**Supplementary Information**

# Csu pili dependent biofilm formation and virulence of *Acinetobacter baumannii*

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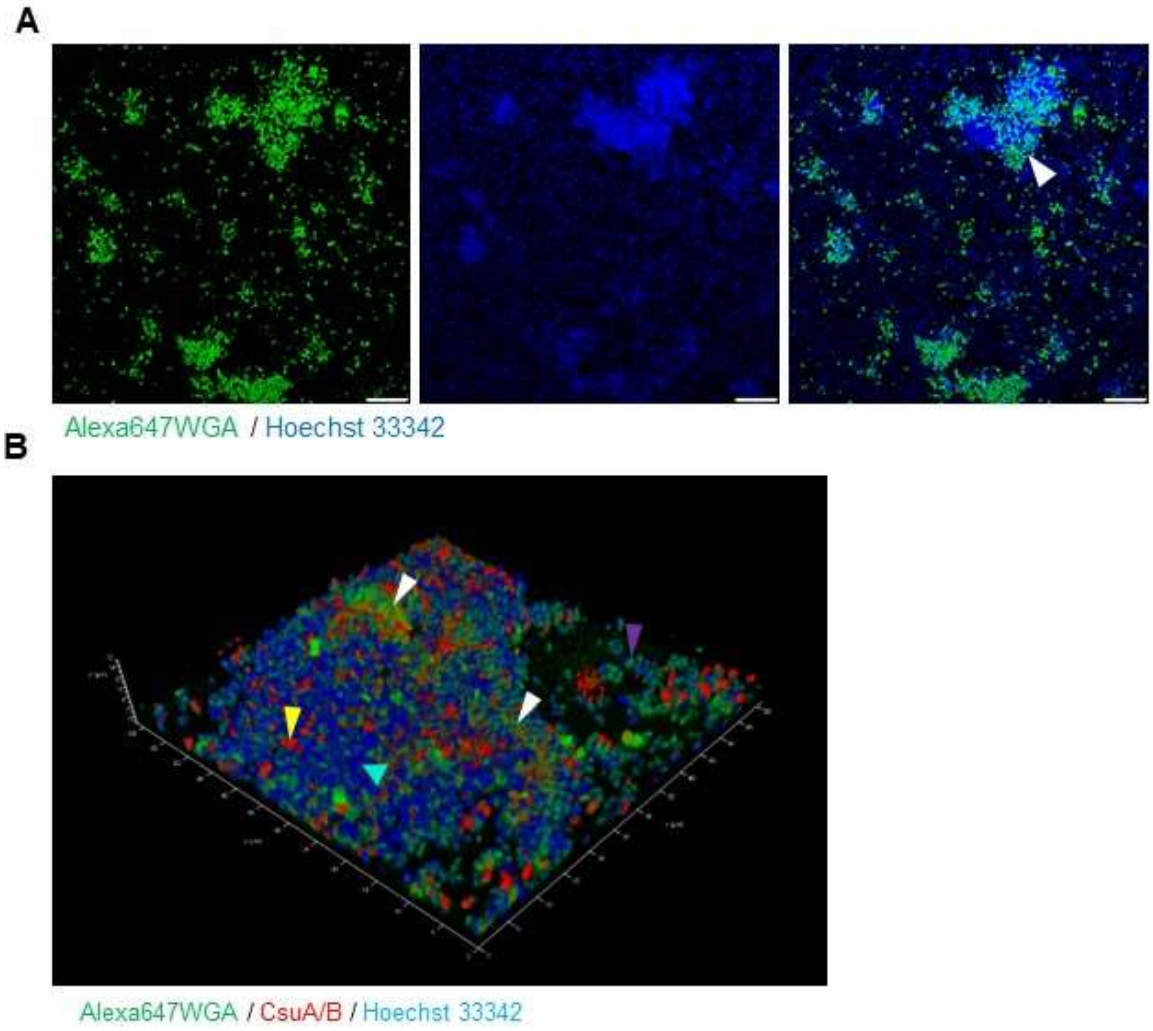
**Supplementary Figures 1-7**

**Supplementary Tables 1-4**

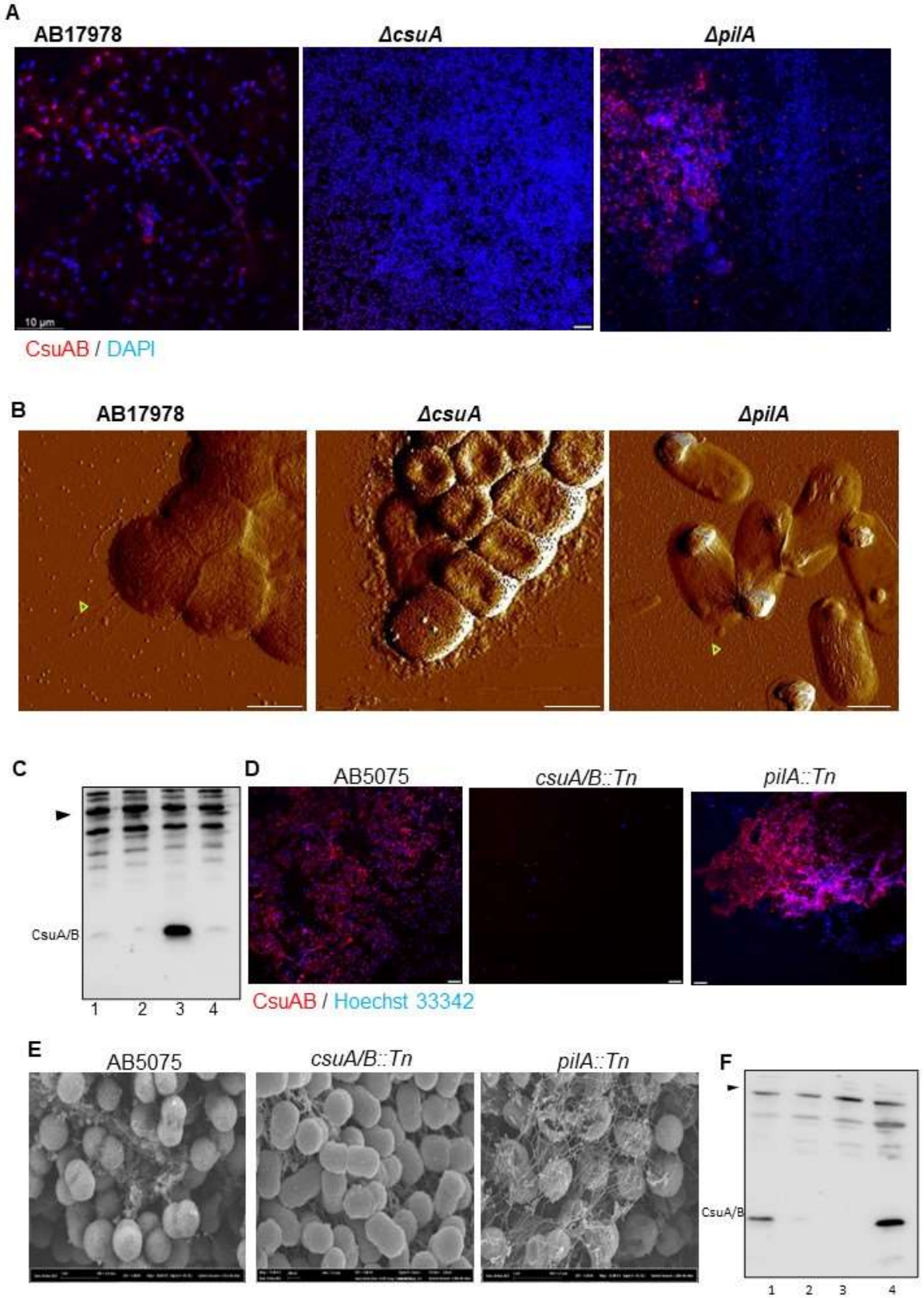
**Supplementary References**

**Supplementary Figure 1**



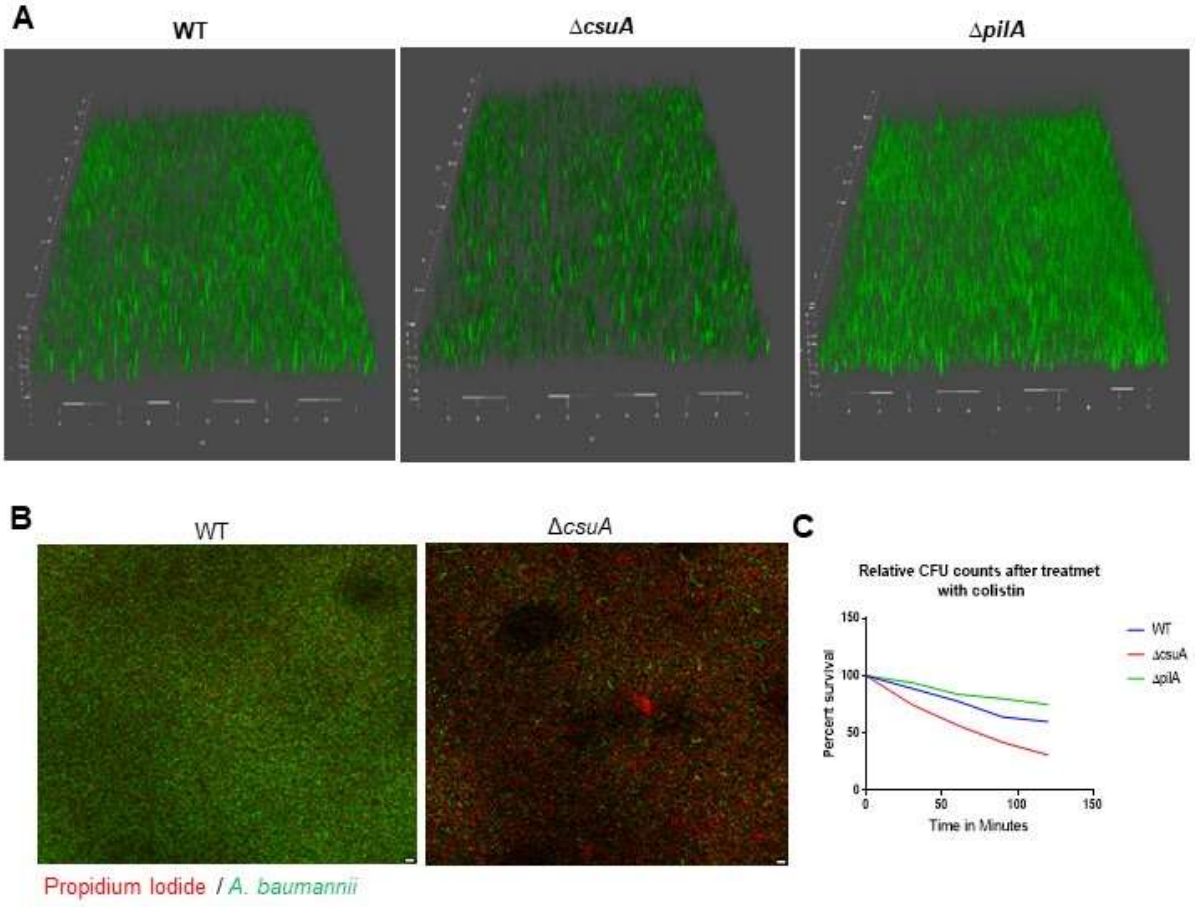
**Supplementary Figure 1:** A single section of biofilm patches formed on a glass surface, followed by labelling with Hoechst 33342 (blue) and Alexa647-WGA (green). Scale bars = 20 μm. (B) Three-dimensional confocal laser microscopic visualization of CsuA/B expression (red), PNAG expression (green) and CsuA-PNAG co-expression within a typical patch of biofilm formed on a glass surface by *A. baumannii* 17978. The arrowheads in white indicate co-localization of Alexa647-WGA (green) and CsuA/B (red), yellow indicates localization of CsuAB (red), cyan indicates Hoechst 33342 (blue) stained cells, and purple shows Alexa647-WGA (green) stained structures. (C) The growth curves of *A. baumannii* 17978 and mutant strains grown in a microtitter plate in LB medium at 37ºC using the built-in temperature control mode of the Spark multimode plate reader (Tecan). Y- axis represents the optical density ($OD_{600}$) measured with an interval of 20 minutes for 16 hours. The experiment was done in triplicate, and the curves were drawn using the mean $OD_{600}$ values.

## Supplementary Figure 2



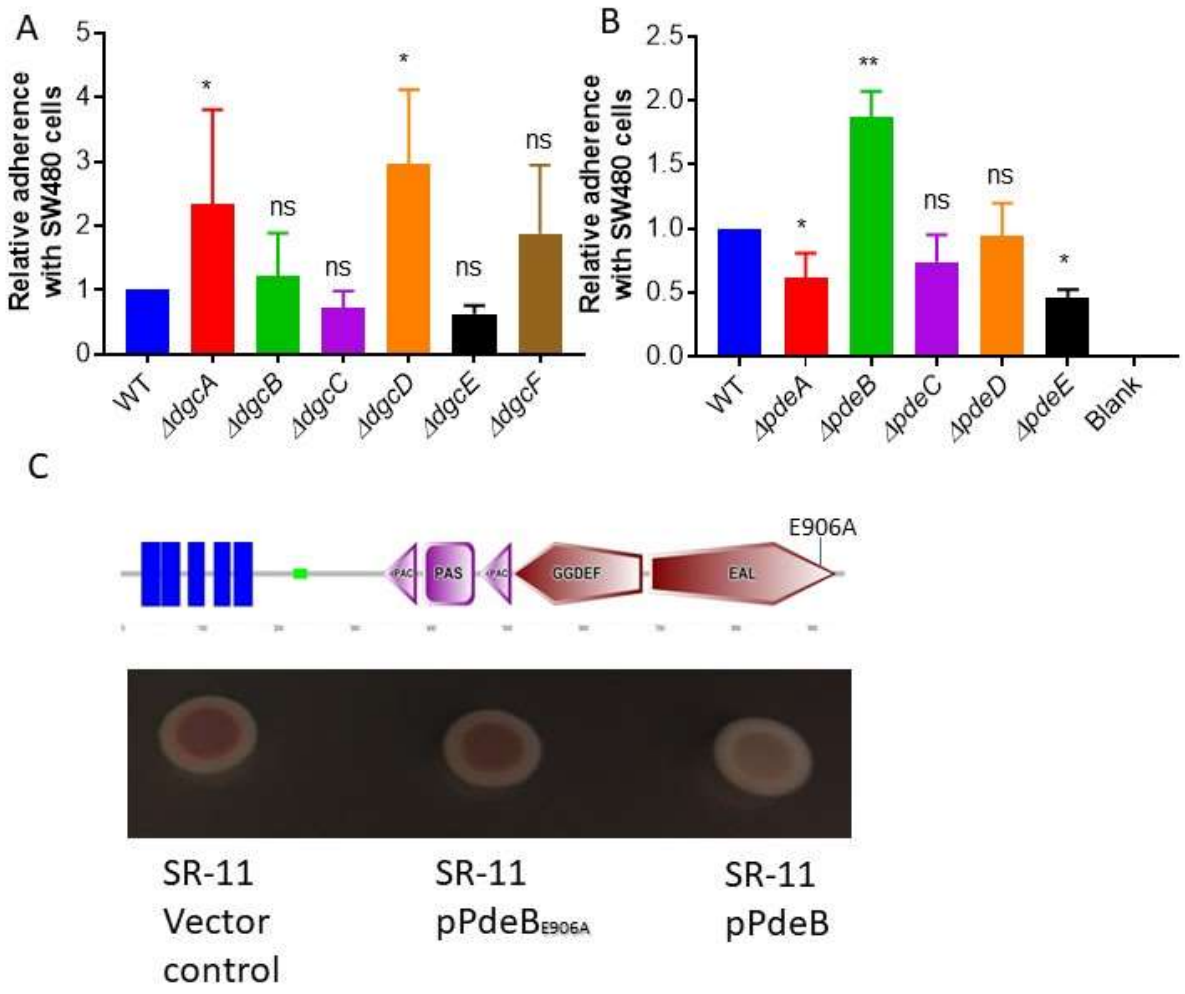
**Supplementary Figure 2:** (A) Confocal laser microscopic imaging of biofilms formed by *A. baumannii* 17978, *ΔcsuA* and *ΔpilA* mutants. Red: CsuA/B immunostained with CsuA/B specific antisera, Blue DAPI. (B) Atomic force microscopic imaging of the samples prepared from the pellicles of *A. baumannii* 17978 wild-type and mutant strains. Scale bars = 1 μm. (C) Western blot analysis of CsuA/B subunit in pellicles formed by *A. baumannii* 17978 and its mutant strains1- AB17978-vector control, 2- Δ*csuA*-vector control, 3- Δ*pilA*-Vector control, 4- Δ*csuA*-pCsuA. The arrowhead indicates equal distribution of proteins. (D) Confocal laser microscopy images of a pellicle sample labelled with CsuA/B antiserum (red) and Hoechst 33342 nuclear stain (blue) illustrating the expression of CsuA/B subunits in the pellicles of *A. baumannii* AB5075 wild-type and transposon mutants. The size bars in the images correspond to 10μm. (E) Scanning electron microscopic images of pellicle formed by *A. baumannii* AB5075 wild type and mutant strains. (F) A representative Western blot image to show expression of the CsuA/B in *A. baumannii* AB5075 wild type strain cultivated after growth at different growth conditions. 1- LB broth after 48 hours at 37ºC. 2- LB plates after 48 hours at 37ºC, 3- LB agar plate lacking NaCl ,4- pellicle formed within LB broth. The arrow-head indicates a nonspecific band as a loading control.

**Supplementary Figure 3**




**Supplementary Figure 3:** (A) Live cell confocal laser microscopic images of biofilms formed by *A. baumannii* 17978 wild-type and mutant strains expressing green fluorescent protein within plastic cells after 24 hours of incubation at 30ºC. (B) Live cell images of biofilms in the plastic cells formed by wild-type and *ΔcsuA* mutant *A. baumannii* 17978 that were treated with 5 µg/ml of colistin for 2 hours. Cells were stained with Propidium Iodide (PI) to visualize dead bacteria within biofilms. The size bars in the images correspond to 5 µm. (C) Comparison of the percent survival of wild-type and mutant strains after treatment of biofilms with colistin, as measured through CFU counts of bacteria on agar plates.

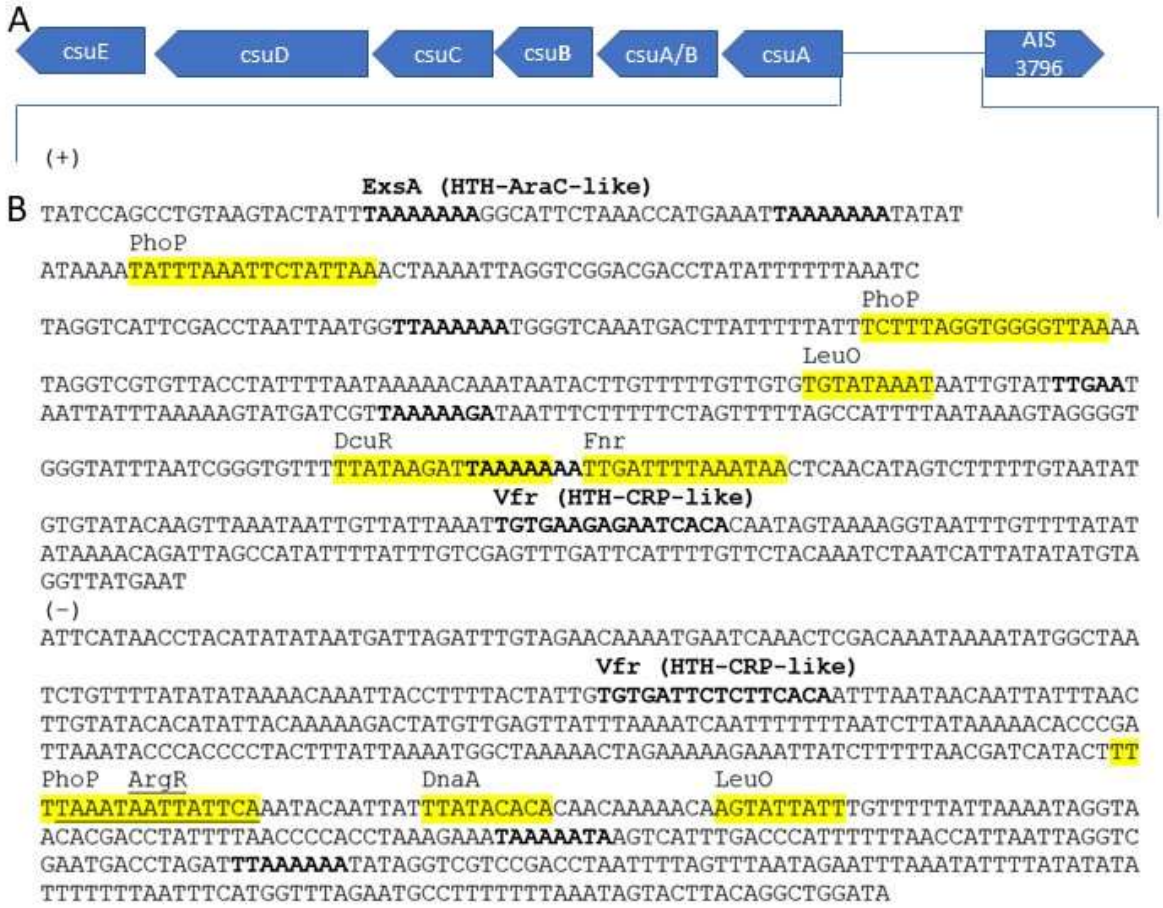
**Supplementary Figure 4**




**Supplementary Figure 4:** Bar chart diagrams illustrating the screening of the individual mutants of 11 GGDEF/EAL proteins of *A. baumannii* 17978 for adherence to epithelial cells A549. Adherence of GGDEF mutants (A) and GGDEF-EAL and EAL mutants (B) to epithelial cells A549. Bars show ± standard deviation between the experiments. Statistical significance is indicated by *p* value that was measured by non-parametric two tailed *t* test. A single asterisk * indicates $p \leq 0{,}05$, two asterisks ** indicate $p \leq 0{,}01$ and "ns" indicates non-significant difference as compared to wild type. (C) Upper panel: Domain architecture of PdeB, alias AIS_2337, highlighting the position of glutamic acid 906, mutated to create a

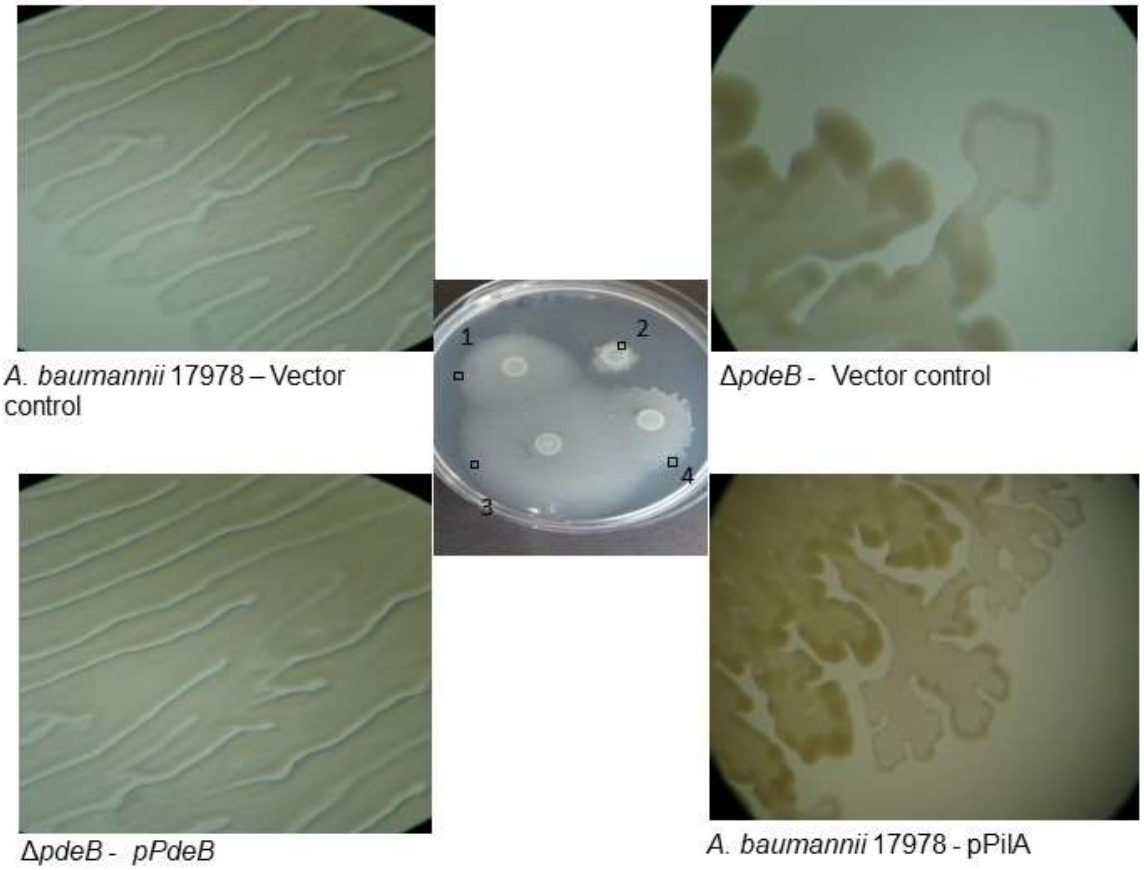
catalytically inactive variant of PdeB. Lower Panel: Image of the Congo red agar plate containing spots of *S. typhimurium* SR-11 grown at 30ºC for 24 hours to illustrate the effect of E906A mutation on the phosphodiesterase activity of PdeB. Wild type PdeB inhibits the formation of the rdar morphotype, whereas $PdeB_{E906A}$ does not suppress rdar morphotype formation**.**

**Supplementary Figure 5**




**Supplementary Figure 5:** (A) Organisation of the *csuABCDE* operon along with the promoter region (B) DNA sequence of the promoter region of *csu* operon highlighting putative binding sites for multiple transcriptional regulators.

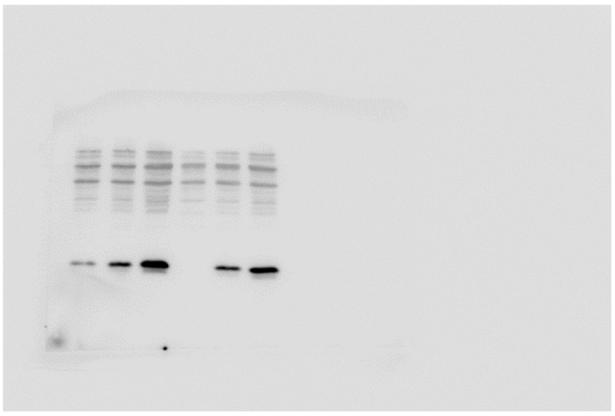
**Supplementary Figure 6**



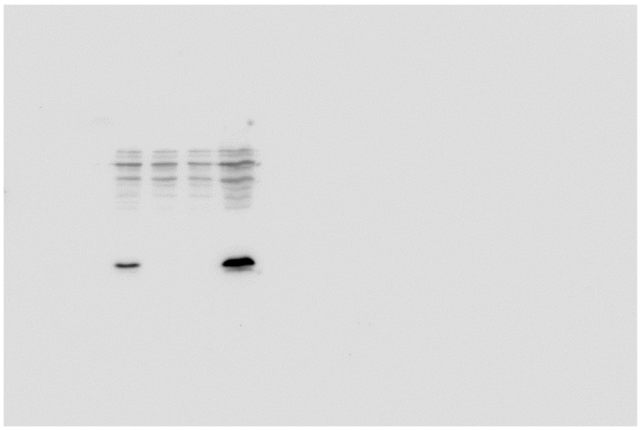

**Supplementary Figure 6:** Stereomicroscopic images of surface-associated motility exhibited by *A. baumannii* 17978 wild-type- vector control (1), Δ*pdeB*- vector control (2) Δ*pdeB* trans complemented strain from the plasmid pPdeB (3) and wildtype with over production of PilA from plasmid pMBB67EH (4).

**Supplementary Figure 7: Full unedited images for Western blots**

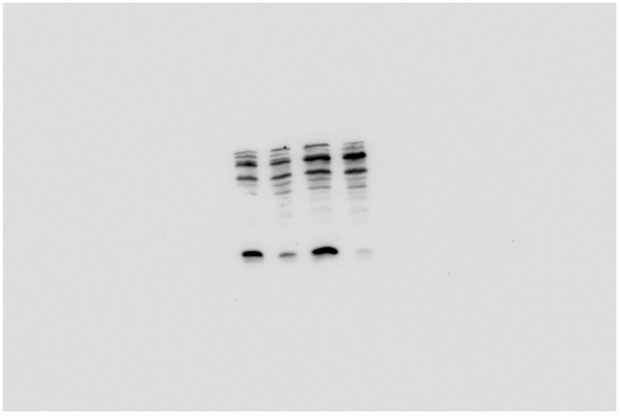
**Unedited gel for Figure 1A**



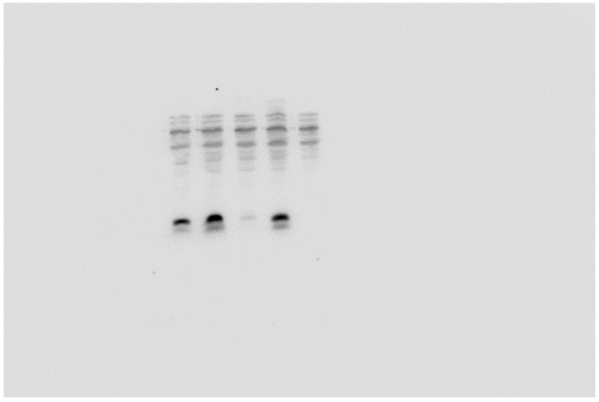
**Unedited gel for Figure 2D**



**Unedited gel for Figure 3B**



**Unedited gel for Figure 5E**



## Supplementary Tables

### Supplementary Table 1: Minimum inhibitory concentration of Colistin and carbenicillin in *A. baumannii* isolates

| Isolate | MIC of colistin | MIC of Carbenicillin |
|---|---|---|
| AB17978 | 0,4 ug/ml | <8 ug/ml |
| AB17978 *ΔcsuA* | 0,4ug/ml | <8 ug/ml |
| AB17978 *ΔcsuC* | 0,4 ug/ml | <8 ug/ml |
| AB17978 *ΔpilA* | 0,4 ug/ml | <8 ug/ml |
| AB5075 | 0,8 ug/ml | >64 ug/ml |
| AB5075 *csuA/B::Tn* | 0,8 ug/ml | >64 ug/ml |
| AB5075 *pilA::Tn* | 0,8 ug/ml | >64 ug/ml |
| AB5075 *pdeB::Tn* | 0,8 ug/ml | >64 ug/ml |

### Supplementary Table 2: *Acinetobacter baumannii* strains used in the study

| Strain ID | Genotype | Reference / Source |
|---|---|---|
| AB17978 | Wild type | ATCC17978 |
| AB17978 *ΔcsuA* | *ΔcsuA:*Km | This study |
| AB17978 *ΔcsuC* | *ΔcsuC:*Km | This study |
| AB17978 *ΔpilA* | *ΔpilA:*Km | This study |
| AB17978 *ΔdgcA* | *ΔAIS_0751:* Km | This study |
| AB17978 *ΔdgcF* | *ΔAIS_3296*: Km | [1] |
| AB17978 Δ*pdeA* | *ΔAIS_1949:* Km | [1] |
| AB17978 *ΔdgcB* | *ΔAIS_1067*: Km | This study |
| AB17978 *ΔpdeC* | *ΔAIS_1254*: Km | This study |
| AB17978 *ΔdgcD* | *ΔAIS_2506*: Km | This study |
| AB17978 *ΔpdeE* | *ΔAIS_2422*: Km | This study |
| AB17978 *ΔpdeD* | *ΔAIS_0546*: Km | This study |
| AB17978 *ΔdgcE* | *ΔAIS_2986*: Km | This study |
| AB17978 *ΔdgcC* | *ΔAIS_1695*: Km | This study |
| AB17978 *ΔpdeB* | *ΔAIS_2337*: Km | This study |
| AB5075 | Wild type | [2] |
| AB5075 *csuA/B::Tn* | *ABUW_1487::Tn10-ATS* | [2] |
| AB5075 *pilA::Tn* | *ABUW_0304::Tn10*-ATS | [2] |
| AB5075 *pdeB::Tn* | *ABUW_*1221*::Tn10*-ATS | [2] |
| Ab-Pak-Pesh-22 | Clinical isolate, $bla_{OXA-68}$, *ampC-79*, Sequence type 23 | [3,4] |
| Ab-Pak-Pesh-37 | Clinical isolate, $bla_{OXA-371}$, *ampC-3*, Sequence type 1106 | [3,4] |
| Ab-Pak-Lah-14 | Clinical isolate, $bla_{OXA-371}$, *ampC-8*, Sequence type 1 | [3,4] |

**Supplementary Table 3: Plasmids used in the study**

| Plasmid | Description | Reference |
|---|---|---|
| pMMB67EH | Vector plasmid for cloning | [5] |
| pAT02 | RecAB cloned in pMBB67EH | Tucker, et al. [6] |
| pCsuA | CsuAx6His cloned in pMBB67EH | This study |
| p3296 | AIS_3296x6His cloned in pMBB67EH | [1] |
| pPdeB | AIS_2337x6His cloned in pMBB67EH | [1] |
| pPilA | PilAx6His cloned in pMBB67EH | This study |

**Supplementary Table 4: Oligonucleotide primers used in the study**

| Primer | 5`-3´ Sequence |
|---|---|
| Primers used to delete genes in *A. baumannii 17978* | |
| A1S_0751_KO_Forward | ATGATTGGTAGTTTTTGGGCCGAATTTGGTTATTGGGTTACTTCAGAGCTTCAAAATGCCTTATTGTTGCTGCCCATTATTTTAAACATCCCCCCTCCGGAATTGCCAGCTGGG |
| A1S_0751_KO_Reverse | TTACAAAGAAATAAAAACTCTATTACGCCCTTGGCGCTTTGCTTGATAGAGCGCATCGTCTGCTTCTTTAAACAAACTTTCAATAGAACGGTTCAGAAGAACTCGTCAAG |
| A1S_1695_ko_f | ATGAAGTTGCAAGGTTCCAATATATTGGGACAGGAACAAATAGATTTATTAACCACACGCGGATTAAATTTCGTATGGTTTCCAAAGCAACTTGAACCGGAATTGCCAGCTGGG |
| A1S_1695_ko_r | TTAAGTTAGATCTACAATTTGTTCTTCATTCAAAGCAATTTGATATTGATTGCGGCCATTTGTTTTTGCCTGATAGAGCGCATGATCAGCATTCAGAAGAACTCGTCAAG |
| A1S_2506_Ko_Forward | ATGGAAACTTTAGATTCTTCAATTTTTGACCTCACCCCAATTCCAATGTGGATTGAAGACTTTAGTGAAGTTAAGCAGTTATTTGACTTATGGAGACCGGAATTGCCAGCTGGG |
| A1S_2506_Ko_Reverse | TTATGGTAGAAGTAATTTCTTGATAATAACTTTTTTCTTTGAATACATATGATGATCTGCGCGTTTTAGCATATCTTCAACTTGTTCATTTTTCAGAAGAACTCGTCAAG |
| A1S_2986_Ko_F | ATGGAAACTTATGCAAAACAAAATTTGCAATTACTTAGCCATACTCTTCTTGAGCGTATTCAACCTGCCGTTGTGTTTAACGATAAGATCACGATCCCGGAATTGCCAGCTGGG |
| A1S_2986_Ko_R | TTAATTCTCTGGTTTATAAATAAACCAATGTTGCTCAGAAGTCTTAGCCTTATACATCGCTTGATCTGCTTGCATAATAAAATCTTCTGGATTCAGAAGAACTCGTCAAG |

| | |
|---|---|
| A1S_0546_ko_F | ATGGGTCATGTTGATTACGATAGCACATTGATCGTCGGCTCTTTTATTGCGGCTGGCGCTATTTGCTATATTGTGATTTCCATGGAGCAGTTAATACCGGAATTGCCAGCTGGG |
| A1S_0546_ko_R | TTAAACAAAATGAGCACCTTGCGACTGAATCAGTTGCCCTACATTTACAGGCATACCCAACAAATAACCTTGGAACTGCTGACAGCCCAAGTTCAGAAGAACTCGTCAAG |
| A1S_2337_ko_F1 | ATGTCTGGCCTTCAGGAAGAATATTTACAGCATCAAACAAAAATTGAGCAGCTTCGCCTCTCAATTCAATATATCCGCTGGTATCTTGTTAGCCTACCGGAATTGCCAGCTGGG |
| A1S_2337_ko_R | TCAAATATGCGTATTTGTTTGTAGATATTGAAGAATCTGATTTGGATGAAGTGGACGGCTAAAGAAAAAGCCTTGTAAAAAATCACATTGCTTCAGAAGAACTCGTCAAG |
| A1S_2422_KO_F | TTTCTATATTACGCTGGTTCGGGCACTGTCATACAGCCGCCTTTTACAGACGGAACAACGTCTTTTGGCTGAGCTTGATACTATTCATACGCATACCCGGAATTGCCAGCTGGG |
| A1S_2422_KO_R | CCATGTTGGCGTAAGCGAGCAATGTGCTGTTGAGCAATTGGGAGATAAGGCTGAAGAGCTTGTTCTGAAAATTGCAAAATCAGTGGGCTTTTTCAGAAGAACTCGTCAAG |
| A1S_1067_KO_f | GTGGCGAATAGGGGAAATGTACATCAATTACTCAAATCTAAAGAAGAAATCGAGTATTTGGTCACTCTACATACCCATCGTTATGCCAATATGCCTTTGCCCCCGGAATTGCCAGCTGGG |
| A1S_1067_KO_R | TTAGGCAATTTCAGCGACTTGTAATGGCTGATAATAAATTTGATTACGGCCAAGCTGTTTTGCCCGGTATAAAGCCTGATCTGCCGCATGAATAAGCCTATTTCAGAAGAACTCGTCAAG |
| A1S_1254_KO_f | ATGGATATCTGTTTCACACTAATTTCCATATTTAGCTTTTTTCTTATTTATATAAACCGTATTTCGGCAGGGATACTTTTATCACAAGCGGTTCTACTGGTTCCGGAATTGCCAGCTGGG |
| A1S_1254_KO_R | TTATTCTTTATCGATCTTGATGGGCAATTGCTTGATAAACTCATTTAAAGACATTGGACAGCCCCATAAATAGCCCTGAAACTCGGTACAACCATTACTTTTTCAGAAGAACTCGTCAAG |
| pilA-ko-f | ATGAATGCACAAAAAGGTTTTACATTAATTGAACTCATGATCGTGGTTGCCATTATCGGTATTTTGGCAGCAATTGCGATTCCGCAATATCAGAAATACACTGTGTAGGCTGGAGCTGCTTC |
| pilA-ko-r | TTAAATTATTGTACAGCCTTTTGGAGCAATTACTGAAGAAATATCTGTTGTTCCTTTAGTAATAGAACATGTCCAGCCACCCACATTTGCATTTACTCCAGCATATGAATATCCTCCTTAGT |
| CsuA_Ko_F | ATGATATTCAATCGTGGTTCGGCATTTATAATTTCTTATTTTTTAATTTCTTTAGTAAATGCGGGTGAAATTGGAGCTAAATTAACTAGTCAAATTGAATCCGGAATTGCCAGCTGGG |
| CsuA_KO_R | TTAAAACTCAATCGTAATTGGTACTATATCTTTATATTCACCCTTAGATATACGACTACCATCATGGGTTGCTTGACCAAAAATATCGATATTCTTTTTATTCAGAAGAACTCGTCAAG |

| | |
|---|---|
| CsuC_ko_F | ATGAACAATTCTGCATTTATTAAAAATGGCATTTTAAAATCTTTTTTATTTGCAAGTACATTATCACTTGTTACACCTGTGATGGCACAAGCAACTTTTCCCGGAATTGCCAGCTGGG |
| CsuC_ko_R | TCATGATGGATCCTCCATTTTGGTGATTTCGATCAATTCTTGTTTAATACCAGAACTGTCCACACCATAAATTTTAGATGTTTTTGACAACTCATTCAGAAGAACTCGTCAAG |
| Primers used to confirm deletion of genes | |
| CsuA_Conrol_FOrward | GGCGATTATAAAGATACTC |
| CsuA_Conrol_Reverse | GAATTGAGCTGAGGATCT |
| CsuC_control_F | CCGTCTGGCTAAATTTTGA |
| CsuC_Control_R | CCACCCGACAATAAAATCA |
| A1S_0751_Control_F | TCGTATGGAGTAATGGCACTCAT |
| A1S_0751_Control_R | GTCGCACCAGTAATTGTGCAAAC |
| A1S_1695_control_F | AGACCATCCATTATGCGGCGTAA |
| A1S_1695_Control_R | GGATGTTGTTCCTCCATTTGCG |
| A1S_2506_control_Forward | CGACCACCCTATTAACCGTTAA |
| A1S_2506_control_Reverse | GGTACTCGTCGTTCTATGGGTAA |
| A1S_2986_Control_F | TTGGCTAAGAACGCGGAGTAA |
| A1S_2986_Control_R | GCACAGCAATGCAATAAAGG |
| A1S_0546- | CAAGCGAACAAAATCGTAGAC |

| | |
|---|---|
| _control_F | |
| A1S_0546-_control_R | GAGTTTAGAAGTGTCAATGTGG |
| A1S_2337_control_F | CGTACTGAAACTGCACCTGTTGTAGC |
| A1S_2337_control_R | GCGGCCTGTTTTAAAGATTGATCGTC |
| A1S_2422_control_F | GCCGCAGCTTCCAATATTAC |
| A1S_2422_control_R | GCTGCTGTAATATTGAATCGC |
| A1S_1067_control_f | GGAGCAGGGTAATAGAGTTCAA |
| A1S_1067_control_r | GGTTCTGCTGTACCTCAATTAG |
| A1S_1254_CONTROL_F | CGCAATAAGGTATAAGCTGAG |
| A1S_1254_CONTROL_R | ACTGCGCTTCAGGTTTCGTCAT |
| pilA-control-f | GCAATACCAGAAAGCTGTAGTTAC |
| pilA-control-rev | CTTAACCCTGCTGCAAAGGCA |
| Inside_Kan_Forw | CTGCTTGCCGAATATCATGG |
| Inside_Kan_Rev | CTCGTCCTGCAGTTCATTC |
| Primers used for cloning | |
| pilA-clon-f | CGTATCTAGAGGGAAAAAGGCTATGAATGCAC |

| | |
|---|---|
| pilA-clon-r | CGTCGAAGCTTTTAATGGTGATGGTGATGGTGAATTATTGTACAG CCTTTTGGAGC |
| Primers used for site directed mutagenesis | |
| pdeB_AG VE_F | GCTAGTTGCAGCAGGTGTGGAAAC |
| pdeB_AG VE_Rev | GTTTCCACACCTGCTGCAACTAGC |

**Supplementary References:**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