g*, 4 °C for 60 minutes. The supernatant was discarded and the membrane pellet was resuspended in IMV preparation buffer containing 15% glycerol, separated into 20 µl aliquots, snap-frozen, and stored at -80 °C.

Bicinchoninic assay and gel electrophoresis

To determine the protein concentration of the IMVs generated, BCA was performed using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific, United States) and the manufacturer's protocol. Based on the determined protein concentrations, 16 µg of each type of IMV was applied on a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel.

Overexpression bands of the wild-type (WT) *Ab*F_1F_O-ATP synthase and the single-point variant *c*T23A IMVs from the SDS polyacrylamide gel at approximately 55 kDa were extracted and validated via Liquid-Chromatographie-Massenspektometrie/Massenspektometrie (LC-MS/MS; short gradient) (Nanyang Technological University, Singapore).

ATP synthesis rate assay

The ATP synthesis rate assay was performed in triplicates for the samples at 0-, 2-, 5-, 10-, 15-, 20-, 25-, and 30 minutes. The IMVs were diluted to a concentration of 10 µg/ml with 2 ml IMV preparation buffer. 25 µl of the diluted IMVs were deposited in a flat-bottomed white 96-well microtiter plate (Corning, United States), having a final concentration of 5 µg/ml, and incubated with 25 µl of assay buffer (20 µM ADP, 500 µM KH_2PO_4, and 2 µM NADH) at each time point. 25 µl of CellTiter-Glo® Luminescent Cell Viability Assay (Promega, United States) was then added into the wells after 30 minutes incubation at room temperature. The 96-well

plate was incubated in the dark for 10 minutes at room temperature before obtaining the luminescence endpoint readings on BioTek Synergy H1 (Agilent, United States).

Growth kinetics in succinate minimal media

E. coli DK8 colonies transformed without or with the pTrc99A plasmid encoding the WT *Ab*F_1F_O-ATP synthase or the *c*-subunit single-point variant *c*T23A and *c*D60A were each inoculated into 5 ml of 2xYT medium supplemented with 50 μg/ml of carbenicillin and incubated overnight at 37 °C, 180 rpm. Cells from the overnight cultures were centrifuged (Eppendorf, Germany) at 4,000 × *g*, 4 °C for 10 minutes and the pellet was washed twice with succinate minimal media (1 mM $MgSO_4$, 0.03% cas-amino acids, 0.3 mM L-isoleucine and L-valine, 6 mM succinic acid disodium salt) as described in Saw et al.[28]. The growth measurement of each cell type was done in a transparent 24-well microtiter plate with lid (Corning, United States), with triplicates for each sample, wherein each well contained the cells at an OD_{600} of 0.1, with 1 ml of succinate minimal media supplemented with 50 μg/ml of carbenicillin. The readings were obtained at 1-hour intervals for 14 hours on BioTek Cytation 5 (Agilent, United States), at 37 °C with 30 seconds of 237 rpm shaking before each reading.

QUANTIFICATION AND STATISTICAL ANALYSIS

ATP synthesis and growth kinetics in succinate minimal media data were obtained from two independent biological samples producing 6 technical replicates each, giving a total of 12 technical replicates. The data was analysed on Microsoft Excel (Microsoft, United States) to determine the mean, SEM, and SD. Error calculations and statistical significance parameters have been described in the Figure 3 legend.

Free energy calculations were carried out in triplicate. These data were used to calculate the means and standard deviations.